SZENT ISTVÁN UNIVERSITY
POSTGRADUATE SCHOOL OF VETERINARY SCIENCES

Immunomodulation of pathogen-host interactions

Doctoral Thesis

By

Dr. Susan Szathmáry

Advisor

Dr. László Stipkovits

Budapest
2005

Szent István University
Faculty of Veterinary Medicine
Postgraduate School of Veterinary Sciences
Budapest, Hungary

Advisor:

…….
Professor László Stipkovits, DVM, Ph.D. D.Sci.
Head of the Mycoplasma Team
Veterinary Medical Research Institute
Hungarian Academy of Sciences
Budapest, Hungary

Prepared in eight copies. This is copy …….of eight.

…….
Dr. Susan Szathmáry

TABLE OF CONTENT

LIST OF ABBREVIATIONS

AP-1:	activated protein-1
APCs:	antigen presenting cells
APTT:	Activated Partial Thromboplastin time
AT:	antithrombin
CFU:	colony forming unit
CHO:	Chinese hamster ovary
chTLR:	chicken TLR
CNBr:	cyanogen bromide
ConA:	Concanavalin A
CpG ODN:	Unmethylated CpG-dinucleotide-containing sequences
CpG:	Unmethylated dinucleotides
CRD:	carbohydrate recognition domain
DC-SIGN:	dendritic cell specific ICAM grabbing non-integrin
DCs:	dendritic cells
DEC-205:	dendritic cell receptor for endocytosis
DI:	deionized
EndoH:	Endoglycosidase H
ET:	Endotoxin
EU:	endotoxin units
FCS:	fetal calf serum
GGPL:	6'-0 phosphocholine-alpha-glycopyranosyl-(1,3)-1,2-diacyl-sn-glycerol
GM-CSF:	granulocyte, macrophage colony stimulating factor
HDV:	Hepatitis Delta Virus
HIV:	human immunodeficiency virus
HSA:	human serum albumin
Hsp:	heat shock protein
IBV:	Infectious Bronchitis Virus
ICAM:	intercellular cell adhesion molecules
IRAK:	IL-1R-associated kinase
IRF:	IFN regulatory factor
ITAM:	immunoreceptor tyrosine-based activation motif
ITIM:	immunoreceptor tyrosine-based inhibition motif
LAL:	Limulus Amoebocyte Lysate
LAM:	Lipoarabinomannan
LAMPs:	Lipid associated membrane proteins
LFA-1:	Leukocyte function associated molecule 1
LP:	lipoproteins
LPG:	lipophosphoglycan
LPS:	lipopolysaccharides
LTA:	Lipoteichoic acid
Mal:	MyD88-adapter-like
MALP-2:	macrophage-activating lipopeptide-2
ManLAM:	mannose-capped lipoarabinomannan
MAPK:	mitogen-activated protein kinase
MARCO:	Macrophage Scavenger Receptor
MBL:	mannose binding lectin
MG:	*M. gallisepticum*
MHCII:	major histocompatibility complex

MnTBAP:	Mn(III)tetrakis(4 benzoic acid)porphyrin
MR:	mannose receptor
MyD88:	myeloid differentiation primary response gene 88
NF-κB:	nuclear factor-κB
NK:	natural killer
Nod2:	nucleotid oligomerization domain 2
NOS:	Nitric oxide synthase
OD:	optical density
PAF-1:	Platelet Activating Factor-1
PAMPs:	pathogen-associated molecular patterns
PBMC:	peripheral blood mononuclear cells
PMN:	polymorphonuclear leukocytes/neutrophils
Poly I:C:	polyinosinic-polycytidylic acid
PP:	Peyer's patches
PRR:	pathogen recognition receptor
PRRSV:	porcine reproductive and respiratory syndrome virus
PT:	Prothrombin time
R-848:	resiquimod
Rip:	receptor interacting protein
RO:	reverse osmosis
RSV:	respiratory syncytial virus
SEA:	soluble egg antigen
SPA:	serum plate agglutination
SR-As:	class A scavenger receptors
SR-Bs:	class B scavenger receptors
TAT:	thrombin-antithrombin
TF:	Tissue Factor
TGF-β:	transforming growth factor-beta
TIR:	Toll/IL-1R homology domain
TIRAP:	TIR domain-containing adapter protein
TLR:	Toll-like receptors
TNF-α:	tumor necrosis factor-α
TOC:	total organic carbon
TRAF6	tumor necrosis factor receptor-associated factor 6
TRT:	Turkey Rhinotracheitis virus
WFI:	Water for Injection

I. SUMMARY

In this thesis we have reviewed the scientific literature related to the subject matters of our work. We have briefly summarized the literature of the innate immune response focusing on the research related to pathogen recognition. These include the Toll-like receptors (TLR) and other pathogen recognition receptors (PRR). We have also reviewed the literature of dendritic cells (DC) with the focus on the pathogen recognition receptor, dendritic cell specific ICAM grabbing non-integrin (DC-SIGN) and the roles of dendritic cell TLRs in induction of Th1 helper response. In addition, we reviewed briefly the literature of the particle based carrier systems as well as the interaction of mycoplasmas with TLRs and *M. gallisepticum* (MG) infection.

In **Experiment 1**, we described the removal of a TLR-4 agonist molecule, endotoxin, from different solutions using affinity technology. The endotoxin concentrations were measured using the Limulus Amoebocyte Lysate (LAL) assay. We have demonstrated endotoxin binding from water, Pseudomonas supernatant, and sa(lt solutions by spiking the samples with known amounts of endotoxin. We have tested the reusability of the affinity resin by cleaning it with NaOH as well as hot water sanitization. Leachables from the affinity resin that could potentially contaminate the solutions were also tested for. We have also investigated if the resin changed the composition of the salt solution.

In **Experiment 2**, we have described the removal of TLR agonist molecules, such endotoxin (TLR-4), peptidoglycan (TLR-2/Nod2), lipopeptide (TLR-2) and bacterial DNA (TLR-9) from blood and plasma using affinity technology. We have tested the efficacy of removal by spiking the anti-coagulated blood and plasma with known amount of TLR agonist and measured their capture under dynamic conditions. We have tested several affinity resins, and the result of the most efficient one is presented here. The removal of endotoxin was tested with the LAL assay, while we have used monocyte activation assay (TNF-α ELISA) for testing the removal of the other TLR agonist molecules. Tissue Factor (TF) assay was used to determine the effect of the removal of TLR agonists on the coagulation part of the innate immune system. We have tested that the affinity resin, while removing the TLR agonist molecules does not have a negative effect on the blood by assaying the parameters of the coagulation, complement activation, hemolysis and cell count.

In **Experiment 3**, we have described mycoplasma capture from solutions, such as serum used in cell culture, by affinity technology. We have used serologically and biochemically different mycoplasmas and affinity resins that are specific for different lipid and carbohydrate moieties on the mycoplasma membranes.

In **Experiment 4**, we have described synergistic effect of TLR agonist molecules, such as peptidoglycan (TLR-2/Nod2) and bacterial DNA (TLR-9) on the stimulation of the innate immune response. Monocyte culture-based activation assay for tumor necrosis factor-α (TNF-α) and Tissue Factor levels (ELISA) were used to demonstrate their effect. We have also determined the effective concentrations of these TLR agonists that synergistically induce TNF-α and Tissue Factor production.

In **Experiment 5**, we have described the preparation of pathogen mimicking microparticles, which included the preparation of an immunoaffinity column using purified polyclonal antibodies from a *M. gallisepticum*-positive sera, the immunoaffinity purification of *M. gallisepticum* antigens and the biochemical modification of the antigens (Endoglycosidase H (EndoH) digestion, Concanavalin A (ConA) adsorption, periodate oxidization and deacylation). In addition we have described the immobilization of antigens and PRR agonists, such as TLR-2, TLR-3, TLR-4, TLR-9 and nucleotid

oligomerization domain 2 (Nod2) agonists to the microparticles. These microparticles are used *in vivo* (Experiment 6) and *in vitro* (Experiment 7).

In **Experiment 6**, we have used a *M. gallisepticum* challenge model in chickens to test the effect of the pathogen mimicking microparticles. We have set up test groups of chickens (10 chickens per group) that were treated with the immunomodulatory microparticles orally 14 days prior to the *M. gallisepticum* challenge or after the challenge. The chickens were challenged with *M. gallisepticum* R_{low}, a highly pathogenic strain of *M. gallisepticum*. Fourteen days after the challenge, the chickens were euthanized and examined for pathological lesions. Samples from different organs were taken for culture for *M. gallisepticum* as well as histopathology. In the challenge experiment, we have examined and compared the effects of PRR agonists, *M. gallisepticum* and *M. gallinarum* membranes, and the immunoaffinity-purified antigens with or without PRR agonist molecules. We have also examined the effect of the different post-transcriptional modifications of the *M. gallisepticum* antigens on the immune response.

In **Experiment 7**, we have used the pathogen mimicking microparticles to study their effect *in vitro* with peripheral blood mononuclear cells (PBMC) and dendritic cells. We have induced monocytes with IL-4 and GM-CSF (granulocyte, macrophage colony stimulating factor) to obtain dendritic cells. We have been able to demonstrate that the microparticles interact with the cells of the innate immune system, such as PBMC and dendritic cells. We have assayed the activation of these cells by testing the level of pro-inflamatory cytokine, TNF-α and anti-inflamatory cytokine, IL-10 induction using ELISA. We have labeled the microparticles with fluorescein and used flow cytometry to show interaction with dendritic cells. We have shown that the microparticles used as an immunomodulator induce changes that are hallmarks of dendritic cells maturation, such as increase in the expression of MHCII (major histocompatibility complex) molecules and CD86 molecules. These were assayed by flow cytometry. Since the dendritic cells are the link between the innate and adaptive immunity, we have been able to show that the microparticles are able to influence both the innate and adaptive immune response.

II. INTRODUCTION

Bacterial and mycoplasmal infections are typically controlled by the use of antibiotics. However, antibiotics are not effective against bacterial toxins and bacteria frequently release large amounts of toxin, such as endotoxin when they perish. Antibiotics are also becoming less effective, due to the increase in antibiotics resistance. According to the 1999 General Accounting Office Report, Food Safety- The Agricultural Use of Antibiotics and Its Implication for Human Health, "the agricultural use of antibiotics is a significant source of antibiotics resistance". Even though it has not warranted restricting antibiotics use in farm animals yet, there is a clear tendency to reduce antibiotics use in these animals. Organic food is becoming increasingly popular. In 1999 the USDA's National Organic Program announced that neither sub-therapeutic nor therapeutic antibiotics would be permitted in organic livestock. The modulation of the innate and adaptive immune response to control both veterinary and human infections is becoming ever more important.

Innate immunity can promptly recognize and respond to pathogenic microorganisms. Adaptive immunity is mediated by B and T lymphocytes. They can recognize antigens with high affinity through rearranged receptors and persist as memory cells for a long time in the host. These features are favorable, but it requires several days to establish adaptive immunity. Therefore, prompt response is necessary especially during the early phase of infection, and this is achieved by innate immunity. Innate immunity in higher animals linked to the adaptive immunity through the dendritic cells.

DCs are unique antigen presenting cells (APCs) that can activate naive T cells. DCs recognize microorganisms, secrete pro-inflammatory cytokines, maturate and acquire T cell stimulatory activity. A group of trans-membrane proteins, called Toll-like receptors, can provide critical signals for these steps linking innate and adaptive immunity. Macrophages and DCs not only capture invading microorganisms by phagocytosis but also recognize a variety of molecular structures expressed on them. These structures are originally called as pathogen-associated molecular patterns (PAMPs). PAMPs are expressed also in non-pathogenic microorganisms, but not in the host. In this sense, they can be regarded as non-self. TLRs can sense infection by recognizing these PAMPs.

Cell wall components from microbial organisms have a strong ability to provoke immune responses. Gram-negative bacteria possess lipopolysaccharides (LPS) in their outer membranes. LPS consists of lipids and polysaccharides and the lipid portion is responsible for the immunostimulatory activity. LPS is the first-identified TLR ligand. Gram-positive bacteria do not carry LPS, but the outer membrane consists of thick layer of peptidoglycan, where a number of lipoproteins (LP) and lipopeptides are embedded. On the other hand, Mycoplasmas lack the cell wall and do not possess either LPS or peptidoglycan. However, these organisms can still exhibit immunostimulatory activity and this activity can be ascribed to a variety of components including lipoproteins or lipopeptides. Most of these components are recognized by TLR-2. Cooperating with TLR-6, TLR-2 can also recognize a synthetic mycoplasmal lipopeptide with endotoxin-like activities, the macrophage-activating lipopeptide-2 (MALP-2).

The aims of the presented experiments were to investigate the efficacy of the innate and adaptive immune responses to pathogens and pathogen-associated molecules, such as endotoxins. We have examined two distinct approaches.

One is aimed at the removal of bacterial toxins, mycoplasma and bacteria from different biologically important solutions, such as salt solutions, pharmaceuticals, water and serum used in the manufacture of pharmaceuticals. This technology has been also applied to remove endotoxin

and other TLR stimulating molecules from blood and other biological fluids. These technologies utilize synthetic ligands based on TLR recognition to capture the immunostimulatory molecules and pathogens. During the development of the capture technologies, we have developed some of the assays and methods utilized in the experiments throughout the work.

The other is aimed at modulating both the innate and the adaptive immune responses. We have utilized a novel "pathogen mimicking" microparticle to influence the function and signaling mechanism of PRR in regulating the immune responses. These particles contain antigens along with selected TLR and other PRR agonists to induce a pathogen-tailored, effective innate and adaptive immune response. *M. gallisepticum* infection has been used as a model system to demonstrate the feasibility of this technology. *In vitro* studies using PBMC and DCs, and animal challenge experiments have been performed.

In the following chapters, I will summarize the background of pathogen recognition and the innate and adaptive immune response, which will be followed by our own experiments.

III. REVIEW OF THE LITERATURE

3.1. Innate Immune Response

The innate immune system provides a critical interface between microorganisms and their hosts. The afferent and efferent limbs of this system defend the host during the initial minutes, hours and days of an infectious challenge, at the time when specific adaptive responses are being generated. Innate immune effector mechanisms help limit infections, induce a heightened state of awareness in the host, and serve as a necessary antecedent to the adaptive immune response. A key feature of the afferent limb of innate immunity lies in its ability to differentiate self from infectious non-self. The cornerstone of this activity is the interaction between pathogen associated molecular patterns (PAMPs) on microorganisms and pattern recognition receptors (PRRs) on host cells (Janeway, 1992; Medzhitov and Janeway, 1997) (Figure 1).

Typical elements of innate immunity involved in controlling infections are:

Proinflamatory response: nuclear factor-κB (NF-κB) mediated, activates many agents of inflammation, increased cytokine production, overstimulation can result in shock;
Cationic host defense peptides: increased production of peptides stimulated by bacterial pathogen associated molecular patterns (PAMPs) and signaling molecules;
Phagocytic cell activation: increased intracellular killing in neutrophils and macrophages (both oxidative and non-oxidative mechanisms enhanced),
Chemotaxis: increased endothel adhesion of phagocytic cells, cell migration to the site of infection, diapedesis;
Extracellular killing mechanism: complement activation, enhanced iron chelation, antimicrobial peptide secretion, production of degradative enzymes;
Infection containment: clot formation via fibrinogen activation;
Wound repair: fibroblast growth and adherence, angiogenesis;
Adaptive immune responses: B- and T-cell activation, often via dendritic cells.

During infection, a major portion of the pathogen is in contact with the host immune system at sites of colonization. This interaction affects the dynamics of the infection and related pathogenic processes. Innate immunity however has a limited capacity to fend off infections and in such scenario the adaptive immune response takes over.

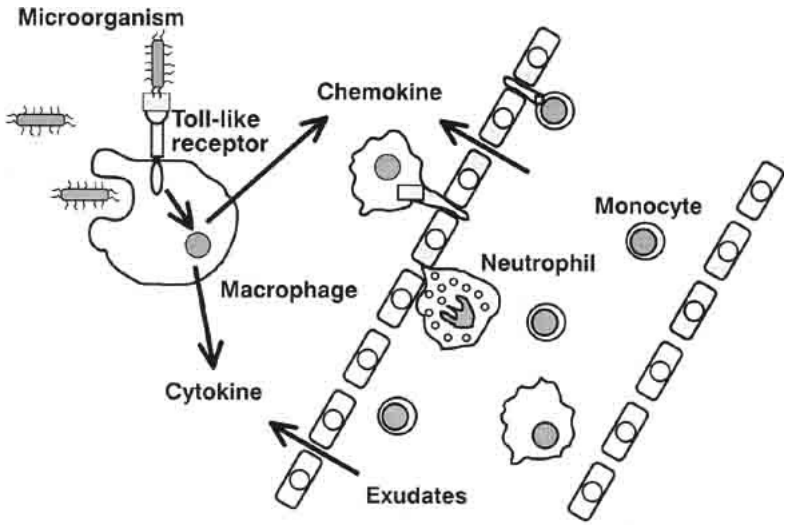


Figure 1. TLRs can activate innate immune cells such as macrophages to produce chemokines or cytokines. TLR activation can lead to production of proinflammatory cytokines (IL-1, IL-6 and TNF-α). These cytokines induce actute phase proteins, chemokines and upregulate expression of adhesion molecules, thereby recruiting inflammatory cells. (Kaisho et al., 2003)

3.1.1. Pathogen Recognition

The interaction of pathogen with the host system is mediated though pathogen pattern recognition receptors (PAMPs). Pattern recognition receptors recognize molecular patterns associated with the surfaces of microbes and apoptotic cells. These receptors act alone and in concert to bind, phagocytose, and transduce cellular signals derived from these molecular patterns. The outcome of these interactions is dependent on the nature of the ligands, and upon the nature and combination of the ligated receptors.

In 1991, Nusslein-Volhard and Wieschaus first described the Toll receptor as a Type 1 trans-membrane receptor that controls dorsal-ventral polarity during embryogenesis in *Drosophila* flies (Stein et al., 1991). Further work revealed that these receptors, which are required for morphogenesis, also control the activity of antimicrobial peptide synthesis in these flies. Toll-deficient flies became exquisitely susceptible to fungal infections but not Gram-negative bacterial infection (Hoffman and Reichhart, 1997). Analogous structures were shown to exist throughout the plant and animal kingdom (Janeway and Medzhitov, 2002). These pattern recognition molecules detect common elements expressed by microbial pathogens and alert the host to the presence of a danger signal within the internal milieu of the host. Their purpose is to sense foreign structural components that might represent an immediate threat and to orchestrate an early, appropriate host defense response. Specific components of prokaryotic cells are immediately recognized generating a specific set of genetically determined synthetic programs (mRNAs for the synthesis of cytokines, enzyme cascades etc.) essential for host survival. It rapidly became evident that humans possess a series of homologous structures now known as the human TLRs (Akira and Hemmi, 2003).

3.1.1.1. Toll-like Receptors

Toll-like receptors are pathogen pattern recognition receptors for micro-organism-derived pathogenic molecules (Akira, 2003). They are the primary sensors of the innate immune system. There are 10 TLRs (TLR 1-10) currently identified (Table 1). Each recognizes one or more specific ligand and performs signal transduction. Newly discovered receptors and receptor interactions are regularly found to be involved in cell activation by bacterial products. Evidence is accumulating that cooperation between TLR receptors comes into play to refine ligand discrimination. Clustering of receptors in lipid rafts has also been found after ligand binding. These large receptor complexes, which are formed among various TLRs and non-TLR moieties, confer a further degree of specificity. The various TLRs or TLR compexes with other receptors, such as CD14 and CD11b/CD18, or dectin-1, will trigger specific intracellular pathways. The signal resulting from the activation of a specific combination of TLRs will induce response best suited for the invading pathogen. Such studies also revealed myeloid differentiation primary response gene 88 (MyD88)-dependent and independent pathways. (O'Neill, 2002a) MyD88 is essential for the stimulation of proinflammatory cytokines such as TNF-α, IL-1β, IL-12, or IL-6, virtually by the entire range of TLR agonists (Wang et al., 2000; Mattson et al., 1993). The protective mechanisms triggered through MyD88 mainly include release of proinflammatory cytokines and of reactive nitrogen and oxygen intermediates (Akira and Hemmi, 2003)

Each TLR is a type-I trans-membrane receptor that has an extracellular, leucine-rich domain and an intracellular portion that contains a conserved region called the Toll/IL-1R homology (TIR) domain, that upon activation results in the recruitment of the MyD88 protein in MyD88-dependent forms of signaling. In response to pathogen binding, the TIR domains recruit adaptor molecules (which also contain TIR domains) to the cytoplasmic side of the activated TLR. This initiates a signaling cascade that eventually leads to the activation of NF-κB,, and other transcription factors, which induce the expression of a wide variety of target proteins including cell-surface proteins and soluble mediators of inflammation (Akira et al., 2001).

A variety of extensive reviews has been published on TLR signaling (Akira et al., 2001; Akira and Hemmi, 2003; Hacker, 2000, O'Neill, 2002; Takeuchi, 2001). Thus, we will only give a short overview on the main events in TLR-mediated signal transduction. Binding of PAMP to a TLR leads to the activation of TIR, forming a signaling complex with MyD88, a cytoplasmic adapter protein, IL-1R-associated kinase (IRAK), and tumor necrosis factor receptor-associated factor 6 (TRAF6). This is followed by activation of the mitogen-activated protein kinase (MAPK) cascade and NF-κB. MyD88 is a universal signaling molecule, as MyD88-deficient cells were found to lack activation of NF-κB and MAPK by all TLR, IL-1 and IL-18. In addition, a MyD88-independent pathway exists for stimulation via TLR-4, as two major biological effects provoked by LPS, cytokine production and co-stimulatory molecule up-regulation, differ in their requirement for MyD88. Since LPS-dependent nuclear translocation of IFN regulatory factor (IRF)-3 is preserved in MyD88-deficient cells (Kawai et al., 2001), IRF-3 activation may contribute to the MyD88-independent pathway. An adapter protein for TLR-4, called TIR domain-containing adapter protein (TIRAP) or MyD88-adapter-like (Mal) associates with TLR-4, but not TLR-9, and seems to be critical for LPS-induced DC maturation (Fitzgerald et al., 2001; Horng et al., 2001). TIRAP/Mal forms homo-or hetero-dimers with MyD88, and associates with IRAK-2, thereby leading to NF-κB activation (Fitzgerald et al., 2001). Although all TLR family members signal via MyD88 and NF-κB, more recent information point to signaling mechanisms unique to each TLR (O'Neill, 2002b).

Table 1. TLRs and their ligands

TLR	**Origin of the ligands**	**Ligands**
TLR1	Gram-positive bacteria	Modulin, lipopeptides
TLR-2	Gram-positive bacteria	Lipoproteins, lipoteichoic acid
	Pseudomonas aeruginosa	Mannuronic acid polimers
	Staphylococcus,	Modulin
	Mycobacterium,	Lipoproteins, lipopeptids, lipoarabinomannan
	Mycoplasma	Lipoproteins, lipopeptides
	Listeria	Heat inactivated bacteria
	Yeasts	Zymozan
	Spirocheta	LPS
TLR-3	Virus	double stranded RNA
TLR-4	Gram-negative bacteria	LPS
	Gram-positive bacteria	Mannuronic acid polymers, lipoteichoic acid
	Plants	Taxol
	Respiratory syntitial virus	F protein
	Host	Hsp60, Hsp70, Fibronectin
TLR-5	Gram-negative bacteria	Flagellin
	Gram-positive bacteria	Flagellin
TLR-6	Gram-positive bacteria	Modulin, tuberculosis factor STF,
TLR-7	virus	Single stranded RNS, Small antiviral compounds
TLR-8	virus	Single stranded RNS, Small antiviral compounds
TLR-9	Bacteria	Non-methylated CpG-DNA

TLR-4 has been the most widely studied of this family of receptors. It is known to recognize lipopolysaccharide from Gram-negative bacteria and lipoteichoic acid from Gram-positive bacteria. TLR-4 also is involved in viral recognition. For example, the F protein of respiratory syncytial virus (RSV) induces pro-inflammatory cytokines by binding to wild type TLR-4 and CD14 (Haynes et al., 2001; Kurt-Jones et al., 2000) whereas mutant C57BL/ 10ScCr mice lacking TLR-4 on their surface were impaired in their ability to eliminate RSV. However, these mice possess mutations not only in TLR-4 but also in IL-12R genes (Poltorak et al., 2001) possibly explaining defective

immunity against RSV. TLR-4 also binds endogenous molecules, such as heat shock protein (Hsp) 60 that induces an inflammatory response in normal mice, but not in C3H/HeJ mice, suggesting the involvement of TLR-4 (Ohashi et al., 2000). Collectively, Hsp are expressed in bacteria as well as in host cells. As Hsp are released from necrotic cells in certain pathological conditions and induce DC maturation (Basu et al., 2000).

TLR-2 binds bacterial lipoproteins/lipopeptides, or mycobacterial components, as well as to lipoarabinomannan, a major cell wall glycolipid derived from *Mycobacterium tuberculosis* (Bulut et al., 2001; Campos et al., 2001; Jones, 2001). In vitro and transfection studies suggested that lipoteichoic acid and, in one study, the Gram-positive organism, Listeria monocytogenes, activate cells via TLR-2 (Flo et al., 2000; Kadowaki et al., 2001). According to some authors, the ability of TLR-2 to bind such a variety of ligands is based on its ability to form heterodimers with other TLR, mainly TLR-6 and TLR1 (Ozinsky et al., 2000; Takeuchi et al., 2001). TLR1 and TLR-6 also bind different lipoproteins and lipopeptides.

The Lps2 mutation identified the role of TIR resistance adaptor protein (TIRAP) in the TLR-3 and TLR-4 MyD88-independent pathway. TIRAP has been discovered as another intracellular player downstream of TLR-2 and TLR-4. A MyD88 independent pathway was also shown to be involved in the regulation of LPS-mediated maturation of DC. TLR-1 and TLR-6 are known to function as the other part of a heterodimer with the TLR-2 receptor.

TLR-3 recognizes double-stranded viral RNA (Alexopoulou et al., 2001). TLR-5 was identified as the receptor for flagellin from Gram negative and positive bacteria, and signaled through MyD88. Curiously TLR5 is not expressed on the apical, but on the basolateral surface in epithelial cells (Gewirtz et al., 2001, Moors et al., 2001). This expression pattern guarantees that invaded, not commensal, organisms can induce inflammatory responses. Thus, TLR5 plays critical roles in mucosal immunity. TLR-7 responds to single stranded RNA and small synthetic immune modifiers such as imiquimod, resiquimod (R-848), bropirimine and loxoribine (Jurk et al., 2002). TLR-9 is known to detect unmethylated bacterial DNA (Chuang et al., 2002). CpG DNA oligonucleotides are currently being investigated for their ability to serve as adjuvant and stimulate human dendritic cells for vaccine development.

TLR-4, TLR-7 and TLR-9 are particularly important with regard to vaccine development as dendritic cells express them. Human TLR-8 was recently identified as a receptor for single stranded RNA and R-848. TLR-7, TLR 8 and TLR-9 have, recently been proposed to be considered as a subgroup in the TLR receptor family, as their ligands are recognized in endosomal/lysosomal compartments (Akira and Hemmi, 2003).

CpG DNA also stimulates immune cells (Krieg, 2000). CpG DNA is largely equivalent to bacterial DNA. Unmethylated CpG-dinucleotide-containing sequences (CpG ODNs) are found much more frequently in bacterial genomes than in vertebrate genomes, whereas the frequency of CpG dinucleotides are suppressed and usually methylated. Methylated CpG ODNs lack immunostimulatory activities. Bacterial DNA and synthetic ODN containing unmethylated CpG-dinucleotide (CpG DNA) stimulate B cell proliferation and activate macrophages and DCs. Genomic DNA from viruses, yeast and insects stimulate mammalian immune cells as well. CpG DNA is known to be an excellent immune adjuvant in various murine disease models and to drive Th1 immune responses. CpG DNA activates the intracytoplasmic signaling molecules such as IRAK, TRAF6, NF-κB and MAPK like other pathogen-derived immunostimulatory components. However, unlike LPS, which can activate TLR-4 at the cell surface, uptake of CpG DNA as well as endosomal maturation is likely to be required for its immunostimulatory activity. Indeed, chloroquine and related compounds that prevent acidification of endosome are shown to inhibit CpG DNA response (Hacker, 2000). Krieg et al. reported that bacterial DNA as well as synthetic

ODN containing a central CpG induces B cell proliferation (Krieg, 2000). They conducted extensive studies to define the DNA sequences with immunostimulatory activities. The most immunostimulatory motif usually has the structure of 5'-purine-purine-CpG-pyrimidine-pyrimidine-3'.

Inversion to GpC or methylation completely abrogates its stimulatory potential, and the regions adjacent to the CpG dinucleotide also affect the immunostimulatory activity. The optimal sequence differs significantly between human and mouse. Mouse cells respond maximally to GACGTT, while for humans the optimal sequence is GTCGTT. Recent studies indicate that immunostimulatory DNAs may exert different immune responses depending on the nucleotide sequence and backbone. There are two major types of immunostimulatory CpG DNAs. One type has an entirely phosphorothioate backbone with CpG dinucleotides. This CpG DNA stimulates B cell proliferation and induces production of IL-6 and IL-12 by monocytes. The other type contains phosphorothioate G-rich sequences at the ends and a phosphodiester palindromic sequences with a CpG dinucleotide in the middle. This CpG DNA preferentially stimulates IFN-γ production by natural killer (NK) cells.

It remains unknown how TLR-9 signaling leads to differential outcomes in response to their ligands. TLR-9 is not expressed on outer cell surfaces, but in endosomes. All DNAs are endocytosed through the pathway not requiring any specific sequences. Only the CpG DNAs trigger TLR-9 signaling in the endosome.

In addition to bacterial DNA, oligodeoxynucleotides (ODN) carrying the CpG motif also stimulate lymphocytes and APC of a variety of species, including ruminants (Brown et al., 1999; Pontarollo, 2002; Rankin et al., 2001; Shoda et al., 2001a,b; Zhang et al., 2001), pigs (Kamstrup et al., 2001), and carnivores (Rankin et al., 2001). This leads to an enhanced antigen presenting activity and maturation of DC, thereby priming antigen-specific Th1 responses (Hartmann et al., 1999; Shirota et al., 2001). Data on the ability to influence the immune response generated in bovine cells using CpG-ODN has been published recently (Brown et al., 1999; Shoda et al., 2001a,b; Stich et al., 1998; Zhang et al., 2001).

Toll like recoptors have been investigated in chickens (Dil and Quersi, 2002). Two types of TLR were cloned from a chicken bursa cDNA library using degenerate primers based on the consensus sequences of mouse and Drosophila Toll and designated as chicken TLR (chTLR) type 1 and type 2. Of the nine human TLRs reported to date, these chTLRs showed the highest homology to human TLR-2. The extracellular regions of type 1 and type 2 contained a distinct approximately 200-amino acid stretch and were 45.3 and 46.3% homologous to that of human TLR-2. The intracellular Toll/interleukin-1R homology domain of type 1 and type 2 was perfectly identical to each other and highly homologous (80.7%) to that of human TLR-2. Both types were widely detected by reverse transcriptase-polymerase chain reaction and immunoblotting in various chicken organs. By reporter gene assay, type 2 and to a lesser extent type 1, selectively signaled the presence of mycoplasma macrophage-activating lipopeptide-2/M161Ag in the human embryonic kidney 293cell system. Co-transfection of type 2 and human CD14 or MD-2 into human embryonic kidney 293 cells allowed the response to *Escherichia coli* lipopolysaccharide, whereas type 1 did not signal LPS or any other microbial components tested. These results indicated that chTLR type 2 covers two major microbe patterns, lipoproteins and LPS, which are regulated by TLR-2 and TLR-4 in mammals (Fukui et al., 2001).

3.1.1.1.1. Interaction of Mycoplasmas with TLRs

Mycoplasmas are known to enhance viral infections. For example, *M. hyopneumoniae* increases the potentiation of porcine reproductive and respiratory syndrome virus (PRRSV) induced pneumonia in dual infected pigs (Thanawongnuwech et al., 2004). In humans, mycoplasmas are known to

enhance human immunodeficiency virus (HIV) replication. This mechanism of activation has been linked to the interaction of mycoplasma with TLRs. Lipid associated membrane proteins (LAMPs) from different mycoplasmas have been shown to interact with TLR1, TLR-2 and TLR-6. For example, purified lipoprotein from *M. penetrans* was able to activate NF-κB through TLR1 and TLR-2, whereas the activation of NF-κB by purified lipoproteins of *M. fermentans* was mediated through TLR-2 and TLR-6. Lipid associated membrane proteins of these mycoplasmas has been shown to activate the long-terminal repeats of immunodeficiency virus through the activation of nuclear NF-κB via TLR1, 2 and 6 (Shimizu et al., 2004).

Mycoplasmas cause chronic inflammation and may able to escape host defense. Hijacking of the TLR signaling by certain pathogens to evade the recognition and elimination by the immune system has been described recently. Several studies to date suggest that TLR-2-dependent mechanism contribute to the evasion or inhibition of the immune response. TLR-2-induced signals in DC preferentially induce a Th2 cytokine pattern (Sundstrom, 2004), which is known to have down-modulatory activity on cellular immunity.

It has been postulated by Wills-Karp et al. (2001) that microbes initially provide the immune system with signals to mature, and later provides signals that instruct regulation of these responses. Based on this model, during chronic infection the continuous microbial stimulation induces the production of regulatory cytokines, such as IL-10 and transforming growth factor β that not only down-regulate Th2 but also Th1 immune responses.

3.1.1.2. Other Pattern Recognition Receptors

Much attention has been focused on the TLR family of trans-membrane signal transducers, whose signal transduction pathways have been dissected in great details (Takeda et al., 2002). A number of other host cell molecules are able to recognize microbial PAMPs, and thus serve as PRRs. In many cases, the recognition abilities of these PRRs have been described in detail, whereas their signal transduction capabilities remain uncertain (Gordon, 2002). Some may indeed even cooperate with TLRs in PAMP recognition or use TLRs as signaling partners: these receptors can, therefore, not truly be termed 'TLR independent'. Some of these PRRs may also mediate anti-inflammatory responses. Many of the PRRs described to date are cell surface receptors expressed by macrophages and dendritic cells – key cellular components of the innate immune system. These cells are strategically located at potential portals of entry for pathogens, and an important component of their armamentarium is their expression of multiple PRRs. They are thus endowed with the capacity to function as an early warning system. Many of these receptors are known to ligate endogenous ligands in addition to their activities in microbial recognition.

Some microbial pathogens are endocytosed and exert their activity directly in the cytoplasm, using the leucine-rich domain of Nod, **Nod-1** and **–Nod-2**, as intracellular Gram-negative bacterial peptidoglycan sensing. However, these various pathways seem to converge towards the nuclear translocation of NF-κB and activation of inflammatory genes and production of cytokines (Althman and Philpott, 2004).

Mindin, an extracellular matrix protein is also a mediator of inflammatory response to several bacterial surface components. Recent studies suggest that innate immunity involves factors independent of TLR signaling, such as mindin and that production of NF-κB or IL-1 are not required to controlling infections (He, 2004).

Table 2. Pathogen Pattern Recognition Receptors

Receptor	**Ligand** (innate immune response)
TLRs 1-10	LPS, flagellin, CpG DNA, dsRNA, etc.
CD14	LPS, peptidoglycan
Complement receptor 3 (CD11b-CD18)	iC3b opsonized particles
Complement receptor 2 (CD21)	iC3b opsonized particles
FcR	"Natural antibodies"
Scavenger Receptor AI/II	LPS, lipoteichoic acid, bacteria
MARCO	bacteria
Integrin $\alpha_x\beta_1$	fibronectin
Dectin 1	β-glycan, yeast, zymosan
Mannose receptor	mannan
DC-SIGN	ManLAM

Other non-TLR pattern recognition receptor molecules include the **scavenger receptors** (Kraal et al., 2000). Chemically modified lipids and lipoproteins are the best described ligands for the class A scavenger receptors (SR-As), these receptors are also capable of interacting with microorganisms as well as apoptotic cells.

The SR-As family member, macrophage scavenger receptor 11 (MARCO11) has bacterial binding properties, but its exact role in host defense remains speculative (Kraal et al., 2000). The class B scavenger receptors (SR-Bs) such as CD36 and related molecules also possess both lipid metabolism and host defense roles (Krieger, 2001). The molecular pattern recognized by scavenger receptors is apparent on the surface of a number of microorganisms. This particular PAMP–PRR interaction appears to result in binding and phagocytosis, but is not clearly linked to a pro-inflammatory response, and does not have a defined signal-transduction pathway.

Multiple PRRs have been implicated in the recognition of unopsonized yeast particles and their components. Previous work has implicated the leukocyte integrin CR3, an unnamed multi-subunit β-glucan receptor (Szabo et al., 1995) and C-type lectin-containing proteins (Stahl and Ezekowitz, 1998) in this process. More recent work, however, has led to the description of a novel β-glucan receptor, termed Dectin-1 that appears to be the major non-opsonic receptor involved in macrophage recognition of yeasts (Brown and Gordon, 2001; Brown et al., 2002). This receptor contains a single extracellular C-type carbohydrate recognition domain (CRD), linked by a transmembrane domain to an intracellular tail containing an immunoreceptor tyrosine-based activation motif (ITAM) (Brown et al., 2003). Whereas Dectin-1 alone is sufficient to mediate phagocytosis and oxidative burst activities, activation of NF-κB and the production of IL-12 and TNF-α require signals from both Dectin-1 and TLR-2 (Brown et al., 2003). It is anticipated that other PRRs may demonstrate similar interactions with TLR molecules.

The **mannose binding lectin** (MBL) is a member of the collectin family of secreted proteins: multimeric structures composed of monomers with C-terminal lectin domains and N-terminal collagen tails. In the case of MBL, 32-kDa monomers assemble as trimers. These trimers then oligomerize further to give rise to hexamers of trimers, thought to be the circulating form of MBL. The MBL lectin or CRD recognizes a micropattern of spatially oriented hydroxyl groups that is present in mannose, fucose, N-acetylglucosamine, and glucose, but not in galactose or sialic acid. Whereas the affinity of individual monomeric CRDs for these ligands is rather low, the multimeric

form of MBL binds with significantly greater avidity to complex ligands bearing repeating subunits of the cognate micropattern. Structural studies have indicated that the spatial conformation of the repeating micropatterns (i.e. a higher-order macro pattern) is required to achieve high avidity binding of MBL (Weis et al., 1992; Sheriff et al., 1994). This macro-pattern or spatial geometry of ligands is found predominantly in microorganisms rather than in mammalian glycoproteins, thus conferring a degree of non-self recognition ability to this pattern recognition molecule. The ability of MBL to bind a variety of bacterial, viral, fungal and protozoal organisms has been well documented (Epstein et al., 1996; Jack et al., 2001). MBL is also able to bind some (but not all) forms of LPS. This latter interaction appears to be complex, as the binding cannot be predicted by the LPS terminal sugar residues, suggesting that spatial conformation within the LPS structure is a critical determinant of MBL binding (Devyatyarova et al., 2000).

The effector functions of MBL include activation of complement and opsonization of target cells or microbes. Indeed the lectin pathway via which MBL activates the complement cascade is an evolutionarily ancient mechanism that is distinct from the classical and alternative activation pathways. This activation is mediated by MBL-associated serine proteases (MASPs), of which MASP2 appears to predominate in activating the lectin pathway (Matsushita et al., 1998). The ability of MBL to facilitate opsono-phagocytosis has been highlighted in recent studies suggesting that apoptotic cells might display a molecular pattern that renders these cells competent to bind MBL (Ogden et al., 2001).

In vitro analysis of macrophage cytokine secretion indicated a diminished pro-inflammatory cytokine response in the MBL-A-deficient mice, suggesting that MBL may modulate cytokine production, and that MBL-associated responses may, under certain conditions, be deleterious to the host (Takahashi et al., 2002). A number of studies have linked low levels of MBL with an increased susceptibility to infection (Summerfield et al., 1995; Summerfield et al., 1997, Garred et al., 1999; Roy et al., 2002). On the other hand, epidemiological studies have indicated a protective role for low MBL levels in infections caused by intracellular pathogens, (Santos et al., 2001; Hoal van Helden et al., 1999) suggesting that intracellular pathogens may benefit from host MBL. This functional duality makes MBL the Jekyll and Hyde of innate immunity (Ezekowitz, 1998).

3.1.1.3. Pathogen Recognition Receptors associated with anti-inflammatory outcomes

Unlike TLRs, other PRRs are frequently associated with anti-inflammatory outcomes, often used to the advantage of the pathogen to overcome the immune system and evade conventional defense mechanisms. The mechanism by which the anti-inflammatory outcome is signaled is unique to each receptor (Mosser and Karp, 1999). Anti-inflammatory cytokine production and autocrine negative feedback result from ligation of certain receptors or after encountering specific pathogens. These receptors typically stimulate release of anti-inflammatory cytokines such as IL-10 and TGF-β (transforming growth factor). Some pathogens interact with the vitronectin receptor avb3 to induce TGF-β, which in turn facilitates their own intracellular growth. (Freiredelima et al., 2000) Similarly, DC-SIGN recognizes mannose residues on some pathogens (Mosser et al., 1992; Tailleux et al., 2003) inducing production of IL-10. This allows these pathogens to actively suppress host immune defenses. These examples demonstrate that ligation of non-TLR PRRs may induce anti-inflammatory cytokine production acting to the advantage of the pathogen and disadvantage of the host.

Recent studies suggest that pathogenic microorganisms can also modulate or interfere with TLR mediated pattern recognition and can use TLRs as an escape mechanism from host defense (Netea et al. 2004). Three major TLR-mediated escape mechanisms have been identified: TLR-2-induced immune suppression, especially through induction of IL-10 release, blockade of TLR recognition and TLR-mediated induction of viral replication (Sing et al., 2002).

Other pathogens and PAMPs recognize or ligate receptors whose intracellular immunoreceptor tyrosine-based inhibition motif (ITIM) directly inhibit host cells. Increasing numbers of such ITIM-bearing receptors are being identified on innate immune cells including macrophages and dendritic cells and it is likely that many pathogens will have evolved to utilize these PRRs. Other PRRs are G protein coupled and, via cAMP, are able to inhibit cytokine production (Braun and Kelsall, 2001). IL-12 is an important immuno-regulatory cytokine required for induction of Th1 responses and which appears to be particularly sensitive to this mechanism of inhibition. Receptors that are linked to such an inhibitory pathway include complement receptors, CD46 (Fugier-Vivier et al., 1997) and others. Some of these recognition molecules, particularly those mediating recognition of apoptotic cells, appear to be not only anti-inflammatory but also capable of actively blocking TLR-induced cytokine production (Voll et al., 1997; Fadok et al., 1998). This latter observation suggests a possible hierarchy of responses to different pattern recognition molecules and emphasizes the possibility for combinatorial variation resulting from co-ligation of multiple receptors.

3.2. Dendritic Cells Connecting the Innate and the Adaptive Immune Response

When the innate host defense mechanisms fail to eliminate the pathogenic microorganisms during the first days of an infection, the host will mount an additional immune response adapted specifically to the particular invading bacteria. This adaptive response is mediated by clonal expansion of B-cell and T-cell populations able to interact specifically with the particular microorganisms. This process is mediated by the presentation of pathogen derived antigenic peptides by antigen-presenting cells (APC) to the T helper cells. DCs are the most effective APC (Figure 2).

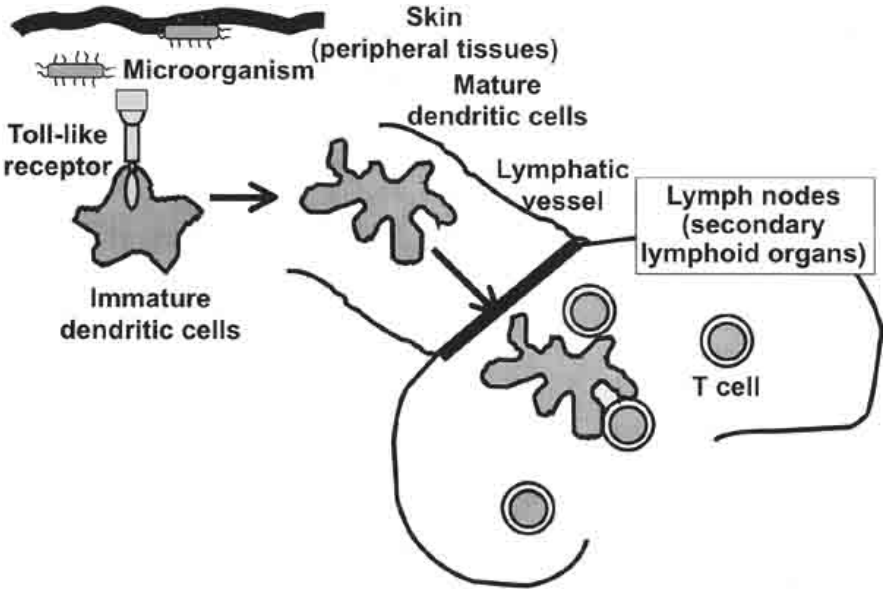


Figure 2. TLR-stimulated DCs migrate from peripheral tissues into secondary lymphoid organs. TLR signaling stimulates DC activation and maturation. Mature DCs loose the ability to endocytose and alter their chemokine receptors. Mature DCs leave peripheral tissues and migrate into lymphoid organs, where they interact with T cells. (Kaisho et al., 2003)

Recently it has been recognized, that dendritic cells are essential to the initiation of adaptive immunity and this knowledge allowed immunologists to design optimized vaccination strategies against poorly immunogenic antigens (Hsu, 1996). DCs originate from precursors of both the myeloid and lymphoid lineages, but are unique for being antigen-presenting cells. DCs are present in every tissue, and during an infection are the first immune cells that enter into contact with the invading pathogen. They are the bridge between the innate and the adaptive immune response. Immature DC express pattern recognition receptors (TLR receptors and lectin domain scavenger

receptors) that bind conserved molecular structures shared by groups of pathogens (Shortman, 2002). Upon activation, immature antigen-capturing DCs differentiate into mature antigen-presenting DCs, able to present antigen in the MHC class-II and class-I contexts, as well as up-regulate the expression of surface co-stimulatory molecules such as CD80 and CD86.

Mature and activated DC migrates to secondary lymphoid organs (lymph nodes, spleen, Peyer's patches), where they translocate to the T-cell areas. Migration of DC to the secondary lymphoid organs is essential to the development of an immune response. The migration of DC and their interaction with and stimulation of T-cells is dependent on cytokines, chemokines and adhesion molecules such as intercellular cell adhesion molecules (ICAMs), Leukocyte function associated molecule 1 (LFA-1) and DC-SIGN (Bleijs, 2001).

3.2.1. DC-SIGN

Several receptors expressed by immature DCs belong to the C-type lectin superfamily, including Langerin (CD207), the mannose receptor (MR; CD206), and DEC-205 (CD205, dendritic cell receptor for endocytosis) (Mitchell, et al., 2001). C-type lectins are characterized by a CRD that interact with proteins with either mannose or galactose side chains in a calcium-dependent manner (Mitchell, et al., 2001). The C-type lectins on DCs have a mannose-type specificity, and binding of mannosylated ligands can be blocked by mannan. However, the number of CRDs present in these lectins differs and the complexity of the mannose groups that they recognize is distinct (Mitchell, et al., 2001).

Recently, a novel C-type lectin has been identified, DC-specific ICAM-grabbing non-integrin (DC-SIGN; CD209), that has a single CRD with mannose-type specificity and is exclusively expressed on DCs, in contrast to the MR and DEC-205, which are also expressed on other cell types (Geijtenbeek et al., 2000a). DC-SIGN functions as cell adhesion receptor mediating both DC migration and T cell activation. Internalization motifs in the cytoplasmic tail of the DC-SIGN indicate a function of DC-SIGN as endocytic receptor. On DCs, DC-SIGN is rapidly internalized upon binding of soluble ligand. Detailed analysis using fluorescence imaging and electron microscopy showed that DC-SIGN-ligand complexes are targeted to late endosomes/lysosomes. Ligands, internalized by DC-SIGN, are efficiently processed and presented to CD4+ T cells. The distinct pattern of expression of C-type lectins on DCs in situ and their non-overlapping antigen recognition profile hints to selective functions of these receptors to allow a DC to recognize a wide variety of antigens and to process these to induce T cell activation.

A common feature of the specific pathogens that interact with DC-SIGN, such as mycobacteria, Leishmania and Helicobacter, is that they cause chronic infections that may last a lifetime, and secondly, that manipulation of theTh1/Th2 balance by these pathogens is central to their persistence. The interaction between these pathogens and DC-SIGN may greatly influence antigen presentation, as well as cytokine secretion by DC, and may thereby contribute to their persistence. For infection with *M. tuberculosis* it has already been demonstrated that the mannose-capped lipoarabinomannan-DC (ManLAM-DC) interaction reduces IL-12 production by DC and shifts the immune response toward Th2, which promotes immune evasion and persistence (Nigou et al., 2001). Likewise, a Th1 to Th2 shift, associated with a decrease in IL-12 concentrations, is crucial to virulence and persistence of *Leishmania mexicana*. Also for Schistosoma mansoni, a Th2 immune response is associated with persistence of the pathogen. soluble egg antigen (SEA) and its major glycan antigen Le^x are able to cause a switch towards a Th2-mediated immuneresponse (Okano et al., 1999). Therefore, these pathogens could have evolved to target DC-SIGN not only to infect DC but also to shift the Th1/Th2 balance in favor of persistence.

Recently, Mitchell et al. (2001) demonstrated that DC-SIGN preferentially recognizes high-mannose oligosaccharides. Thus, DC-SIGN and the MR recognize different mannose moieties,

whereas the MR has a high affinity for antigens with single mannose groups, and DC-SIGN binds more complex mannose residues.

Despite similarities of C-type lectins on DCs and possible redundancy, the specificity for ligands can differ between these lectins. The complexity of mannose structures recognized, the number of mannose groups per ligand, their branching and spacing on the ligand, as well as additional interactions other than carbohydrates may especially differ. Recently, Mitchell et al. (2001) demonstrated that DC-SIGN preferentially recognizes high-mannose oligosaccharides. In eukaryotes, cell membrane-bound mannose residues are predominantly present in complex-type N-linked glycoproteins and probably also on viruses, such as HIV. This is in contrast to single terminal-situated mannose residues that are not recognized by DC-SIGN but are bound by mannose-binding protein and the MR. DC-SIGN has a much higher affinity for ICAM-3 compared with mannosylated BSA illustrates that whereas DC-SIGN recognizes complex mannose residues in specific arrangements on the surfaces of select glycoproteins, the MR recognizes end-standing single mannoses often present on microorganisms. Instead of being complementary receptors, C-type lectins are functionally distinct on DCs and have distinct recognition profiles to bind specific ligands and pathogens with high affinity. Comparison of distribution of DC-SIGN expression with that of Langerin, DEC-205, and the MR revealed that only DC-SIGN and the MR, which have distinct antigen recognition profiles, are expressed on DCs at the same places in the body, whereas the other C-type lectins are differentially expressed on subsets of DCs.

3.2.1.1. DC-SIGN is a pathogen receptor with broad specificity

The identification of novel carbohydrate structures recognized by DC-SIGN lead to a more detailed analysis of the binding of DC-SIGN to human pathogens that express mannose or fucose-containing glycans. The gram-negative bacterium *Helicobacter pylori*, which induces peptic ulcers and gastric carcinoma (Appelmelk et al., 2000), and the worm parasite *Schistosoma mansoni* (the causal agent of schistosomiasis) both express Le^x (Srivatsan et al., 1992). In *H. pylori*, Le^x is present on surface-located lipopolysaccharide, while in S. mansoni Le^x is expressed at all stages of the parasite, including SEA (Srivatsan et al., 1992). Indeed, both Le^x-positive pathogen structures, *H. pylori* LPS and *S. mansoni* SEA,were strongly bound by DC-SIGN expressed by transfectants and the binding was completely inhibited by anti-DC-SIGN antibodies (Appelmelk et al., 2004). Moreover, whole *H. pylori* bacteria were also specifically bound by DC-SIGN (Appelmelk et al., 2004). Investigation of mannose-containing pathogens demonstrated that the mannose-capped surface lipo-phosphoglycan (LPG) expressed by *Leishmania mexicana*, a unicellular parasite that causes leishmaniasis (Appelmelk et al., 2004), and *Mycobacterium tuberculosis* antigens also interact with DC-SIGN-Fc, whereas no binding of DC-SIGN to three clinically relevant Gram-negative bacterial human pathogens (*Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa*) or to Gram-positive *Staphylococcus aureus* was observed (Appelmelk et al., 2004). These findings indicate that binding of DC-SIGN to pathogens is selective, and that the carbohydrate specificity of DC-SIGN governs a broader pathogen recognition than HIV-1, Ebola and CMV (Alvarez et al., 2002, Colmenares et al., 2002, Geijtenbeek et al., 2000b).

3.2.1.2. Mannosylated lipid binding to DC-SIGN suppresses DC function

Lipoarabinomannan (LAM) glycolipids are present in the mycobacterial cell wall, but are also secreted from phagosomes following macrophage ingestion of *M. tuberculosis* (Chatterjee and Khoo, 1998, Xu et al., 1994, Sturgill-Koszycki et al., 1994). The presence of anti-LAM antibodies in sera of tuberculosis patients suggests that LAM is released in vivo (Chatterjee and Khoo, 1998). Thus, mycobacteria within macrophages can affect bystander immune cells and modulate the immune response. DCs are critical in that they mediate cellular immune responses against mycobacteria. It has been demonstrated that secreted ManLAM targets DC-SIGN on DC to suppress DC functions (Geijtenbeek et al., 2003c). Triggering of TLR on DC induces DC maturation, resulting in release of cytokines and up-regulation of accessory molecules for efficient

stimulation of T lymphocytes (Kadowaki et al., 2001, Jarossay et al., 2001). DC maturation by LPS is mediated through TLR-4, which generates intracellular signaling via the transcription factor Nf-kB (Akira et al., 2001). Mycobacteria such as *M. bovis* BCG also induce DC maturation (Tsuji et al., 2000) and *M. bovis* BCG can mediate the observed maturation through TR2 and TLR-4 signaling (Tsuji et al., 2000). Strikingly, both *M. bovis* BCG- and LPS-induced maturation of DC was specifically blocked by ManLAM but not by AraLAM (Geijtenbeek et al., 2003a). This inhibition by ManLAM is mediated through DC-SIGN, since antibodies against DC-SIGN abrogated this effect and can completely restore strong DC maturation by both *M. bovis* BCG and LPS. These results suggest that DC-SIGN, upon binding ManLAM, delivers a signal that interferes with the *M. bovis* BCG-induced signals presumably generated by TLR-4. These results suggest that pathogen binding to DC-SIGN may mediate intracellular signaling. Moreover, ManLAM binding to DC-SIGN resulted in an induction of IL-10 by LPS-acti-vated DC (Geijtenbeek et al., 2003b). The ManLAM-induced production of IL-10 could contribute to the virulence of mycobacteria, since IL-10 impairs the ability of DC to generate Th1 responses by blocking up-regulation of co-stimulatory molecules and IL-12 production (Redpath et al., 2001). Moreover, *M. bovis* BCG-infected DC produced high levels of IL10, demonstrating that mycobacteria induce IL-10 both through direct infection and by influencing bystander DC by ManLAM secretion (Geijtenbeek et al., 2003c). Recently, it was demonstrated that ManLAM inhibits the IL-12 production by LPS-matured DC (Nigou et al., 2001). The authors suggested that MR is involved in ManLAM binding, but DC-SIGN may also be involved in this pathway (Geijtenbeek et al., 2003a). Nigou et al. hypothesized that pathogen receptors could interfere with TLR signaling upon pathogen recognition, modulating the cellular immune responses against pathogens (Nigou et al., 2001). The data from Geijtenbeek et al. (2003b) further support this hypothesis. Thus, shifting the balance between TLR and C-type lectin signaling may be a general principle by which pathogens suppress the immune response (Engering et al., 2002).

In humans, *M. tuberculosis* may target DC-SIGN to suppress cellular immune responses, since both immature DC and IL-10–treated DC are not only less efficient at stimulating T cell responses but can also induce a state of antigen-specific tolerance (Jonuleit et al., 2000, Steinbrink et al., 1999). The results obtained with the mildly virulent *M. bovis* BCG strain indicate that the mechanism of immunosuppression may not directly contribute to the virulence and persistence of virulent *M. tuberculosis* strains as compared to less virulent strains. However, differences between the interaction of DC-SIGN with virulent and avirulent mycobacteria strains will have to be investigated in more detail, in order to determine whether some strains are more efficient in targeting DC-SIGN and thus suppressing immune responses than others.

3.2.2. Toll-like receptors and T-helper-1 responses

Depending on the local cytokine environment and the antigen, cellular T-helper (Th1) and humoral antibody mediated Th2-oriented immune responses are triggered to various degrees (Shortman, 2002). TLR activation induces the maturation of APCs with increased cell surface antigen presentation and expression of CD80/CD86. CD80 and CD86 are members of the B7 family that specially interact with CD28 on T cells, and in so doing they provide a critical co-stimulatory signal that is required for the activation and differentiation of antigenically naıve T cells, particularly those of the CD4+T lineage. This co-stimulation also increases APC activation and maturation in an indirect manner, in which CD40-ligand (CD154; which is expressed on activated CD4+ T cells) in turn binds to CD40 on the APC, resulting in the production by APCs of cytokines and increases in B7 expression. These APCs, mainly dendritic cells, acquire the ability to activate antigenically naıve CD4+T cells and induce their differentiation into either Th1 or Th2 cells, depending on the cytokine milieu and other poorly understood factors.

Adaptive immune responses of the Th1 type are driven by proinflamatory cytokines, such as IL-12, IL-23, and IL-27, and are characterized by production of IFN-γ and generation of IgG2a isotype

antibodies. In contrast, Th2-type responses are driven by anti-inflamatory cytokines, such as IL-4 and other poorly understood factors, and are associated with IL-4, IL-5, and IL-13 production and antibodies of the IgE isotype and the IgG1 subclass of IgG. TLR ligands generally induce APCs to produce IL-12, and this is believed to constitute the main mechanism by which they favor Th1-type adaptive immune responses.

This global view is strongly supported by studies conducted in MyD88-deficient mice. MyD88 is an intracellular adapter for signaling that is downstream of all TLRs, and mice that are deficient in this adapter can be viewed as being deficient in TLR signaling (Schnare et al., 2001). However, MyD88 gene disruption also impacts on signaling by the IL-1 family members (O'Neill et al., 2002a). In addition, other adapter molecules in this signaling pathway exist (such as TIRAP, see below), as does a MyD88-independent signaling pathway for certain TLRs (e.g. induction of B7 co-stimulatory molecule expression in response to TLR-4 activation) (Yamamoto, 2002a). In addition to showing impaired APC responses to several TLR agonists, MyD88-deficient mice showed an inability to generate Th1-type immune responses to antigen in the presence of complete Freund's adjuvant (containing heat-killed *Mycobacterium tuberculosis*, components of which can activate TLRs such as TLR-2 and TLR-4). In contrast, Th2 responses (in the presence of alum as the adjuvant, which induces predominantly Th2 responses) were completely preserved.

These findings were confirmed and extended to the context of microbial infection in a study conducted by Jankovic et al. (2002). In MyD88-deficient mice, those investigators demonstrated that interferon-γ-producing CD4+T cells failed to develop in response to a microbial antigen inoculation. The Th1 CD4+T cell development in response to the microbial antigens was unaffected by the absence of IL-12 (using IL-12 deficient mice); IL-12 is traditionally believed to be the master factor that regulates the initiation of Th1 responses, although other cytokines, such as IL-23 and IL-27, may also play a similar role (Robinson and O'Garra, 2002). In contrast, MyD88 deficiency resulted in a CD4+T cell response that defaulted to a Th2 type. These results suggest that MyD88 signaling is involved in the initial commitment of naive CD4+T cells to differentiate into Th1 or Th2 lineage cells. Thus, microbial interaction with TLRs constitutes a critical early checkpoint in CD4+T cell differentiation, and points to a crucial role for TLRs in the qualitative determination of adaptive immune responses. TIRAP (also known as MAL) is another adapter that was recently shown to be important for TLR-2 and TLR-4 responses.

The involvement of TLR signaling pathways in Th1-type responses was also demonstrated using receptor interacting protein (Rip)2-deficient mice (Kobayashi et al., 2002; Chin et al., 2002). Rip2 (also termed RICK, CARDIAK, CCK, and Ripk2) is a serine/threonine kinase that is involved in TNF-α-induced nuclear factor-κB activation. Rip2 was demonstrated to become associated with TLR-2 transiently in response to peptidoglycan stimulation, and to be important for TLR-induced cytokine production. Immunized Rip2-deficient mice produced less IgG2a and IFN-γ in ex-vivo assays than did wild-type mice. In addition, Rip2-deficient CD4+T cells displayed impaired proliferation in response to T cell receptor engagement, and when differentiated in vitro into Th1 cells produced lesser amounts of IFN-γ. These results demonstrate that, similar to MyD88, Rip2 is necessary for the development of Th1-type responses. However, it is unclear in this in vivo experimental system whether Rip2 is required for optimal Th1 responses as part of TLR signaling in cells such as APCs, or as part of the T cell receptor-signaling pathway in T cells.

TLRs may also potentially regulate T cell immunity directly. Human CD4+T cells express a variety of TLRs (Muzio et al., 2000; Hornung et al., 2002; Zarember and Godowski, 2002), with TLR1, TLR-2, TLR3, TLR5, TLR-9, and TLR-10 being the predominant forms, and this probably also applies to mice. Although TLR expression in CD4+ T cells is generally lower than in monocytes, TLR3 and TLR-9 were expressed to similar degrees in both cell types. Furthermore, MyD88 was equally expressed at high levels in CD4+ and CD8+ T cells, B cells, and monocytes. Therefore, it is

plausible that TLR signaling pathways may play a direct role in CD4+ T cell activation and differentiation, in addition to their well-characterized effects on APCs. The strongest evidence for this comes from studies conducted with CpG oligodeoxynucleotides and human T cells. It has long been known that bacterial DNA and DNA containing unmethylated CpG motifs act as adjuvants, stimulate Th1 responses, and induce/exacerbate pathologies in disease models that are Th1-dependent (such as murine experimental autoimmune encephalomyelitis and colitis) (Ichikawa et al., 2002; Obermeier et al., 2002). These effects are widely believed to be mediated by TLR-9 (Hemmi, 2000). CpG DNA was found to act directly as a co-stimulator for T cell activation and increased IFN-γ production in response to antigenic stimulation (Iho et al., 1999; Bendigs et al., 1999) in the absence of APCs. Although the role of TLR-9 in those studies was not demonstrated, it is highly likely that this is the molecule mediating these Th1-enhancing effects of CpG DNA.

In addition to TLR signaling pathway molecules, TLRs themselves have also been shown to regulate adaptive Th1-type immune responses. Most studies reporting these findings suggest that this is achieved by enhancing antigen presentation. Bacterial lipoproteins are activators of TLR-2 (in combination with TLR1), and their synthetic derivatives are increasingly used as vaccine adjuvants. In a quest to elucidate the mechanism of action, human peripheral blood mononuclear cells were stimulated with synthetic lipopeptides, and T cell proliferation as well as IFN-γ (but not IL-4) production was demonstrated (Sieling et al., 2003). Antibody neutralization demonstrated that these stimulatory effects were mediated via TLR-2 and not TLR-4, and only required T cells and monocytes.

The dose of antigen has also been shown to direct the Th1/Th2 differentiation, with high doses stimulating preferentially the Th1 response and low doses the Th2 response (Boonstra, 2003). The delineation of how APC process antigens in the immune system during an infection has influenced the design of vaccines. Carrier devices displaying antigenic proteins and DNA vaccines have been shown to be taken up by immature dendritic cells and lead to an efficient immune response in the control of pathogenic microorganisms (Beyer, 2001). DC therefore represents a main target of development for adjuvant and delivery systems in vaccines.

Mucosal DC specifically provide an important first-line of defense by ingesting foreign invaders *via* both pinocytosis and receptor-mediated endocytosis. DCs play a critical role in mucosal immunity as bodily mucosa act like a barrier between the inside and the outside of the body. DC can be found on the lining of the respiratory tract and of the gut. Langerhans' cells are a population of DC found in the skin and mucosa. M cells transport antigens to the underlying lymphoid follicle that is the immuno-inductive site of the gut. Similar nasal and bronchus associated lymphoid tissues have been described in the respiratory tract (Yuki, 2003). This system is important in the gastrointestinal tract (Jones, 1994), but in the airways, the underlying DC network may be even more important (Pabst, 1990). The mucosal immune system, particularly the oral and gut immune system, has developed a way to discriminate between highly pathogenic substances and exogenously administered antigens that are poorly immunogenic.

Although much has been learned about DC, their precursors and various DC subtypes have been proposed, the full degree of functional complexity and plasticity of DC renders difficult predictions about the effect of a specific immunomodulator on DC and subsequent Th1 and Th2 responses. However, some of the results obtained for DCs matured *in vitro* might be extrapolated to mature DCs isolated from the lymphoid organs since they display similar characteristics (Shortman, 2002).

DCs express different sets of TLRs. TLR-4 may be required for the induction of an optimal Th2 responses to antigen of non-pathogenic sources, suggesting a role for TLR ligands, such as LPS derived from commensal bacteria or endogenously derived ligands, in maturation of the innate immune system before pathogen exposure (Dabbagh et al., 2002). In humans, but not mice, TLR-9

is selectively expressed on plasmacytoid DC, but not on myeloid DC (Jarrossay et al., 2001; Sparwasser et al., 1998, 2000), yet its expression is not restricted to DC. Taken together, these observations show that TLR are crucial not only in the early phase of infection when innate immunity is important, but also links innate and adaptive immunity throughout the entire course of the host defense response.

The potential of the mycoplasma lipopeptide MALP-2 to modulate DC via TLR-2 for clinical application has been studied *in vitro* (Weigt, 2003). MALP-2 treatment of DC induced the expression of CD80, CD86 and the release of bioactive TNF-α and IL-10, as well as the proliferation of autologous lymphocytes and the production of IL-4, IL-5 and IFN-γ by the latter. These features correlate with an ability to stimulate T-cells and therefore suggest a possible effect of MALP-2 on DC *in vivo*.

3.3. Particle-based carrier systems

When antigens applied orally, they must pass un-degraded through the stomach and the upper intestines. Such degradation is unlikely to occur through a nasal or genital route of administration. Subsequently, the antigen must be taken up through the intestinal epithelium, so the antigenic epitopes associated with the pathogenicity of the microorganism can be adsorbed and subsequently presented to the immune-competent cells by the antigen-presenting cells. The immune competent cells are located in the epithelium, the lamina propria or beneath the basal membrane. Therefore, the components must be formulated with carriers taking them through this barrier. When bound to particulate carriers, it is generally accepted that the antigens can be transported over the barrier by the M-cells in the Peyer's patches (PP) (Kraehenbuhl, 2000).

Several different particle-based systems have been used experimentally as carrier for different antigens (Ermak, 1998). Chitosan, poly-DL-lactic acid, or polyacryl starch micro particles have previously been described as a drug carrier system (Laakso, 1986). In one study, it was observed that the starch micro-particles with covalently bound human serum albumin (HSA), as a model antigen, functioned as a strong adjuvant in mice, when administered parenterally, however, the micro particles alone were not immunogenic (Degling, 1995).

Premature antigen breakdown or release has hampered the development of particle-based drug carrier technologies. This is the likely explanation why in the published literature a high dose of antigen is still required to achieve comparable responses to the injected counterpart. This makes them uneconomical to use and therefore the utility particle carriers as adjuvant has been questioned (Brayden, 2001). Besides the poor utilization of antigens, the other main criticism refers to the poor capacity of M-cells in the Peyer's patches to transport particles and the insufficient immune response induced in humans. The epithelial M-cells of the PP are known to allow the transport of certain bacteria, viruses and protozoa from the intestines (Neutra et al., 1992). Several studies have shown that a size-dependent uptake with a maximal diameter of 10µm can occur by the M-cells and Caco-2 cells (Ebel, 1990). It should be pointed out, though, that the uptake through the PP is most likely dependent on the structure and the possible adhesive properties of the carrier adjuvant, too. Natural polysaccharides have intrinsic mucoadhesive properties, which may improve their interaction with different mucosal membranes and facilitate uptake.

Recent information on the uptake of particles by M-cells and the different types of dendritic cells present in the PPs and their vicinity may provide an understanding of the mechanisms involved. Beier (Beier, 1998) followed the uptake and kinetics of baker's yeast cells (*Saccharomyces cerevisiae*) into PP, assuming it as an inert model for transport through the mucosa. A typical time dependency compatible with the transport to different types of phagocytosing, antigen-processing macrophages or dendritic cells was found for the distribution of the yeast cells in the M-cells, the

intercellular pocket below the M-cells and the space beneath the basal membrane. Depending on where the DCs are located, they were found to have different functions in the PP microenvironment producing Th1 or Th2-directing cytokines upon activation (Iwasaki 2001). The cytokine and chemokine microenvironment will then subsequently decide the differentiation of the Th-cells to Th1 and Th2 subsets, respectively (Pulendran, 1999), and affect the survival or apoptosis of T-cells, as well (Kellermann, 2001).

In addition, the differentiation of the B-cells and the homing of the mucosal plasma cells are regulated by separate cytokines (lL-6 in addition to TGF-β, IL-4 and lL-5) and the specific homing receptor, a_4b_7 (Kantele, 1999). It can thus be concluded that there seem to be mechanisms available in the mucosa by which mucosal immunomodulation can induce a more differentiated immune response, better mimicking the response to a natural infection than obtained after parenteral application. Further information is needed, however, on the role of the antigen-presenting cells involved in the phagocytosis of micro-particles and their contribution to the differentiation of the T-cells and B-cells, to be able to control the Th1/Th2 profile of the immune response.

DC tolerance can be a problem that can be overcome by the simultaneous administration of adjuvants. Cholera Toxin, the most potent mucosal adjuvant described, as well as other toxins (Gluck, 2000) are not well tolerated and their negative effects preclude their use in vaccination in humans (Levine, 1983). Other adjuvants, such as unmethylated CpG rich bacterial oligonucleotides, represent an efficient and well-tolerated alternative (Klinman, 2003). CpG DNA up-regulates the expression of MHC-II and co-stimulatory molecules on DC and has been found to promote a productive *in vivo* Th1 response (Chu, 1997; Lipford, 1997; Davis 1998; Zimmermann, 1998). However, CpG rich bacterial oligonucleotides showed poor pharmacokinetics and caused splenomegaly in an animal model (Storni et al., 2004). Subsequent cytokine production and expression of cell surface molecules play a major role in the stimulation of mucosal immunity (Williamson, 1999; Sierro, 2001). DCs were also powerfully stimulated by Flt-3 ligand and IL-1α co-administered with an otherwise tolerogenic orally delivered antigen (Williamson, 1999).

The characteristics of the immunogen bound to a particle carrier has been found to influence the interaction with receptors in the mucosa. Some pathogens have organelles on the bacterial surface, which mediate the attachment of the bacteria to host tissues and facilitate the invasion of the host.

3.4. Mycoplasma

Mycoplasmas represent the smallest known living microorganism. Mycoplasmas are prokaryotes with definite phenotypical characteristics. From a pathological point of view, these are the following: the lack of cell wall, and a very limited genome of 470-677 kpb which makes them highly dependent on their host for survival. This causes molecular biological and histological changes in host cells.

Mycoplasmal infection results in the production of cytokines, which distorts the immune system and prevents an effective response to other pathogens. It has been shown that *M. hyopneumoniae* has a powerful effect to predispose *Pasturella multocida* (Ciprian et al., 1988) and *Actinobacillus pleuropneumoniae* (Caruso and Ross, 1990). It has also been demonstrated, that when *M. hyopneumoniae* vaccination is carried out in pigs, it reduces the potentiation of PRRSV (porcine respiratory and reproductive syndrome virus) induced pneumonia in dual infected pigs and reduces the infection and the effect of *Pasturella multocida, Actinobacillus pleuropneumoniae* and other bacterial complications.

Mycoplasma is among the most persistent of all pathogens. Infected host can be treated with antibiotics. Even after clinical symptoms disappear as a result the successful antibiotics treatment,

mycoplasma can be still found shedding (Stipkovits, 2002). To successfully treat systemic mycoplasmal infection, several cycles of antibiotic treatment are required. The most effective way to control mycoplasmal infection is vaccination. However, there are shortcomings in the currently available anti-mycoplasmal vaccines.

So far, mycoplasmal antigens used in new "prototype" vaccines (ISCOM) represented multiple, undefined proteins, suggesting that the use of a mixture of antigens might be a key factor in stimulating a protective immune response. Indeed, mycoplasmal subunit vaccines composed of only one defined antigen did not show satisfactory protective effect (Abusugra and Morein, 1999; King et al. 1997, Sundquist et al. 1996). In mice, immunization with the single immunodominant cytoadhesive protein P1 of *M. pneumoniae,* as vaccine candidate did not induce a protective immune response and, in some cases, rather resulted in adverse reactions (Jacobs et al. 1988). This illustrates one of the major challenges in vaccine design, that is, the definition of antigens, which are able to generate a specific, protective host immune response. Compared to conventional killed or live vaccines, the main advantage in using characterised, defined vaccine components is the possibility of isolating the antigens that are inducing a protective immunity from those responsible for side-effects (Liljeqvist and Stahl, 1999; Hansson et al., 2000). The most defined immunogenic vaccine components are epitopes, which are recognized by B-cells and/or T-cells and which represent the minimal amino-acid sequences necessary to achieve an immune response. Mapping of immunogenic epitopes within known proteins has been a major field of research in vaccine development.

In mycoplasmas, epitopes located within surface-exposed proteins responsible for cell attachment and/or invasion were proposed as promising candidates for vaccine components since host antibodies directed against these component are predicted to prevent colonization of the host mucosal surfaces by the pathogen. In *M. gallisepticum*, four proteins displaying adhesive features have been identified that may play a major role in the initial attachment of the pathogen, via its so-called tip-structure, to the host cells (Winner et al., 2003). The absence of two of these components in a mutated strain has been shown to correlate with a reduced adhesive capacity and with a reduced pathogenic activity (Papazisi 2002a,b).

3.4.1. *M. gallisepticum*

The wall-less bacterium MG induces chronic respiratory disease (CRD) and arthritis in chickens, as well as infectious sinusitis in turkeys that result in major health problems for the poultry industry and cause severe economic losses worldwide (Stipkovits, 2001). The infections caused by *M. gallisepticum* are responsible for significant economical losses in the poultry industry and for their ability of promoting other pathogenic infections (*E. coli*) thereby causing additional losses (Stipkovits, 1968). In commercial poultry production, 5-30% of the flocks are infected with *M. gallisepticum* in the developed countries while the infection rate is 60-70% in the developing countries. *M. gallisepticum* infection causes losses as a result of condemned and downgraded carcasses at slaughter, reduced egg production, poor feed conversion and high medication costs. Therefore, it is considered as one of the most costly disease of poultry worldwide.

In an attempt to control MG infections, several inactivated (MG bacterins) and live vaccines were developed (Whithear, 1996) and (Kleven, 1998) which presented several drawbacks. For instance, killed whole-cell MG bacterins were able to reduce the severity of the disease, but did not prevent colonization of the chicken respiratory tract and apparition of lesions in inoculated tissues (Elfaki et al., 1993; Whithear, 1996). Commercial available live vaccines remarkably differ from each other in terms of post-vaccination reactions, serological responses and persistence in the upper respiratory tract (Whithear, 1990; Kleven, 1998; Turner and Kleven, 1998) and because of the inherent safety problems (reversion to virulence), they have been approved in only a few countries. This is the case of the F strain, which reduces the decline in egg production and has been used to displace

endemic strains in multiple-age flocks. However, its inherent residual virulence in young chickens and in turkeys is a major disadvantage (Whithear, 1996).

Although the preferred method for the control of mycoplasma in poultry flocks is eradication of the organism, vaccination is another possible means to control the disease. An attenuated strain of mycoplasma has been used to vaccinate chickens by respiratory exposure. However, the attenuated strain does cause mild, transitory disease. Furthermore, the attenuated vaccine persists in the environment and has the potential of reverting to a virulent strain.

The U.S. Department of Agriculture has approved several vaccines comprising inactivated or killed mycoplasma, but, because these vaccines are administered parenterally, mucosal immunity is not adequately stimulated. Even though losses are reduced by vaccination, infection and the persistence of pathogenic strains is evident. Adequate stimulation of the mucosal immunity should best be achieved by inoculation at the mucosal surfaces. However, numerous attempts exposing respiratory mucosae to killed mycoplasma have failed. Traditional adjuvants cannot be used with mucosal vaccines because they would cause excessive irritation of the extremely sensitive respiratory and conjunctival mucosae.

IV. OWN EXPERIMENTS

The aims of the presented experiments were to investigate the efficacy of the innate and adaptive immune responses to pathogens and pathogen-associated molecules, such as endotoxins. We have examined two distinct approaches.

One is aimed at the removal of bacterial toxins, mycoplasma and bacteria from different biologically important solutions, such as salt solutions, pharmaceuticals, water and serum used in the manufacture of pharmaceuticals. This technology has been also applied to remove endotoxin and other TLR stimulating molecules from blood and other biological fluids. These technologies utilize synthetic ligands based on TLR recognition to capture the immunostimulatory molecules and pathogens. During the development of the capture technologies, we have developed some of the assays and methods utilized in the experiments throughout the work.

The other is aimed at modulating both the innate and the adaptive immune responses. We have utilized a novel "pathogen mimicking" microparticle to influence the function and signaling mechanism of PRR in regulating the immune responses. These particles contain antigens along with selected TLR and other PRR agonists to induce a pathogen-tailored, effective innate and adaptive immune response. *M. gallisepticum* infection has been used as a model system to demonstrate the feasibility of this technology. *In vitro* studies using PBMC and DCs, and animal challenge experiments have been performed.

4.1. EXPERIMENT 1: ENDOTOXIN REMOVAL FROM VARIOUS SOLUTIONS

4.1.1. Introduction

In vitro, bacteria release bacterial membrane components when growing in water, salt solution or culture media (Hoekstra et al., 1976) or when incubated in serum or blood (Hellman et al., 2000). *In vivo*, these bacterial compounds, such as endotoxin, are the messengers that signal the presence of a foreign organism by binding to the TLR, considered the sensors of the innate immune system present on endothel and epithel cells, monocytes, macrophages and dendritic cells. The innate immune system is the first line of defense against invading pathogens.

Endotoxin (ET) has been known for many decades to play a critical role in the initiation of inflammatory response. ET binds to the CD14 receptor, which associates with TLR-4, and leads to the activation of monocytes and other cells carrying these receptors. The binding results in transcriptional changes in the nucleus of activated cells, and the synthesis of different inflammatory compounds, such as Platelet Activating Factor-1 (PAF-1), pro-inflammatory cytokines such as TNF-α, IL-1 or IL-6 leading to a systemic inflammatory response. Even at the molecular level, the overlap of gene expression changes when dendritic cells are exposed to ET or whole *E. coli* remarkably amounts to 88 %, suggesting that ET by itself can almost mimic the effect of a whole Gram- bacteria (Huang et al., 2001).

Endotoxin is introduced during infection as part of the Gram-negative bacteria, however, iatrogenic introduction of endotoxin through contaminated water, salt solutions or biopharmaceuticals also happens. Endotoxin is of increasing concern in biotechnology. Endotoxin, also referred as LPS, is composed of hydrophobic fatty acid and hydrophilic carbohydrate domains (Sharma, 1986). The primary hydrophobic domain, known as lipid A, carries many of the biological activities associated with LPS. Lipid A contains fatty acid chains attached to a phosphorylated disaccharide. The lipid composition of lipid A exhibits strain-specific variations. The core is made of carbohydrates, some of which are phosphorylated and ethanolaminylated. The repeat units are trisaccharides, the number of which varies among the different strains.

The chemical nature of LPS makes pyrogen removal problematic (Sharma, 1986). LPS is unusually thermostable and fairly insensitive to pH changes. High concentrations of acids or bases are necessary to destroy LPS within a reasonably short time. The size of LPS varies depending on the environment; reported molecular weights range from 2 kDa to several million daltons. Also, naturally occurring LPS has a Stokes radius smaller than the purified endotoxin typically used to qualify filters (Harding, 1990). This adds to the uncertainty of developing effective LPS removal methods. The heterogeneity of LPS is substantial, and its implications are not fully appreciated in the area of endotoxin removal.

Besides LPS, Gram-negative bacteria release peptides such as exotoxin A (from Pseudomonas), peptidoglycans, muramyl peptides, and other still unidentified substances. These bacterial products act similarly to LPS in terms of inducing the secretion of cytokines. The molecular size of these products also varies greatly. Peptidoglycans and LPS are large molecules, whereas LPS subunits and muramyl peptides may be as small as 2000 Da. In the supernatant of Pseudomonas, a major group of waterborne bacteria, a pyrogen with a very low molecular weight (<1000 Da) recently was identified (Mahiout, 1991; Massion 1994). In actual final-product samples, a mixture of pyrogens always is present, suggesting that no single modality of pyrogen removal may be adequate.

We describe here a novel technology for a simple, rugged, and economical method to remove endotoxin from water. This method can also be used to effectively remove pyrogenic substances from pharmaceutical salt solutions, such as dialysate.

4.1.2. Materials and Methods

Preparation of Endotoxin using Pseudomonas maltophilia supernatant
Pseudomonas maltophilia was obtained from ATCC, and the bacterial culture broth from Difco Laboratories (Detroit, MI). The culture was grown at 37^{o}C and LPS in the supernatant was monitored periodically by the LAL assay. Total culturing time was 48 h. The culture was refrigerated at 4^{o}C and centrifuged at 2,000xg. Then, 20 µg/ml Gentamicin (Sigma, St. Louis, MO) and 5 µg/ml Ciprofloxacin (Bayer, West Haven, CT) were added to the supernatant. The supernatant was sterile-filtered and aliquoted for freeze storage. Sterility of the filtrate was ascertained using agar-plate culture. No colony growth was observed.

ClarEtox adsorbent
ClarEtox (Sterogene Bioseparations, Carlsbad, CA) is an affinity adsorbent designed to remove endotoxin though the Lipid A moiety. The backbone is a rigidized natural polysaccharide matrix. The proprietary functional ligand catalyzes its own coupling to the beads via a stable linkage. Endotoxin in aqueous solutions frequently form polymeric, high molecular weight complexes which, in this case, facilitate its binding to the adsorbent. ClarEtox reversibly binds endotoxin, such that during treatment with hot water, >85°C, endotoxin is solubilized and removed. ClarEtox can also be regenerated with 1M NaOH.

Static binding of endotoxin to ClarEtox
Two 5 ml polypropylene columns were filled with 1 ml of ClarEtox and measured by suction drying. Resin was washed with 8 bed volumes of reverse osmosis (RO) water, drained and then washed with 8 bed volumes of 1 M NaOH. Columns were filled with 3 ml of 1M NaOH and rocked overnight at room temperature. Columns were placed in a laminar flow hood, drained, and washed with LAL reagent water (Charles River) until pH 6-7. Columns were suction-dried and the resins transferred to pyrogen-free, flint glass tubes and capped. Lyophilized LPS derived from *E.*

coli 055:B5 was reconstituted with 5 ml of LAL reagent water from an unused 30 ml bottle and vortexed for 5 minutes.

Reconstituted lyophilized LPS was transferred to a 30 ml bottle. This was vortexed for 5 min. Then, 2 ml was added to each tube and 500 µl was added to a third tube as a reference. The two samples were rocked for 30 min at room temperature and then centrifuged at 250xg for 3 min. Supernatant was removed and transferred to clean T100 tubes and tested either immediately or stored at below -10^{o}C overnight. Samples and references were vortexed for 2 min and diluted 10^6 fold in LAL reagent water in three serial dilutions of 10^2, vortexing 2 min between each dilution.

A four-log standard curve of 5 EU to 0.005 EU was prepared using Control Standard Endotoxin. Samples and standards were measured by the kinetic turbidimetric LAL assay (Charles River). Endotoxin binding is determined by subtracting the total endotoxin in supernatant from the total endotoxin added for both samples. The results of the two samples were averaged for total binding.

Endotoxin removal from reverse osmosis water
The ClarEtox resin was packed into a test column (9 ml bed volume). The column was sanitized with 1 M NaOH at 2-8^{o}C overnight. The water treatment procedure was carried out with a flow rate of RO water of 11 ml/min. The RO water was passed through the column up to a total of 12,000 bed volumes. The experiment was run for 91.5 hours.

Endotoxin adsorption from Pseudomonas supernatant
About 500 ml of 1M NaOH was pumped into a 500 ml column containing ClarEtox resin and allowed to stand overnight. The column and the tubings were then rinsed with reverse osmosis water to neutrality. The bicarbonate concentrate was spiked with sterile Pseudomonas culture supernatant. The binding was tested at flow rates of 500 ml/min and 800 ml/min. Samples were taken hourly before and after ClarEtox. Endotoxin in the samples was measured by the kinetic turbidimetric LAL assay.

Endotoxin challenge of ClarEtox
This experiment determined if ClarEtox worked efficiently under the conditions of extreme endotoxin loads. Samples were taken pre- and post-ClarEtox for base endotoxin level determination before spiking. The salt solution (dialysate) was spiked with endotoxin. Endotoxin levels in the reconstituted dialysate ranged between an average of 35 EU/ml to 193 EU/ml in the different runs. Samples of the spiked dialysate were taken for endotoxin analysis.
Samples were taken at this time pre- and post-ClarEtox for beginning endotoxin levels (T=0). For the 9h endotoxin challenge experiment, test samples were taken to determine endotoxin levels pre- and post-ClarEtox at T=0min, T= 5min, T=60min, and every 30min to the end of the challenge. For the 20h endotoxin challenge experiment, samples were taken at T=0min and every 60 minutes for the first 15h and every 30min after 15h.

Sanitization of ClarEtox by 90^{o}C hot water
A cumulative exposure method was used to test the effect of repeated hot water sanitization cycles on the ClarEtox adsorbent. A 25h exposure was calculated to be equivalent to 100 regenerations. ClarEtox resin, 600ml, was suctioned dry and placed in a glass reactor followed by 2L of reverse osmosis water.

The reactor was equipped with a thermometer and a reflux condenser. The reactor was placed in a heating mantle and the entire apparatus was wrapped in aluminum foil except for the thermometer and reflux condenser. The heating temperature was adjusted within the range of 85^{o}C to 100^{o}C. The system was heated for 25h. At the end of the incubation period, the resin was tested for static

endotoxin binding capacity as well as dynamic endotoxin binding. A ClarEtox column was prepared using the heated resin for a 20h challenge to determine its effectiveness.

Sanitization of ClarEtox by NaOH

The use of sodium hydroxide (NaOH) is a method of choice in pharmaceutical processes for removing endotoxin (15-17). A used ClarEtox resin was flushed with 4L of reverse osmosis water at 500 ml/min using a peristaltic pump. Sanitization was performed with 2L of 1M NaOH by recirculating the NaOH at 500ml/min for 15 minutes. After 15min, the tubing was clamped on one side of the device. The NaOH was removed by rinsing ClarEtox with RO water at 500ml/min until the pH was equivalent to the incoming water's pH.

Since endotoxin inactivation is temperature-dependent, the following time schedules were applied for NaOH treatment:

1. At 2 - 8°C for 12 to 14 hours,
2. At room temperature for 5-6 hours, or
3. At 37°C for 2 hours.

Leachables by total organic carbon (TOC)

The US Pharmacopoiea prescribes TOC testing as a way to determine dissolved organic contaminant in water. To test the stability of the resin, ClarEtox was washed with reverse osmosis water at 500 ml/min. The reverse osmosis water was run for 15 minutes after which samples were taken pre- and post-ClarEtox at the start and at 180min for the three hours test and after 30min for the 1/2h test.

A sample was also taken directly from the reverse osmosis water port. Samples were collected by opening the stopcocks and letting the water run through for 20sec. The stopcock was closed and attached to a sterile 20ml syringe via a luer-lock port. A sample was taken into the syringe, the content of which was transferred into a TOC sampling jar. Subsequently, the samples were analyzed for TOC content.

4.1.3. Results

Pressure-flow characteristics of ClarEtox

The pre-ClarEtox pressures remained within the range of 0-5 psi. The post-ClarEtox pressure remained constant at 0 psi.

TOC leaching test

Results indicated that ClarEtox did not produce any leachables that would affect safety and efficacy of the device (Table 3). The TOC result obtained was below the level allowed for Water-for-Injection (0.5mg/L).

Table 3. Total Organic Carbon (TOC) testing results with ClarEtox

Sample	Time (min)	TOC (mg/L)
RO Port		0.40
Pre-ClarEtox	0	2.32
Post-ClarEtox	0	1.32
Pre-ClarEtox	180	0.47
Post-ClarEtox	180	0.41

Endotoxin challenge

Dialysate was spiked to an average of 37EU/ml with *Pseudomonas maltophilia* supernatant and applied to a ClarEtox device. A total of 270L of dialysate was passed through the device over the 9h time period containing a total endotoxin of 1 x 10^7EU. An average of 20,000 EU/ml resin were bound. The experiment was terminated after 9h without exhausting the column (Table 4). ClarEtox is capable of binding more endotoxin than what was loaded on in both the 9h and the 20h challenge experiments (Table 4). A 60h challenge study was also performed and showed that ClarEtox can be continually used for 6 days without regeneration (not shown).

Table 4. Results of the Endotoxin Challenge Test on ClarEtox

	9hr test		20 hr test	
Time (min)	Pre-ClarEtox (EU/ml)	Post-ClarEtox (EU/ml)	Pre-ClarEtox (EU/ml)	Post-ClarEtox (EU/ml)
3 hour Average	35.13±0.007	0.009±0.007	25.9±0.0	<0.015±0.0
6 hour Average	38.75±0.016	0.020±0.016	0.0±0.0	<0.015±0.0
9 hour Average	37.00±0.022	0.030±0.022	25.2±0.007	<0.011±0.007
20 hour Average	23.3±0.008	<0.016±0.008		

Dialysate conductivity and ionic composition with on-line ClarEtox

Measurements of conductivity were performed during the endotoxin challenge experiment. The conductivity was read pre- and post-ClarEtox at each time point of the endotoxin challenge assay. The conductivity test was performed and recorded immediately after sample collection. The results indicated insignificant changes in conductivity pre- and post-ClarEtox (Table 5). For dialysate ion composition, pre-ClarEtox and post-ClarEtox samples were collected after the initial stabilization (approximately 15-20min) of the dialysis machine (T=0min). The dialysis machine was run for 180min. Samples were analyzed at the UCSD Medical Center's Clinical Chemistry Laboratory for ion composition. All results were within less than 2% difference indicating that ClarEtox did not affect the ion composition of dialysate (Table 6).

Table 5. ClarEtox 's effect on dialysate conductivity during the endotoxin challenge assay

Timepoint (min)	Conductivity Pre-ClarEtox (mS/cm)	Conductivity Post-ClarEtox (mS/cm)	Percent Difference (%)
0	16.82	16.75	0.4
60	16.82	16.46	2.0
120	16.91	16.91	0.0
180	16.97	16.88	0.5
240	17.11	17.00	0.6
300	17.14	17.05	0.5
360	17.03	17.02	0.1
420	17.11	17.14	0.2
480	16.93	16.99	0.4
540	17.09	17.22	0.8

Table 6. Results of the dialysate ion composition test upon contact with ClarEtox

Dialysate Analyte	Pre-ClarEtox (T=0)	Post-ClarEtox (T=0)	Pre-ClarEtox (T=180)	Post-ClarEtox (T=180)	Percent Difference (%)
Chloride	106	106	105	105	0
Bicarbonate	37	37	38	38	0
Potassium	2.0	2.0	2.0	2.0	0
Calcium	5.2	5.2	5.1	5.2	1.9
Magnesium	1.2	1.2	1.2	1.2	0
Sodium	142	142	142	142	0

Endotoxin removal from reverse osmosis and deionized (DI) waters

The purpose of this experiment was to determine if ClarEtox could efficiently remove endotoxin from reverse osmosis water. Endotoxin levels in the RO water varied between 1-20EU/ml. After passing through ClarEtox the endotoxin levels were between >0.005EU/ml to 0.01 EU/ml until 10,000L water per L of resin passed through the column. With 100 regenerations, ClarEtox could produce 1,000,000L of purified water per L of resin.

A deionized water depyrogenation experiment was also carried out with ClarEtox. Deionized water was passed through ClarEtox column at 500ml/min for 32h. Endotoxin levels in the deionized water varied between 0.1-52EU/ml. Post-column endotoxin levels varied from <0.005-0.01EU/ml. ClarEtox effectively removed endotoxin from the deionized water at a high throughput.

The results indicate that ClarEtox efficiently and consistently decreases endotoxin in RO water. An average of 408,236 EU were loaded onto the column over the 91.5 hours. This results shows the endotoxin binding capacity of ClarEtox is 45,359EU/ml resin. When deionized water was treated, a breakthrough point has not been reached. However, the results of the RO water treatment can be used as a reference point for deionized water, too.

Hot water sanitization of ClarEtox

Results indicated that ClarEtox works effectively after 100 hot water sanitization cycles. An average of 193 EU/ml dialysate was loaded on the 100 times heat-sanitized resin column for the first nine hours of the challenge. This was a total of 57,900,000 EU loaded over the 9h period. The total endotoxin units that were not bound over the 9h are 18,000 EU. There was a three logs endotoxin reduction in all the post-ClarEtox samples. The average post result was 0.06EU/ml dialysate.

4.1.4. Discussion

At present, the main bacteriological and pyrogen control technology for water and salt solution applications is ultrafiltration that has not become a widely used method because of technological and cost considerations. Ultrafiltration requires a prefiltration of the fluid stream that adds to the cost of this unit operation. Recent studies also indicated the presence of a low molecular weight (<1000 Da) pyrogen(s) in Pseudomonas supernatant (Klein et al., 1990). Ultrafilters cannot remove such low molecular weight bacterial product(s).

Bicarbonate containing solutions have the tendency to form a precipitate on standing, a process that accelerates membrane fouling. ClarEtox is insensitive to the particulate matter typically present in bicarbonate solution.

ClarEtox was developed to achieve control of bacteria and bacterial products in water and salt solutions. ClarEtox is a novel adsorbent that represents an unexplored direction in bacterium control due to its particle based matrix as well as its surface chemistry that uses a synthetic mimetic ligand based on the ligand binding extracellular pocket of TLR-4 to bind bacteria and bacterial products. The data demonstrated that ClarEtox has a high capacity to remove TLR analog bacterial toxins. The high stability of ClarEtox allows for extended periods of usage. This makes ClarEtox appropriate for the removal of bacteria and bacterial products from water and salt solution.

However, water systems can exhibit sudden fluctuations in bacterial and endotoxin levels. This is why pharmaceutical water systems are tested daily for endotoxin and if specified levels are exceeded, the system is shut down for sanitization. Water for Injection (WFI) must meet stringent purity criteria specified in the Pharmacopoieas. In dialysis, up to thousands of times the amount of water come in contact with the patient's bloodstream than during the administration of a typical parenteral pharmaceutical. During dialysis, assuming the use of 120L of dialysate per session and 0.5EU/ml endotoxin present, a total of 60,000EU may be passing through the dialyzer. If just 1% of this amount crosses into the bloodstream, it essentially would be twice the amount of endotoxin currently allowed for single doses of parenteral pharmaceuticals by the FDA. Even in the area of parenteral medicines, the wisdom of permitting the presence of any endotoxin is a debated issue (Grandics, 2000).

ClarEtox has a very high endotoxin binding capacity ensuring that temporary breakdowns in the sanitization controls in a dialysis center would not pose an undue risk to dialysis patients. The high stability of ClarEtox allows for extended periods of usage. This makes ClarEtox optimal for the depyrogenation of dialysate and water for dialysis and reuse.

4.2. EXPERIMENT 2: A NOVEL METHOD FOR REMOVING TLR ACTIVATING MOLECULES USING AFFINITY CAPTURE TECHNOLOGY

4.2.1. Introduction

Bacterial compounds are the messengers that signal the presence of a foreign organism by binding to the TLR, considered the sensors of the innate immune system. The same molecules and receptors, which are able to eliminate an infection, can paradoxically over-stimulate the innate immune system and create a systemic inflammatory response syndrome and lead to septic shock. ET has been known for many decades to play a critical role in the initiation and development of sepsis, but as our understanding of both Gram-negative and Gram$^-$positive sepsis expands, it is clear that ET is only one of the actors in sepsis (Horn et al., 2000). Increasing evidence suggests that various bacterial membrane components from both Gram-negative and positive bacteria play a critical role in sepsis. The underlying molecular mechanisms have similarities. For instance, the main genes induced in monocytes by LPS, *S. aureus* or PepG overlap, with chemokines being the most induced, but the level of induction differs between stimuli.

These molecules have in common that they are all pro-inflammatory and bind to the CD14 receptor, which associates to various receptors of the same family, the TLRs on monocytes, macrophages and dendritic cells (Akira: 2003). There are currently 10 TLR reported. Each recognizes one or more specific ligand and performs signal transduction. The membrane receptors and downstream factors involved in the signal transduction mechanisms have been discovered by knock-out, complementation and overexpression strategies, as well as more global random germline mutagenesis studies. TLR were originally found to be associated with CD14 (Yamamoto, 2002a), then also with MD-2 (Shimazu et al., 1999). Evidence is accumulating that cooperation between TLR receptors (such as TLRs 1/2 and 6/2) comes into play to refine ligand discrimination. Clustering of receptors in lipid rafts has also been identified after ligand binding. Such studies also revealed MyD88-dependent and independent pathways (Akira and Hemmi, 2003). Some microbial pathogens can also be endocytosed and exert their activity directly in the cytoplasm, using the leucine-rich domain of Nod-1 and Nod-2 as intracellular Gram$^-$ bacterial PepG sensing for instance (Girardin et al., 2003a). However, the core signaling converge towards the nuclear translocation of NF-*k*B and activation of inflammatory genes and production of cytokines, with in addition the IFN-α and IFN-γ induction pathway downstream of TLR-3 and TLR–4.

TLR-4 has been the most widely studied member of this family of receptors. It is known to recognize LPS from Gram-negative bacteria (Akira and Hemmi, 2003), whereas TLR-2 binds PepG, bacterial lipoproteins/lipopeptides, or mycobacterial components (Hellman and Warren, 2001). TLR-9 is known to detect unmethylated bactDNA (Hemmi et al., 2000a). This illustrates that the innate immune system has developed a way to identify some specific pathogenic molecules. In addition, because of the cooperation between TLR receptors and the common denominators in proximal events of TLR activation, it is not surprising to observe synergistic effects between microbial pathogenic compounds (De Kimpe et al., 1995 and our data). This synergy has been proposed as a contribution to the pathogenesis of sepsis, particularly in mixed bacterial infections.

Relevant to this project, the role of ET, PepG and bactDNA will be reviewed in relation to sepsis, as far as their presence and release in the blood, their pro-inflammatory effect, pro-coagulant effect and synergistic properties.

4.2.1.1. Endotoxin

The chemical structure of the endotoxin consists of a polysaccharide and a lipid A portion. The polysaccharide, which varies from one bacterial species to another, is made up of the O-specific

chain (built from repeating units of three to eight sugars) and the two-part core. Lipid A virtually always includes two glycosamine sugars modified by phosphate and variable number of fatty acids. The Kdo (3-deoxy-D-manno-2-octulosonic acid), which is found in all endotoxins, links the polysaccharide to the lipids. The Lipid A portion of the endotoxin, which is the least variable in structure is responsible for the inflammatory effect of the endotoxin through the stimulation of TLR-4 (Rietschel and Brade, 1992). The endotoxic property of Lipid A, carries to negatively charged phosphate groups and six acyl chain residues in a defined asymmetric distribution. The two negative charges of Lipid A, represented by the two phosphate groups are essential for agonistic as well as for antagonistic activity (Seydel et al., 2004).

4.2.1.2. Peptidoglycan

PepG, a polymer of alternating N-acetyl glucosamine and N-acetylmuramic acid cross-linked by short peptides, is a component corresponding to about 70 % of Gram+ and 20 % of Gram- bacteria cell walls. PepG can activate leukocytes, generate pro-inflammatory cytokines and cause Systemic Inflammatory Response Syndrome (Mattson et al., 1993). PepG can stimulate monocytes to produce IL-1, IL-6 and TNF-α (Heumann et al., 1994). Bacterial LP, made by all bacteria and composed of N-terminal lipo-amino acid, N-acyl-S-diacylglyceryl cysteine which are often associated with PepG. LP has been shown to activate B-cells and macrophages (Bessler et al., 1985). LP from *E. coli* induces TNF-α and IL-6 production in peritoneal exudate macrophages from LPS-responsive and LPS non-responsive mice. An analogue of the immunologically active LP activates the release of IL-1, IL-6, TNF-α and NO. LP is the most abundant protein in the outer membrane of the bacteria of the *Enterobacteriaceae* family.

4.2.1.3. Bacterial DNA

BactDNA in and of itself can induce septic shock and death in sensitive mouse and synergizes with LPS (Sparwasser et al., 1997). Moreover, bactDNA sensitizes mice and increases toxicity to LPS (Cowdery et al., 1996). CpG DNA induces the production of TNF-α in macrophages in vitro (Sparwasser et al., 1997).

Hellman and co-workers reported that bacterial fragments containing LPS and at least 3 outer membrane proteins (outer membrane protein A, PepG-associated LP, murein LP) were released from an *E. coli O18* strain into normal serum and into the blood of rats with experimental Gram- sepsis and later in an infected burn wound model of Gram- sepsis in rats. Circulating bacterial toxins have also been identified in animal models of sepsis (Hellman and Warren, 2001) and in septic patients. ET concentration in blood has been shown to be a good predictor of mortality in patients with non-*Enterobacteriaceae* bacteria-induced sepsis. PepG was only detected in the blood of septic patients compared to control. As part of the treatment of septic patients, antibiotic administration is a primary measure. β-lactam antibiotics have been shown to induce the release of Lipoteichoic acid (LTA) and PepG from *S. aureus* by about 6-fold and 72-fold, respectively and to be associated with increased leukocytes and endothelial chemokine secretion and granulocytes adhesiveness. The fact that in some animal experiments antibiotics have not been administered has been proposed as an explanation for the different results obtained when tested in human clinical trials, which include antibiotics therapy.

4.2.2. Materials and Methods

Resin preparation

Various resins have been made to achieve both efficient bacterial toxins removal and good hemocompatibility. The final resin has a 4 % agarose as base matrix, the bacterial toxin binding ligand and a surface modification. The ligand consists of 3 tertiary amines that confer a positive charge to the molecule, available to interact with the negatively charged phosphate groups of ET. In addition, the presence of 2 condensed rings confers hydrophobic properties enhanced with a

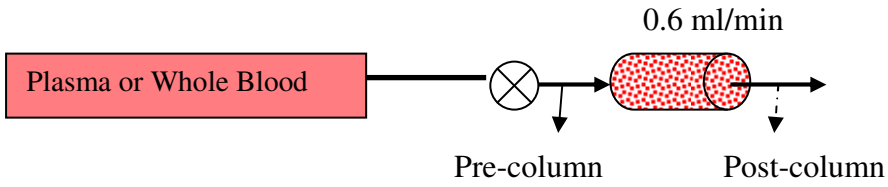
halogen substitution. This allows for hydrophobic interactions with the acyl chains of the lipid A portion of ET. This ligand was found to be an efficient capture ligand of ET, PepG and bactDNA. However LTA or coagulation factors were not bound. LPs bound weakly compared to the other compounds.

The optimal bead size (100-200 µm) minimized hemolysis of red cells when using whole blood while maintaining a large adsorption surface. Polyethylene glycol (OH-PEG-COOH, MW:3,400 from Shearwater, Huntsville, AL) was also immobilized to improve biocompatibility as it has been shown in other systems (Alcantar et al., 2000; Uchida et al., 2000; Sirolli et al., 2000; Deible et al., 1999)

Dynamic circulation of plasma or whole blood on the affinity column
Column depyrogenation
A 1.5 ml glass column was packed with resin. The glass column was pretreated with Sigmacoat (Sigma, St Louis) in order to remove negative charge on the surface of the glass, which would induce the intrinsic coagulation pathway. The column was decontaminated using 1M NaOH overnight at room temperature and returned to neutrality the next day by running ET free LAL water (Charles River, Charleston, SC). Sample ports were also soaked in 1M NaOH overnight and rinsed in LAL water.

Priming of the column for whole blood circulation
When whole blood was used, about 15 ml of Ringer solution from which ET have been removed using ClarEtox.

Dynamic flow through the affinity column, choice of anticoagulant and spiking with bacterial toxins



Whole blood was obtained from healthy volunteers. It was placed into a syringe and loaded onto the column at a flow rate of 0.6 ml/min using a syringe pump (model 22 from Harvard Apparatus, Holliston, MA). Plasma was run through the column using a peristaltic pump.
Samples were taken at regular intervals via the sample ports placed right upstream and downstream of the column using sterile needles and syringes, or a single pre-column sample was obtained from the loaded fluid.

For ET removal, heparinized blood or plasma (Heparin 5U/ml) was spiked with ET. Plasma was either purchased from Bioresource Technologies (Fort Lauderdale, FA) or prepared in house from blood from healthy volunteers by spinning the blood at 1,800xg for 20 minutes at 12°C. The resulting supernatant was sterile filtered with a 0.22 µm cut-off filter.

The preparation of ET from bacterial culture and plasma are described bellow. The level of ET chosen is representative of ET levels in the blood of septic patients (Kodama, 1993; Opal et al., 1999). The use of citrate was prevented by the interference of citrate with the LAL assay. When monocyte activation was used as a read-out of ET, PepG or bactDNA removal, citrate was used. Various concentrations of bacterial toxins were tested as described further in the results section. For coagulation studies involving Prothrombin time (PT) and Activated Partial Thromboplastin time

(APTT), and for hematology analysis, Sodium Citrate (10 mM) was used as an anticoagulant. For Fibrinogen concentration or thrombin-antithrombin (TAT) assays, heparin or citrate was used.

Endotoxin was prepared by the method described in Own Experiment 1. Purified LPS and PepG were obtained from Sigma and bactDNA from KM Biomedical (Aurora, OH).

ET removal: measurement by LAL assay

The amount of ET was measured by Limulus Amaebocyte Lysate (LAL) assay after optimization of this assay for ET measurement in plasma (6). Briefly, the assay was performed under a laminar flow hood. Heparinized plasma samples were diluted 10 to 67 times in order to reduce inhibitory or enhancing effects of the plasma and meet the criteria of recovery (see below). Samples were then heat-inactivated at 75°C for 15 minutes, and vortexed for 2 minutes. A 5.0 to 0.005 EU/ml standard curve was prepared with Certified Control Standard ET (Charles River, Charleston, SC). Samples were loaded onto 96 well plates. For each sample four aliquots were processed. Two of them were spiked for positive control. Turbidimetry was read for 1 hour in a Bio-Tek Elx808 microplate reader. Data were analyzed using the Biolise Software.

Bacterial toxins removal: measurement in peripheral blood mononuclear cell or monocyte by TNF-α induction and Tissue Factor expression assays: Induction of TNF-α secretion by monocytes or PBMC was used as an in vitro assay system for determination of pyrogen-induced inflammatory reactions. Indeed, TNF-α is an important early mediator of host responses to pyrogen contamination, and is induced in vitro by challenge with ET, PepG or bactDNA. TNF-α is the only endogenous mediator capable of triggering the entire spectrum of metabolic, hemodynamic, tissue and cytokine cascade responses of septic shock (Shalaby et al., 1986, Tracey et al., 1993). Tissue Factor is an initiator of coagulation as in DIC and was used as an indicator of pyrogen-induced procoagulant reaction.

PBMC were prepared by Ficoll separation of peripheral blood and plated in 1 ml RPMI-1640 2X + 6% fetal calf serum (FCS) and treated with 1 ml plasma to be tested. TNF-α was then measured from the culture supernatant after 5 hours incubation. When monocytes were used, they were obtained after the removal of non-adherent cells as follows. The mononuclear cells were incubated at a density of 10^6 cells/well in 6-well plates, overnight in RPMI-1640 (containing 100 U/ml Penicillin/Streptomicin and 2 mM Glutamine) supplemented with 20% FCS. Adherent cells were then incubated in 1 ml RPMI-1640 (2X) containing 6 % FCS + 1 ml plasma from pre- and post-column samples. Cells supplemented with 1 ml unspiked plasma were used as baseline TNF-α production, which was substracted from each sample value. TNF-α was measured from the monocytes culture supernatant after 24 hour incubation, using an ELISA-kit according to manufacturer's instructions (Cell Sciences, Norwood, MA).

TF expression in monocytes was also evaluated from PBMC cultures treated for 5 hours and after removing non-adherent cells. The Imubind kit from American Diagnostica (Greenwich, CT) was used.

Analysis of activation of coagulation

Any thrombin formed as the result of activation of the coagulation cascade will be rapidly inactivated by antithrombin (AT) to form TAT complexes in the presence of heparin. This provides a sensitive and specific means to detect evidence of activation of coagulation cascade. Plasma samples collected were assayed for TAT complexes by ELISA (Enzyme Research Lab, South Bend, IN).

Analysis of consumption of coagulation factors
Possible consumption of coagulation factors was assessed by PT and APTT. PT assay was performed by incubating 50 µl of plasma at 37 ºC for 3 minutes. Then, 100 µl of Innovin - recombinant human Tissue Factor thromboplastin (Baxter Diagnostics Inc., Deerfield, IL), pre-warmed at 37 ºC was added to initiate clotting. APTT assay was performed by incubating 50 µl of plasma and 50 µl of APTT reagent (Organon Teknika, Durham, NC) at 37 ºC for 5 minutes. Then, 50 µl of 25 mM $CaCl_2$, pre-warmed at 37 ºC was added to initiate clotting. A semi-automated coagulation instrument (ST4, Diagnostica Stago, Parsipanny, NJ) was used.

Analysis of Fibrinogen
Fibrinogen concentration is measured using the enzyme Atroxin (Sigma, St Louis), which catalyzes the formation of fibrin from fibrinogen. A standard clotting curve is obtained from normal plasma.

Analysis of hemolysis and cell counts
Hemolysis was evaluated from the color of the plasma in pre- and post-samples. Blood cell count including erythrocytes, leukocytes with differential count, platelets and hematocrit were determined with an automated cell counter from pre-column and post-column citrated blood samples. Hematology profiles were done using a Hemavet cell counter (Model 850 FS, CDC Technologies Inc., Oxford, CT). Five samples were taken over the course of the experiment at about 4 minutes intervals via sample ports.

Analysis of complement activation
The ELISA test from Research Diagnostics (Flanders, NJ) was used to measure the level of C3a-desArg in pre- and post-column plasma samples.

Analysis of cell activation
Platelet and Leukocytes (monocytes and neutrophils) activation assays: Markers of cell activation previously described for the study of extracorporeal devices, were followed (Bosch et al., 1987; 1999). Platelet activation was assessed by an ELISA assay for plasma β-thromboglobulin (β-TG) (Diagnostica Stago, Parsippany, NJ) to detect degranulation. Monocyte activation was assessed by measuring TNF-α by ELISA. Monocytes were prepared by Ficoll separation of blood (5 ml), and removal of non-adherent cells as described above. Adherent cells were incubated in RPMI-1640 containing 3 % FCS. Each sample was tested in duplicate culture wells. The conditioned media was collected after 5 hours to measure TNF-α. This time point was found to be when the maximum TNF-α was measured in the conditioned media after stimulation with LPS (not shown). The TNF-α sandwich ELISA kit from Cell Sciences (Norwood, MA) was used. Conditioned media from each culture well was tested in duplicate in the ELISA test. Neutrophil activation was determined from whole blood samples by a specific ELISA for polymorphonuclear leukocytes/neutrophils (PMN)-Elastase (Cell Sciences).

4.2.3. Results

Bacterial toxins removal
ET can be reliably measured by the LAL assay. In addition, we opted to use a biological assay to assess the removal of ET and other bacterial compounds. Various effects of the later on PBMC have been reported. Since we had initially focused on ET we used monocytes, but we later used PBMC for PepG and bactDNA as other blood cells are known to play a role in TNF-α production, used as an indicator of inflammation. In addition, we used TF expression by monocytes as an indicator of procoagulant activity and a surrogate marker of removal of bacterial toxins. After assessing the removal of bacterial toxins in water (up to 95 % removal for PepG and up to 40 % for bactDNA), we first analyzed the removal of individual factors present in plasma or whole blood.

Moreover, after obtaining evidence of synergistic activity between some of these molecules in these assay systems, we measured the removal of combinations of these factors.

Table 7. Removal of ET from various bacterial species in plasma using a 1.5 ml column

Source of ET	ET removal by a capture resin (%)
Pseudomonas aeruginosa purified ET	20.38
Salmonella typhimurium purified ET	17.25
Escherichia coli O55:B5 purified ET	43.95
Escherichia coli O55:B5 culture supernatant	33.78
Pseudomonas maltophilia culture supernatant	53.40

ET removal

Variable structures and aggregation states of ET are likely to affect the removal efficiency. While monomers of ET have a molecular weight of 10-20 kDa, ET forms aggregates of 0.2 to 1 million Da. Large aggregates limit the accessibility of ET to the immobilized ligand. The aggregation state of ET is known to be different in size and mass between different bacterial species, which may partly explain the different removal efficiencies for different ET. LPS from different sources (Table 7) display different critical aggregation concentrations.

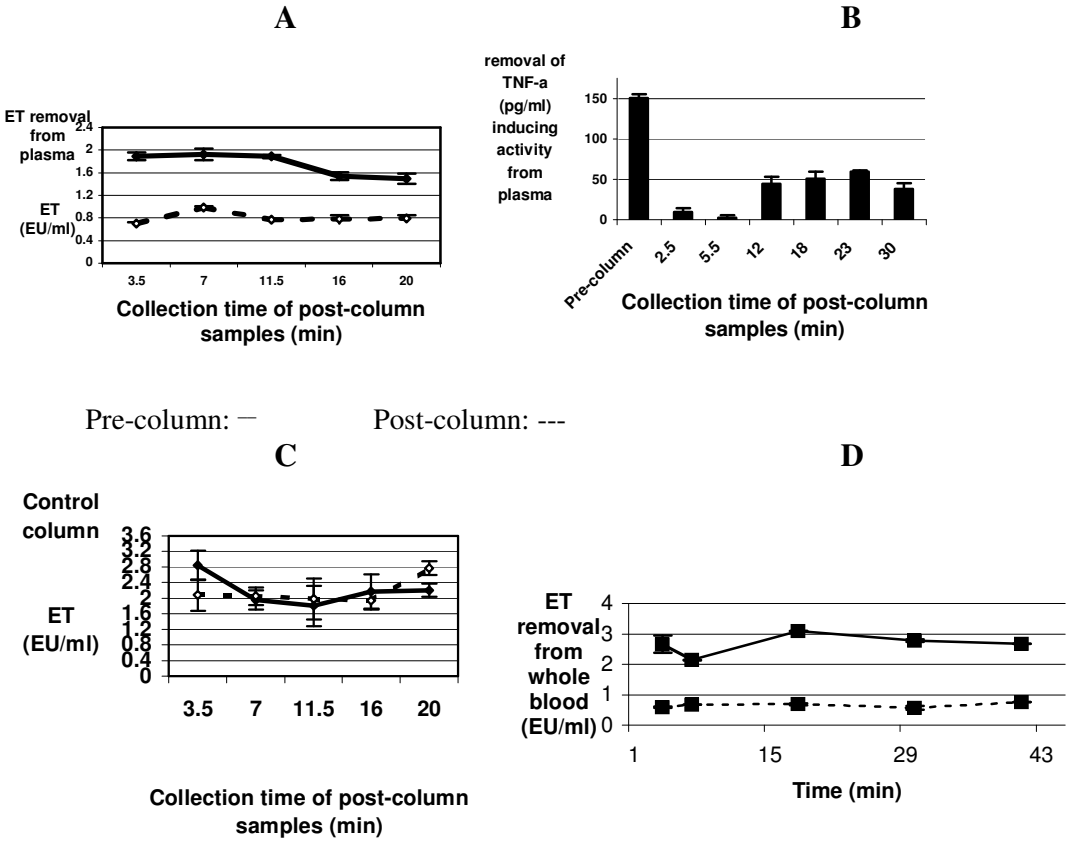


Figure 3. Time course of removal of *P. maltophilia* culture supernatant ET from plasma and whole blood, by the LAL assay and monocyte activation assay

ET was removed by 50 % from plasma (Figure 3, A) and 75 % from whole blood (Figure 3, D) using the LAL assay. This was accompanied by a >68% decrease in TNF-α (inducing activity in plasma (Figure 3, B). A control resin did not significantly bind ET (Figure 3, C).

Peptidoglycan removal

PepG initial concentration was 10 μg/ml. Horn et al. (Horn, 2000) discussed the structural homology between lipid A and PepG, which could explain the binding of PepG. PepG is recognized by the intracellular Nod proteins (Girardin et al., 2003a; Girardin et al., 2003b), which could explain why when PepG was spiked into blood, the TNF-α inducing activity in subsequent PBMC culture was much lower than when PepG was spiked into plasma. PepG was removed with 81% efficiency from whole blood (Figure 4, A) and 53 % from plasma (Figure 4, B).

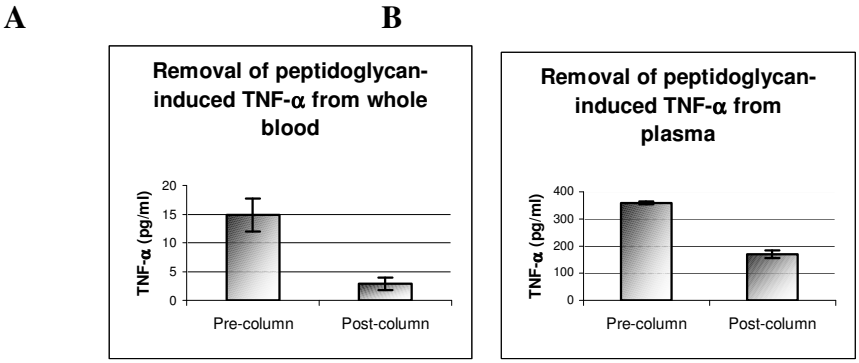


Figure 4. Removal of peptidoglycan from blood and plasma

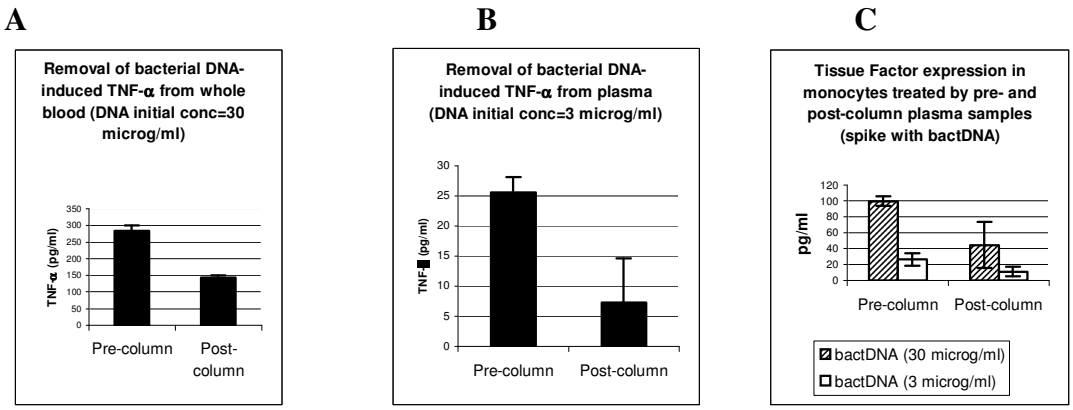


Figure 5. Bacterial DNA removal

Bacterial DNA removal

The decrease of TNF-α was 49% from whole blood (Figure 5, A) and 71% from plasma (Figure 5, B). Although the mechanism of binding was not elucidated, it is likely due to the polyanionic nature of DNA. No detectable TF expression was detected in untreated cells. No significant difference was measured in the amounts of total proteins in monocytes lysates between pre- and post-column samples. When TF was analyzed, running plasma spiked with DNA through the column induced a 55 and 59 % decrease in TF expression in monocytes (when bactDNA was present at 30 μg/ml and 3 μg/ml, respectively) (Figure 5, C).

Lipopeptide removal

We tested in a single experiment the removal of a synthetic analogue of lipoprotein, $Pam_3SerLysCys$. It was removed by 20 % when tested in the TNF-α induction assay.

Removal of bacterial toxins acting synergistically

It is known that microbial compounds synergize to induce TNF-α. Therefore, combinations of PepG, DNA and ET were applied to the resin at different concentrations. The TNF-α inducing activity and TF level in monocytes were measured.

Combination 1: PepG:10 µg/ml, DNA: 30 µg/ml, ET: 0.5 EU/ml; *combination 2*: PepG: 10 µg/ml DNA: 30 ng/ml ET: 0.5 EU/ml; *combination 3*: PepG 10 ng/ml DNA 30 µg/ml ET: 0.5 EU/ml; *combination 4*: PepG 10ng/ml DNA 30 ng/ml ET: 0.5 EU/ml. Combination#4 was not tested in whole blood.

Removal of bacterial toxins acting synergistically

It is known that microbial compounds synergize to induce TNF-α. Therefore, combinations of PepG, DNA and ET were applied to the resin at different concentrations. The TNF-α inducing activity and TF level in monocytes were measured (Figure 6, A, B, C).

Combination 1: PepG:10 µg/ml DNA: 30 µg/ml ET: 0.5 EU/ml; *combination 2*: PepG: 10 µg/ml DNA: 30 ng/ml ET: 0.5 EU/ml; *combination 3*: PepG 10 ng/ml DNA 30 µg/ml ET: 0.5 EU/ml; *combination 4*: PepG 10ng/ml DNA 30 ng/ml ET: 0.5 EU/ml. Combination#4 was not tested in whole blood.

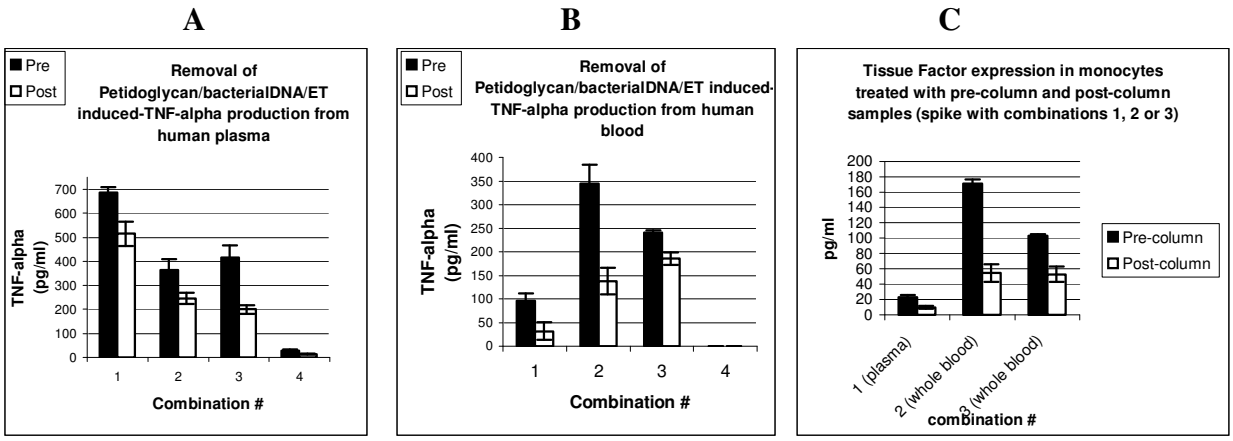
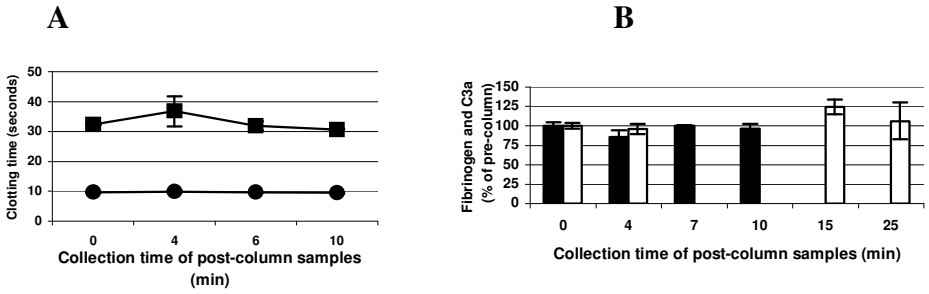


Figure 6. Peptidoglycan, Bacterial DNA and Endotoxin removal from blood and plasma

The TNF-α and TF inducing activity were significantly decreased for all the tested combinations.

Hemocompatibility

The affinity resin was tested for its hemocompatibility with plasma and whole blood (Figure 7, A, B, C, D, E).



PT (●); APTT (■) Fibrinogen ■; C3a □

Figure 7. Hemocompatibility with plasma and whole blood

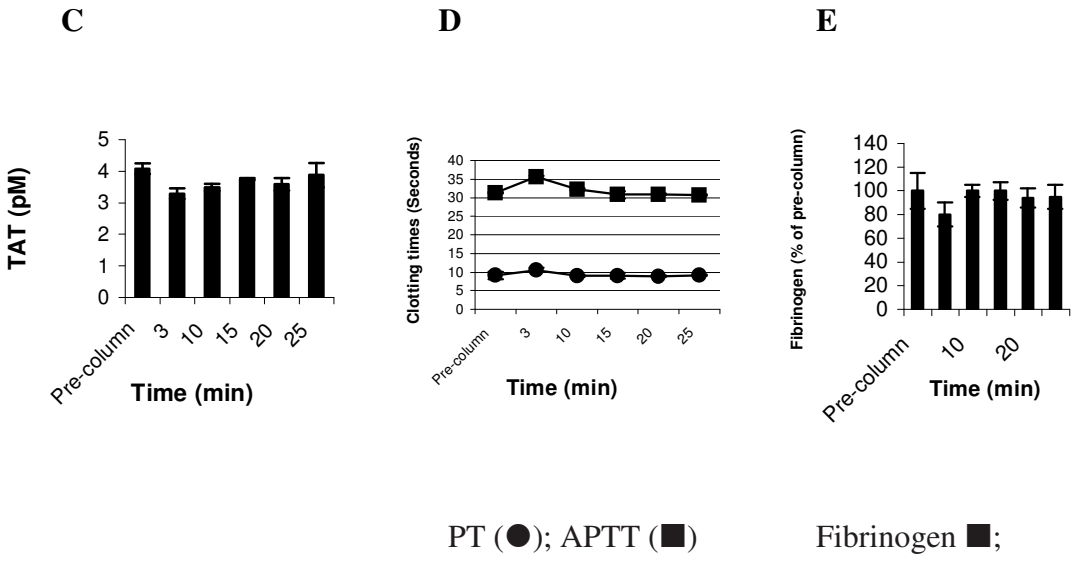


Figure 7. Hemocompatibility with plasma and whole blood

PT and APTT were measured in plasma prepared from whole blood and were unchanged after contact of plasma or whole blood with the resin. There was no loss of coagulation factors. Fibrinogen or C3a in pre-and post-samples was not changed by the flow of plasma or whole blood through the resin.

Analysis of complement and cell activation

The effect of the capture resin was analyzed in parallel with the effect of an empty column (Figure 8, A, B, C).

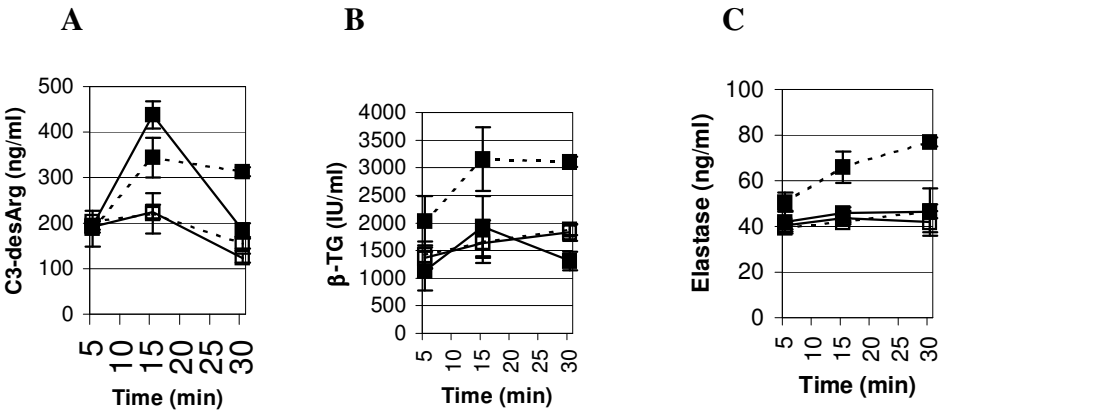


Figure 8. Complement and cell activation results

These data indicate no induction of coagulation, no loss of fibrinogen, no binding of coagulation factors, no activation of complement in plasma and minor activation (maximum 37 % compared to empty column) in blood, small activation of platelets and neutrophils (maximum 65 % and 66 % compared to empty column),

No hemolysis occurred after the blood ran through the column. For each time point, blood counts were performed, and no significant difference was measured between pre- and post-column samples or between time points. The following table 8 represents the average of the 5 time points (Table 8).

Table 8. Cell counts pre- and post column from whole blood

	Pre-column	**Post-column**		**Pre-column**	**Post-column**
Leukocytes			**Thrombocytes**		
WBC(K/µl)	7.84+/-0.61	7.74+/0.39	PLT (K/µl)	105.01+/-18.42	111.47+/-10.76
NE (K/µl)	4.35+/-0.62	3.96+/0.34	MPV (fl)	7.92+/0.34	8.03+/0.29
LY (K/µl)	1.74+/-0.13	1.88+/0.07	**Erythrocytes**	4.97+/0.28	4.34+/0.37
MO (K/µl)	0.56+/-0.05	0.63+/0.08	RBC (M/µl)	14.95+/0.79	13.59+/0.56
EO (K/µl)	1.10+/-0.23	1.23+/0.23	HB (g/dl)	42.93+/2.52	37.43+/2.97
BA (K/µl)	0.09+/-0.02	0.05+/0.02	HCT (%)	94.83+/0.57	95.03+/1.45
NE (%)	60.49+/0.59	56.09+/2.69	MCV (fl)	33.14+/0.96	34.62+/2.69
LY (%)	24.71+/1.04	26.71+/1.16	MCH (pg)	38.43+/1.16	40.04+/2.68
MO (%)	7.93+/-0.76	9.07+/-1.17	MCHC (g/dl)	18.38+/0.21	18.39+/0.14
EO (%)	15.75+/3.51	17.44+/3.04	RDW (%)		
BA (%)	1.12 +/- .23	0.68+/-0.25			

4.2.4. Discussion

Efficient ET, PepG and bactDNA removal from human plasma and whole blood was achieved.
Opal *et al.* (1999) found that survivors of septic shock had on average half the amount of ET than non-survivors in the systemic circulation at the onset of severe sepsis and septic shock. The median level ET level in survivors was 230 pg/ml and the median level for non-survivors was 515 pg/ml. Although this level may vary during the course of the septic episode and this is not directly indicative of the clinical benefit of ET removal, the difference between survivors and non-survivors levels is within the range of reduction achieved by our device. The binding capacity of the resin was such that *in vitro*, for 41 minutes at 0.6 ml/min, the resin removed ET with a consistent and substantial efficiency. It is important to note that there was no indication of saturation of the column. On the current basis, the volume of blood for an average adult being 5 liters, a maximum of 2 columns of about 150 ml would be sufficient to treat the entire volume of blood, while maintaining a reasonably low extracorporeal volume in a clinical size device. In this setting, the duration of the procedure would be around 1 hour and 20 minutes using a flow rate of 100 ml/min.

No haemostatic change (inhibition or activation of coagulation) from plasma or whole blood was measured. Minimal cell activation occurred, less than by other clinical devices. It is common for hemodialysis membranes routinely used in the clinic to elicit comparable or greater levels of activation (Bosch et al., 1987). In addition, the level of C3a also rose 37-fold in the DALI extracorporeal system (Bosch et al., 1999). The highest elastase level measured here (78 ng/ml) is still within the range found in healthy individuals. Transient increases in β-TG release in whole blood through the DALI extracorporeal system, followed by a return to initial values have been reported. Bosch et al. (1999) have measured up to 73 % increase in β-TG release in otherwise uneventful clinical sessions. Platelets were also activated by 31 % and neutrophils by 600 % by the avidin-agarose adsorption device for anti-tumor radiolabeled antibodies used in the clinic (Bosch et al., 2000). No cell loss, blood clot or hemolysis in whole blood was observed.

4.3. EXPERIMENT 3: BINDING OF MYCOPLASMAS TO SOLID PHASE ADSORBENTS

4.3.1. Introduction

Infection with pathogenic mycoplasmas can significantly affect livestock production. They have a pronounced affinity for mucosal tissues and consequently show a predilection for the respiratory system, mammary gland, serous membranes and urogenital tract (Frey, 2002). In cattle, the most important disease is pleuropneumonia caused by *M. mycoides subsp. mycoides SC*, pneumoarthritis and mastitis caused by *M. bovis* (Pfützner and Sachse, 1996). In sheep and goat, infectious agalactia can be found frequently, caused by *M. agalactiae*. In swine, *M. hyopneumoniae* causes pneumonia (Ross, 1990), while in chicken and turkey *M. gallisepticum* and *M. synoviae* are responsible for respiratory disease and arthritis (Kleven, 1990). One of the methods to control economic losses associated with mycoplasmal diseases is vaccination (Elfaki et al., 1993; Stipkovits et al., 2004). In order to produce an effective vaccine, the concentration of mycoplasma cells in a vaccine dose is important. The concentration of mycoplasmas by filtration is plagued by the problem of fouling of the ultrafilters and ultracentrifugation is impractical on an industrial scale.

Over the past decades, cell culture has become an indispensable tool for modern biomedical research. With the increased use of cell cultures, the need to address mycoplasmal contamination of media components has become ever more significant. While bacterial and fungal infections of cell cultures are relatively easy to detect, to prevent and to control, contamination with mycoplasmas represents a much bigger problem in terms of incidence, detectability, prevention, and eradication attempts. It was estimated that between 5 to 35% of cell cultures in current use are infected with mycoplasma. The sources of mycoplasmal contamination in tissue culture are the animal serum and enzymes of animal origin (Rottem and Barile, 1993).

Numerous pathogens utilize surface receptors capable of recognizing host cell surface glucoseaminoglycans, which are targeted during the coloniziation of host organisms. Sulfated carbohydrate polymers mimic cell surface glucoseaminoglycans and thus may provide a modality to bind such pathogens. These polymers could thus become the basis for the removal of various pathogens including mycoplasmas.

The pMGA lipoprotein is the major antigen of *M. gallisepticum* (Markham et al., 1992). This lipoprotein appears to be one of the hemagglutinins of this organism. Similar lipoproteins are present in other mycoplasmas, such as *M. hyorhinis* (Rosengarten and Wise, 1990), *M. arthritidis* (Washburn et al., 1998), *M. hominis* (Olson et al., 1991) and *M. fermentans* (Theiss and Wise, 1997). *M. fermentans* through its M161Ag surface lipoprotein serves as a potent cytokine inducer for monocytes/macrophages, maturing dendritic cells and activates host complement. The 2-kDa macrophage activating lipopeptide-2 (MALP-2) has been identified as a ligand for Toll-like receptor-2 (Nishiguchi et al., 2001; Takeuchi et al., 2000).

We have investigated several different approaches for capturing mycoplasma on chromatography resins. The cell membrane of mycoplasma contains several alkali-labile glycophospholipids, one of them is 6'-0 phosphocholine-alpha-glycopyranosyl-(1,3)-1,2-diacyl-sn-glycerol (GGPLI), the other is GGPLIII, the major GGPL of *M. fermentans* (Matsuda et al., 1997; Ben-Menachem et al., 2001). MALP-2 is a macrophage activating 2 kDa lipopeptide (Nishiguchi et al., 2001), that expresses potent endotoxin-like activity and whose lethal toxicity is comparable to LPS. MALP-2 is available as a synthetic active stereoisomer (Mulhradt et al., 1997). MALP-2 appears to have structural features in common to LPS such as a palmitoyloxy hydrophobic domain that can provide a basis for capturing it.

These phospho-amino membrane components could potentially be captured by functional groups with hydrophobic characteristics attached to solid phase adsorbents, such as ClarEtox (Sterogene

Bioseparations, Inc. Carlsbad, CA) where the capture ligand is based on the lipid structures binding to TLR-2 and TLR-4, such as of lipoteichoic acid from Gram-positive bacteria and endotoxin from Gram-negative bacteria (lipopolysaccharide). Different bacterial components (lipoteichoic acid, lipoarabinomannan, peptidoglycan etc.) bind through the TLR-2 similarly to MALP-2 of *M. fermentans*. ClarEtox also captures lipoteichoic acid, lipoarabinomannan and peptidoglycan, and even though mycoplasmas do not contain such components, the binding of mycoplasma membrane components to TLR-2 indicates that similar structural components are present in the mycoplasma membrane. The purpose of our study was to examine if these resins could capture mycoplasmas. We have used several mycoplasma strains that represent a wide variety of origins and characteristics.

4.3.2. Materials and Methods

Mycoplasma culture
Glucose containing Medium B (Erno and Stipkovits, 1973) was used for the growth of glucose fermenting *M. fermentans, M. synoviae, M. gallisepticum* as well as *M. bovis* and likewise arginine-containing medium was used for culturing arginine splitting *M. hominis, M. buccale* and *M. arthritidis* strains. Ten ml medium was inoculated with 0.5 ml of inoculum and incubated for 48 hours. Subsequently, culture was spun down at 10,000x*g* for 10 minutes. Sediment was re-suspended and washed with sterile PBS (20 mM phosphate, 145 mM NaCl, pH 7.2) 3 times. Final sediment was re-suspended in 10ml sterile PBS and loaded onto 1ml columns. Prior to loading, mycoplasma count was determined by serial dilution (1:10) and plating on agar plates. Plates were incubated for 7 days at 37^{o}C in a 5% CO_2 incubator.

Column preparation
One ml bed volume glass columns were packed with various resins and stored at 4^{o}C in 20% ethanol. The columns were sanitized in 1M NaOH overnight. Next day the column was washed with sterile deionized water until the pH changed to neutral, and equilibrated with five bed volumes of sterile PBS.

Cell culture
A 100 ml sample of a cell culture-tested batch of fetal bovine serum was run through a 5 ml Acticlean Etox column. This batch of ligand-treated serum was tested against Chinese hamster ovary (CHO), VERO (African green monkey kidney cell), and Sp2 (mouse myeloma) cell lines.

Binding of Mycoplasmas to chromatography resins
Mycoplasma suspensions were incubated with different resins (Acticlean Etox, ClarEtox, Heparin-Actigel, Sulfated Hiflow and SulfEtox) for up to 20 minutes under static conditions. The static binding capacity per ml resin is the difference between the mycoplasma load and the unbound mycoplasma in the supernatant. Pre and post column samples were transferred to sterile tubes with PBS and a serial of 1:10 dilutions were made and 10µl aliquots from each dilution were plated on agar plates. The plates were read for up to 7 days. The samples were prepared in parallels of two.

Time course of binding of various mycoplasmas to ActiClean Etox resin
The binding of the mycoplasmas was tested under dynamic conditions. Mycoplasma suspensions prepared as described above were applied at a flow rate of 1 column volume per minute. The binding capacity of the column is the difference between the initial CFU/ml (colony forming unit) loaded on the column and the CFU/ml in the flow-through. Pre and post column samples were collected at 1 minute and 5 minutes aseptically. Samples were assayed for mycoplasma count by serial dilution.

Binding of *M. fermentans* to various chromatography resins

Three ml of *M. fermentas* culture (48 hours, 2 x 10^9 CFU/ml) was added to each containing chromatography resin column containing Heparin Actigel, Sulfated HiFlow, Acticlean Etox, SulfEtox, ClarEtox, Dextran sulfate silica and shaken for 20 minutes. The resin supernatants were collected into sterile tubes. Three ml of PBS was added to each resin and the supernatants were collected into the same tube. A series of 10-fold dilution of the supernatants were prepared with glucose containing growth medium ranging from 10^{-1} to 10^{-7} in 2 ml volume. The tubes were incubated at 37^{o}C for 48 hours and the colour change in the tubes was recorded. As a control, 3 ml PBS was added to the culture and serial 10-fold dilutions were prepared and incubated along the samples.

The capture of mycoplasmas from Fetal Bovine Serum by ActiClean Etox resin

The binding of three mycoplasmas (*M. bovis, M. hominis, M. buccale)* to Acticlean Etox was tested under dynamic conditions. Mycoplasma cultures were prepared as described above. Subsequently, culture was spun down at 10,000 x *g* for 10 minutes. Sediment was re-suspended and washed with sterile PBS (20 mM phosphate, 0.145 M NaCl, pH 7.2) 3 times. Final sediments were re-suspended in 10ml Fetal Bovine Serum (FBS) and applied to 1 ml ActiClean Etox at a flow rate of 1 column volume per minute. Pre- and post-column samples were collected at every 5 minutes aseptically. Samples were assayed for mycoplasma count by serial dilution.

4.3.3. Results

In Table 9, the binding efficiency of various resins for several mycoplasma strains is presented. In case of *M. hominis, M. buccale* and *M. bovis*, all 4 resins were efficient, reducing mycoplasma content by 5-7 logs, while in case of *M. fermentans* all resins except Sulfated Hiflow gave 6 logs of mycoplasma reduction.

Table 9. Binding of Mycoplasmas to chromatography resins

Mycoplasma strains	Initial CFU/ml	ClarEtox supernatant CFU/ml	Sulfated Hiflow supernatant CFU/ml	SulfEtox supernatant CFU/ml	ActiClean Etox supernatant CFU/ml
M. hominis	1 x 10^5	0	0	0	0
M. buccale	1 x 10^5	0	0	0	0
M. bovis	1 x 10^7	0	0	0	0

Table 10 shows that some mycoplasmas are eliminated from the treated suspension by 3-8 logs. At very high concentrations, the reduction of mycoplasma concentration is about 6-8 logs. After one column volume, the flow-through contained no mycoplasmas and this was maintained even at five column volumes. At five column volumes, the resins had bound 5 x 10^3 to 5 x 1.2 x 10^8 cfu.

Table 10. Time course of binding of various mycoplasmas to ActiClean Etox resin

Mycoplasma strain	Initial CFU/ml	Supernatant after 1 minute adsorption CFU/ml	Supernatant after 5 minutes adsorption CFU/ml
M. hominis	1×10^5	0	0
M. buccale	3×10^5	0	0
M. gallisepticum	1.2×10^8	0	0
M. fermentans	2×10^6	0	0
M. bovis	1×10^3	0	0
M. arthritidis	1×10^5	0	0
M. synoviae	1×10^3	0	0

Table 11 shows that *M. fermentans* binds efficiently through its lipid moiety to ClarEtox, through the polysaccharide moiety to Acticlean Etox. It is also able to bind to sulfated polysaccharides such as Heparin-Actigel, Dextran sulfate-silica, Sulfated Hiflow and SulfEtox.

Table 11. Binding of *M. fermentans* to various chromatography resins

Resin	Flow-through after 48 hours of culture (color changing units per ml)
Heparin Actigel	10^4
Sulfated HiFlow	10^2
Acticlean Etox	10^2
SulfEtox	10^2
ClarEtox	10^2
Dextran sulfate silica	10^3
Control	10^7

The results reflect more efficient binding to homo-polysaccharide-based resins (Dextran sulfate-silica, Sulfated Hiflow and SulfEtox) over hetero-polysaccharide-based resin, such as Heparin-Actigel.

The results shown in Table 12 demonstrate that mycoplasmas, which are frequently found in tissue culture, can efficiently be captured using Acticlean Etox. We have also examined the effect of Acticlean Etox on the cell growth-promoting properties of fetal bovine serum on different cell lines. The results were within 5% of each other and the control. This suggests that the process did not change the cell growth-promoting properties of fetal bovine serum (not shown).

Table 12. The capture of mycoplasmas from Fetal Bovine Serum by ActiClean Etox resin

Mycoplasma strain	Initial CFU/ml	Supernatant after 5 minutes adsorption (CFU/ml)
M. hominis	1×10^5	0
M. buccale	1×10^5	0
M. bovis	1×10^3	0

4.3.4. Discussion

Many mycoplasma species contain phospholipids in the form of phosphatidylglycerol. It takes up 15-80% of the de novo synthesized lipids (Rottem, 1980). Glycolipids constitute a significant portion of membrane lipids. Lipoglycans represent a special type of lipopolysaccharides that are structurally composed of a long oligosaccharide chain covalently linked to diglyceride. They exhibit a variety of biological activities, e.g. immunogenicity and antigenic specificity specific adhesion to mammalian cells (Smith et al., 1976). ActiClean Etox binds LPS through the polysaccharide portion and ClarEtox through the lipid moieties. Since mycoplasmas bound efficiently to ActiClean Etox, it indirectly also confirmed that mycoplasma membranes contain endotoxin-like molecules (Galanos et al., 2000). The efficient binding of the mycoplasmas to ClarEtox is in agreement with the literature that the membranes of mycoplasmas contain high portion of different lipoproteins and lipopeptides (Smith et al., 1976). M. fermentans MALP-2 also has a similar structure to some of the pyrogenic molecules, such as lipoteichoic acid, since both binds to TLR-2. MALP-2 lipid portion that imparts the macrophage stimulatory activity has a structural similarity to that seen in LPS endotoxin from Gram-negative bacteria. In both types of compounds the lipid moiety, in the case of LPS, the Lipid A, for mycoplasma lipoprotein the diacylated N-terminus, is responsible for the macrophage stimulatory activity, whereas the rest of the molecule provides antigenic variation (Brade et al., 1999). ClarEtox was designed to bind endotoxin (LPS), lipoteichoic acid and phosholipids (Amoureux et al., 2004).

Invasion of mycoplasmas is associated with adhesion as well as host cells receptors that mediate the specific interaction of the mycoplasmas with the host cells. It is likely that surface proteins and lipids that facilitate the adhesion process will have an effect on invasion (Athamna et al., 1996; Zhang et al., 1994; Olson and Gilbert, 1993; Krivan et al., 1989). It has been shown that bacterial and viral invasion is based on the ability of several pathogens to attach to fibronectin or sulfated polysaccharides (Christensen et al., 2001). Fibronectin binding activity was detected in case *M. penetrans* (Giron et al., 1996). This organism binds selectively immobilized fibronectin and a 65 kDa fibronectin-binding protein was identified in this mycoplasma. We have tested several chromatographic resins based on different sulfated polysaccharide structures, such as Heparin-Actigel, Dextran sulfate-silica, Sulfated Hiflow and SulfEtox. The results reflect more efficient binding to homo-polysaccharide-based resins (Dextran sulfate-silica, Sulfated Hiflow and SulfEtox) over the hetero-polysaccharides-based resin, such as Heparin-Actigel.

The results indicate that chromatographic resin capture could be a new approach to eliminate mycoplasmas from cell culture reagents. It could replace ultrafiltration, which is difficult and slow considering the small size of the mycoplasmas and the viscosity of serum. This method could be also useful for the concentration of antigens, upon the development of specific recovery conditions.

4.4. EXPERIMENT 4: PEPTIDOGLYCAN AND BACTERIAL DNA SYNERGISTICALLY INDUCE IMMUNE RESPONSE

4.4.1. Introduction

Microbial components are key activators of the innate immune system. They were found to induce TNF-α and TF. Lipopolysaccharide is known to play a critical role in inducing inflammation. However, other TLRs ligands have been recognized to be involved (Akira and Hemmi, 2003). PepG, a main component of bacterial cell walls, can activate leukocytes, generate pro-inflammatory cytokines and cause systemic inflammatory response syndrome through the activation of Nod2. PepG was also shown to mediate platelet aggregation in *S. aureus* septicemia, and induce TF in monocytes and display pro-coagulant activity (Mattson et al., 1993; Kessler et al., 1991). BactDNA induces the production of TNF-α in macrophages through TLR-9 *in vitro*. BactDNA leads to inflammation, septic shock and death in sensitive mice (Sparwasser et al., 1997). Because of the cooperation between TLRs and the common denominators downstream of TLR activation, synergistic effects between microbial pathogenic compounds have been observed. Whereas Lipoteichoic acid can cause only moderate hypotension, PepG co-administered with LTA induces multiorgan dysfunction and death in rats (De Kimpe et al., 1995; Kengatharan et al., 1998). BactDNA increases the toxicity of LPS in mice. Synergistic effects between agonists of TLRs and Nod receptors, such as bactDNA and PepG, have not been reported. Moreover, although IL-1, induced by bacterial toxins including bactDNA, has been shown to induce TF in endothelial cells (Bevilacqua et al., 1984; Nawroth et al., 1986), the effect of bactDNA on the regulation of TF in monocytes has not been described.

The goal of this study was to investigate the effect of bactDNA and PepG on the induction of a marker of the innate immune system such as inflammation and TNF-α in PBMC, and of a coagulation marker, TF, in monocytes We also wanted to test the hypothesis that bactDNA and PepG can exert synergistic effects and determine the effective concentration of these PRR agonist molecules for further *in vivo* studies using microparticles.

4.4.2. Materials and Methods

TNF-α induction assay

TNF-α secretion was measured in PBMC, prepared from peripheral blood (Amoureux et al., 2004) and treated with PepG (Sigma, St Louis, MI), bactDNA (KM Biomedicals, Aurora, OH; LPS<0.2 EU/ml measured by the *Limulus* amoebcyte lysate assay (Amoureux et al., 2004), or both. The TNF-α concentration was measured by ELISA (Amoureux et al., 2004) from the culture supernatant after 5 hours incubation.

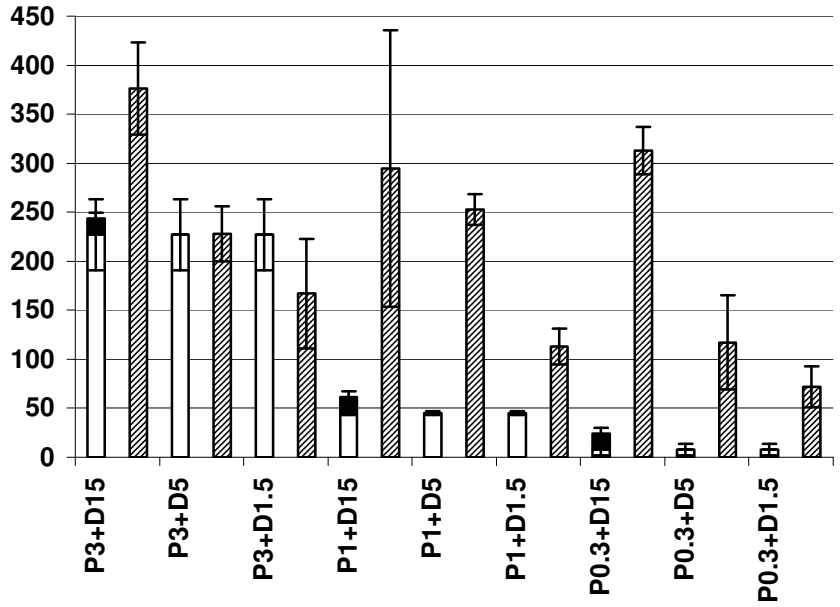
Tissue Factor induction assay

TF in monocytes was evaluated from the corresponding PBMC cultures used for TNF-α analysis. TF was extracted from the adherent cells with Triton-X100 as recommended by the manufacturer for TF ELISA (Imubind kit, American Diagnostica, Greenwich, CT).

4.4.3. Results

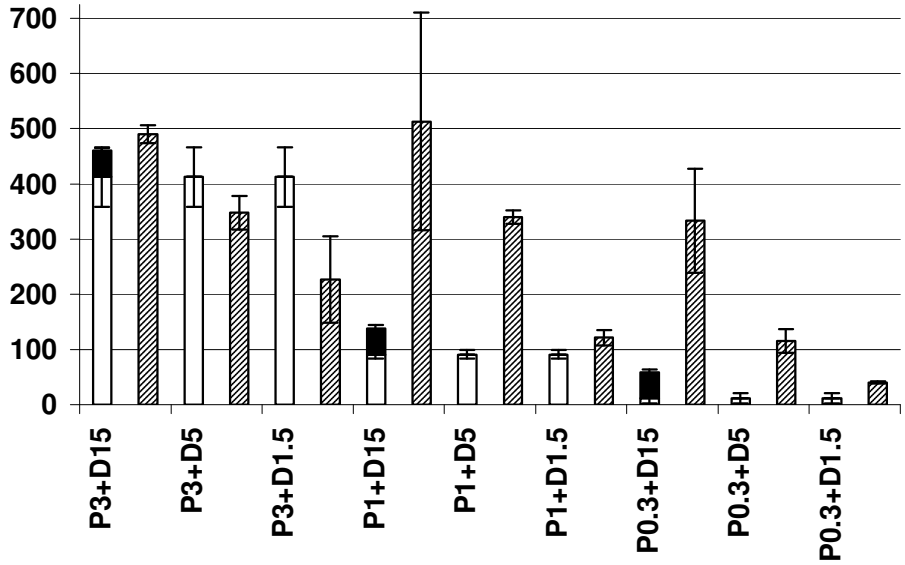
Both PepG and bactDNA applied individually to PBMC were able to induce TNF-α (Figure 9) and TF in a dose-response fashion (Figure 10). In presence of high concentration of PepG (3 μg/ml), adding bactDNA did not generate more TNF-α or TF, except at 15μg/ml bactDNA for TNF-α. However, when lower concentrations of PepG were used, treatment with bactDNA and PepG indicated that these 2 components acted in synergy, even when bactDNA alone did not induce

detectable TNF-α or TF by itself. The induction of TNF-α and TF were up to 15-and 10-fold higher, respectively, when PepG and bactDNA were present in conjunction, than when adding the effect of the 2 compounds alone.



Peptidoglycan –white bar, bacterial DNA-black bar, both applied simultaneously-hatched bar

Figure 9. TNF-α secretion by monocytes



Peptidoglycan –white bar, bacterial DNA-black bar, both applied simultaneously-hatched bar

Figure 10. Monocytic Tissue Factor concentrations

4.4.4. Discussion

These results demonstrate the concomitant induction of TNF-α and TF by PepG and bactDNA in PBMC, and a synergistic effect between the two molecules. Although the molecular mechanism for this synergy remains to be elucidated, it could involve direct effects on common downstream signaling pathways, or indirect effects mediated by secreted compounds following PepG or bactDNA action.

The increase in TF expression on monocytes by bactDNA is a new finding and may have significance in disseminated intravascular coagulation. An increase in TF present on circulating monocytes (Giesen and Nemerson, 2000), elicited by PepG and/or bactDNA could have local as well as systemic effects. TNF-α is an important early mediator of host responses to pyrogens. These results are also significant in as much as the cross talk between inflammation and coagulation is an aspect of sepsis pathology to consider in the quest of an effective therapy. The synergistic effects of bactDNA and PepG also have implications for the pathogenesis of sepsis, where each molecule, although present at low concentrations *in vivo*, is likely to amplify the effect of another one.

We have also determined the effective concentration range of the PRR agonist molecules. This information is utilized in the subsequent experiments.

4.5. EXPERIMENT 5: IMMUNE MODULATION OF MYCOPLASMA – HOST INTERACTION

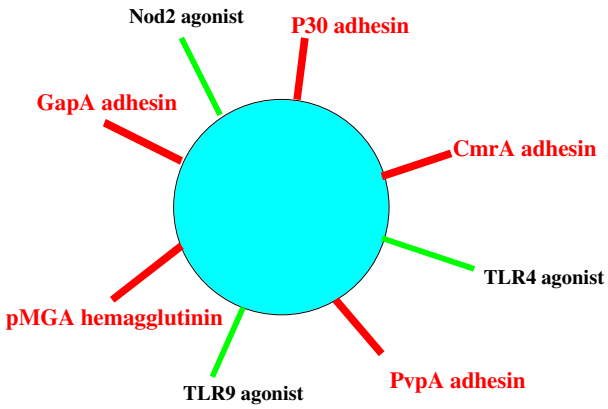
4.5.1. Introduction

The aim of the following experiments is to investigate the mycoplasma-host interaction and to influence the innate and adaptive immune response to *M. gallisepticum* infection.

It has been shown that chickens have TLRs for pathogen recognition (Fukui et al., 2001). Mycoplasmas interact with TLRs on different cells and hide intra-cellular and induce chronic infection (Shimizu et al., 2004; Stipkovits, 2001). Intracellular pathogens require a robust Th1 response for clearance (Dabbagh et al., 2002; Ichikawa et al., 2002) The interaction of mycoplasmas with dendritic cells TLRs determines Th1 vs. Th2 balance. We have utilized the function and signaling mechanism of PRR, such as TLR agonists in regulating the immune response to *M. gallisepticum*. *M. gallisepticum* antigens along with TLR and other PRR agonists immobilized to the surface of microparticles. These microparticles were used to induce effective innate and adaptive immune response. The covalent linkage between antigen and the particles assures that the same antigen-processing cells take up both the TLR agonist adjuvant and the antigen. Also, the size of the agarose micro-particles (<5µm) makes them suitable for their entry into the Peyer's patches. *M. gallisepticum* infection has been used as a model system to demonstrate the feasibility of this technology. *In vitro* studies using PBMC and DCs, and animal challenge experiments have been performed.

Outline of the concept
Microparticle in the size of a pathogen is used as a carrier.
On the surface of the particle immobilized several pathogen representative molecules.
On the surface of the particle co-immobilized different PRR agonists to influence the Th1/Th2 balance.

We have examined this concept in chickens with *M. gallisepticum*



"Mycoplasma mimicking" microparticle

.

4.5.2. Materials and Methods

Preparation of microparticles
Agarose microparticles in the 1-10µm ranges have been produced by Sterogene Bioseparations, Inc. (Carlsbad, CA) and tested using a Saturn DigiSizer 5200 (Micromeritis Instrument Corp).

Culturing of *Mycoplasma gallisepticum* (MG)

One ml of mycoplasma growth medium (BEG) was added to the lyophilized form of *M. gallisepticum* (K781R-16P) and 100 µl of this suspension was placed into 1 ml BEG medium and placed in the incubator at 37°C. The remaining stock was frozen and stored at –70°C. The growth of the mycoplasma was monitored by the colour change. The mycoplasma was also plated on agar plates and then the colonies were counted. Ten to fifteen litres of mycoplasma culture was grown by transferring the infected cultures into fresh media. Subsequently, the culture was centrifuged at 10,000x*g* for 30 min. Washing of the pellet with PBS was performed (20 min at 10,000xg) until the OD_{280} of the supernatant was below 0.2. The pellet was re-suspended in 20ml of PBS.

Preparation of immunoaffinity column for MG antigen purification

Step 1.

To 64 ml of serum from *M.gallisepticum* infected chickens, 128 ml DI water was added. The pH of the solution was adjusted to 4.5 using glacial acetic acid. Sixty four ml of CAP-8 Precipitating Solution (Sterogene Bioseparations, Inc. Carlsbad, CA) was added to the mixture. The solution was stirred for 30 minutes at room temperature and then centrifuged at 10,000xg for 15 minutes. The supernatant was decanted into a container and the pellet washed with 20 ml of 20 mM Na acetate, pH 4.8 buffer. The supernatant was filtered by using 0.22µm syringe filter.

Step 2.

SP Thruput Plus cation exchange resin, 10 ml (Sterogene Bioseparations, Inc. Carlsbad, CA) was suspended in 30 ml of 1M NaOH for 10 minutes and then washed with DI water to neutrality. Subsequently, it was washed with 100 ml of 0.5M sodium acetate, pH 4.8 and with 200 ml of DI water. The resin was equilibrated with 150 ml of 20 mM sodium acetate, pH 4.8 and packed into a column by using 20 mM acetate, pH 4.8. The supernatant from Step 1 was loaded onto the column at 3ml/min flow rate and the column washed with 20 mM sodium acetate, pH 4.8. The flowthorough and wash were combined. The OD_{280} was measured against 20 mM sodium acetate, pH 4.8. The column was eluted with 50 mM sodium phosphate, 300 mM NaCl, pH 8.0 and OD_{280} of the eluent was measured.

Step 3.

To 20 ml of Actigel ALD activated resin (Sterogene Bioseparations, Carlsbad, CA) the purified anti-*M.gallisepticum* chicken IgG solution (eluent) was added at 10 mg/ml followed by 10.5 ml of 1M sodium cyanoborohydride (ALD Coupling Solution, Sterogene Bioseparations, Carlsbad, CA). The suspension was gently mixed for 20 hours at 2-8°C followed by an extensive wash with DI water. The resin was stored in PBS, at pH 7.0 and 2-8°C.

Purification of MG antigens

***M. gallisepticum* surface antigens** (Figure 11)

Gene	Product	Molecular Weight
mgc1(gapA)	GapA adhesin	105 kDa
mgc2	P30 adhesin	32.7 kDa
mgc3(cmrA)	CmrA adhesin	116 kDa
pvpA	PvpA adhesin (variable)	40 kDa
pMGA	hemagglutinin (variable)	67 kDa

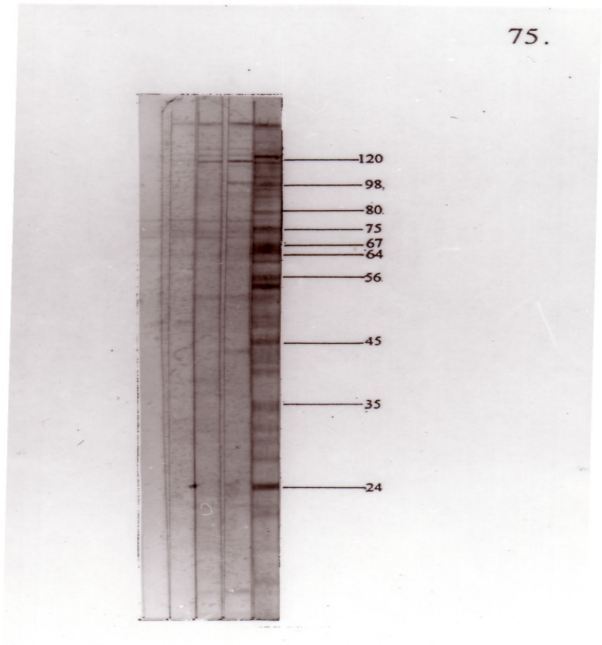


Figure 11. Western Blot of *M. gallisepticum* antigens

Step 1.
To 20 ml of washed MG concentrate, 0.2g Mega-10 detergent was added and mixed for 20 hour at room temperature. After 20 hours incubation, 1ml of Triton-X 100 was also added to the suspension and mixing continued for another hour at room temperature. Subsequently, it was centrifuged at 10,000xg for 10 minutes. The supernatant was separated from the pellet and 200 ml of PBS, pH 7.2 was added to the supernatant. The MG protein solution was kept at 2-8°C for 5 days.
Step 2.
The detergent treated antigen was purified by a modification of the method described by Szathmary et al., (1991). In QuickPure System instead of anti-kappa chain antibody capture ligand, the capture ligand is anti-MG-chicken IgG immobilized to Actigel. A 20 ml anti-MG-chicken IgG Actigel column was equilibrated with 100 ml of PBS, pH 7.2 at 3ml/min flow rate. The MG-protein solution was loaded onto the column at 8-15ml/min. The flow-through was collected. The column was washed with 200 ml of PBS, pH 7.2 at 8-15ml/min and eluted with 20 ml of 0.1M Citric acid, pH 2.5 at the same flow rate. The pH of the eluate was immediately adjusted to 7.2 by using 2M Tris. This purification was repeated until the flow-through exhausted. All eluates were combined and concentrated. The concentrated solution was dialyzed against 5L of PBS, pH 7.2 at 2-8°C overnight. Bradford's protein assay was performed to determine the concentration of purified antigen.

Treatment of MG antigens

1. Endoglycosidase H digestion
To 53.6 ml of antigens (about 1.5 mg) 2.5 Units of enzyme was added and incubated at 37^{o}C overnight. The next day the mixture was passed through a chilled Mannan-Actigel column (5 ml) and the flow through was collected. This sample was designated Endo H Antigens.

2. Periodate treatment
To 53.6 ml of antigens (about 1.5 mg) solid sodium periodate was added to a final concentration of 15mM and after mixing kept chilled for 1 hour. Glycerin in a two-fold molar access was added and the sample incubated for another hour. Dialysis against PBS was performed overnight. The dialyzed sample is designated Periodate Antigens.

3. Removal of ConA binding antigens
Purified antigens (about 1.5 mg in 53.6 ml) were passed through a 2 ml of Con A-Actigel column. The flow-through was collected. This sample is designated Con A flowthrough. The bound antigens were eluted with 1M alpha-methyl mannoglucoside in 50mM TRIS, pH 9.5. This sample is designated Con A elution.

4. MG antigen deacylation
To 27ml of antigens (about 0.65 mg) 8 ml of 1 M NaOH and 10mg of pentadecanoic acid were added and the solution was gently agitated at 70^{0}C for 45min. The pH was then adjusted to 8.0 and the precipitate removed by centrifugation at 3,000 rpm for 5min. This sample was designated Deacylated Antigens.

Activation of microparticles and the coupling of MG antigens
Particles were activated by cyanogen bromide (CNBr) activation method. Briefly, to 30 ml of agarose microparticles, 30 ml of 2M sodium carbonate solution was added and kept in an ice bath for 3-5 minutes without mixing. Then, 1.5g CNBr was weighed and dissolved into 9 ml acetonitrile. Immediately, the CNBr solution was added to the resin mixture and vigorously mixed on an ice bath for 2 minutes. Subsequently, it was washed with 500 ml of ice-cold water by spinning down at 9,000xg, 2°C for 5 minutes. To 15mls of CNBr activated microparticles, the purified MG antigen solution was added at pH 8.0. The solution was gently mixed at 2-8°C for 20 hours. The

supernatant was separated by centrifugation and the resin washed with 10 bed volumes of DI water. The coupled resin was stored in LAL water at 2-8°C. Bradford's protein assay was used to measure unbound protein in supernatant. The antigens were immobilized at the concentration of 50µg/0.2ml microparticles.

Immobilization of PRR agonists to the microparticles

Agonist	**Pathogen Recognition Receptor (PRR)**
LPS	TLR-4
Bacterial DNA	TLR-9
Peptidoglycan	Nod2 or TLR-2
Poly I:C	TLR3

TLR agonists 2, 4 and 9 were mixed together at 2µg/0.2 ml resin, 10µg/0.2 ml resin and 2µg/0.2 ml resin, respectively in 0.1 M $NaHCO_3$ at pH 8 and immobilized to CNBr-activated microparticles according to above. The reaction was allowed to run overnight at 2-8^{0}C. The supernatant was separated by centrifugation and resin washed thoroughly with LAL grade water which is also the storage medium.

Immobilization of MG antigen with the combination of PRR agonists to the microparticles
MG antigens were coupled under conditions described above for 1h. Subsequently, the TLR agonist mixture, described above was added and the reaction was allowed to proceed overnight at 2-8^{0}C. The supernatant was separated by centrifugation and resin washed thoroughly with LAL grade water which is also the storage medium.

4.5.3. Results

Preparation of microparticles
The data below shows that the particle distribution is 75% is below 5µm, 24% is 5-10µm and 1% is above 10µm (Figure 12).

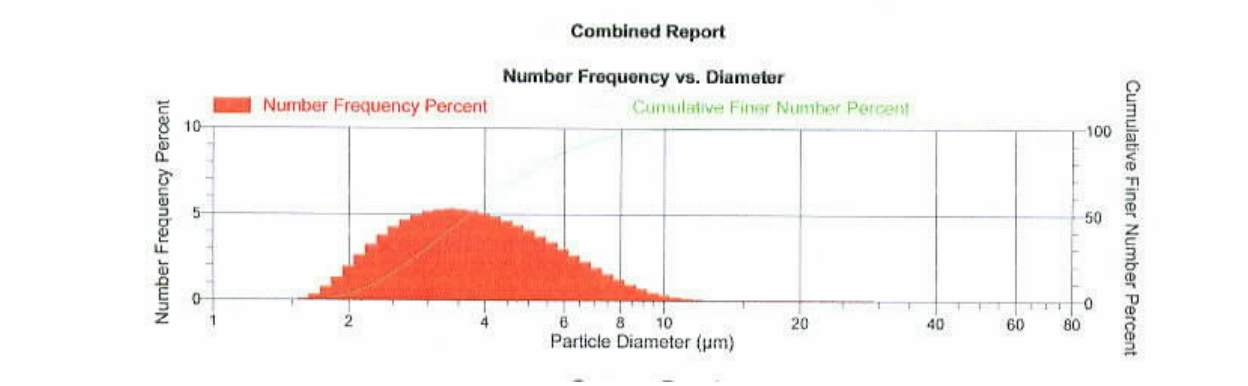


Figure 12. Microparticle size distribution

Purification of polyclonal antibodies
Concentration of protein in the crude MG chicken solution after the precipitation was 1,593 mg. (OD280= 0.286 (1/30 dilution), Vol= 260ml, Conc= 1,593.0 mg)

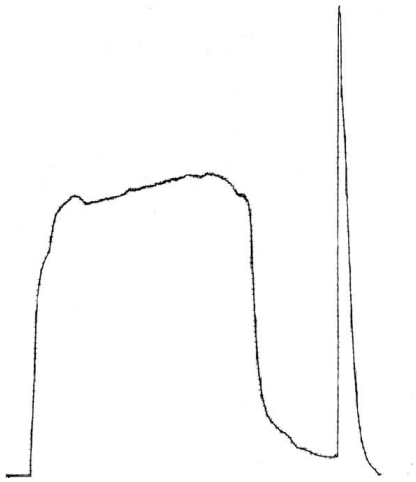


Figure 13. Ion-exchange chromatogram of the polyclonal antibody solution

The large peak is the flow-through and the polyclonal antibody is eluted in the second peak fractions (Figure 13). (Eluent: OD_{280}= 0.172 (1/20 dilution), Vol= 86ml, Conc= 211mg) We have recovered 211 mg of polyclonal chicken antibodies.

Preparation of the immunoaffinity resin

The chicken polyclonal antibodies were coupled to Actigel at 9.4 mg antibody per ml resin. (Unbound: OD_{280}= 0.356, Vol= 90ml, Conc= 23mg) The coupling level was calculated by subtracting the unbound protein amount from the starting amount and divided by the volume of the activated resin (211 – 23) / 20 = 9.4mg/ml.

$$\text{Coupling level (mg/ml)} = \frac{\text{Starting concentration – unbound}}{\text{the volume of activated resin}}$$

Purification of MG antigen

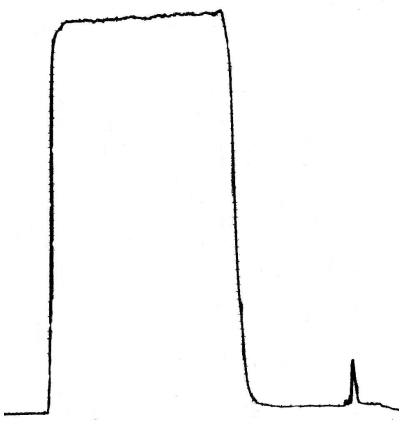


Figure 14. Immunoaffinity chromatogram of MG antigen

The large peak is the flow-through and the MG antigen is eluted in the second peak fractions (Figure 14). MG antigen concentration in the combined peak fractions is 1.1 mg based Bradford protein assay. (11µg/ml x 100ml = 1.1 mg)

Antigen treatments

Below in Table 13 is a summary of MG antigen recoveries from the different antigen treatments.

Table 13. Antigen treatments

	OD_{280nm}	Protein (mg/ml)	Total protein (mg) (% of recovery)
Initial antigens	0.1005	0.0284	1.52*
Con A flow-through	0.0818	0.0179	1.00 (66)
Con A elution	0.0045	0.0084	0.088 (5.7)
Endo H antigens	0.0660	0.0210	1.81 (72)
Periodate antigens	0.1110	0.0296	1.67 (100)

For Endo H treatment, the initial amount of antigens treated was 2.51 mg. (53.6 ml of this preparation and 40.3 ml of a previous *M. gallisepticum* preparation at 0.0246 mg/ml).

Antigen coupling

The MG antigen was coupled to the microparticles at 50 µg and at 250 µg per ml microparticle. The coupling efficiency was calculated by subtracting the unbound protein amount from the starting amount and divided by the volume of the activated microparticles. The coupling efficiency was typically in the 80-90% range based on Bradford protein assay.

$$\text{Coupling efficiency (µg/ml)} = \frac{\text{Starting concentration – unbound ligand}}{\text{the volume of activated microparticles}}$$

PRR agonist coupling

The PRR agonists were coupled to the microparticles respectively at 10 µg, 50 µg and at 125 µg per ml microparticle. The coupling efficiency was calculated based on the formula above. It was typically in the range of 70-80%.

Antigen coupling with PRR agonists

The MG antigen was coupled to the microparticles at 50 µg and at 250 µg per ml microparticle along with the PRR agonists that were coupled respectively at 10 µg, 50 µg and at 125 µg per ml microparticle. The coupling efficiency was calculated based on the formula above. It was typically in the range of 70-80%.

Final microparticle compositions

The following microparticle compositions have been prepared:
microparticles with PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose)
microparticles with *M. gallisepticum* affinity purified antigen (10µg/dose)
microparticles with *M. gallisepticum* affinity purified antigen (50µg/dose)
microparticles with *M. gallisepticum* affinity purified antigen (10µg/dose) and PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose)
microparticles with *M. gallisepticum* affinity purified antigen (50µg/dose) microparticles with PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose)

microparticles with *M. gallisepticum* membrane (10^7/dose)
microparticles with *M. gallisepticum* membrane (10^7/dose) and PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose)
microparticles with *M. gallinarum* membrane (10^7/dose)

microparticles with *M. gallisepticum* antigen Con A eluate on beads.

microparticles with *M. gallisepticum* affinity purified ConA adsorbed antigen (10 µg)
microparticles with *M. gallisepticum* affinity purified Endo H digested antigen (10 µg)
microparticles with *M. gallisepticum* affinity purified perjodate oxidized antigen (10 µg)
microparticles with *M. gallisepticum* affinity purified ConA adsorbed antigen (50 µg)
microparticles with *M. gallisepticum* affinity purified Endo H digested antigen (50 µg)
microparticles with *M. gallisepticum* affinity purified perjodate oxidized antigen (50 µg)

microparticles with *M. gallisepticum* affinity purified Con A adsorbed antigen (10 µg)+ PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose)
microparticles with *M. gallisepticum* affinity purified Con A adsorbed antigen (50 µg)+ PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose)

microparticles with *M. gallisepticum* affinity purified Endo H digested antigen (10 µg)+ PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose)
microparticles with *M. gallisepticum* affinity purified Endo H digested antigen (50 µg)+ PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose)

microparticles with *M. gallisepticum* affinity purified perjodate oxidized antigen (10 µg)+ PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose)
microparticles with *M. gallisepticum* affinity purified perjodate oxidized antigen (50 µg)+ PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose)

microparticles with *M. gallisepticum* affinity purified ConA adsorbed antigen (10 µg) +PRR 3,4,9(*E. coli* LPS: 2 µg , bacterial DNA: 10 µg, polyinosinic-polycytidylic acid (Poly I:C) 25 µg per dose)

microparticles with *M. gallisepticum* affinity purified deacylated antigen (10 µg) +PRR 3,4,9(*E. coli* LPS: 2 µg , bacterial DNA: 10 µg, Poly I:C 25 µg per dose)
microparticles with *M. gallisepticum* affinity purified deacylated antigen (10 µg) +PRR 2,4,9(*E. coli* LPS: 2 µg , bacterial DNA: 10 µg, 2µg peptidoglycan per dose)

microparticles with *M. gallisepticum* affinity purified perjodate oxidized (+) and deacylated antigen (10 µg) + PRR 3,4,9 (*E. coli* LPS: 2 µg , bacterial DNA: 10 µg, Poly I:C 25 µg per dose)

4.5.4. Discussion

We have been able to prepare pathogen mimicking microparticles in the desired combinations. These microparticles have been used both in the animal studies and in the in vitro experiments with mononuclear cells and dendritic cells. (Experiments 6 and 7)

4.6. EXPERIMENT 6: ANIMAL STUDIES

4.6.1. Introduction

The aim of this study is to investigate the feasibility and efficacy of pathogen mimicking microparticles to modulate the host immune response to the mycoplasmal infection. We also aim to determine the required dose and composition of the pathogen mimicking microparticles required to effectively prevent the infection and treat chickens already infected with *Mycoplasma gallisepticum*. *Mycoplasma gallisepticum* causes a respiratory disease in chickens and turkeys. The disease in chickens is characterised by snicking and tracheal rales. It is a primary pathogen but disease is increased in severity if other respiratory pathogens (IBV, TRT, NDV, *Escherichia coli*) are present at the same time (Weinack, 1984). In turkeys, conjunctivitis and swelling of the infraorbital sinuses are usually more evident (Stipkovits, 2001). The mycoplasma can cause a drop in egg production and the quality of eggs may be reduced as well as the fertility and hatchability, in breeders. The mycoplasma may also cause leg lesions, such as swelling of the joints and difficulty in walking. This is a result of the inflammatory response of the host in the synovial membranes as well as excess synovial fluid production. Its incidence worldwide is well documented. The current treatment of the disease consists of antibiotics, including macrolides, tetracylines, aminoglycosides, fluoroquinolones and pleuromutilins.

We have used a chicken challenge model system using the pathogenic *M. gallisepticum* R_{low} strain. *Mycoplasma gallisepticum* can cause significant inflammation in the air-sac and peritoneum which is accompanied by colonization of the trachea, air-sac as well as the lungs. Mycoplasma can also be detected frequently from inner organs. Efficacy of treatment is assessed using clinical scores, performance figures, gross pathology scores (trachea, lung, and air sac), histological scores of respiratory tissues, isolation/detection of *Mycoplasma gallisepticum* as comparisons.

4.6.2. Materials and Methods

Challenge strain
Fresh broth culture of virulent R-strain of *M. gallisepticum* was used in the challenge. The titer is 7.0 $\log_{10}$ CFU/ml. The place of origin, clinical condition isolated from and history of this organism is available.

Animals
Species: Chicken, Sex: male, Breed: commercial broiler breeder chickens, Ross, Origin: Bábolna Agriculture Company, Status: *M. gallisepticum* (MG) and *M. synoviae* (MS) free (based on negative results of serological monitoring of parent stock as well as on negative results of testing for maternal antibodies in one day chicks by ELISA). Age: Chickens arrive at the laboratory at one day of age, treated at three days of age or at 18 days of age and are raised until 28 days of age.
Identification: individually identified using numbered (colored) wing tags in wings.
Care and maintenance: daily, Feed: commercial feed, available *ad libitum*. Water: local tap drinking water, available *ad libitum*.

Setting-up of the groups
Chicks were placed in the poultry research isolation facility at one day of age and grown until commencement of the experiment under the conditions outlined in the specific experiment. Chicks from the same flock were bled and the resulting serum is tested for antibodies to MG or MS in order to determine their status. Only chicks shown to be MG and MS negative are used.

On D-1 the chickens are allocated to one of the groups (10 birds/group). The chicken's individual body weight is recorded. The chickens are allocated to each of the groups such that the average

body weight of the chickens in each of the group will be not be markedly different. Each bird is identified with a colored and numbered wing tag.
The groups for each experiment are described under the section for the appropriate experiment.

Timeline
Day–1: Setting-up of the groups. Sacrificed 10 chickens for ELISA assay, PCR and culturing of *M. gallisepticum* and *M. synoviae* to confirm that the experimental chickens are negative for maternal antibodies and the presence of *M. gallisepticum* and *M. synoviae*.
Day 0: Treatment of groups with the microparticles prior to challenge.
Day 14: Challenge of groups.
Day 15: Treatment of groups with the microparticles post-challenge.
Day 28: Euthanasia, necropsy, and plating for the isolation of *Mycoplasma gallisepticum* from specified organs, trachea, air sac and lung. Histological examinations of trachea and lung were performed.
Days D14 and D28: Chickens were bled in order to obtain serum to be tested for MG-specific antibodies using a serum plate agglutination (SPA) test and blocking ELISA.

Treatment
On Day 0, chickens were treated with different composition of the microparticles, 0.2 ml in 1 ml PBS per animal. The post-challenge treatment groups were treated on day 15.
The treatment of the groups for each experiment is described under the section for the appropriate experiment.

Blood sampling and serology
Ten one-day old chicks are bled and the resulting serum tested for the presence of maternal antibodies against *M. gallisepticum* and *M.synoviae* using ELISA kits manufactured by Diagnosticum Rt. Before the start of the study (D-1), before the challenge and again at the end of study, all birds were bled and sera was tested by SPA using *M.gallisepticum* Nobilis antigen and a MYGA ELISA kit manufactured by Diagnosticum Rt., (Budapest, Hungary).
SPA test of each sample is given a score from 0 to 4 according to the agglutination rate.
The reading of the test is according to the following scheme: 0 = negative, 1 = suspect, 2= positive, 3 = strong positive reaction.
ELISA results are evaluated using average OD (optical density) values of the groups. Data from the serological monitoring of the parent flock is available.

Body weight
Body weights are determined on day 1 (D-1) (before setting the groups), before challenge and at the end of experiment. Body weight gains were used to evaluate the efficacy of the treatment and to calculate the feed conversion rate in groups.

Challenge
On day 14 (D14), the groups of chickens, are challenged using a fresh broth culture of the virulent R-strain of *M. gallisepticum*, at a titre of about 7.0 log10 CFU/ml.
Ten ml of this fresh broth culture will be administered to each of these groups using a spray technique developed by this laboratory. Briefly, the birds will be placed in a 0.220 cubic meter isolation unit. Ten ml of fresh *M. gallisepticum* R-strain culture will then be sprayed, under a pressure of 1 atmosphere, for about a 100 second duration and the chickens left exposed for about 20 minutes.

Clinical follow-up
The clinical examination of all birds is carried out on daily basis as part of the routine care and maintenance. Any sick or dead birds are recorded. Histological samples are taken from dead birds in order to try and confirm a diagnosis.
Nasal discharge (N), increase lacrymation (L), congestion of conjunctival vessels (C), respiratory rales (R), and difficulties in breathing (B) are recorded for each bird, when present.
Specific gross lesions are also recorded for dead birds.
The post challenge clinical signs was scored as follows: 0= healthy, 1= with specific signs, 2= dead with specific lesions.

Euthanasia and pathology
At the end of the experimental study, all groups are euthanised. Each bird is necropsied and scored for gross lesions associated with MG. The presence of exudate in the trachea, left and right thoracic air sacs and peritoneum are recorded. Lesion are scored according to the following:
In trachea: 0= no exudates, 1= slight redness and small quantity of exudates, 2 = redness of mucous membrane, exudates.
Left and right air sacs: 0= no lesion, 1=serous exudates, 2= serous exudates with small pieces of fibrin, 3 = serous, fibrinous exudates, slightly thickened air sac wall, 4 = lots of fibrinous exudates, very thickened air sac wall.
Peritoneum: 0= no exudates, 1=serous exudates, 2= serous exudate with small pieces of fibrin, 3 = serous-fibrinous exudates, 4 = lots of fibrinous exudates

MG isolation
During necropsy examination, trachea, thoracic air sacs, liver, lung, spleen, kidney and heart were aseptically sampled using swabs. Materials from the swabs were then plated onto mycoplasma agar (MA) and incubated at 37°C in a 5% CO_2 incubator. Plates were observed for mycoplasma on days 2, 4, and 7, and then at weekly intervals for a maximum of three weeks. Positive colonies were tested by PCR to identify *M.gallisepticum and M.synoviae*.

Statistical Evaluation
Data from clinical signs, gross pathological lesions and re-isolation of mycoplasmas from organs is evaluated by chi-square test. Body weight and ELISA were analysed by variance, one-way ANOVA.

4.6.3. Animal Challenge Study I

4.6.3.1. Aim of the study

The goal of this experiment is to test the effect of microparticles, and microparticles with PRR agonists and *M. gallisepticum* affinity purified antigen and the *M. gallisepticum* affinity purified antigen co-immobilized with PRR agonists. We also wanted to compare the effect of the microparticles applied prior or after challenge.

Groups

The groups were set up as follows:

G1-G5 treatment with microparticle compositions prior to challenge

- **G1** = treated orally with microparticles (0.2ml/chicken) only and challenged
  G2 = treated orally with microparticles with *M. gallisepticum* affinity purified antigen (10µg/dose) (0.2ml/chicken) and challenged
- **G3** = treated orally with microparticles with PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) (0.2ml/chicken) and challenged
- **G4** = treated orally with microparticles with *M. gallisepticum* affinity purified antigen (10µg/dose) and PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) (0.2ml/chicken) and challenged

G5-G6 treatment with microparticle compositions post-challenge

- **G5** = challenged and treated orally post-challenge with microparticles with M. gallisepticum affinity purified antigen (10µg/dose) and PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) (0.2ml/chicken)
- **G6** = challenged and treated orally post-challenge with microparticles with PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) (0.2ml/chicken)

G7 and G8 positive and negative controls

- **G7** = challenged and non-treated (control +)
- **G8** = non-challenged and non-treated (control -)

4.6.3.2. Results

Necropsy

There were no lesions in the non-treated, nonchallenged chickens (Figure 15). Subsequent to MG challenge, significant pathological lesions were recognized in the air-sac and the peritoneum (Figures 16, 17). However, significant reduction in lesion scores was recorded in the groups treated with particles plus PRR agonists ($p<0.01$), particles plus purified antigen ($p<0.001$) (Figure 19) and purified antigen plus PRR agonists ($p<0.001$) in comparison with the control non-treated, challenged group as well as the group treated with particles only (Table 14). These results are also true when the animal were treated after the challenge.

Re-isolation of Mycoplasma

Mycoplasma can be re-isolated frequently from the inner organs of the non-treated, infected control chickens. Significant reduction in re-isolation rate (from inner organs) was noticed in groups treated with particles plus purified antigen with or without PRR agonists in comparison to the non-treated control ($p<0.05$-0.001) group. The group treated with particles only was significantly higher than the one treated with particle with antigen plus PRR agonists ($p<0.05$) (Table 14). These results are also true when the animal were treated after the challenge.

Table 14. Comparison of pre and post challenge treatments

Groups	Lesion Scores (%)	Efficacy	Re-isolation (%)	Efficacy
Control -	0	N/A	0	N/A
Control +	(76) 100%	0	(14) 100%	0
Orally 2 weeks prior to challenge				
particles only	(65) 85.5%	14.5%	(10) 71.4%	28.6%
particles + 10 µg A (antigen)	(36) 47.4%	52.8%	(5) 35.7%	64.3%
particles + PRR agonists 2,4,9	(54) 71.1%	28.9%	(8) 57.1%	42.9%
10µg A + PRR agonists 2,4,9	(19) 25.0%	75%	(2) 14.3%	86.7%
Orally after the challenge				
particles + PRR agonists 2,4,9	(56) 73.7%	26.3%	(8) 57.1%	42.9%
10µg A + PRR agonists 2,4,9	(29) 38.2%	61.8%	(2) 14.3%	86.7%

4.6.3.3. Discussion

Our results showed that the particles alone did not protect the chickens from peritonitis and air-sacculitis caused by *M. gallisepticum* nor did it prevent the colonization of organs by mycoplasma.

However, when particles were coated with PRR agonist without antigen, colonization of organs with mycoplasma was reduced and the scores of pathological lesions were reduced.

When purified antigen was added to the particles coated with PRR agonists, the colonization of organs reduced significantly and scores of pathological lesions was low. This effect was more pronounced when the microparticles were introduced mucosally and before challenge, but similar positive effect was noticed when the microparticles were administered mucosally after challenge.

The experimental data confirmed our hypothesis that a pathogen mimicking microparticle containing PRR agonist molecules alone or with antigen could influence the protective immune response upon challenge regardless if it was applied prior to or after the challenge. The microparticles containing PRR agonist had a smaller effect most likely through the induction of innate immune response, which is not antigen-specific. However, the dose of particle applied was not sufficient to protect against a high dose of pathogen challenge. This was expected, as innate immune response is alone not sufficient in case of a high dose of infectious agents. Microparticles containing PRR agonists along with immuno affinity purified antigen had a more pronounced protective effect regardless if it was applied prior or after the challenge. The addition of PRR agonists to the antigen has improved the protective response.

4.6.4. Animal Challenge Study II

4.6.4.1. Aim of the study

The goal of this experiment was to test if the increased dose of antigen would further improve the protective effect of the pathogen mimicking microparticles. We also wanted to examine the effect of *M. gallisepticum* membrane attached to the microparticles with or without PRR agonists. We also wanted to see the effect of the mycoplasma membrane isolated from a non-pathogenic mycoplasma, *M. gallinarum* attached to the microparticles. This would allow us to better understand the mycoplasma-host interaction.

Groups

The groups were set up as follows:

G1-G5 treatment with microparticle compositions prior to challenge

- **G1** = treated orally with microparticles with *M. gallisepticum* affinity purified antigen (10µg/dose) (0.2ml/chicken) and challenged
  G2 = treated orally with microparticles with *M. gallisepticum* affinity purified antigen (50µg/dose) (0.2ml/chicken) and challenged
- **G3** = treated orally with microparticles with *M. gallisepticum* membrane (10^7/dose) (0.2ml/chicken) and challenged
- **G4** = treated orally with microparticles with *M. gallisepticum* membrane (10^7/dose) and PRR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) (0.2ml/chicken) and challenged
- **G5 =** treated orally with microparticles with *M. gallinarum* membrane (10^7/dose) (0.2ml/chicken) and challenged

G6 and G7 positive and negative controls

- **G6** = challenged and non-treated, (control +)
- **G7** = non-challenged and non-treated, (control -)

4.6.4.2. Results

Necropsy

Subsequent to MG challenge, significant pathological lesions were recognized in the air-sac and the peritoneum. However, significant reduction in lesion scores was recorded in the groups treated with 10 µg antigen plus PRR agonists ($p<0.001$) and 50 µg purified antigen plus PRR agonists ($p<0.01$) in comparison with the control non-treated, challenged group. When the group was treated with *M. gallisepticum* membrane immobilized to the particles, the pathological lesions in some of the chickens was more pronounced than in the challenge group itself (Figure 18). The addition of the PRR agonists to the *M. gallisepticum* membrane-containing particle did not change the outcome. Membrane isolated from the non-pathogenic *M. gallinarum* also did not have any protective effect (Table 15).

Re-isolation of Mycoplasma

Mycoplasma can be re-isolated frequently from the inner organs of the non-treated, infected control chickens. Significant reduction in re-isolation rate (from inner organs) was noticed in groups treated with 10µg purified antigen with PRR agonists in comparison to the non-treated control ($p<0.01$) group. However, there was significantly lower re-isolation rate ($p<0.01$) between the group treated with 50µg purified antigen with PRR agonists and the control. When the group was treated with *M. gallisepticum* membrane immobilized to the particles the re-isolation rate of *M. gallisepticum* from the organs in some cases was higher than in the challenge group itself. The addition of the PRR agonists to the *M. gallisepticum* membrane containing particle did not change the effect. Membrane isolated from the non-pathogenic *M. gallinarum* also did not have any protective effect (Table 15).

Table 15. The effect of mycoplasma membrane compared to the purified antigen

Groups	Lesion Scores (%)	Efficacy	Re-isolation (%)	Efficacy
Control -	0	N/A	0	N/A
Control +	(76) 100%	0	(14) 100%	0
10µgA+PRR agonists 2,4,9	(33) 43.4%	57.6%	(3) 21.4%	79.6%
50µgA+PRR agonists 2,4,9	(55) 72.4%	28.6%	(4) 28.6%	71.4%
M.gallisepticum membrane	(80) 105.3%	-5.3%	(11) 78.6%	22.4%
M.gallinarum membrane	(75) 98.7%	1.3%	(17) 121.4%	-21.4%
M.gallisepticum membrane +PRR agonists 2,4,9	(72) 94.7%	5.3%	(11) 78.6%	21.4%

A=antigen

4.6.4.3. Discussion

Mycoplasmas cause chronic inflammation and may able to escape host defense. The treatment with the *M. gallisepticum* membrane prevented the appropriate immune response to develop against the challenge with the pathogen and allowing a more pronounced infection. The non-pathogenic *M. gallinarum* membrane had even more pronounced negative effect on the immune response. One explanation may be is that the pathogenic mycoplasmas are more effective inducing immune response through the activation of the inflammatory cytokines. The fact, that the 50µg dose of antigen was less protective than the 10µg dose, indicates that post-transcriptional modifications on the purified antigen may be involved in the escape of host-defense.

DC-SIGN has been implicated in the escape mechanism of pathogens. DC-SIGN is a C-type lectin specific for high-mannose containing lipid molecules. Mycoplasma membranes are composed of high proportions of lipids and different mycoplasmas have been shown to bind the Concanavalin A affinity resin, indicating the presence of mannose on the mycoplasma surface. In the following experiment, we will examine if mannose is involved in the immune-escape mechanism of mycoplasmas.

4.6.5. Animal Challenge Study III

4.6.5.1. Aim of the study

The goal of this experiment is to examine if mannose post-transciptional modifications participate in the ability of mycoplasmas to establish chronic infections. Mycoplasmas have been shown to bind to ConA (Kahane and Tully, 1976). We have used different approaches to remove mannose by removing mannose-containing antigens using a Concanavalin A affinity resin or by using Endo H to remove mannose residues. EndoH is highly specific for high-mannose modifications, such as those that participate in DC-SIGN binding. We have also used sodium periodate under different conditions to oxidize sensitive sugars. The specifics of each method are the following:

Concanavalin A: High mannose-type oligosaccharides bind very tightly to Concanavalin A
Endo H: Cleaves asparagine-linked hybrid or high mannose oligosaccharides, but not complex oligosaccharides. It cleaves between two N-acetylglycosamine residues in the diacethylchitobiose core of the oligosacchraride, generating a truncated sugar molecule with one N-acetylglycosamine residue remaining on the asparagine.
Periodate: Oxidize mannan. Oxidized mannan, due to the presence of aldehydes binds to the mannose receptor, not the scavenger receptor. After internalization there is a rapid access to the class I pathway via endosomes, not lysosomes leading to rapid and efficient MHC class I presentation to CD8+ cells and a preferential Th1 response.

Upon infection, *M. gallisepticum* induces the synthesis of nitric oxide (NO). Nitric oxide synthase (NOS) in T cells regulate the T cell death and immune memory (Vig, 2004). We wanted to examine if we could improve the immune response against *M. gallisepticum* by blocking nitric oxide synthesis. Blocking Nitric oxide can be achieved by using Amino Guanidine (100 mg/kg), which is an inducible NOS inhibitor or by Pentoxyphillin, which blocks Reactive Nitrogen Intermediates mediated trophic signal withdrawal death pathway or by MnTBAP (Mn(III)tetrakis(4 benzoic acid)porphyrin), a peroxinitric scavenger (Vig, 2004). In this experiment we have used Amino Guanidine, intra peritoneal for 7 days.

Groups
The groups were set up as follows:
G1 = non-challenged and non-treated (control -)
G2 = challenged and non-treated (control +)
Treated 2 weeks prior challenge:
G3 = treated orally with microparticles with *M. gallisepticum* affinity purified antigen (10µg/dose) and TLR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) (0.2ml/chicken) and challenged
G4 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* affinity purified antigen (50µg/dose) and TLR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) and challenged
G5 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* affinity purified ConA adsorbed antigen (10 µg) and challenged
G6 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* affinity purified Endo H digested antigen (10 µg) and challenged
G7 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* affinity purified perjodate oxidized antigen (10 µg) and challenged
G8 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* affinity purified Con A adsorbed antigen (10 µg)+ TLR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) and challenged

G9 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* affinity purified Con A adsorbed antigen (50 µg)+ TLR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) and challenged
G10 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* affinity purified Endo H digested antigen (10 µg)+ TLR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) and challenged
G11 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* affinity purified Endo H digested antigen (50 µg)+ TLR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) and challenged
G12 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* affinity purified perjodate oxidized antigen (10 µg)+ TLR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) and challenged
G13 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* affinity purified perjodate oxidized antigen (50 µg)+ TLR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) and challenged
G14 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* affinity purified Endo H digested antigen (10 µg)+ TLR agonists (10µg bacterial DNA, *E. coli* + 2µg *E. coli* LPS and 2µg peptidoglycan/dose) plus amino guanidine ip. for 7 days and challenged
G15 = treated orally with microparticles (0.2ml/chicken) with *M. gallisepticum* antigen Con A eluate on beads and challenged.

4.6.5.2. Results

4.6.5.2.1. Results of comparison of differently treated antigens (10µg)

Necropsy

Subsequent to MG challenge, significant pathological lesions were recognized in the air-sac and the peritoneum. However, significant reduction in lesion scores was recorded in the groups treated with 10 µg antigen plus PRR agonists ($p<0.001$). This was further improved significantly by changing the post-transcriptional modifications of the antigens (Figures 20, 21, 23). The best protection was achieved when the mannosylated antigen was either oxidized by Periodate ($p<0.001$) or removed by Concanavalin A ($p<0.001$). These results were better than the highly specific removal of the mannosyl modification from the antigen by Endoglycosidase H (Table 16).

Re-isolation of Mycoplasma

Mycoplasma can be re-isolated frequently from the inner organs of the non-treated, infected control chickens. Significant reduction in re-isolation rate (from inner organs) was noticed in groups treated with 10µg purified antigen with PRR agonists ($p<0.05$). However, there was significantly lower re-isolation rate ($p<0.001$) in the group treated with antigen depleted on Concanavalin A and immobilized with PRR agonists. Similar result was achieved in the group treated with EndoH and amino guanidine ($p<0.001$). Endo H treatment gave a slight improvement over the unmodified antigen, whereas periodate treatment had shown no further improvement (Table 16).

Table 16. Comparison of differently treated antigens (10µg)

Groups	Lesion Scores (%)	Efficacy %	Re-isolation (%)	Efficacy %
Control -	0	100%	0	100%
Control +	(92) 100%	0	(12) 100%	0
10 µg A + PRR agonists 2,4,9	(39) 42.4%	57.6%	(4) 33.3%	67.7%
ConA 10 µg A + PRR agonists 2,4,9	(14) 15.2%	84.8%	(1) 8.3%	91.7%
EndoH 10 µgA + PRR agonists 2,4,9	(24) 26.1%	73.9%	(3) 25%	75%
Periodate 10 µg A + PRR agonist 2,4,9	(18) 19.6%	89.4%	(4) 33.3%	67.7%
EndoH 10 µg A + PRR agonists 2,4,9 + amino guanidine	(12) 13.0%	87%	(1) 8.3%	91.7%

4.6.5.2.2. Results of comparison of differently treated antigens (50µg)

Necropsy

Subsequent to MG challenge, significant pathological lesions were recognized in the air-sac and the peritoneum. However, reduction in lesion scores was recorded in the groups treated with 50 µg antigen plus PRR agonists ($p<0.001$). This was significantly improved by changing the post-transcriptional modifications of the antigens. The best protection was achieved when the mannosylated antigen was removed by Concanavalin A. This result was slightly better then the highly specific removal of the mannosyl modification from the antigen by Endoglycosidase H. The effect of the periodate oxidization was comparable to the EndoH treatment. Antigen eluted from the Concanavalin A has shown reduction in inflammation (Table 17).

Re-isolation of Mycoplasma

Mycoplasma can be re-isolated frequently from the inner organs of the non-treated, infected control chickens. Reduction in re-isolation rate (from inner organs) was noticed in groups treated with 50µg purified antigen with PRR agonists. However, there was significantly lower re-isolation rate ($p<0.001$) in the group treated with antigen depleted on Concanavalin A and immobilized with PRR agonists. Similar result was achieved in the group treated with EndoH and Periodate. Antigen eluted from the Concanavalin A has shown no reduction in re-isolation rate as compared to the non-treated, challenged control (Table 17).

Table 17. Comparison of differently treated antigens (50µg)

Groups	Lesion Scores	Efficiency	Re-isolation	Efficiency
Control -	0	N/A	0	N/A
Control +	(92) 100%	0	(12) 100%	**0%**
50µgA + PRR agonists (2,4,9)	(52) 56.5%	43.5%	(8) 66.6%	**33.4%**
Con A 50µgA + PRR agonists (2,4,9)	(10) 10.9%	89.1%	(1) 8.3%	**91.7%**
EndoH 50µg A + PRR agonists(2,4,9)	(17) 18.5%	81.5%	(0) 0%	**100%**
Periodate 50µg A + PRR agonists(2,4,9)	(21) 22.8%	78.2%	(2) 16.7%	**83.3%**
Con A eluted antigen	(53) 57.6%	42.4%	(12) 100%	**0%**

4.6.5.2.3. Results of comparison of different amounts of antigens

Necropsy

Subsequent to MG challenge, significant pathological lesions were recognized in the air-sac and the peritoneum. However, reduction in lesion scores was recorded in the groups treated with antigen. This was significantly improved by changing the post-transcriptional modifications of the antigens ($p<0.001$). The protection was improved by the increase in the antigen dose, as opposed to the native antigen when the increased dose reduced the protective effect. The best protection was achieved regardless of the antigen dose in groups treated by microparticles where the mannosylated antigen was removed by Concanavalin A ($p<0.001$). This result was slightly better than the highly specific removal of the mannosyl modification from the antigen by Endoglycosidase H (Endo H). The effect of the periodate oxidization was comparable to the EndoH treatment (Table 18).

Re-isolation of Mycoplasma

Mycoplasma can be re-isolated frequently from the inner organs of the non-treated, infected control chickens. Reduction in re-isolation rate (from inner organs) was noticed in groups treated with purified antigen. However, there was significantly lower re-isolation rate ($p<0.001$) in the group treated with antigen depleted on Concanavalin A regardless of the antigen dose. Similar result was achieved in the group treated with EndoH, however it was dose dependent and the protective effect increased with the increased antigen dose (Table 18).

Table 18. Comparison of different amounts of antigens

Groups Antigen+PRR agonists 2,4,9	Lesion Scores %	Efficacy %	Re-isolation %	Efficacy %
10 µg native	(39) 42.4%	58.6%	(4) 33.3%	67.7%
50µg native A	(52) 58.7%	43.3%	(8) 66.6%	33.4%
10 µg A Con A	(14) 15.2%	84.8%	(1) 8.3%	91.7%
50µg A ConA	(10) 10.7%	89.3%	(1) 8.3%	91.7%
10 µg A EndoH	(24) 26.1%	73.9%	(3) 25%	75%
50µg A EndoH	(17) 18.5%	81.5%	(0) 0%	100%
10 µg A Periodate	(24) 26.1%	73.1%	(4) 33.3%	67.7%
50µg A Periodate	(15) 16.3%	83.7%	(2) 16.7%	84.3%
Control +	(92) 100%	0	(12) 100%	0
Control -	0	N/A	0	N/A

4.6.5.2.4. Results of the effect of PRR agonists

Necropsy

Subsequent to MG challenge, significant pathological lesions were recognized in the air-sac and the peritoneum. However, reduction in lesion scores was recorded in the groups treated with the antigen improved by changing the post-transcriptional modifications of the antigens ($p<0.001$). However, by adding the PRR agonist molecules to the microparticles containing the modified antigen, the

protection has been significantly improved in each treatment group ($p<0.001$). The best protection was achieved when the mannosylated antigen was removed by Concanavalin A treatment and PRR agonist were added to the microparticle. This result was slightly better than achieved by microparticles containing periodate modified antigen with PRR agonist molecules or the EndoH treated antigen combined with the PRR agonist molecules (Table 19).

Re-isolation of Mycoplasma

Mycoplasma can be re-isolated frequently from the inner organs of the non-treated, infected control chickens. Reduction in re-isolation rate (from inner organs) was noticed in groups treated with modified antigen immobilized to the microparticles. However, there was significantly lower re-isolation rate ($p<0.001$) in all of the groups treated with antigen and co-immobilized with PRR agonists regardless of the antigen modification. The lowest re-isolation rate was in the group treated with ConA depleted antigen immobilized along with PRR agonists molecules (Table 19).

Table 19. Effect of PRR agonists

Groups 10µg Antigen	**Lesion Scores %**	**Efficacy %**	**Re-isolation %**	**Efficacy %**
ConA	(32) 34.8%	65.2%	(3) 25%	75%
ConA +PRR agonists 2,4,9	(14) 15.2%	84.8%	(1) 8.3%	91.7%
EndoH	(46) 50%	50%	(5) 41.7%	58.3%
EndoH+PRR agonists 2,4,9	(24) 26.1%	73.9%	(3) 25%	75%
Periodate	(21) 22.8%	77.2%	(7) 58.3%	41.7%
Periodate+PRR agonists 2,4,9	(18) 19.6%	80.4%	(4) 33.3%	67.7%
Control +	(92) 100%	0	(12) 100%	0
Control -	0	N/A	0	N/A

4.6.5.3. Discussion

The results of these experiments showed that the removal of immune suppressive antigens by ConA affinity chromatography from the immunoaffinity purified antigen pool has markedly improved the protective effects of the microparticle composition. In addition, the chemical modification (Periodate) or enzymatic breakdown (EndoH) of the sugar containing post-transcriptional modifications on the antigens also led to an improved protective immunity.

Conversely, we have also found that the administration of the isolated immune suppressive antigens coupled to microparticles lead to a reduced inflammatory response along with persistence of the mycoplasma in the inner organs. Mycoplasmas have been shown to bind to Concanavalin A affinity chromatography resin indicating that mannosylated molecules are present in the mycoplasma membrane (Kahane, 1976). It seems that these mannosylated components in the *M. gallisepticum* are participating in the escape mechanism of this pathogen, resulting in immune suppression and the development of tolerance to the pathogen. We have also demonstrated that proper antigen characteristics can help to shift the immune response towards either protection or tolerance.

4.6.6. Animal Challenge Study IV

4.6.6.1. The aim of the study

Recent studies suggest that pathogenic microorganisms can modulate or interfere with TLR mediated pattern recognition and can use TLRs as an escape mechanism from host defense (Netea, 2004). Three major TLR-mediated escape mechanisms have been identified: TLR-2-induced immune suppression, especially through induction of IL-10 release, blockade of TLR recognition and TLR-mediated induction of viral replication (Sing, 2002). Mycoplasma membrane components have been shown to interact with TLR-2 (also TLR1 and TLR-6) (Shimizu, 2004). TLR1 and TLR-6 are highly homologous to TLR-2 in structure. The functional association of TLR1, 2 and 6 has been demonstrated (Akira 2003). TLR1 and TLR-6 are involved in the discrimination of subtle differences between triacyl and diacyl lipopeptides through interaction with TLR-2 (Takeda, 2002). The aim of this experiment is to test if the lipid modifications on the mycoplasma antigens contribute to the escape mechanism of this pathogen.

In this experiment we have deacylated the lipid post-transcriptional modifications on the immunoaffinity purified MG antigen. We have also changed the composition of the PRR agonists by replacing peptidoglycan with Poly I:C, a TLR3 agonist, if the commercial peptidoglycan preparation is not properly deacylated, part of the peptidoglycan might participate in TLR-2 signaling.

Groups

The groups were set up as follows:

G1. Non-treated, non-challenged (control –)

G2. Non-treated, challenged (control +)

Orally 2 weeks prior challenge

G3. Affinity purified ConA adsorbed antigen (10 µg) +PRR 3,4,9

G4. Affinity purified deacylated antigen (10 µg) +PRR 3,4,9

G5. Affinity purified perjodate oxidized (+) and deacylated antigen (10 µg) +PRR 3,4,9

Orally 1 day after challenge

G6. Affinity purified ConA adsorbed antigen (10 µg) + PRR 3,4,9

G7. Affinity purified deacylated antigen (10 µg) +PRR 3,4,9

G8. Affinity purified perjodate oxidized (+) and deacylated antigen (10 µg) +PRR 3,4,9

PRR 3,4,9 = *E. coli* LPS: 2 µg, bacterial DNA: 10 µg, Poly I:C 25 µg per dose.

4.6.6.2. Results of effect of deacylation of antigen

Necropsy

Subsequent to MG challenge, significant pathological lesions were recognized in the air-sac and the peritoneum. However, reduction in lesion scores was recorded in the groups treated with antigen improved by changing the post-transcriptional modifications of the antigens ($p<0.001$). There was no significant difference between the different treatment groups(Figures 22, 24, 25, 26). The results are similar if the treatment was applied prior or after the challenge (Table 20).

Re-isolation of Mycoplasma

Mycoplasma can be re-isolated frequently from the inner organs of the non-treated, infected control chickens. Reduction in re-isolation rate (from inner organs) was noticed in the groups treated with modified antigen immobilized to the microparticles. However, re-isolation rate was higher in the group treated with deacylated antigen containing microparticles over the groups treated with microparticles where the antigen was modified on both the mannosylated site as well as the lipid moiety. The lowest re-isolation rate was in the group treated with ConA depleted antigen immobilized along with PRR agonists molecules. There was no significant difference between the groups treated prior or after the challenge (Table 20).

Table 20. Effect of deacylation of the antigen

Groups **All 10 µg antigen**	**Necropsy score**	**Efficiency %**	**Re-isolation**	**Efficiency %**
Control -	0	N/A	0	N/A
Control +	(78) 100%	0	(23) 100%	0
Orally 2 weeks prior challenge				
ConA + PRR agonists 3,4,9	(18) 23.1%	76.9%	(3) 13%	87%
Deacylated + PRR agonists 3,4,9	(30) 38.5%	61.5%	(10) 43.5%	56.5%
Periodate (++) + deacylated + PRR agonists 3,4,9	(21) 26.9%	73.1%	(4) 17.4%	82.6%
Orally after challenge				
ConA + PRR agonist 3,4,9	(26) 33.3%	66.7%	0	100%
Deacylated + PRR agonists 3,4,9	(17) 21.8%	78.2%	(9) 39.1%	60.9%
Periodate (++) + Deacylated + PRR agonists 3,4,9	(19) 24.4%	75.6%	(6) 26.1%	73.9%

4.6.6.3. Discussion

Based on the re-isolation rate, which was higher in the group treated with deacylated antigen containing microparticles over the groups treated with microparticles where the antigen was modified on both the mannosylated site as well as the lipid moiety, we have concluded that ConA might have removed the mannosylated lipid, as mannosylation is frequently found on lipid modifications. Periodate combined with deacylation could have effected both the mannosylation and the lipid portion of the molecule. This is also reflected in the results. Since there was no significant difference between the groups treated prior or after the challenge, it appears that it is possible to achieve a protective immunity even post-infection with these compositions.

The results of these experiments showed that the removal or blocking of immune suppressive antigens or antigenic determinants from the affinity purified antigen pool has markedly improved the protective effects of the microparticle composition. In addition, the removal of and the chemical modification of the sugar containing post-transcriptional modifications on the antigens also led to an improved protective immunity. In addition, lipid moieties on antigens were chemically removed that also led to marked immune protective effects.

We have demonstrated that with proper antigen modifications it is possible to shift the immune response towards protection.

4.6.7. Animal Challenge Study V

4.6.7.1. The aim of the study

The aim of this experiment is to compare the effect of the different PRR agonist composition of the microparticles.

Groups

The groups were set up as follows:
G1. Control –
G2. Control +
Orally 2 weeks prior challenge
G3. Affinity purified ConA adsorbed antigen (10 µg) +PRR 2,4,9
G4. Affinity purified deacylated antigen (10 µg) +PRR 2,4,9
G5. Affinity purified ConA adsorbed antigen (10 µg) +PRR 3,4,9
G6. Affinity purified deacylated antigen (10 µg) +PRR 3,4,9
PRR 2,4,9 = *E. coli* LPS: 2 µg, bacterial DNA: 10 µg, peptidoglycan: 2 µg per dose.
PRR 3,4,9 = *E. coli* LPS: 2 µg, bacterial DNA: 10 µg, Poly I:C 25 µg per dose.

4.6.7.2. Results of the comparison of the effect of different PRR agonist composition

Necropsy

Subsequent to MG challenge, significant pathological lesions were recognized in the air-sac and the peritoneum. However, reduction in lesion scores was recorded in the groups treated with antigen improved by changing the post-transcriptional modifications of the antigens ($p<0.001$). There was no significant difference between the different PRR agonists compositions when the antigen was treated by ConA, however, the PRR 2,4,9 composition gave better protection over the PRR 3,4,9 composition when the antigen was deacylated (Table 21).

Re-isolation of Mycoplasma

Mycoplasma can be re-isolated frequently from the inner organs of the non-treated, infected control chickens. Reduction in re-isolation rate (from inner organs) was noticed in groups treated with modified antigen immobilized to the microparticles. However, re-isolation rate was higher in the group treated with deacylated antigen containing microparticles over the groups treated with microparticles where the antigen was modified on both the mannosylated site as well as the lipid moiety, such as the ConA treatment. Overall the PRR 2,4,9 composition gave better protection over the PRR 3,4,9 composition ($p<0.05$) (Table 21).

Table 21. Comparison of the effect of different PRR agonist composition

Groups **All 10 µg antigen**	**Necropsy score**	**Efficiency %**	**Re-isolation**	**Efficiency %**
Control -	0	100	0	100%
Control +	(78) 100%	0%	(23) 100%	0
ConA +PRR agonists 2,4,9	(19) 24.4%	75.6%	(5) 21.7%	78.3%
ConA + PRR agonists 3,4,9	(18) 23.1%	76.9%	(3) 13%	87%
Deacylated + PRR agonists 2,4,9	(19) 24.4%	75.6%	(2) 8.7%	91.3%
Deacylated + PRR agonists 3,4,9	(30) 38.5%	61.5%	(10) 43.5%	56.5%

4.6.7.3. Discussion

The results of these experiment showed that the different composition of the PRR agonists has an effect on the immune protection along with the removal or blocking of immune suppressive antigens or antigenic determinants from the affinity purified antigen pool. We have demonstrated

that with proper antigen modifications along with the combination of PRR agonist molecules it is possible to shift the immune response towards protection.

Safety Evaluation
After treatment, prior to challenge the treated chickens were examined daily to evaluate the safety of the microparticle compositions. The chickens were found to be clinically healthy, and showed no side effects from the treatments. The feed and water consumption of the birds did not change as compared to the non-treated groups. The microparticle compositions seemed to be safe, but further evaluation is needed.

4.6.8. Summarized discussion of the animal studies

A significant degree of protection was achieved against the infectious strain of *Mycoplasma gallisepticum* when the animals were treated prior to the mycoplasmal challenge. The level of protection achieved with our mycoplasma mimicking microparticles was similar to what was described in the literature for *M. gallisepticum* vaccines (Whithear, 1990, Kleven, 1998). We could not compare our microparticles to the commercial vaccines in our challenge studies, as we have used chickens of three-days of age, whereas the commercial vaccines are not applicable at this age (Whithear, 1990, Hildebrand, 1983).

In addition, the characteristic pathological symptoms were not observed on the challenged animals when the microparticles were applied post-challenge, indicating that immunomodulation could be an effective method to treat infected birds. This is significant because of the widespread antibiotic resistance of various strains of microorganisms and the growing public and regulatory concerns about allowing the use of antibiotics profilactically in farm animals.

In addition, we have found that just a small amount of antigen (10µg/bird) was sufficient to elicit protection by this method. This suggests that the antigen remains effective while traversing the gut in the animals and that it is delivered to the immune cells in an efficacious manner. The results also confirm that the pathogen recognition systems are similar in chickens that have been described in more details in mammals (Fukui, 2001, Brown, 1999). The results show that these microparticles can be used in developing rational approach to modulate immune responses as we have demonstrated protective effects. This is a new approach, as using traditional adjuvants, it was not possible to make killed vaccines that can be applied mucosally, as the components of the adjuvants cause irritation on the mucosal membranes (Ben-Yedidia, 1997).

Nevertheless, we did not find differences in ELISA titers between the different treatment groups. However, it is known that young animals do react slowly serologically to antigen stimulus (Stipkovits, 2004). We have focused our evaluation to the pathological lesions that develops early (around 10-14 days) after the mycoplasma challenge. The evaluation of protection based on pathological lesions is important. The level of serum antibody is not a good marker of protection, as the antibodies are not protective against mycoplasma infection.

We wanted to gain a better understanding of the mechanism of action of the pathogen mimicking microparticles. However, due to the lack of a variety of immunological reagents for chickens, an *in vitro* system using human cells has been set-up both to study the innate immune response and through the use of dendritic cells, the initiation of adaptive immune response. Such systems have been used to evaluate vaccines and adjuvants (Yuki, 2003, Zimmermann, 1998, Williamson, 1999) and may have a predictive value as to immune response produced by the immunologically active composition. The *in vitro* studies are presented in the next section, Own Experiment 7.

4.6.9. Pathological lesions of the experimental chickens

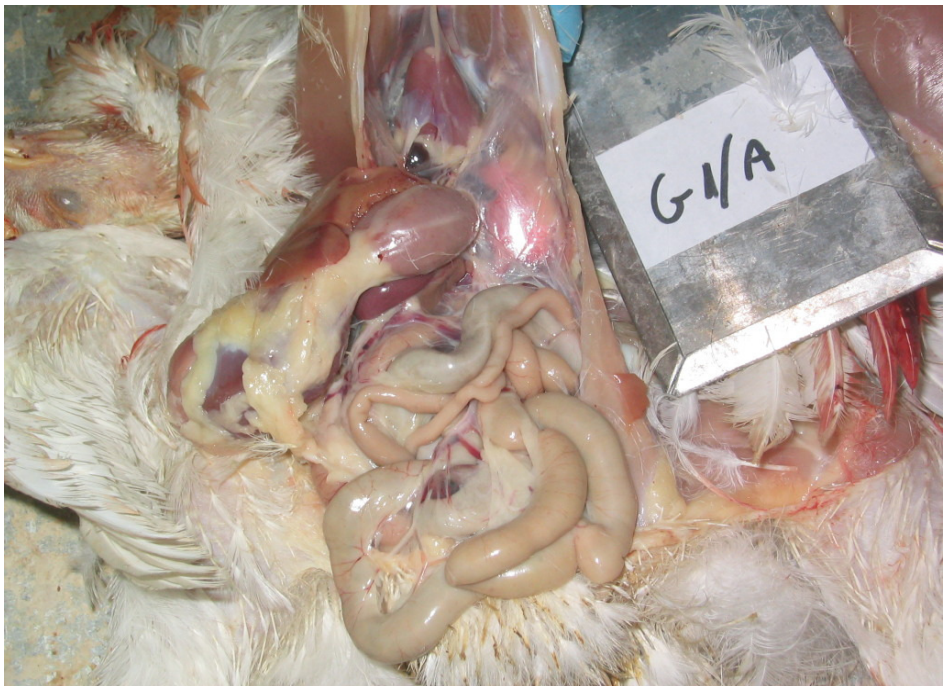


Figure 15. Non-challenged, non-treated chicken (negative control).
No airsacculitis and no peritonitis.

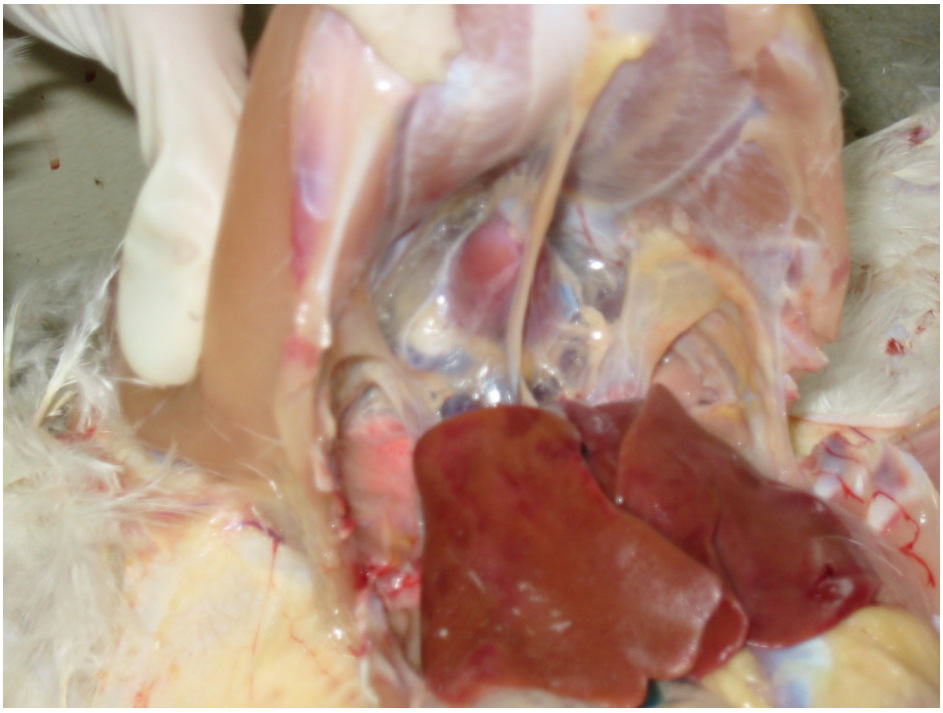


Figure 16. *M. gallisepticum* challenged and non-treated chicken (positive control)
Airsacculitis, thickness of air sac walls and fibrinous exudates are in thoracic air sac.

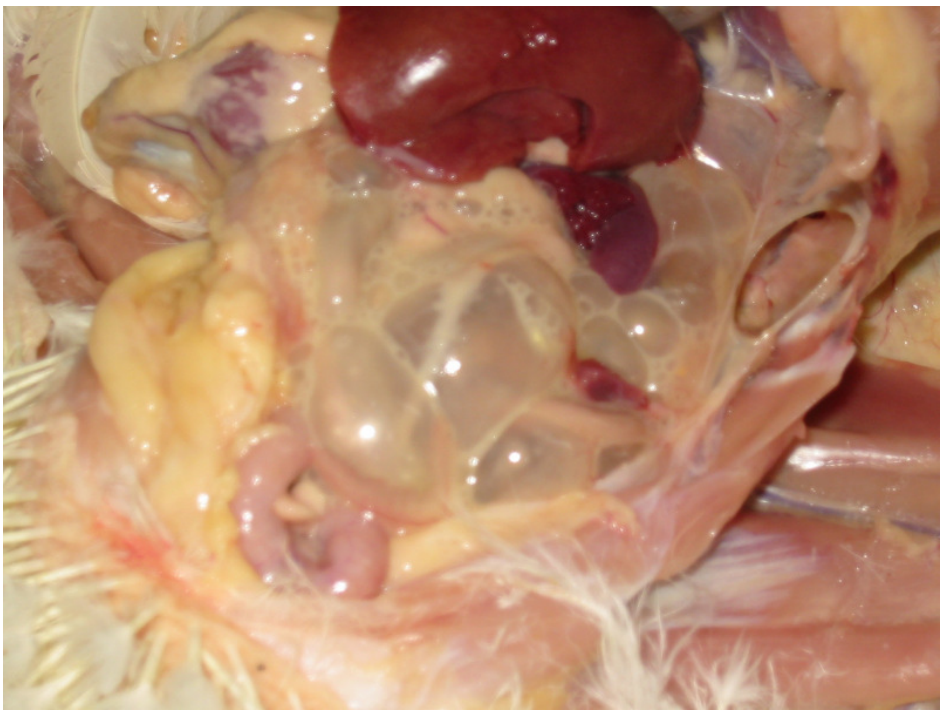


Figure 17. *M. gallisepticum* challenged and non-treated chicken showing peritonitis, accumulation of serous-fibrinous exudates in peritneum.

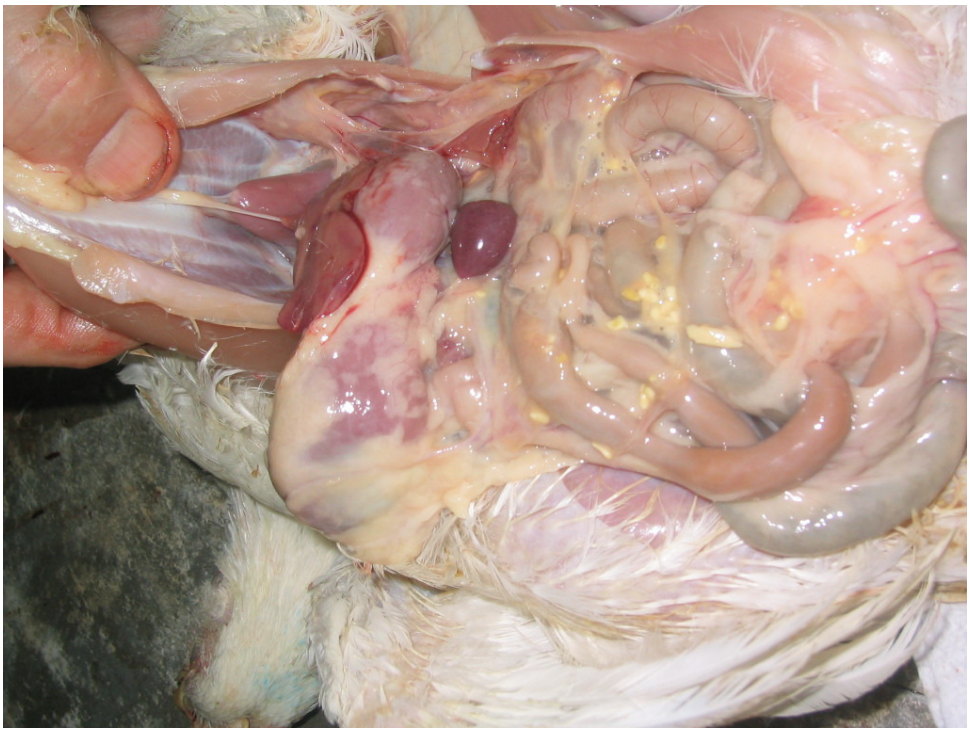


Figure 18. Chicken treated with M. gallisepticum membrane and challenged with *M. gallisepticum*. Peritonitis, accumulation of serous-fibrinous exudates in peritneum.

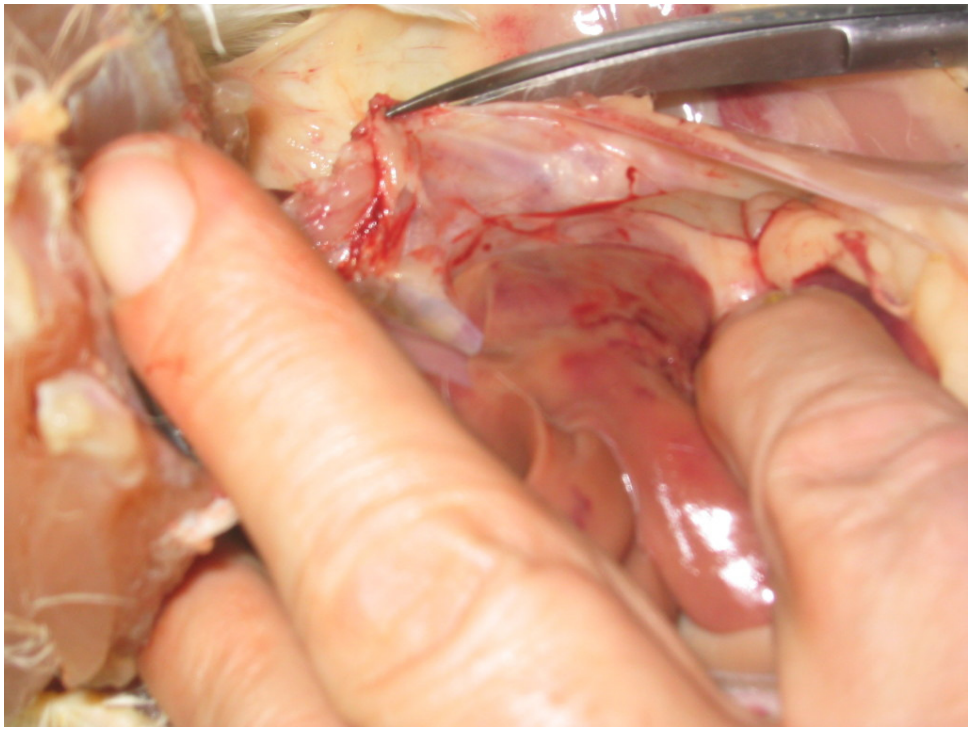


Figure 19. Chicken treated with antigen (10 µg) and challenged with *M. gallisepticum*. Airsacculitis and thickened air sac walls.

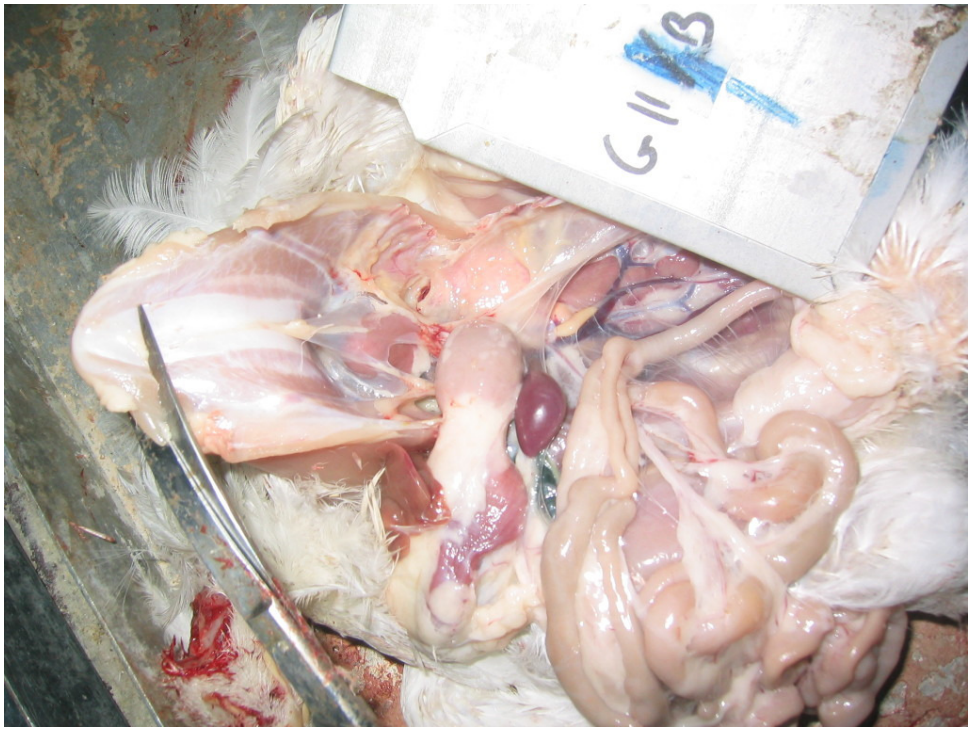


Figure 20. Chicken treated with Con A modified antigen (10 µg) + PRR agonists (2,4,9) and challenged with *M. gallisepticum*. No airsacculitis and no peritonitis.

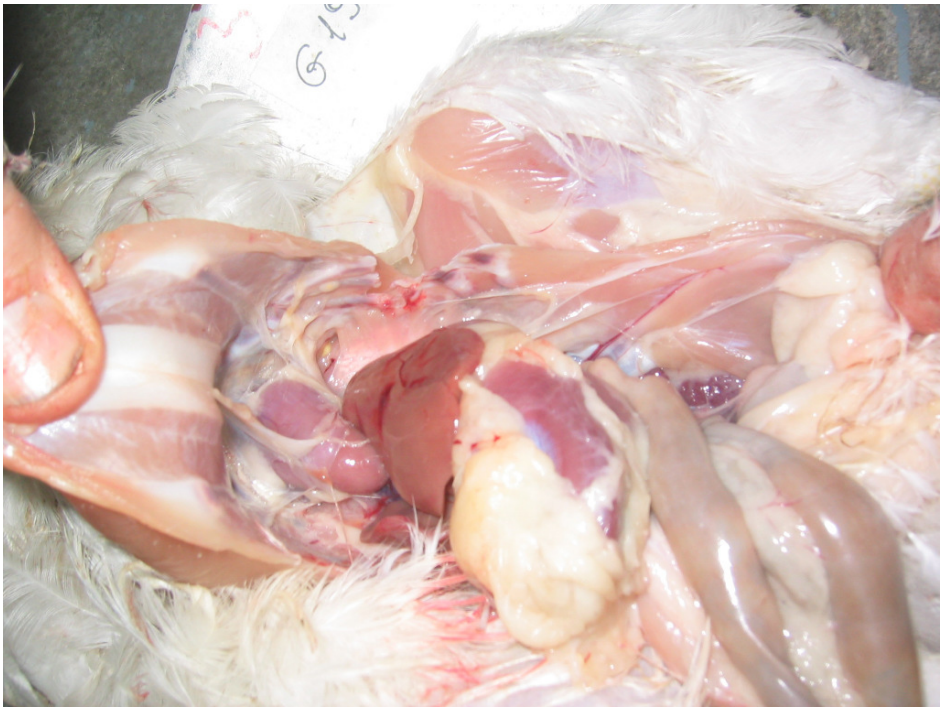


Figure 21. Chicken treated with periodate oxidized antigen (10 µg) + PRR agonists (2,4,9) and challenged with *M. gallisepticum*. No airsacculitis and no peritonitis.

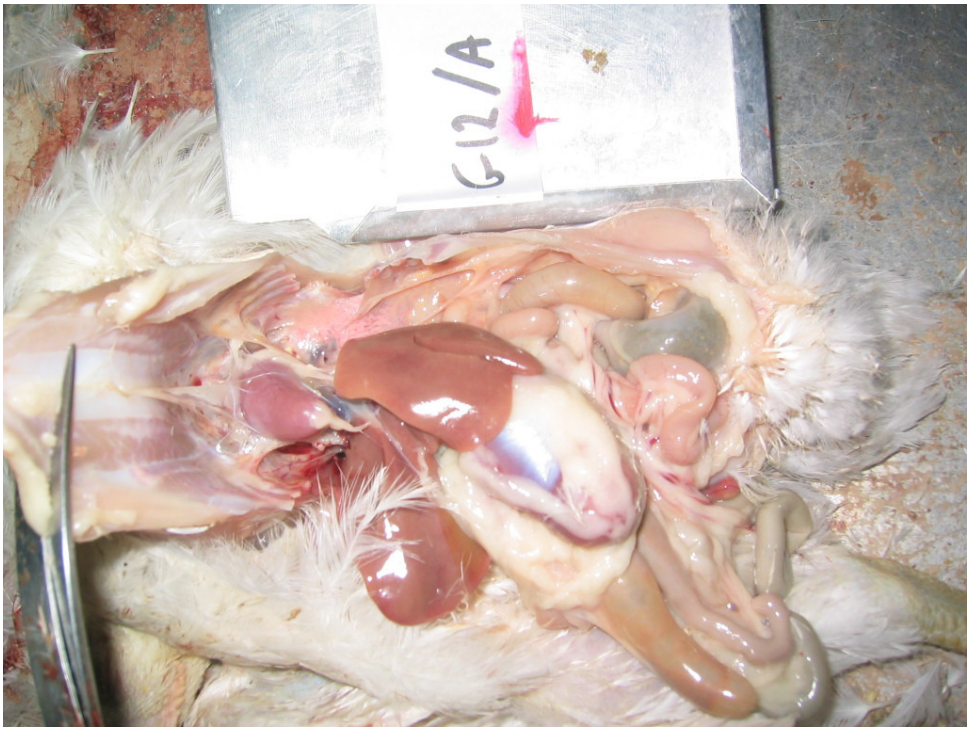


Figure 22. Chicken treated with deacylated antigen (10 µg) + PRR agonists (3,4,9) and challenged with *M. gallisepticum*. No airsacculitis and no peritonitis.

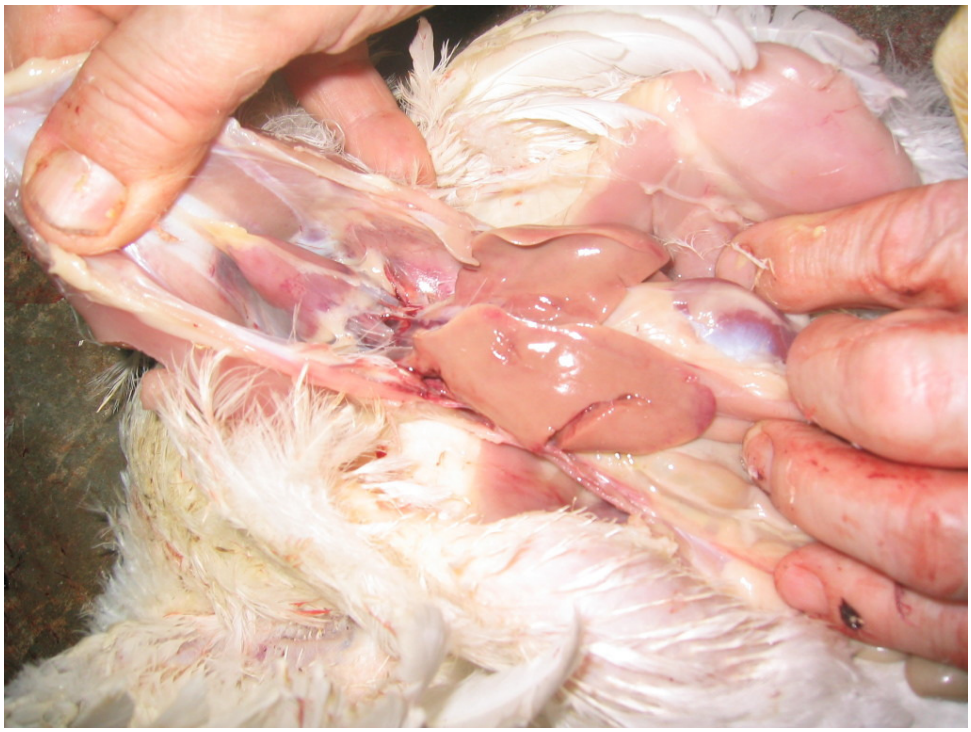


Figure 23. Chicken treated with EndoH treated antigen (10 µg) + PRR agonists (2,4,9) and challenged with *M. gallisepticum*. No airsacculitis and no peritonitis.

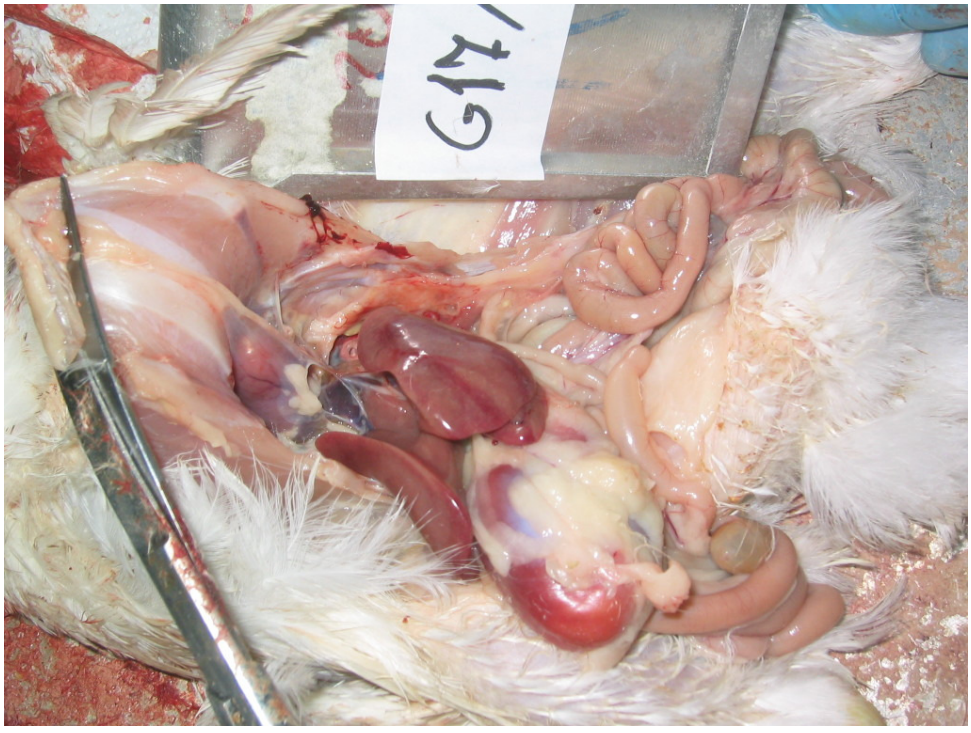


Figure 24. Chicken challenged with *M. gallisepticum* and treated with periodate oxidized antigen (10 µg) + PRR agonists (3,4,9). No airsacculitis and no peritonitis.

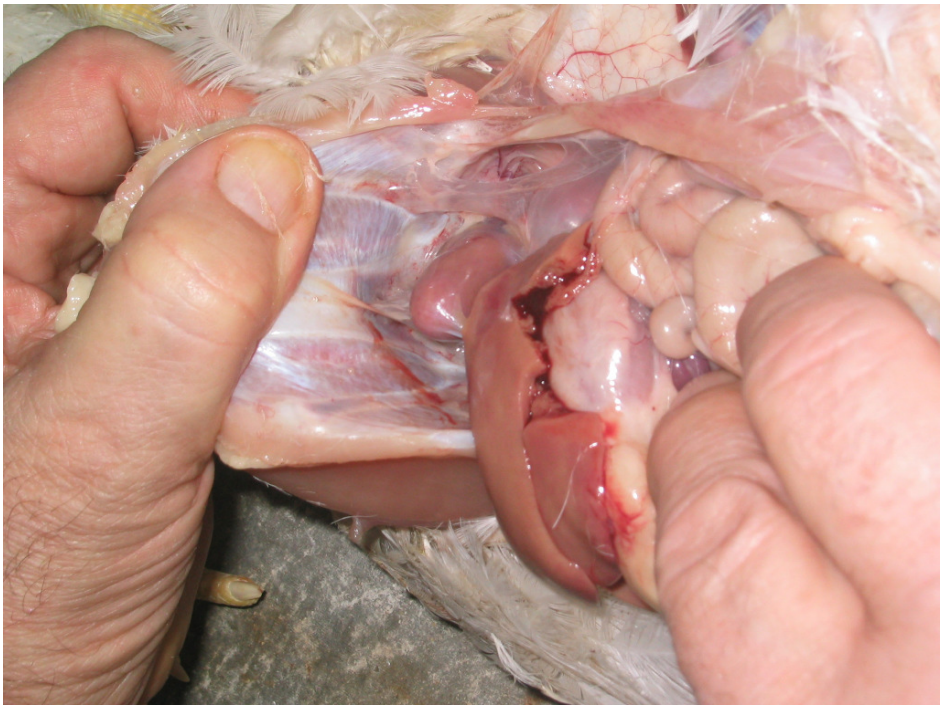


Figure 25. Chicken challenged with *M. gallisepticum* and treated with Con A adsorbed antigen (10 µg) + PRR agonists (3,4,9) and. No airsacculitis and no peritonitis.

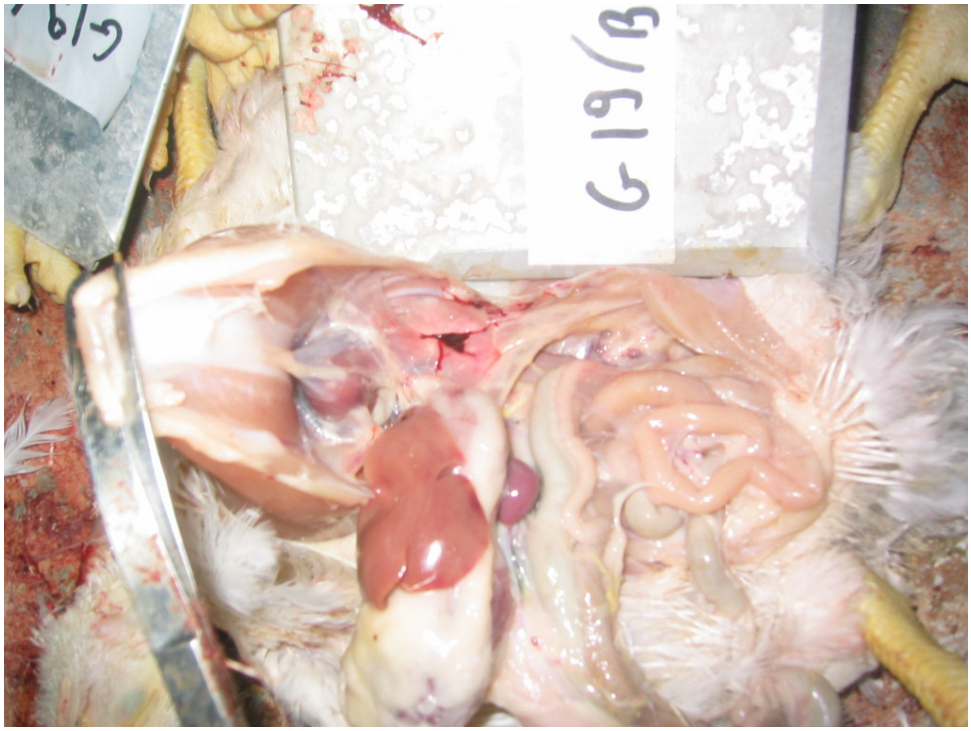


Figure 26. Chicken challenged with *M. gallisepticum* and treated with deacylated antigen (10 µg) + PRR agonists (3,4,9) and. No airsacculitis and no peritonitis

4.7. EXPERIMENT 7: *IN VITRO* STUDIES

4.7.1. Introduction

The innate immune system is the first line of defense against invading pathogens and is, evolutionary speaking, the original immune system. Bacterial compounds are the messengers that signal the presence of a foreign organism by binding to the PRR, such as the TLRs and Nod receptors, considered the sensors of the innate immune system present in monocytes, macrophages and dendritic cells (Akira and Hemmi, 2003; Girardin et al., 2003b). TLRs mediate inflammatory responses. TLRs can activate innate immune cells such as macrophages to produce chemokines or cytokines. TLR activation can lead to production of proinflamatory cytokines (IL-1, IL-6 and TNF-α̃). These cytokines induce acute phase proteins, chemokines and up-regulate expression of adhesion molecules, thereby recruiting inflammatory cells.

Dendritic cells are key to the establishment of adaptive immunity and bridge the innate immunity with the adaptive immune response. TLR signaling can activate and direct DCs to support Th1 cell differentiation. TLR signaling can induce up-regulation of co-stimulatory molecule expression, which functions to stimulate T cell expansion together with antigen presentation. TLR signaling can also induce production of IL-12 and IL-18, which can instruct naive T cells (CD4+) to differentiate into Th1 cells and secrete IFN-γ and participate in cellular immunity, or Th2 cells to release Il-4, IL-10 and IL-13 and participate in humoral immunity. Antigen presentation is critical but not sufficient for T cell clonal expansion. However, TLR signaling up-regulates co-stimulatory molecules that are mandatory for T cell proliferation.

In this study, we have used agonists of these receptors immobilized on microparticles to modulate the immune response. These molecules have in common that they are all pro-inflammatory. Their core signaling mechanism converges towards the nuclear translocation of NF-*k*B and activation of inflammatory genes and production of cytokines, such as TNF-α (Heumann et al., 1994; Sparwasser et al., 1997), with, in addition, a participation in dendritic cell maturation (Kaisho et al., 2001).

TLR-4 has been the most widely studied member of this family of receptors. It is known to recognize LPS from Gram-negative bacteria (Hoshino et al., 1999), whereas TLR-2 binds bacterial lipoproteins/lipopeptides, or mycobacterial components (Hellman, 2001), PepG exclusively interact with its intracellular receptors Nods (Travassos et al., 2004). TLR-9 is known to detect unmethylated bactDNA (Hemmi et al., 2000b). In addition, because of the cooperation between TLR receptors and the common denominators in proximal events of TLR activation, it is not surprising to observe synergistic effects between microbial pathogenic compounds (Takada et al., 2002; Yang et al., 2001). We have demonstrated in a previous experiment that two of the PRR agonists used in this study, PepG and bactDNA induce TNF-α in synergy (Own experiment 4).

In order to understand the biological effect of the microparticles as immunomodulators, we studied their effect on immune cells that are well characterized in an *in vitro* system. We used PBMC as a target to assess the effect of the immunomodulant on the innate immune system, and dendritic cells as a model of antigen presenting cells, which make the link between the innate and the adaptive immune response.

Sterilization of the immunomodulatory microparticle is required for studying its effects in cell culture. Sterilization and destruction of bacteria or fungi for particles to which fragile ligands are attached is difficult. Typical sterilization conditions require extremes of pH (e.g., 1M NaOH) or high temperature that is detrimental to delicate proteins or other biological signaling molecules. Chemical sterilization with formaldehyde or glutaraldehyde may change the epitope conformation and presentation on antigens. Mild oxidizing agents such as diluted peroxide or chlorine generators

suffer from the same deficiency. Ethyleneoxide sterilization may produce the same effect and can only be performed only by use of special equipment. Therefore, we have chosen a chemical sterilization agent that has been shown is capable of destroying both Gram negative and Gram-positive microorganisms over a prolonged period of time. Residual reagent can be removed with dilute alcohol that is not expected to affect the immunological activity of immobilized proteins.

4.7.2. Materials and methods

Sterile Microparticle Preparation
Agarose-based microparticles (1-10 µm diameter) were used to immobilize purified *M. gallisepticum* antigens and Toll-like receptor agonists along with Nod receptors agonists, including Peptidoglycan (PepG), Lipolysaccharide (LPS), bacterial DNA (bactDNA) and Poly I:C (poly-inosilic acid:poly-citidylic acid) (all from Sigma). Antigens were immobilized at 50 µg/ml to microparticles while PepG at 10 µg/ml, LPS at 10 µg/ml, bactDNA at 50 µg/ml and poly I:C at 125 µg/ml ml. The beads were sterilized by a 6-day incubation with a 4-fold excess of a chemical sterilant after which the beads were washed with an excess of 50% grain alcohol, and re-suspended in PBS as a 2 % solution. Sterility has been tested by plating the microparticles on agar plate and by incubating 100 µl 2% microparticle suspension in 1.5 ml of RPMI-1640 for up to 14 days.

Preparation of fluorescein-labeled microparticles
Particles were activated by cyanogen bromide (CNBr) activation method. Briefly, to 30 ml of agarose microbeads, 30 ml of 2M sodium carbonate solution was added and kept in an ice bath for 3-5 minutes without mixing. Then, 1.5g CNBr was weighed and dissolved into 9 ml acetonitrile. Immediately, the CNBr solution was added to the resin mixture and vigorously mixed on an ice bath for 2 minutes. Subsequently, it was washed with 500 ml of ice-cold water by spinning down at 9,000xg, 2°C for 5 minutes. To the CNBr activated microparticles, 0.1% fluorescein isothiocyanate solution was added at pH 8.0. The solution was gently mixed at 2-8°C for 20 hours. The supernatant was separated by centrifugation and the resin washed with 10 bed volumes of DI water. The coupled resin was stored in sterile LAL water at 2-8°C.

Peripheral blood mononuclear cells and dendritic cells cultures
Peripheral blood mononuclear cells were prepared by Ficoll separation of peripheral blood from healthy volunteers as previously described (Amoureux et al., 2004). Mononuclear cells were plated at a density of 2.10^6 cells in 1.5 ml RPMI-1640 (Irvine Scientific, Irvine, CA), supplemented with 100 U/ml Penicillin/Streptomycin, 2 mM Glutamine and 10 % FCS in 6-well tissue culture plates. The cells were treated for 18 hours with different microparticle preparations, control microparticles on which no antigen or adjuvant was immobilized, and microparticles on which *Mycoplasma gallisepticum* antigens depleted of ConA binding antigens were immobilized (ConA Ag) with or without the following PRR agonist combinations: 2,4,9: PepG, LPS, and bactDNA (Sigma, St Louis, MI) or PRR agonist combinations 3,4,9: poly I:C, LPS, and bactDNA.

Dendritic cells were derived from monocytes. After plating PBMC as described above, the non-adherent cells were removed the next day and the adherent cells were cultured in RPMI+10 % FCS + IL-4 (100 ng/ml)+GM-CSF (80 ng/ml) for 7 days and the media was renewed every 2-3 days (Figure 27). For both PBMC and dendritic cell cultures, 150 µl of the 2 % bead solution described above was applied to 1.5 ml of culture. Cultures were performed in duplicate.

The interaction of fluorescein-labeled microparticles with DCs
PBMC were prepared by Ficoll separation of peripheral blood from healthy volunteers as previously described (Amoureux et al., 2004). Mononuclear cells were plated at a density of 2×10^6 cells in 1.5 ml RPMI-1640 (Irvine Scientific, Irvine, CA), supplemented with 100 U/ml Penicillin/Streptomycin, 2 mM Glutamine and 10 % FCS in 6-well tissue culture plates. The cells

were treated for 24 hours with fluorescein-labeled microparticles. Dendritic cells were trypsinized and centrifuged for 10 minutes at 2000 rpm. Duplicate cultures were pooled for further processing. The pellet was re-suspended in 100µl of PBS containing 2 % FCS for flow cytometry processing (Figure 28).

TNF-α ELISA

TNF-α was measured from the culture supernatant using ELISA after 18 hours treatment and elimination of non-adherent cells by centrifugation as described (Amoureux et al., 2004). The TNF-α ELISA kit from Cell Sciences (Norwood, MA) was used. The coefficient of variation for the TNF-α ELISA was less than 8.9 % for duplicate measurements of a single culture. The lowest detection limit is 10 pg/ml.

Flow cytometry

Dendritic cells were trypsinized and centrifuged for 10 minutes at 2000 rpm. Duplicate cultures were pooled for further processing. The pellet was re-suspended in 100µl of PBS containing 2 % FCS. 20 µl of anti-MHCII antibody labeled with fluorescein isothiocyanate and anti-CD86 labeled with phycoerythrin (both from BD Biosciences) were added to the cells, which were incubated for 15 minutes on ice. The cells were washed with PBS+2 % FCS and re-suspended in 100µl of PBS+ 2 % FCS for flow cytometry processing. As a control, the isotype matched control antibody was added to the corresponding cell suspension.

4.7.3. Results

The sterility test confirmed that we were able to prepare sterile microparticles. We have been also able to induce the differentiation of monocytes into dendritic cells.

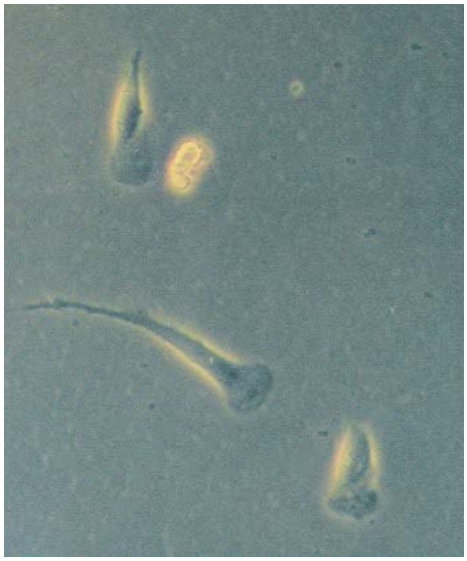


Figure 27. Monocyte derived dendritic cells
(Brightfield microscopy 20X)

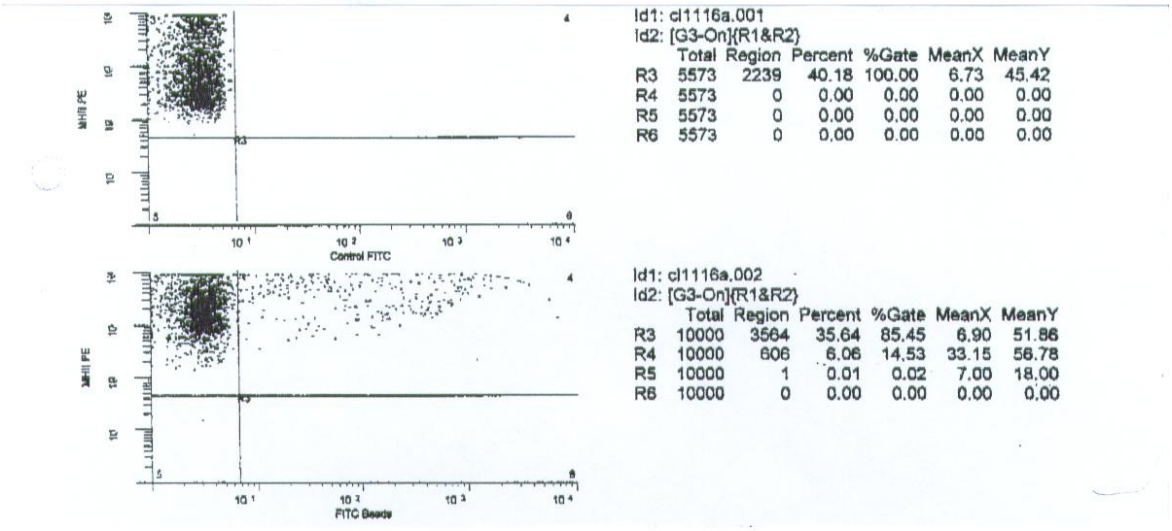


Figure 28. Flow cytometry analysis of the interaction of fluorescein-labeled microparticles with Dendritic cells

The results show that the microparticles labeled with fluorescein isothiocyanate is interacting with the DCs. Because of the phagocytic nature of the DCs it is most likely that the particles are phagocytosed, however, this has been confirmed by blocking the phagocytosis by cytochalasin B.

The interaction of the microparticles with the innate immune system

The results depict the effect of the microparticles on TNF-α secretion by PBMC. Control microparticles did not induce a detectable secretion of TNF-α in the PBMC conditioned media. The other three types of microparticles (*M. gallisepticum* antigens after depletion of ConA binding antigens and similar antigens co-immobilized with PepG, LPS, bactDNA and poly I:C) induced a significant TNF-α secretion by PBMC, compared to control microparticles. In addition, the presence of bacterial compounds as immunomodulator generated 4.6-fold (for combination 2,4,9) and 3-fold (for combination 3,4,9) more TNF-α as compared to the antigens alone (Figure 29, A). These results indicate that the microparticles do interact with PBMC and are able to activate TLRs and Nod receptors to generate a physiological response in accordance with the known effect of these molecules on cells of the immune system.

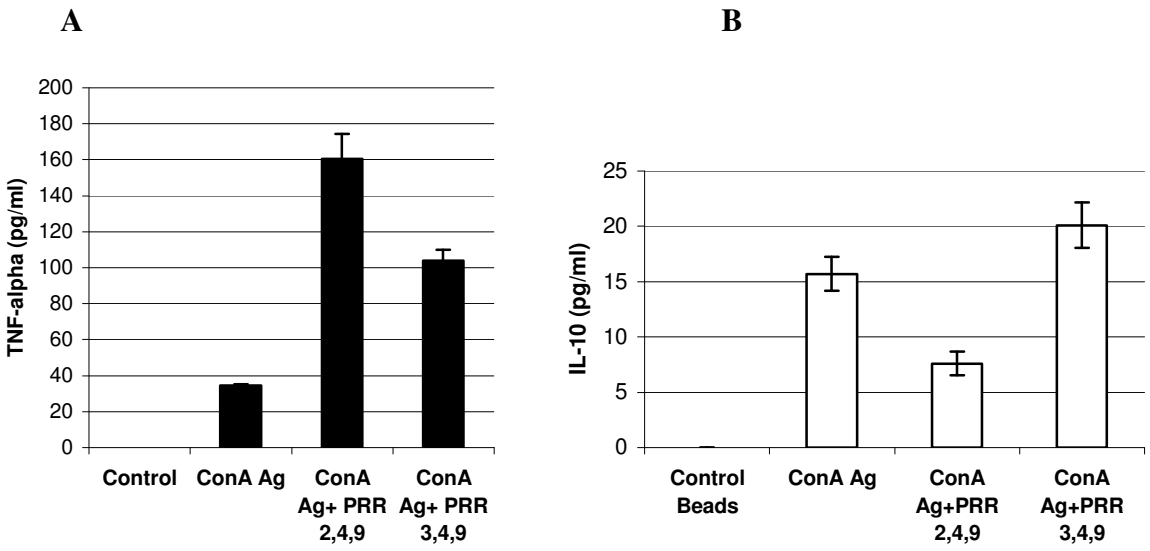


Figure 29. Effect of beads on monocytes

The effect of the microparticles on anti-inflamatory cytokine, IL-10 secretion by PBMC is shown in Figure 29, B. Control microparticles did not induce a detectable secretion of IL-10 in the PBMC conditioned media. The other three types of microparticles (*M. gallisepticum* antigens after depletion of ConA binding antigens and similar antigens co-immobilized with PepG, LPS, bactDNA and poly I:C) induced IL-10 secretion by PBMC, compared to control microparticles. In addition, the presence of bacterial compounds as immunomodulators that can skew the immune response toward pro-inflamatory cytokines reduced the amount of IL-10 production by over 2-fold (PRR 2,4,9).

However, the addition of PRR 3,4,9 did not result in the reduction of IL-10 level as compared to the antigens alone (Figure 30). These results indicate that the microparticles do interact with PBMC and are able to activate TLRs and Nod receptors to generate a physiological response in accordance with the known effect of these molecules on cells of the immune system.

TNF-α measured by ELISA from the culture supernatant of PBMC after 18 hours of treatment with different microparticle preparations: control microparticles (control) onto which no antigen or PRR agonist was immobilized, and microparticles to which *M. gallisepticum* antigens depleted of ConA binding antigens were immobilized (ConA Ag) with or without the following PRR agonist combinations: 1) PepG, LPS, bacterial DNA (bactDNA) (Sigma, St Louis, MO) (ConA Ag + PRR 2,4,9) or 2) poly I:C, LPS, bactDNA (ConA Ag + PRR 3,4,9) (Figure 30). The ratio of TNF-α and IL-10 is shown in Figure 31.

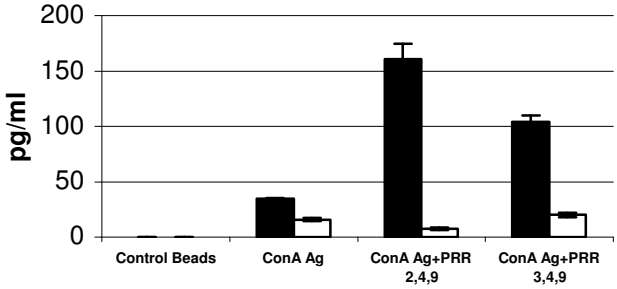


Figure 30. Cytokines induced in PBMC

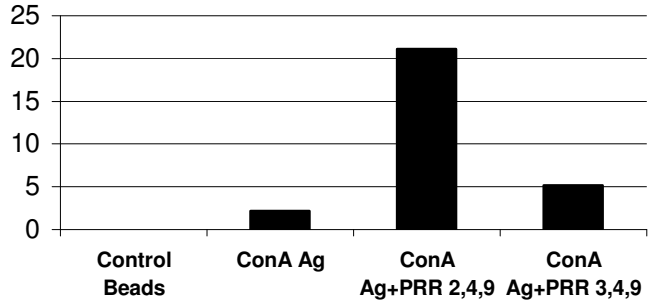


Figure 31. Ratio of TNF-α/IL-10 in PBMC

To further characterize the cellular response to the immunomodulator microparticle, we exposed dendritic cells to the microparticles. We observed an up-regulation of the MHCII molecule and the co-stimulatory molecule CD86 (B7-2). The presence of either PRR agonist combinations seems necessary to measure an MHCII increase at the surface of dendritic cells after 18 hours of treatment. Since the MHCII presence at the cells surface is a dynamic process, a different incubation time with ConA Ag beads may be needed to observe an increase in MHCII at the cell surface. MHCII allows presentation of antigens to T-cells and CD86 interacts with CD28 on T-cells. Concomitant antigen presentation by dendritic cells and B7-co-stimulatory ligand (such as CD86) interaction with CD28 results in T-cell activation. These *in vitro* data indicate that the microparticles induce changes that are hallmarks of dendritic cells maturation and that are required for induction of innate and adaptive immunity (Figure 32).

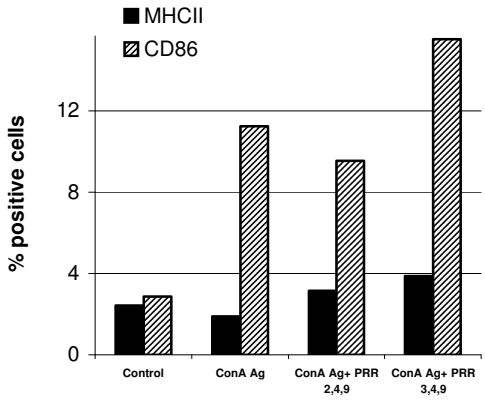


Figure 32. Induction of MHC-II and CD86 in dendritic cells

MHCII and CD86 expression on the surface of dendritic cells after 18 hours exposure to different microparticle preparations: control microparticles (control) to which no antigen or PRR agonist was immobilized, and microparticles to which *M. gallisepticum* antigens depleted of ConA binding antigens were immobilized (ConA Ag) with or without the following PRR agonist combinations: 1) PepG, LPS, bacterial DNA (bactDNA) (Sigma, St Louis, MI) (ConA Ag + PRR 2,4,9) or 2) poly I:C, LPS, bactDNA (ConA Ag + PRR 3,4,9).

The results show the effect of the microparticles on TNF-α secretion by DCs. Control microparticles did not induce a detectable secretion of TNF-α in the conditioned media. The other three types of microparticles (*M. gallisepticum* antigens after depletion of ConA binding antigens

and similar antigens co-immobilized with PepG, LPS, bactDNA and poly I:C) induced a significant amount of TNF-α by DCs, compared to control microparticles. Interestingly, the presence of bacterial compounds as immunomodulator generated less TNF-α as compared to the antigens alone (Figure 33). These results indicate that the microparticles do interact with DCs and are able to activate TLRs and Nod receptors to generate a physiological response in accordance with the known effect of these molecules on cells of the immune system.

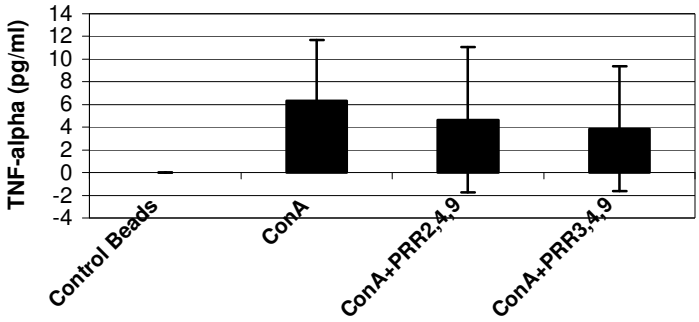


Figure 33. TNF-α production by dendritic cells

The effect of the microparticles on anti-inflamatory cytokine, IL-10 secretion by DC is shown in Figure 34. Control microparticles did not induce a detectable secretion of IL-10 in the DC conditioned media. The other three types of microparticles (*M. gallisepticum* antigens after depletion of ConA binding antigens and similar antigens co-immobilized with PepG, LPS, bactDNA and poly I:C) induced IL-10 secretion by the DCs, compared to control microparticles. In addition, the presence of bacterial compounds as immunomodulators that can skew the immune response toward pro-inflamatory cytokines reduced the amount of IL-10 production significantly (PRR 2,4,9).

However, the addition of PRR 3,4,9 resulted in further reduction of IL-10 level (Figure 34). These results indicate that the microparticles do interact with the DCs and are able to activate TLRs and Nod receptors to generate a physiological response in accordance with the known effect of these molecules on cells of the immune system.

TNF-α measured by ELISA from the culture supernatant of DC after 18 hours of treatment with different microparticle preparations: control microparticles (control) onto which no antigen or PRR agonist was immobilized, and microparticles to which *M. gallisepticum* antigens depleted of ConA binding antigens were immobilized (ConA Ag) with or without the following PRR agonist combinations: 1) PepG, LPS, bacterial DNA (bactDNA) (Sigma, St Louis, MO) (ConA Ag + PRR 2,4,9) or 2) poly I:C, LPS, bactDNA (ConA Ag + PRR 3,4,9). The ratio of TNF-α and IL-10 is shown in Figure 35.

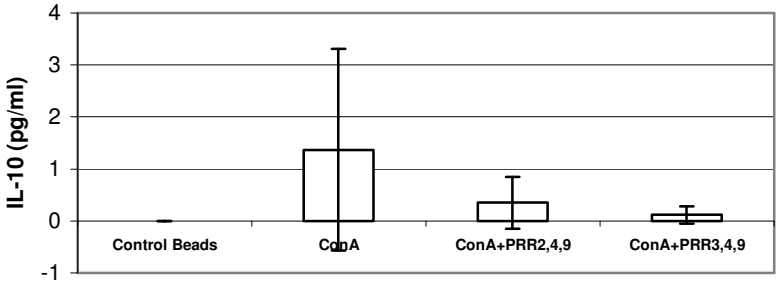


Figure 34. IL-10 production by dendritic cells

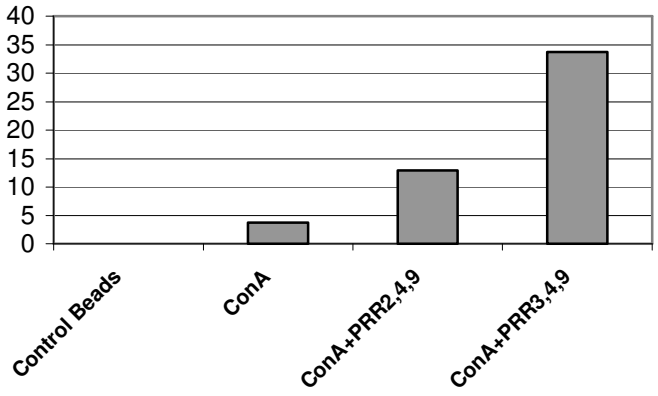


Figure 35. Ratio of TNF-α and IL-10 produced by dendritic cells

4.7.4. Discussion

We have been able to show *in vitro* that the microparticles interact with the innate and adaptive immune system. The pathogen mimicking microparticles were able to up-regulate the MHCII molecule and the co-stimulatory molecule CD86 (B7-2) on dendritic cells. The presence of either PRR agonist combinations seems necessary to up-regulate MHCII, which allows presentation of antigens to T-cells and CD86, which interacts with CD28 on T-cells. Dendritic cells take up pathogens and sense the infection mainly through TLRs. The TLR signaling then activates the dendritic cells to mature (Dabbagh, 2002).

These in vitro data indicate that the pathogen mimicking microparticles have been taken up by the dendritic cells as they induce changes that are hallmarks of dendritic cells maturation and that are required for induction of innate and adaptive immunity. The microparticles that included the PRR/TLR agonist molecules induced significant up-regulation of the co-stimulatory molecules, which is mandatory for T cell proliferation (Shortman, 2002, Kantele, 1999).

Pro-inflammatory and anti-inflamatory cytokine balance has an effect on the skewing of the immune response (Beyer, 2001, Boonstra, 2003). In the in vitro experiment we were able to show that the different PRR agonist molecules influenced the pro-inflamatory versus anti-inflammatory cytokine balance. The combination of ConA modified antigen with the PRR agonist 2,4,9 gave the highest ratio of pro versus anti-inflammatory cytokine balance. This same combination was the most protective in our in vivo challenge experiment, as well. We have also confirmed that the *in vitro* system using dendritic cell and PBMC could be used for preliminary evaluation of the effect of different combinations of immunomodulators on the skewing of the immune response (Spellberg, 2001).

V. FINAL CONCLUSIONS

The innate immune system is the first line of defense against invading pathogens and is, evolutionary speaking, the original immune system. Bacterial compounds are the messengers that signal the presence of a foreign organism by binding to PRR such as the TLRs and Nod receptors, DC-SIGN, etc., considered the sensors of the innate immune system present in monocytes, macrophages and dendritic cells (Akira and Hemmi, 2003; Girardin et al., 2003b). These TLRs have in common that their core signaling mechanism converges towards the nuclear translocation of NF-*k*B and activation of genes and production of cytokines, such as TNF-α (Heumann et al., 1994; Sparwasser et al., 1997), in addition to a participation in dendritic cell maturation (Kaisho et al., 2001). Dendritic cells provide the cellular link between the innate and adaptive immune responses.

In this research, we have used two approaches to manipulate the innate immune system either by removing activators of these receptors or using them as immunomodulators immobilized on microparticles as part of a pathogen mimicking system containing mycoplasmal antigens. We have focused our research on the activators of TLRs 2, 3, 4, 9 and Nod2.

TLR-4 has been the most widely studied member of this family of receptors. It is known to recognize LPS from Gram-negative bacteria (Hoshino et al., 1999), whereas TLR-2 binds PepG, bacterial lipoproteins, lipopeptides, or mycoplasmal components (Hellman and Warren, 2001), although it has been recently suggested that PepG could exclusively interact with its intracellular receptors Nods (Travassos et al., 2004). TLR-9 is known to detect unmethylated bactDNA (Hemmi et al., 2000). TLR-3 is activated by viral RNA or poly I:C. In addition, because of the cooperation between TLR receptors and the common denominators in proximal events of TLR activation, it is not surprising to observe synergistic effects between microbial pathogenic compounds (Takada et al., 2002; Yang et al., 2001).

In the first part of this research, we have demonstrated that removing bacterial compounds by affinity methods could affect the innate immune system *in vitro*. The use of the particle-based affinity resins is a novel approach to address these problems.

In the second part, we have showed that a pathogen mimicking microparticle, using PRR/TLR agonists could be used to prevent pathological lesions and colonization induced by *M. gallisepticum* infection or successfully treat a pre-established infectious disease *in vivo*. Our pathogen mimicking microparticles represents a novel approach that has not been described in the scientific literature.

Several different particle-based systems have been used experimentally as carrier for different antigens (Ermak, 1998). Chitosan, poly-DL-lactic acid, or polyacryl starch micro particles have previously been described as a drug carrier system (Laakso, 1986). ISCOM particles and liposomes have been used as prototype vaccines (Abusugra and Morein, 1999). The limitations of the particles prepared by precipitation are that the conditions for the precipitation can differ for different antigens. The particle must break down before the antigen is released. The incorporation of different antigens and PRR agonist into the surface is not feasible. This is also true for ISCOM and liposomes. In case of polyacrylamide particles, the biocompatibility is a potential problem along with the fact that the breakdown of the particles in the dendritic cells has not been demonstrated.

Our pathogen mimicking microparticles are made of the natural polysaccharide of galactose polymers. This provides excellent biocompatibility and they are readily biodegradable. The immobilization chemistry allows the immobilization of several antigens along with different combinations of PRR agonist molecules in a controlled and highly reproducible fashion. The covalent linkage between antigen and the particles assures that the same antigen-processing cells take up both the TLR agonist adjuvant and the antigen. The size of the agarose microparticles

(<5µm) makes them suitable for their entry into the Peyer's patches and into immune cells, such as dendritic cells.

In order to understand the biological effect of the immunomodulatory microparticles, we have studied their effect on immune cells that are well-characterized *in vitro* systems. We used PBMC as a target of the PRR component of the pathogen mimicking microparticle, and dendritic cells as a model of antigen presenting cells, which make the link between the innate and the adaptive immune response.

The results presented here indicate that the microparticles interacted with PBMC and DC and were able to activate PRR (TLRs and Nod receptors) to generate a physiological response in accordance with the known effect of these molecules on cells of the immune system.

We have observed an up-regulation of the MHCII molecule and of the co-stimulatory molecule CD86. The presence of PRR agonist combinations seemed necessary to measure an MHCII increase on the surface of dendritic cells. MHCII allows presentation of antigens to T-cells and CD86 interacts with CD28 on T-cells. Concomitant antigen presentation by dendritic cells and B7-co-stimulatory ligand (such as CD86) interaction with CD28 results in T-cell activation.

Our *in vitro* data indicate that the microparticles used as an immunomodulator induce changes that are hallmarks of dendritic cells maturation and that are required for induction of innate and adaptive immunity. Because of the dynamic interactions of TLR agonists, DC and T-cells, we are aware of the limitations of such *in vitro* system. These *in vitro* data give some insights into the mechanism of action of the immunomodulatory microparticles, *in vivo*. The microparticle composition that showed the highest pro-inflammatory/anti-inflammatory cytokine ratio and produced the highest increase in the level of co-stimulatory molecules on dendritic cells was also the most protective in our *in vivo* challenge experiment.

VI. RESEARCH ACHIEVEMENTS

Our achievements can be summarized as follows:

We have demonstrated successful removal of the TLR-4 agonist molecule, endotoxin from solutions using a novel affinity technology.

The TLR agonist molecules induce the innate immune response resulting in the production of among others, TNF-α and Tissue Factor. We have demonstrated efficient removal of TLR-2, TLR-4, TLR-9 and Nod2 agonist molecules, such as peptidoglycan, endotoxin, lipoprotein and bacterial DNA from blood and plasma using a novel affinity technology. We have shown the reduction in the level of innate immune response relevant molecules, such as TNF-α and Tissue Factor without negatively effecting cell count or coagulation.

We have demonstrated mycoplasma capture from solutions, such as serum used in cell culture, by a novel method using affinity chromatography without affecting the growth supporting properties or the composition of the serum.

We have demonstrated synergistic effect of peptidoglycan and bacterial DNA on the stimulation of the innate immune response. TNF-α and Tissue Factor levels were increased more significantly by the combination of these TLR agonist molecules and, the effective concentrations of these TLR agonists that induces TNF-α and Tissue Factor production were established in a monocyte activation assay. This is a new observation that has not been described in the literature.

We have prepared novel pathogen mimicking microparticle compositions using *M. gallisepticum* immunoaffinity purified antigens and different combinations of PRR/TLR (TLR 3,4,9 and TLR 4,9-Nod2) agonists. These immunomodulatory microparticles were tested in a *M. gallisepticum* challenge model in chickens.

We have shown that the addition of PRR agonist molecules to microparticles induced immune response and when antigen was added, increased the *M. gallisepticum* specific protective immunity. We have demonstrated the effect of the different post-transcriptional modifications of the mycoplasmal antigens that seems to participate in the pathomechanism of *M. gallisepticum* infection. We have demonstrated that by removing these post-transcriptional modifications using Endoglycosidase H, Concanavalin A, sodium periodate,as well as deacylation, we were able to achieve protective immunity. We demonstrated that is it possibleto achieve protective immunity not only preventatively, but also therapeutically, post-infection. This has not been described for veterinary vaccines. We have also shown that the antigens, which contain these post-transcriptional modifications, are able to induce immune tolerance against *M. gallisepticum* infection.

We have demonstrated the effect of these immunomodulatory microparticles *in vitro* with PBMC and dendritic cells. We have demonstrated that the microparticles interact with the cells of the innate immune system, such as PBMC and dendritic cells. We have shown that the immunomodulatory microparticles induce changes that are hallmarks of dendritic cells maturation. Since dendritic cells are the link between the innate and adaptive immunity, we have been able to show that the microparticles can influence both the innate and adaptive immune response.

VII. LIST OF RELATED PUBLICATIONS

Articles

Amoureux, M.C., Hegyi, E., Le, D., Grandics, P., Tong, H., **Szathmary, S**. (2004) A new method for removing endotoxin from plasma using hemocompatible affinity chromatography technology, applicable for extracorporeal treatment of septic patients. J. Endotoxin Res. 10:85-95.

Amoureux, M.C., Rajapakse, N., Hegyi, E., Le, D., Grandics, P., **Szathmary, S**. (2004) Endotoxin removal from whole blood by a novel adsorption resin: efficiency and hemocompatibility. Int. J. Artif. Organs. 27:480-487.

Amoureux, M.C., Rajapakse, N., **Szathmary, S**. (2005) Peptidoglycan and bacterial DNA synergistically induce inflamation and coalgulation markers. Mediators of Inflamation. (accepted)

Biró, J., Erdei, N., **Szathmary, S**., Stipkovits, L. (2004) *Mycoplasma bovis* kimutatása különböző PCR módszerekkel. Magyar Állatorvosok Lapja, 126:626-630.

Grandics, P., **Szathmary, S**., Szathmary, Z., O'Neill, T. (1991) Integration of cell culture with continuous, on-line sterile downstream processing. Ann. N Y. Acad. Sci. 646:322-333.

Grandics, P., Szathmary, Z., **Szathmary, S**. (1990) A novel immunoaffinity chromatography system for the purification of therapeutic proteins. Ann. NY. Acad. Sci. 589:148-156.

Szathmary, S., Grandics, P. (1990) Bioreactor integration with downstream processing. Nature Bio/technology. 8:924-925.

Szathmary, S., Grandics, P. (1991) A method for purifying IgM, IgA, IgE, IgG1, and other monoclonal antibodies. Am. Biotechnol. Lab. 9:31-33.

Szathmary, S., Hegyi, E., Amoureux, M.C., Rajapakse, N., Chicorka, L., Szalai, G., Reszegi, K., Derbyshire, Z., Paluh, J., Dodson, B., Grandics, P. (2004) Characterization of the DialGuard Device for Endotoxin Removal in Hemodialysis. Blood Purif. 22:409-415.

Szathmary, S., Pitlik, E., Stipkovits, L. (2005) A Toll-like receptorok szerepe a szervezet védekező mechanizmusában. Magyar Állatorvosok Lapja (submitted)

Szathmary, S., Rajapakse, N., Székely, I., Pitlik, E., Biró, J., Erdei, N., Stipkovits, L. (2004) Binding of Mycoplasmas to Solid Phase Adsorbents. Acta Vet. Hung. (submitted)

Tamic, L., **Szathmary, S**., Grandics, P., Markland, F.S. Jr. (1986) The goat blood binder of triamcinolone acetonide does not interfere with glucocorticoid receptor purification from mammary tissue. J. Recept. Res. 6:475-487.

Abstracts published in journals

Amoureux, M,C., Rajapakse, N., Hegyi, E., Le, D., **Szathmary, S**., Tong, H., George, N. and Grandics, P. (2003) Endotoxin Removal From Human Plasma And Blood By A Hemocompatible, Affinity-Based Extracorporeal Technology For The Treatment Of Sepsis. J. Am.Soc.Nephrology, American Society of Nephrology Meeting, San Diego. Oral presentation

Amoureux, M.C., Rajapakse, N., Hegyi, E., Le, D., **Szathmary, S**., Grandics, P. (2004) Bacterial toxins removal from human plasma and blood by a hemocompatible, affinity-based extracorporeal

technology for the treatment of sepsis. J. Am.Soc.Nephrology. American Society of Nephrology Meeting, St Louis. Oral Presentation

Grandics, P., Amoureux, M.C., Rajapakse, N., Hegyi, E., Tong, H., George, N. and **Szathmary, S**., (2003) Endotoxin-Free Dialysate Using DialGuard™ J.Am.Soc.Nephrology. American Society of Nephrology Meeting, San Diego. Oral Presentation

Szathmary, S., Amoureux, M.C., Chicorka, L., Hegyi, E., Tong, H. and Grandics, P. (2003) A novel method for producing sterile water for injection (WFI) on-line. J. Am. Soc. Nephrology. American Society of Nephrology Meeting, San Diego. Oral Presentation

Proceedings

Stipkovits, L., Biro, J., **Szathmary, S**., Klein, U. (2004) Sensitivity testing of mycoplasma pathogens to antimicrobials. Proceeding of IOM, Athens, USA. Poster

Szabo, I., **Szathmary, S**., Stipkovits, L. (2004) Detection of maternal antibodies to M. hyopneumoniae in swine of farrowing-fattening type of farm. Proceeding of 18th Congress of IPDS. Hamburg. Poster

VIII. ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Laszlo Stipkovits, for introducing me the science of mycoplasmology, for teaching me how to design and conduct reliable animal studies and showing me the importance of being precise, critical and attentive to the smallest details. I really enjoyed his support and our inspiring brain storming sessions. I am thankful to the Head of the Veterinary Medical Research Institute, Balazs Harrach, who gave me the opportunity to complete my studies at the Institute. I am also grateful to Judit Biro, Noemi Erdei, Ibolya Szekely, Agnes Tuboly, Nandani Rajapakse, Marie-Claude Amoureux, Warren Chester, James Harris, Hung Tong and Peter Grandics for the discussions and all the help and support. I am also thankful for the help of Tibor Bacsko and Zoltan Hargittai with the animal studies. Finally, I would like to say thanks to my colleagues and family, especially my daughter, who tolerated and supported me in the hard days of thesis- and article-writing, and at the times when I spent my life, the long days and weekends in the lab being deeply involved with mycoplasmas.

IX. LIST OF REFERENCES

Abusugra, I., and Morein, B. (1999). Iscom is an efficient mucosal delivery system for *Mycoplasma mycoides* subsp. mycoides (MmmSC) antigens inducing high mucosal and systemic antibody responses. FEMS Immunol. Med. Microbiol. 23. 5-12.

Akira, S., Takeda, K. and Kaisho, T. (2001). Toll-like receptors. critical proteins linking innate and acquired immunity. Nat Immunol. 2. 675–80.

Akira, S. and Hemmi, H. (2003). Recognition of pathogen-associated molecular patterns by TLR family. Immunol Letters. 85. 85-95.

Alcantar, N. A., Aydil, E. S. and Israelachvili, J. N. (2000). Polyethylene glycol-coated biocompatible surfaces. J Biomed Mater Res. 51. 343-351.

Alexopoulou, L., Holt, A. C., Medzhitov, R. and Flavell, R.A. (2001). Recognition of double-stranded RNA and activation of NFkappaB by TOLL-like receptor 3. Nature. 413, 732-738.

Althman, R. and Philpott, D. (2004). Innate immunity via Toll-like receptors and Nod proteins. Curr. Opn. Microbiol. 7. 25-32.

Alvarez, C. P., Lasala, F., Carrillo, J., Muniz, O., Corbi, A. L. and Delgado, (2002). R. C-type lectins DC-SIGN and LSIGN mediate cellular entry by Ebola virus in cis and in trans. J Virol. 76. 6841–4.

Amano, K. and Fukushi, K. (1984). Electron microscopic studies of endotoxin treated with alkaline and acid reagents. Microbiol Immunol. 28. 161-168.

Amoureux ,M. C., Hegyi, E., Le, D., Grandics, P., Tong, H. and Szathmary, S. (2004). A new method for removing Endotoxin from plasma using a hemocompatible affinity chromatography technology, applicable for extracorporeal treatment of septic patients. J Endotoxin Res. 10. 85-95.

Amoureux, M. C., Rajapakse, N., Stipkovits, L. and Szathmary, S. (in press). Peptidoglycan and bacterial DNA induce inflammation and coagulation markers in synergy, Mediators Inflammation.

Appelmelk, B. J., Monteiro, M. A., Martin, S. L., Moran, A. P. and Vandenbroucke-Grauls, C. M. (2000). Why Helicobacter pylori has Lewis antigens. Trends Microboil. 8. 565–70.

Appelmelk, B. J., van Die, I., van Vliet, S. J., Vanden-broucke-Grauls, C. M. J. E., Geijtenbeek, T. B. and van Kooyk, Y. (inpresss). The dendritic cell C-type lectin DC—SIGN binds to surface glycans of the human pathogens *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Schistosoma mansoni* and *Leishmania mexicana*. J Immunol.

Athamna, A., Kramer, M. R. and Kahane, I. (1996). Adherence of *Mycoplasma pneumoniae* to human alveolar macrophages. FEMS Immunol Med Microbiol. 15. 135-141.

Aurell, C. A. and Wistrom, A. O. (1998). Critical aggregation concentrations of Gram negative bacterial lipopolysaccharides (LPS). Biochem Biophys Res Commun. 253. 119-123.

Baggerman, C., Pathmamanoharan, C., Spies, F., Joosten, J.G. and Junginger, H.E. (1985). Physicochemical properties of endotoxins in large volume parenterals. J Pharm Pharmacol. 37. 521-527.

Banchereau, J. and Steinman, R. M. (1998). Dendritic cells and the control of immunity. Nature 392. 245–52.

Basu, S., Binder, R.J., Suto, R., Anderson, K.M., Srivastava, P.K. (2000) Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappaB pathway. Int. Immunol. 12. 1539-1546.

Bates, D. W., Parsonnet, J. and Ketchum, P. A., (1998). Limulus amebocyte lysate assay for detection of endotoxin in patients with sepsis syndrome. AMCC Sepsis Project Working Group. Clin Infect Dis. 27. 582-591.

Beier, R. (1998). Kinetics of particle uptake in the domes of Peyer's patches. Am J Physiol. 275. 130-7.

Bendigs, S., Salzer, U. and Lipford, G. B. (1999). CpG-oligodeoxynucleotides co-stimulate primary T cells in the absence of antigen-presenting cells. Eur J Immunol. 29. 1209–1218.

Ben-Menachem, C., Zahringer, U. and Rottem, S. (2001). The phosphocholine motif in membranes of *Mycoplasma fermentans* strains. FEM Microbiol Lett. 199. 137-141.

Ben-Yedidia, T. and Arnon, R. (1997). Design of peptide and polypeptide vaccines. Curr. Opin. Biotechnol. 8. 442-448.

Bessler, W. G., Cox, M., Lex, A., Suhr, B., Wiesmuller, K. H. and Jung, G. (1985). Synthetic lipopeptide analogs of bacterial lipoprotein are potent polyclonal activators for murine B lymphocytes. J Immunol. 135. 1900-1905.

Bevilacqua, M. P., Pober, J. S., Majeau, G. R., Cotran, R. S. and Gimbrone, M. A. Jr. (1984). Interleukin 1 induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. J Exp Med. 16. 618-623.

Beyer, T. (2001). Bacterial carries and Virus-like-Particles an antigen delivery devices. Role of dendritic cells in antigen presentation. Curr. Drug Targets-Infect. Disord. 1. 287-302.

Bird, A.P. and Taggart, M. H., (1980). Variable patterns of total DNA and rDNA methylation in animals. Nucleic Acids Res. 8. 1485-1497.

Bleijs, D. A. (2001). DC-SIGN and LFA-1. a battle for ligand. Trends Immunol. 22. 457-63.

Boonstra, A. (2003). Flexibility of mouse classical and Plasmacytoid-derived dendritic cells in directing T helper 1 and 2 cell development. Dependency on antigen dose and differential Toll-like Receptor Ligation. J. Exp. Med. 197. 101-9.

Bosch, T., Schmidt, B., Spencer, P. C., Samtleben, W., Pelger, M., Baurmeister, U. and Gurland, H. J. (1987). Ex vivo biocompatibility evaluation of a new modified cellulose membrane. Artif Organs. 11. 144-8.

Bosch, T., Schmidt, B., Kleophas, W., Otto, V. and Samtleben, W. (1999). LDL hemoperfusion—a new procedure for LDL apheresis. biocompatibility results from a first pilot study in hypercholesterolemic atherosclerosis patients. Artif Organs. 21. 1060-5.

Bosch, T., Lennertz, A., Duhr, C., Fink, E. and Samtleben, W. (2000). Ex vivo biocompatibility of avidin-agarose. a new device for direct adsorption of biotinylated antibodies from human whole blood. Artif Organs. 24. 696-704.

Brade, H., Opal, S. M., Vogel, S. M., Morrison, D. C. eds. (1999). Endotoxin in health and disease. Marcel Dekker, New York.

Braun, M. C. and Kelsall, B. L. (2001). Regulation of interleukin12 production by Gproteincoupled receptors. Microbes Infect. 3. 99–107.

Brayden, D. J. (2001). Oral vaccination in man using antigens in particles. current status. Eur J Pharm Sci. 14. 183-9.

Brown, W. C., Suarez, C. E., Shoda, L. K. and Estes, D. M. (1999). Modulation of host immune responses by protozoal DNA. Vet. Immunol. Immunopathol. 72. 87-94.

Brown, G. D. and Gordon, S. (2001). A new receptor for beta glucans. Nature. 413. 36–37.

Brown, G. D., Herre, J., Williams, D. L., Willment, J. A., Marshall, A. S. J. and Gordon, S. (2003). Dectin1 mediates the biological effects of bglucans. J Exp Med. 197. 1119–1124.
Brown, G. D., Taylor, P. R. and Reid, D. M. (2002). Dectin1 is a major βglucan receptor on macrophages. J Exp Med. 196. 407–412.

Bulut, Y., Faure, E., Thomas, L., Equils, O.and Arditi, M. (2001). Cooperation of TOLL-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein. role of TOLL-interacting protein and IL-1 receptor signaling molecules in TOLL-like receptor 2 signaling. J. Immunol. 167. 987-994.

Campos, M. A., Almeida, I. C., Takeuchi, O., Akira, S., Valente, E. P., Procopio, D. O., Travassos, L. R., Smith, J. A., Golenbock, D. T. and Gazzinelli, R. T. (2001). Activation of TOLL-like receptor2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J. Immunol. 167. 416-423.

Caruso, J. P. and Ross, R. F. (1990). Effect of *Mycoplasma hyopneumoniae* and *Actinobacillus (Haemophillus) pleuropneumoniae* infections on alveolar macrophage functions in swine. Am. J. Vet. Res. 51. 227-231.

Chatterjee, D. and Khoo, K. H. (1998). Mycobacterial lipoarabinomannan. an extraordinary lipoheteroglycan with profound physiological effects. Glycobiology. 8. 113–20.

Chin, A. I., Dempsey, P. W. and Bruhn, K. (2002). Involvement of receptor-interacting . protein 2 in innate and adaptive immune responses. Nature. 416. 190– 194.

Christensen, N. D., Reed, C. A., Culp, T. D., Hermonat, P. L., Howett, M. K., Anderson, R.A. and Zaneveld, J.D. (2001). Papillomavirus microbicidal activities of high molecular weight cellulose sulfate, dextrane-sulfate and polystyrene sulfonate. Antimicrob. Agent Chemother. 45. 3427-3432.

Chu, R. S., (1997). CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J.Exp Med. 186. 1623-31.

Chuang, T. H., Lee, J., Kline, L., Mathison, J. C. and Ulevitch, R. J. (2002). TOLL-like receptor 9 mediates CpG-DNA signaling. J. Leukoc. Biol. 71. 538-544.

Ciprian, A., Pijoan, C., Cruz, T., Camacho, J., Tortora, J., Colmenares, G., Lopez-Revilla, R. and de la Garza, M. (1988). Mycoplasma hyopneumoniae increases the susceptibility of pigs to experimental Pasturella multocida pneumonia. Can. Vet. Res. 52. 434-438.

Clark, M. A. (2001). Exploiting M cells for drug and vaccine delivery. Adv Drug Deliv Rev. 50. 81-106.

Colmenares, M., Puig-Kroger, A., Pello, O. M., Corbi, A. L. and Rivas, L. (2002). Dendritic cell (DC)-specific intercellular adhesion molecule 3(ICAM-3)-grabbing nonintegrin (DC-SIGN, CD209) ,a C-type surface lectin in human DCs, is a receptor for Leishmania amastigotes. J Biol Chem. 277. 36766–9.

Cowdery, J. S., Chace, J. H., Yi, A. K. and Krieg, A. M. (1996). Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. J Immunol. 156. 4570-4575.

Dabbagh, K., Dahl, M. E., Stepick-Biek, P. and Lewis, D. B. (2002). TOLL-like receptor 4 is required for optimal development of TH2 immune responses. role of dendritic cells. J. Immunol. 168. 4524-4530.

Davis, H. L., (1998). CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol. 160. 870-76.

De Kimpe, S. J., Kengatharan, M., Thiemermann, C. and Vane, J. R. (1995). The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. Proc Natl Acad Sci USA. 92. 10359-10363.

Degling, L. (1995). Biodegradable microspheres. XVIII. The adjuvant effect of polyacryl starch microparticles with conjugated human serum albumin. Vaccine. 13. 629-36.

Deible, C. R., Petrosko, P., Johnson, P. C., Beckman, E. J., Russell, A. J. and Wagner, W. R. (1999). Molecular barriers to biomaterial thrombosis by modification of surface proteins with polyethylene glycol. Biomaterials. 20). 101-109.

DevyatyarovaJohnson, M., Rees, I. H., Robertson, B. D., Turner, M. W., Klein, N. J. and Jack, D. L. (2000). The lipopolysaccharide structures of Salmonella enterica serovar typhimurium and Neisseria gonorrhoeae determine the attachment of human mannosebinding lectin to intact organisms. Infect Immun. 68. 3894–3899.

Dil, N. and Qureshi, M. A. (2002). Differential expression of inducible nitric oxide synthase is associated with differential TOLL-like receptor-4 expression in chicken macrophages from different genetic backgrounds. Vet. Immunol. Immunopathol. 84. 191-207.

Dzionek, A., Sohma, Y., Nagafune, J., Cella, M., Co-lonna, M., Facchetti, F., Gunther, G., Johnston, I., Lanzavecchia, A., Nagasaka, T., Okada, T., Vermi, W., Winkels, G., Yamamoto, T., Zysk, M., Yamaguchi, Y. and Schmitz, J. (2001). BDCA-2, a novel plasmacytoid dendritic cell-specifict ype II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. J Exp Med. 194. 1823–34.

Ebel, J. P. (1990). A method for quantifying particle absorption from the small intestine of the mouse. Pharm Res. 7. 848-51.

Elfaki, M. G., Kleven, S. H., Jin, L. H. and Ragland, W. L. (1993). Protection against airsacculitis with sequential systemic and local immunization of chickens using killed *Mycoplasma gallisepticum* bacteria with iota carrageenan adjuvant. Vaccine. 11. 311-317.

Engering, A., Geijtenbeek, T. B., van Vliet, S. J., Wijers, M., van Liempt, E., Demaurex, N., Lanzavecchia, A., Fransen, J., Figdor, C. G., Piguet, V. and van Kooyk, Y. (2002). The dendritic cell-specific adhesion receptor DCSIGN internalize santigen for presentation to Tcells. J Immunol. 168. 2118–26.

Epstein, J., Eichbaum, Q., Sheriff, S. and Ezekowiz, R. A. B. (1996). The collectins in innate immunity. Curr Opin Immunol. 8. 29–35.

Ermak, T. H. (1998). Microparticle targeting to M cells. Adv Drug Deliv Rev. 34. 261-283.

Erno, H. and Stipkovits, L. (1973). Bovine mycoplasmas. Culture and biochemical studies. Acta Vet. Scand. 14. 436-449.

Ezekowitz, R. A. B. (1998). Genetic heterogeneity of mannosebinding proteins. the Jekyll and Hyde of innate immunity? Am J Hum Genet. 62. 6–9.

Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y. and Henson, P. M. (1998). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGFbeta, PGE_2, and PAF. J Clin Invest. 101. 890–898.

Fitzgerald, K.A., Palsson-McDermott, E.M., Bowie, A.G., Jeff-eries, C.A., Mansell, A.S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M.T., McMurray, D., Smith, D.E., Sims, J.E., Bird, T.A., O'Neill, L.A. (2001) Mal (MyD88-adapter-like) is required for TOLL-like receptor-4 signal transduction. Nature 413.78-83.

Flo, T. H., Halaas, O., Lien, E., Ryan, L., Teti, G., Golenbock, D. T., Sundan, A. and Espevik, T. (2000). Human TOLL-like receptor 2 mediates monocyte activation by Listeria monocytogenes, but not by group B streptococci or lipopolysaccharide. J. Immunol. 164. 2064-2069.

Flo, T. H., Ryan, L., Latz, E., Takeuchi, O., Monks, B. G., Lien, E., Halaas, O., Akira, S., Skjak-Braek, G., Golenbock, D. T. and Espevik, T. (2002). Involvement of TOLL-like receptor (TLR) 2 and TLR-4 in cell activation by mannuronic acid polymers. J. Biol. Chem. 277. 35489-35495.

FreiredeLima, C. G., Nascimento, D. O. and Soares, M. B. P. (2000). Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. Nature. 403. 199–203.

Frey, J. (2002). Mycoplasmas of Animals in Molecular Biology and Pathogeneicity of Mycoplasmas, Ed. Rasin and Herrmann, Kluver Academic/Plenum Publishers, New York

FugierVivier, I., ServetDelprat, C., Rivailler, P., Rissoan, M. C. and Liu ,Y. J. (1997). RabourdinCombe C. Measles virus suppresses cellmediated immunity by interfering with the survival and functions of dendritic and T cells. J Exp Med. 186. 813–823.

Fukui, A., Inoue, N., Matsumoto, M., Nomura, M., Yamada, K., Matsuda, Y., Toyoshima, K. and Seya, T. (2001). Molecular cloning and functional characterization of chicken toll-like receptors. A single chicken toll covers multiple molecular patterns. J Biol Chem. 276. 47143-9.

Galanos, C., Gumenscheimer, M., Muhlradt, P., Jirillo, E. and Freudenberg, M. (2000). MALP-2, a Mycoplasma lipopeptide with classical endotoxic properties. end of an era of LPS monopoly? J Endotoxin Res. 6. 471-4766.

Garred , P., Pressler, T. and Madsen, H. O. (1999). Association of mannosebinding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. J Clin Invest. 104. 431–437.

Garred, P., Madsen, H. O. and Halberg, P. (1999). Mannosebinding lectin polymorphisms and susceptibility to infection in systemic lupus erythematosus. Arthritis Rheum. 42. 2145–2152.

Geijtenbeek, T. B. H., Kwon, D.S., Torensma, R., van Vliet, S. J, van Duijnhoven, G. C. F., Middel, J., Cornelissen, I. L., Nottet, H. S., Kewal, Ramani, V. N., Littman, D. R., Figdor, C. G. and van Kooyk, Y. (2000). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell. 100. 587–97.

Geijtenbeek, T. B. H., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C. F., Adema, G. J., van Kooyk, Y. and Figdor, C. G.(2000). Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 100. 575–85.

Geijtenbeek, T. B. H., Krooshoop, D. J., Bleijs, D. A., van Vliet, S. J., van Duijnhoven, G. C., Grabovsky, V., Alon, R., Figdor, C. G. and van Kooyk, Y. (2000). DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. Nat Immunol. 1. 353–7.

Geijtenbeek, T. B. H., van Vliet, S. J., Koppel, E. A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C. M., Appelmelk, B. and van Kooyk, Y. (2003). Mycobacteria target DC-SIGN to suppress dendritic cell function J Exp Med. 197. 7–17.

Gewirtz, A. T., Navas, T. A., Lyons, S., Godowski, P. J. and Madara, J. L., (2001). Cutting edge. bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proin¯ammatory gene expression. J. Immunol. 167. 1882-1885.

Giesen, P. and Nemerson, Y. (2000). Tissue Factor on the loose. Semin Thromb Hemost. 26. 379-384.

Girardin, S. E, Boneca, I. G., Carneiro, L A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M. K., Labigne, A., Zahringer, U., Coyle, A. J., DiStefano, P. S., Bertin, J., Sansonetti, P. J. and Philpott, D. J. (2003). Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science. 300. 1584-87.

Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D. J. and Sansonetti, P. J. (2003). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem. 278. 8869-72.

Giron, J. A., Lange, M. and Baseman, J. B. (1996). Adherence, fibronectin binding and induction of cytoskeleton reorganization in cultured human cells by *M. penetrans*. Infect Immun. 64. 197-208.

Gluck, R., (2000). Safety and immunogenicity of intranasally administered inactivated trivalent virosome-formulated influenza vaccine containing escherichia coli heat-labile toxin as a mucosal adjuvant. J. Infect. Dis. 181. 1129-32.

Gordon, S. (2002). Pattern recognition receptors. doubling up for the innate immune response. Cell. 111. 927–930.

Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M., Dustin, M. L. (1999). The immunological synapse. a molecular machine controlling T cell activation. Science. 285. 221–7.

Grandics, P. (2000). Pyrogens in parenteral pharmaceuticals. Pharm Technol. 4. 26-34.

Hacker, H. (2000) Signal transduction pathways activated by CpG-DNA. Curr. Top. Microbiol. Immunol. 247. 77-92.

Hansson, M., Nygren, P. A., and Stahl, S. (2000). Design and production of recombinant subunit vaccines. Biotechnol. Appl. Biochem. 32. 95-107.

Harding, G. B. (1990). Endotoxin and Bacterial Contamination of Dialysis Center Water and Dialysate. A Cross-Sectional Survey, Int. J. Artif. Organs. 13. 39-43.

Hartmann, G., Weiner, G.J., Krieg, A.M. (1999) CpG-DNA: a potent signal for growth, activation, and maturation of human dendritic cells. Proc. Natl. Acad. Sci. U.S.A. 96. 9305-9310.

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M. and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by TOLL-like receptor 5. Nature. 410. 1099-1103.

Haynes, L.M., Moore, D.D., Kurt-Jones, E.A., Finberg, R.W., Anderson, L.J., Tripp, R.A. (2001) Involvement of TOLL-like receptor 4 in innate immunity to respiratory syncytial virus. J. Virol. 75. 10730-10737.

He, Y. W. (2004). The extracellular matrix protein mindin is a pattern recognition molecule for microbial pathogens, Nat. Immunol. 5. 88-97.

Hellman, J., Loiselle, P. M., Zanzot, E. M., Allaire, J. E., Tehan, M. M., Boyle, L. A., Kurnick, J. T. and Warren, H. S. (2000). Release of gram-negative outer-membrane proteins into human serum and septic rat blood and their interactions with immunoglobulin in antiserum to Escherichia coli J5. J Infect Dis. 181. 1034-43.

Hellman, J., Warren, H. S. (2001). Outer membrane protein A (OmpA), peptidoglycan-associated lipoprotein (PAL), and murein lipoprotein (MLP) are released in experimental Gram-negative sepsis. J Endotoxin Res. 7. 69-72.

Hemmi, H., Takeuchi, O. and Kawai, T. (2000). A Toll-like receptor recognizes bacterial DNA. Nature. 408. 740–745.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000). A TOLL-like receptor recognizes bacterial DNA. Nature. 408. 740-745.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K. and Akira, S. (2002). Small anti-viral compounds activate immune cells via the TLR7MyD88-dependent signaling pathway. Nat. Immunol. 3. 196-200.

Heumann, D., Barras, C., Severin, A., Glauser, M. P. and Tomasz, A. (1994). Gram-positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. Infect Immun. 62. 2715-2721.

HoalVan Helden, E. G., Epstein, J. and Victor, T. C. (1999). Mannosebinding protein B allele confers protection against tuberculous meningitis. Pediatr Res. 45. 459–464.

Hoekstra, D., van der Laan, J. W., de Leij, L. and Witholt, B. (1976). Release of outer membrane fragments from normally growing Escherichia coli. Biochim Biophys Acta. 455. 889-99.

Hoffman, J.A., Reichhart, J.H. (1997) *Droshophila* Immunity. Trends Cell Biol. 7:309-316

Horn, D. L., Morrison, D. C., Opal, S. M., Silverstein, R., Visvanathan, K. and Zabriskie, J. B. (2000). What are the microbial components implicated in the pathogenesis of sepsis? Report on a symposium. Clin Inf Dis. 31. 851-858.

Horng, T., Medzhitov, R. (2001). Drosophila MyD88 is an adapter in the Toll signaling pathway. Proc Natl Acad Sci USA. 98. 12654-12658.

Hornung, V., Rothenfusse, S. and Britsch, S. (2002). Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol. 168. 4531–4537.

Hoshino, K., Takeuchi, O. and Kawai, T. (1999). Cutting edge. Toll-like receptor 4 (TLR-4)-deficient mice are hyporesponsive to lipopolysaccharide. evidence for TLR-4 as the Lps gene product. J Immunol. 162. 3749-3752.

Hsu, F. J. (1996). Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells.Nat Med. 2. 52-8.

Huang, Q., Liu, D., Majewski, P., Schulte, L. C., Korn, J. M., Young, R. A., Lander, E. S. and Hacohen, N. (2001). The plasticity of dendritic cell responses to pathogens and their components. Science. 294. 870-5.

Ichikawa, H. T., Williams, L. P. and Segal, B. M. (2002). Activation of APCs through CD40 or Toll-like receptor 9 overcomes tolerance and precipitates autoimmune disease. J Immunol. 169. 2781–2787.

Igietseme J. U. (2003). Contemporary approaches to designing and evaluating vaccines against Chlamydia. Expert Rev Vaccines. 2. 129-46.

Iho, S., Yamamoto, T., Takahashi, T. and Yamamoto, S. (1999). Oligodeoxynucleotides containing palindrome sequences with internal 5'-CpG-3' act directly on human NK and activated T cells to induce IFN-gamma production in vitro. J Immunol. 163. 3642–3652.

Iwasaki, A. (2001). Unique functions of CD11b+, CD8 alpha+, and double-negative Peyer's patch dendritic cells. J Immunol. 166. 4884-90.

Jack, D. L., Klein, N. J. and Turner, M. W. (2001). Mannosebinding lectin. targeting the microbial world for complement attack and opsonophagocytosis. Immunol Rev. 180. 86–99.

Jacobs, E., Drews, M., Stuhlert, A., Buttner, C., Klein, P. J., Kist, M., and Bredt, W. (1988). Immunological reaction of guinea-pigs following intranasal *Mycoplasma pneumoniae* infection and immunization with the 168 kDa adherence protein. J. Gen. Microbiol. 134. 473-479.

Janeway, Jr. C. A. (1992). The immue system evolved to discriminate infectious nonself from noninfectious self. Immunol. Today. 13. 11-16.

Janeway, Jr. C. A. and Medzhitov, R. (2002). Innate immune recognition. Ann. Rev. Immunol. 20. 197-216.

Jankovic, D., Kullberg, M. C. and Hieny, S. (2002). In the absence of IL-12, $CD4^+$ Tcell . responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in an IL-1setting. Immunity. 6. 429–439.

Jarrossay, D., Napolitani, G., Colonna, M., Sallusto, F. and Lanzavecchia, A. (2001). Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur J Immunol. 31. 3388–93.

Jones BD. (1994). Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J Exp Med. 180. 15-23.

Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. and Enk, A. H. (2000). Induction of interleukin10-producing, non-proliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. J Exp Med. 192. 1213–22.

Jurk, M., Heil, F., Vollmer, J., Schetter, C., Krieg, A.M., Wagner, H., Lipford, G., Bauer, S. (2002) Human TLR7or TLR-8 independently confer responsiveness to the antiviral compound R-848. Nat. Immunol. 3. 499.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R. W., Kastelein, R. A., Bazan, F. and Liu, Y. J. (2001). Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med. 194. 863–9.

Kahane, I. and Tully, J. G. (1976). Binding of plant lectins to Mycoplasma cells and membranes. J. of Bacteriology. 128. 1-7.

Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K. and Akira, S. (2001). Endotoxin-induced maturation of MyD88-deficient dendritic cells. J Immunol. 166. 5688-94.

Kaisho, T. and Akira, S. (2003). Regulation of dendritic cell function through Toll-like receptors. Current Molecular Medicine. 3. 373-385.

Kamstrup, S., Verthelyi, D. and Klinman, D.M. (2001). Response of porcine peripheral blood mononuclear cells to CpG-containing oligodeoxynucleotides. Vet. Microbiol. 78. 353-362.

Kantele, A. (1999). Differential homing commitments of antigen-specific T cells after oral or parenteral immunization in humans. J Immunol. 162. 5173-7.

Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P.F., Sato, S., Hoshino, K., Akira, S. (2001) Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J. Immunol. 167. 5887-5894.

Kellermann, S. A. (2001). The Peyer's patch microenvironment suppresses T cell responses to chemokines and other stimuli. J Immunol. 167. 682-90.

Kengatharan, K. M., De Kimpe, S., Robson, C., Foster, S. J. and Thiemermann, C. (1998). Mechanism of gram-positive shock. identification of peptidoglycan and lipoteichoic acid moieties essential in the induction of nitric oxide synthase, shock, and multiple organ failure. J Exp Med. 188. 305-315.

Kessler, C. M., Nussbaum, E. and Tuazon, C. U. (1991). Disseminated intravascular coagulation associated with *Staphylococcus aureus* septicemia is mediated by peptidoglycan-induced platelet aggregation. J Infect Dis. 164. 101-107.

King, K. W., Faulds, D. H., Rosey, E. L., and Yancey, R. J., Jr. (1997). Characterization of the gene encoding Mhp1 from *Mycoplasma hyopneumoniae* and examination of Mhp1's vaccine potential. Vaccine. 15. 25-35.

Kitchens, R. L. and Munford, R. S. (1998). CD14-dependent internalization of bacterial lipopolysaccharide (LPS) is strongly influenced by LPS aggregation but not by cellular responses to LPS. J Immunol. 160. 1920-1928.

Kitchens, R. L., Thompson, P. A., O'Keefe, G. E. and Munford, R. S. (2000). Plasma constituents regulate LPS binding to, and release from, the monocyte cell surface. J Endotoxin Res. 6. 477-482.

Kitchens, R. L., Thompson, P. A., Viriyakosol, S., O'Keefe, G. E. and Munford, R. S. (2001). Plasma CD14 decreases monocyte responses to LPS by transferring cell-bound LPS to plasma lipoproteins. J Clin Invest. 108. 485-493.

Klein, E., Pass, T., Harding, G. B., Wright, R. and Millon, C. (1990). Microbial and endotoxin contamination in water and dialysate in the central United States. Artif Organs. 14. 85-94.

Kleven, S. H. (1990). Summary of discussion of Avian *Mycoplasma* Team, IRPCM, IOM. Avian Pathol. 19. 795-800.

Kleven, S. H. (1998). Pen trial studies on the use of live vaccines to displace virulent *Mycoplasma gallisepticum* in chickens. Avian Dis. 42. 300-6.

Klinman, D. M. (2003). CpG DNA as a vaccine adjuvant. Expert Rev Vaccines. 2. 305-15.

Kobayashi, K., Inohara, N. and Hernandez, L. D. (2002). RICK/Rip2/CARDIAK . mediates signalling for receptors of the innate and adaptive immune systems. Nature. 416. 194–199.

Kodama, M. (1993). Hemoperfusion using a polymyxin B immobilized fiber column for the removal of endotoxin. In. Levin J, Alving CR, Munford RS, Stutz PL, ed. Bacterial endotoxin. recognition and effector mechanisms. 389-398.

Kraal, G., van der Laan, L. J., Elomaa, O. and Tryggvason, K. (2000). The macrophage receptor MARCO. Microbes Infect. 2. 313–316.

Kraehenbuhl, J. P. (2000). Epithelial M cells. differentiation and function. Annu Rev Cell Dev Biol. 16. 301-32.

Krieg, A. M. (2000). The role of CpG motifs in innate immunity. Curr. Opin. Immunol. 12. 35-43.

Krieger, M. (2001). Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. J Clin Invest. 108. 793–797.

Krivan, H. C., Olson, L .D., Barile, M. F., Ginsburg, V. and Roberts, D. D. (1989). Adhesion of *Mycoplasma pneumoniae* to sulfated glycolipids and inhibition by dextran sulfate. J. Biol. Chem. 264. 9283-9288.

Kurt-Jones, E.A., Popova, L., Kwinn, L., Haynes, L.M., Jones, L.P., Tripp, R.A., Walsh, E.E., Freeman, M.W., Golenbock, D.T., Anderson, L.J., Finberg, R.W. (2000) Pattern recognition receptors TLR-4 and CD14 mediate response to respiratory syncytial virus. Nat. Immunol. 1. 398-401.

Laakso, T. (1986). Biodegradable microspheres. IV. Factors affecting the distribution and degradation of polyacryl starch microparticles. J Pharm Sci. 75. 962-7.

Levine, M. M. (1983). New knowledge on pathogenesis of bacterial enteric infections as appilied to vaccine development. Microbiol. Rev. 47. 510.

Liljeqvist, S. and Stahl, S. (1999). Production of recombinant subunit vaccines. protein immunogens, live delivery systems and nucleic acid vaccines. J. Biotechnol. 73. 1-33.

Lipford, G. B. (1997b). CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen. A new class of vaccine adjuvants. Eur. J. Immunol. 27. 2340-44.

Mahiout, A. (1991). Production of Endotoxin and Its Related Subfragments in Contaminated Bicarbonate Dialysate, J. Am. Soc. Nephrol. 2. 337.

Mahom, B. P. (2001). The role design of vaccine adjuvants for mucosal and neonatal immunization. Curr. Med Chem. 8. 1057-75.

Markham, P. F., Glew, M. D., Brandon, M. R., Walker, J. D. and Whithear, K. G.(1992). Characterization of a major hemagglutinin protein from *Mycoplasma gallisepticum*. Infect. Immun. 60. 3885-3891.

Markham, P. F., Glew, M. D., Whithear, K. G. and Walker, J. D. (1993). Molecular cloning of a member of the gene family that encodes pMGA, a hemaglutinin of *Mycoplasma gallisepticum*. Infect. Immun. 61. 903-909.

Massion, P. P. (1994). Novel Pseudomonas Product Stimulates Interieukin-8 Production in Airway Epithelial Cells In Vitro, J. Clin. Invest. 93. 26-32.

Matsuda, K., Ishizuka, I. and Kasama, T. (1997). Structure of a novel phosphocholine containing aminoglycoglycerolipid of *Mycoplasma fermentans*. Biochim. Biophys. Acta.. 1349. 1-12.

Matsushita, M., Endo, Y., Nonaka, M. and Fujita, T. (1998). Complementrelated serine proteases in tunicates and vertebrates. Curr Opin Immunol. 10. 29–35.

Mattsson, E., Verhage, L., Rollof, J., Fleer, A., Verhoef, J. and vanDijk, H. (1993). Peptidoglycan and teichoic acid from Staphylococcus epidermidis stimulate human monocytes to release tumour necrosis factor-alpha, interleukin-1 beta and interleukin-6. FEMS Immunol Med Microbiol. 7. 281-287.

Mattsson, E., Herwald, H., Bjorck, L. and Egesten, A. (2002). Peptidoglycan from *Staphylococcus aureus* induces Tissue Factor expression and procoagulant activity in human monocytes. Infect Immun. 70. 3033-3039.

Medzhitov, R. and Janeway, Jr. C. A. (1997). Innate Immunity. the virtues of a nonclonal system of recognition. Cell. 91. 295-298.

Mitchell, D. A., Fadden, A. J. and Drickamer, K. (2001). A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. subunit organization and binding to multivalent ligands. J. Biol. Chem. 276. 28939.

Moors, M.A., Li, L., Mizel, S.B. (2001) Activation of interleukin-1 receptor-associated kinase by gram-negative flagellin. Infect. Immun. 69. 4424-4429.

Mosser, D. M., Springer, T. A. and Diamond, M. S. (1992). Leishmania promastigotes require opsonic complement to bind to the human leukocyte integrin Mac1 (CD11b/CD18). J Cell Biol. 116. 511–520.

Mosser, D. M. and Karp, C. L. (1999). Receptor mediated subversion of macrophage cytokine production by intracellular pathogens. Curr Opin Immunol. 11. 406–411.

Mulhradt, P. F., Kiess, M., Meyer, H., Sussmuth, R. and Jung, G. (1997). Isolation,structure elucidation, and synthesis of a macrophage stimulatory lipopeptide from *Mycoplasma fermentans* acting at picomolar concentration. J. Exp. Med. 185. 1951-1958.

Muzio, M., Bosisio, D. and Polentarutti, N. (2000). Differential expression and regulation of toll-like receptors (TLR) in human leukocytes. selective expression of TLR3 in dendritic cells. J Immunol. 164. 5998–6004.

Nawroth, P. P., Handley, D. A., Esmon, C. T. and Stern, D. M. (1986). Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity. Proc Natl Acad Sci USA. 83. 3460-3464.

Netea, M. G., Van der Meer J. W. M. and Kullberg, B. J. (2004). Toll-like receptors as an escape mechanism from the host defence. Trends in Microbiology. 12. 484-488.

Neutra, M. R. (1992). Transepithelial transport and mucosal defence I: the role of M cells. Trends Cell Biol. 2. 134-8.

Nigou, J., Zelle-Rieser, C., Gilleron, M., Thurnher, M. and Puzo, G. (2001). Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells. evidence for a negative signal delivered through the mannose receptor. J Immunol. 166. 7477–85.

Nishiguchi, M., Matsumoto, M. and Takao, T. (2001). *Mycoplasma fermentans* lipoprotein M161 Ag-induced cell activation is mediated by Toll-like receptor 2. Role of N-terminal hydrophobic portion in its multiple functions. J. Immunol. 164. 2610-2616.

Novitsky, T. J. (1998). Limitations of the Limulus amebocyte lysate test in demonstrating circulating lipopolysaccharides. Ann N Y Acad Sci. 851. 416-421.

O'Neill, L. A. (2002). Signal transduction pathways activated by the IL-1 receptor/toll-like receptor superfamily. Curr Top Microbiol Immunol. 270. 47–61.

O'Neill, L.A. (2002). TOLL-like receptor signal transduction and the tailoring of innate immunity. a role for Mal? Trends Immunol. 23. 296-300.

Obermeier, F., Dunger, N. and Deml, L., (2002). CpG motifs of bacterial DNA exacerbate colitis of dextran sulfate sodium-treated mice. Eur J Immunol. 32. 2084–2092.

Ogden, C. A., deCathelineau, A. and Hoffmann, P. R. (2001). C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J Exp Med. 194. 781–795.

Okano, M., Satoskar, A. R., Nishizaki, K., Abe and M., Harn, Jr. D. A. (1999). Induction of Th2 responses and IgE is largely due to carbohydrates functioning as adjuvants on Schistosoma mansoni egg antigens. J Immunol. 163. 6712–7.

Olson, L. D., Shane, S. W., Karpas, A .A., Cuningham, T. M., Probst, P. S. and Barile, M. F.(1991). Monoclonal antibodies to surface antigens of a pathogenic *Mycoplasma homin*is strain. Infect. Immun. 59. 1683-1689.

Olson, L. D. and Gilbert, A. A. (1993). Characteristics of *Mycoplasma hominis* adhesion. J. Bacteriol. 175. 3224-3227.

Ohashi, K., Burkart, V., Flohe, S., Kolb, H. (2000) Cutting edge: heat shock protein 60 is a putative endogenous ligand of the TOLL-like receptor-4 complex. J. Immunol. 164. 558-561.

Opal, S. M., Scannon, P. J. and Vincent, J. L. (1999). Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. J Infect Dis. 180. 1584-1589.

Ortiz, A. M. (1981). The immunosuppressive effect of *Mycoplasma meleagridis* on nonreplicating antigens. Avian Dis. 25. 954-63.

Ozinsky, A., Underhill, D.M., Fontenot, J.D., Hajjar, A.M., Smith, K.D., Wilson, C.B., Schroeder, L., Aderem, A. (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between TOLL-like receptors. Proc. Natl. Acad. Sci. U.S.A. 97, 13766-13771.

Pabst, R. (1990). Is the bronchus-associated lymphoid tissue (BALT) an integral structure of the lung in normal mammals, including humans? Am J. Respir. Cell Mol. Biol. 3. 131-5.

Papazisi, L. (2002). A modified live *Mycoplasma gallisepticum* vaccine to protect chickens from respiratory disease. Vaccine. 20. 3709-19.

Papazisi, L. (2002). GapA and CrmA coexpression is essential for *Mycoplasma gallisepticum* cytadherence and virulence. Infect Immun. 70. 6839-45.

Pfützner, H. and Sachse, K. (1996). *Mycoplasma bovis* as an agent of mastitis, pneumonia, arthritis and genital disorders in cattle. Rev. Sci. Technol. 15. 1477-1494.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Huffel, C.V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in TLR-4 gene. Science 282. 2085-2088.

Pontarollo, R. A., Rankin, R., Babiuk, L. A., Godson, D. L., Griebel, P. J., Hecker, R., Krieg, A. M. and van Drunen Little - van den Hurk, S. (2002). Monocytes are required for optimum in vitro stimulation of bovine peripheral blood mononuclear cells by non-methylated CpG motifs. Vet. Immunol. Immunopathol. 84. 43-59.

Pridmore, A. C., Wyllie, D. H., Abdillahi, F., Steeghs, L., van der Ley, P., Dower, S. K. and Read, R. C. (2001). A lipopolysaccharide-deficient mutant of Neisseria meningitidis elicits attenuated cytokine release by human macrophages and signals via TOLL-like receptor (TLR) 2 but not via TLR-4/MD2. J. Infect. Dis. 183. 89-96.

Pulendran, B. (1999). Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. Proc Natl Acad Sci U S A. 2. 96. 1036-41.

Rankin, R., Pontarollo, R., Ioannou, X., Krieg, A. M., Hecker, R., Babiuk, L. A. and van Drunen Littel-van den Hurk, S. (2001). CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. Antisense Nucl. Acid Drug Dev. 11. 333-340.

Razin, S., Yogev, D. and Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. Microbiol. Mol. Biol. Rev. 62. 1094-1156.

Redpath, S., Ghazal, P. and Gascoigne, N. R. (2001). Hijacking and exploitation of IL-10 by intracellular pathogens. Trends Microbiol. 9. 86–92.

Rietschel, E. T. and Brade, H. (1992). Bacterial Endotoxins. Sci. Am. 8. 54-61.

Robinson, D. S. and O'Garra, A. (2002). Further checkpoints in Th1 development. Immunity. 16. 755–758.

Rosengarten, R. and Wise, K. S. (1990). Phenotypic switching in Mycoplasmas. Phase variation of diverse surface lipoproteins. Science. 247. 315-318.

Ross, R. F. (1990). Proc. Mycoplasma Pneumoniae Symposia SmithKline Beecham Animal Health, Aimes, Iowa, 116.

Rottem, S. (1980). Membrane lipids of mycoplasmas. Biochim. Biophys. Acta. 604. 65-90.

Rottem, S. and Barile, M. F. (1993). Beware of mycoplasmas. Tibtech. 11. 143-151.

Roy, S., Knox, K. and Segal, S. (2002). MBL genotype and risk of invasive pneumococcal disease. a case-control study. Lancet. 359. 1569–1573.

Sallusto, F., Cella, M., Danieli, C. and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment. downregulation by cytokines and bacterial products. J Exp Med. 182. 389–400.

Santos, I. K., Costa, C. H. and Krieger, H. (2001). Mannanbinding lectin enhances susceptibility to visceral leishmaniasis. Infect Immun. 69. 5212–5215.

Schnare, M., Barton, G. M. and Holt, A. C. (2001). Toll-like receptors control activation of adaptive immune responses. Nat Immunol. 2. 947–950.

Schweizer, M. and Peterhans, E. (2001). Noncytopathic bovine viral diarrhea virus inhibits double-stranded RNA-induced apoptosis and interferon synthesis. J. Virol. 75. 4692-4698.

Seydel, U., Schromm, A. B., Brade, L., Gronow, S., Andra, J., Muller, M., Koch, M. H. J., Fukase, K., Kataoka, M., Hashimoto, M., Kusumoto, S. and Brandenburg, K. (2004). Physicochemical characterization of carboxymethyl lipid A derivatives in relation to biological activity. J. Endotox. Res. 10. 308-309.

Shalaby, M. R., Pennica, D. and Palladino, M. A. Jr. (1986). An overview of the history and biologic properties of tumor necrosis factors. Spring Semin Immunopathol. 9. 33-7.

Sharma, S. K. (1986). Review. Endotoxin Detection and Elimination in Biotechnology, Biotechnol. Appl. Biochem. 8. 5-22.

Sheriff, S., Chang, C. Y. and Ezekowitz, R. A. B. (1994). Human mannosebinding protein carbohydrate recognition domain trimerizes through a triple alphahelical coiledcoil. Nature Struct Biol. 1. 789–794.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. and Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med. 189.1777-82.

Shimizu, T., Kida, Y. and Kuwano, K. (2004). Lipid-associated membrane proteins of *Mycoplasma fermentans and M. penetrans* activate human immunodeficiency virus long-terminal repeats through Toll-like receptors. Immunology. 113. 121-9.

Shirota, H., Sano, K., Hirasawa, N., Terui, T., Ohuchi, K., Hattori, T., Shirato, K., Tamura, G. (2001) Novel roles of CpG oligodeoxynucleotides as a leader for the sampling and presentation of CpG-tagged antigen by dendritic cells. J. Immunol. 167. 66-74.

Shoda, L. K., Kegerreis, K. A., Suarez, C. E., Mwangi, W., Knowles, D. P. and Brown, W.C. (2001a). Immunostimulatory CpG-modified plasmid DNA enhances IL-12, TNF-alpha, and NO production by bovine macrophages. J. Leukoc. Biol. 70. 103-112.

Shoda, L. K., Kegerreis, K. A., Suarez, C. E., Roditi, I., Corral, R. S., Bertot, G. M., Norimine, J. and Brown, W. C. (2001b). DNA from protozoan parasites *Babesia bovis*, *Trypanosoma cruzi*, and *T. brucei* is mitogenic for B lymphocytes and stimulates macrophage expression of interleukin-12, tumor necrosis factor alpha, and nitric oxide. Infect. Immun. 69. 2162-2171.

Shortman, K. (2002). Mouse and human dendritic cell subtypes. Nat. Rev. Immunol. 2. 151-61.

Sieling, P. A., Chung, W. and Duong, B. T. (2003). Toll-like receptor 2 ligands as adjuvants for human Th1 responses. J Immunol. 170. 194–200.

Sierro, F. (2001). Flagellin Stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells. PNAS. 98. 13722-727.

Simecka, J. W. (1998). Mucosal immunity of the gastrointestinal tract and oral tolerance. Adv Drug Deliv Rev. 34. 235-259.

Sing, A., Rost, D., Tvardovskaia, N., Roggenkamp, A., Wiedemann, A., Kirsching, C. J., Aepfelbacher, M. and Heesemann, J. (2002). Yersinia V-antigen exploits Toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. J Exp Med. 196. 1017-1024.

Sirolli, V., Di Stante, S., Stuard, S., Di Liberato, L., Amoroso, L., Cappelli, P. and Bonomini, M. (2000). Biocompatiblity and functional performance of a polyethylene glycol acid-grafted cellulosic membrane for hemodialysis. Int J Artif Organs. 23. 356-364.

Smith, P. F., Langworthy, T. A. and Mayberry, W. R. (1976). Distribution and composition of lipopolysaccharides from mycoplasmas. J. Bacteriol. 125. 916-922.

Sparwasser, T., Miethke, T., Lipford, G., Erdmann, A., Hacker, H., Heeg, K. and Wagner, H. (1997). Macrophages sense pathogens via DNA motifs. induction of tumor necrosis factor-alpha-mediated shock. Eur J Immunol. 27. 1671-1679.

Sparwasser, T., Koch, E. S., Vabulas, R. M., Heeg, K., Lipford, G. B., Ellwart, J. W. and Wagner, H. (1998). Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur. J. Immunol. 28. 2045-2054.

Sparwasser, T., Vabulas, R. M., Villmow, B., Lipford, G. B. and Wagner, H. (2000). Bacterial CpG-DNA activates dendritic cells in vivo. T helper cell-independent cytotoxic T cell responses to soluble proteins. Eur. J. Immunol. 30. 3591-3597.

Spellberg, B. and Edwards, J. E., Jr. (2001). Type 1/Type 2 immunity in infectious diseases. Clin. Infect. Dis. 32. 76-102.

Srivatsan, J., Smith, D. F. and Cummings, R. D. (1992). The human blood fluke Schistosoma mansoni synthesizes glycoproteins containing the Lewis X antigen. J Biol Chem. 267. 20196–203.

Staag A. J.(1998). Protection against ascending infection of the genital tract by Chamydia Trachomentis is associated with recruitment of major histocompatibility complex class II antigen-presenting cells into uterine tissue. Immunol Infect. 66. 3535-44.

Stahl, P. D. and Ezekowitz, R. A. B. (1998).The mannose receptor is a pattern recognition receptor involved in host defense. Curr Opin Immunol. 10. 50–55.

Stein, D., Roth, S., Vogelsang, E., Nusslein-Volhard, C. (1991) Cell 65:725-735

Steinbrink, K., Jonuleit, H., Muller, G., Schuler, G., Knop, J. and Enk, A. H. (1999). Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. Blood. 93. 1634–42.

Stich, R. W., Shoda, L. K., Dreewes, M., Adler, B., Jungi, T. W. and Brown, W. C. (1998). Stimulation of nitric oxide production in macrophages by *Babesia bovis*. Infect. Immun. 66. 4130-4136.

Stipkovits, L. (1968). Az Escherichia Coli szerepe es jelentosege a hazi tyuk megbetegedeseiben, Budapest, Hungary, Kand. Disszertacio.

Stipkovits, L. (2001). Proceedings XII International Congress of the World Veterinary Poultry Association, Cairo, Egypt. p50-58.

Stipkovits, L. (2002). Possibilities of control of *Mycoplasma-pneumonia* in swine, Magy. Ao. Lapja 124. 337-341.

Stipkovits, L., Molnár, T., Glávits, R., Szabó, I. and Thevasagayam, S. (2004). Newer opportunities in the control of mycoplasma pneumonia. Magy. Áo Lapja, 126. 625-331.

Storni, T., Ruedl, C., Schwartz, R. A., Renner, W. A.. and Bachmann, M. F. (2004). Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic effects. J. Immunol. 172. 1777-1785.

Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J. and Russell, D. G. (1994). Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPa-se. Science. 263. 678–81.

Summerfield, J., Ryder, S., Sumiya, M., Thursz, Gorchein, A. and Monteil, M. (1995). Mannose binding protein gene in mutations associated with unusual and severe infections in adults. Lancet. 345. 886–889.

Summerfield, J. A., Sumiya, M., Levin, M. and Turner, M. W. (1997). Association of mutations in mannose binding protein gene with childhood infection in consecutive hospital series. BMJ. 314. 1229–1232.

Sundaram, R., Dakappagari, N. K. and Kaumaya, P. T. (2002). Synthetic peptides as cancer vaccines. Biopolymers. 66. 200-216.

Sundquist, B. G., Czifra, G. and Stipkovits, L. (1996). Protective immunity induced in chicken by a single immunization with *Mycoplasma gallisepticum* immunostimulating complexes (ISCOMS). Vaccine. 14. 892-897.

Sundstrom, J. B. (2004). Signaling Through Toll-like receptors triggers HIV-1 replication in latently infected mast cells. J. Immunol. 172. 4391-4399.

Szabo, T., Kadish, J. L. and Czop, J. K. (1995). Biochemical properties of the ligand binding 20kDa subunit of the βglucan receptors on human mononuclear phagocytes. J. Biol. Chem. 270. 2145–2151.

Szathmary, S. and Grandics, P. (1991). A method for purifying IgM, IgA, IgE, IgG1, and other monoclonal antibodies. Am. Biotechnol. Lab. 9. 31-33.

Tailleux, L., Schwartz, O. and Herrmann, J. L. (2003). DCSIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. J. Exp. Med. 197. 121–127.

Takada, H., Yokoyama, S. and Yang, S. (2002). Enhancement of endotoxin activity by muramyldipeptide. J. Endotoxin Res. 8. 337-42.

Takahashi, K., Gordon, J. and Liu, H. (2002). Lack of mannose binding lectin A enhances survival in a mouse model of acute septic peritonitis. Microbes Infect. 4. 773–784.

Takeda, K., Takeuchi, O. and Akira, S. (2002). Recognition of lipopeptides by Toll-like receptors. J. of Entotoxin Res. 8. 459-463.

Takeuchi, O., Kaufmann, A., Grote, K., Kawai, T., Hoshino, K., Morr, M., Muhlradt, P. F. and Akira, S. (2000). Cutting edge preferentially the R-stereoisomer of the mycoplasmal lipopeptide macchrophage-activating lipopeptide-2 activates immune cells through a Toll-like receptor 2- and MyD88-dependent signaling pathway. J. Immunol. 164. 554-547.

Thanawongnuwech, R., Thacker, B., Halbur, P. and Thacker, E. L. (2004). Increased production of proinflammatory cytokines following infection with porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae*. Clin. Diagn. Lab. Immunol. 11. 901-8.

Theiss, P. and Wise, K. S. (1997). Localized frameshift mutation generates selective, high-frequency phase variation of a surface lipoprotein encoded by a *Mycoplasma* ABC transporter operon. J. Bacteriol. 179. 4013-4022.

Tracey, K. J. and Cerami, A. (1993). Tumor necrosis factor: an update review of its biology. Crit. Care Med. 21. 415-22.

Travassos, L. H., Girardin, S. E., Philpott, D. J., Blanot, D., Nahori, M. A., Werts, C. and Boneca, I. G. (2004). Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep. 5. 1000-6.

Tsuji, M., Kawaoka, Y., Naiki, M. and Yanagawa, R. (1981). Isolation of antigenically active components from leptospiral serovar-specific lipopolysaccharide antigen by alkaline treatment. Microbiol. Immunol. 25. 949-957.

Tsuji, S., Matsumoto, M., Takeuchi, O., Akira, S., Azuma, I., Hayashi, A., Toyoshima, K. and Seya, T. (2000). Maturation of human dendritic cells by cell wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guerin. involvement of toll-like receptors. Infect. Immun. 68. 6883–90.

Turner, K. S. and Kleven, S. H. (1998). Eradication of live F strain mycoplasma gallisepticum vaccine using live ts-11 on a multiage commercial layer farm. Avian Dis. 42. 404-7.

Uchida, Yamato, M., Ito, E., Kwon, O. H., Kikuchi, A., Sakai, K. and Okano, T. (2000). Two different types of nonthrombogenic surfaces. PEG suppresses platelet adhesion ATP-independently but HEMA-St block copolymer requires ATP consumption of platelets to prevent adhesion. J. Biomed. Mater. Res. 50. 585-590.

Vig, M., Srivastava, S., Kandpal, U., Sade, H., Lewis, V., Sarin, A., George, A., Bal, V., Durdik, J. M. and Rath, S. (2004). Inducible nitric oxide synthase in T cells regulates T cell death and immune memory. J. of Clinical Investigation. 113. 1734-1741.

Voll, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalden, J. R. and Girkontaite I. (1997). Immunosuppressive effects of apoptotic cells. Nature. 390. 350–351.

Wang, Z. M., Liu, C. and Dziarski, R. (2000). Chemokines are the main proinflammatory mediators in human monocytes activated by *Staphylococcus aureus*, peptidoglycan, and endotoxin. J. Biol. Chem. 275. 20260-20267.

Washburn, L. R.,Weaver, K. E., Weaver, E. J., Donelan, W. and Al-Sheboul, S. (1998). Molecular characterization of *Mycoplasma arthritidis* variable surface protein MAA2. Infect. Immun. 66. 2576-2586.

Weigt, H. (2003). Synthetic mycoplasma-derived lipopeptide MALP-2 induces maturation and function of dendritic cells. Immunobiology. 207. 223-33.

Weinack, O. M. (1984). Influence of *Mycoplasma gallisepticum*, infectious bronchitis, and cyclophosphamide on chickens protected by native intestinal microflora against Salmonella typhimurium or Escherichia coli. Avian Dis. 28. 416-25.

Weis, W. I., Drickamer, K. and Hendrickson, W. A. (1992). Structure of a Ctype mannosebinding protein complexed with an oligosaccharide. Nature. 360. 127–134.

Whithear, G. H. (1990). Immunogenicity of a temperature sensitive mutant *Mycoplasma gallisepticum* vaccine. Aust. Vet. J. 67. 168-74.

Whithear, G. H. (1996). Control of avian mycoplasmoses by vaccination. Rev. Sci. Tech. 15. 1527-53.

Williamson, E., (1999). Modulating dendritic cells to optimize mucosal immunization protocols. J. Immunol. 163. 3668-75.

Wills-Karp, M., Santeliz, J. and Karp, C. L. (2001). The germless theory of allergic disease revisiting the hygene hypothesis. Nat. Rev. Immunol. 1. 69-75.

Winner, F., Markova, I., Much, P., Lugmair, A., Siebert-Gulle, K., Vogl, G., Rosengarten, R. and Citti, C. (2003). Phenotypic switching in *Mycoplasma gallisepticum* hemadsorption is governed by a high-frequency, reversible point mutation. Infect. Immun. 71. 1265-1273.

Wright, S. D. (1990). CD 14, a Receptor for Complexes of Lipopolysaccharide (LPS) and LPS Binding Protein, Science. 249. 1431-1433.

Xu , S., Cooper, A., Sturgill-Koszycki, S., vanHeyningen, T., Chatterjee, D., Orme, I., Allen, P. and Russell, D.G. (1994). Intracellulart rafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages. J. Immunol. 153. 2568–78.

Yamamoto, H. (2002). Direct hemoperfusion with polymyxin B-immobilized fiber improves shock and hypoxemia during endotoxemia in anesthetized sheep. J. Endotoxin Res. 8. 419-426.

Yamamoto, M., Sato, S. and Hemmi, H. (2002). Essential role for TIRAP in activation . of the signalling cascade shared by TLR-2 and TLR-4. Nature. 420. 324– 329.

Yang, S., Tamai, R., Akashi, S., Takeuchi, O., Akira, S., Sugawara, S. and Takada, H. (2001). Synergistic effect of muramyldipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture. Infect. Immun. 69. 2045-53.

Yuki, Y. (2003). New generation of mucosal adjuvants for the induction of protective immunity. Rev. Med. Virol. 13. 293-310.

Zarember, K. A. and Godowski, P. J. (2002). Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J. Immunol.168. 554–561.

Zhang, Q., Young, T. F. and Ross, R. F. (1994). Glycolipid receptors for attachment of *Mycoplasma hyopneumoniae* to porcine respiratory ciliated cells. Infect. Immun. 62. 4367-4373.

Zhang, Y., Shoda, L. K., Brayton, K. A., Estes, D. M., Palmer, G. H. and Brown, W. C. (2001). Induction of interleukin-6 and interleukin12 in bovine B lymphocytes, monocytes, and macrophages by a CpG oligodeoxynucleotide (ODN 2059) containing the GTCGTT motif. J. Interferon Cytokine Res. 21. 871-881.

Zimmermann, S. (1998). CpG oligodeoxynucleotides trigger protective and curative Th 1 responses in lethal murine lishmaniasis. J. Immunol. 160. 3627-30.