Review article

Adjuvants modulating mucosal immune responses or directing systemic responses towards the mucosa

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Abstract – In developing veterinary mucosal vaccines and vaccination strategies, mucosal adjuvants are one of the key players for inducing protective immune responses. Most of the mucosal adjuvants seem to exert their effect via binding to a receptor/or target cells and these properties were used to classify the mucosal adjuvants reviewed in the present paper: (1) ganglioside receptor-binding toxins (cholera toxin, LT enterotoxin, their B subunits and mutants); (2) surface immunoglobulin binding complex CTA1-DD; (3) TLR4 binding lipopolysaccharide; (4) TLR2-binding muramyl dipeptide; (5) Mannose receptor-binding mannan; (6) Dectin-1-binding ß 1,3/1,6 glucans; (7) TLR9-binding CpG-oligodeoxynucleotides; (8) Cytokines and chemokines; (9) Antigen-presenting cell targeting ISCOMATRIX and ISCOM. In addition, attention is given to two adjuvants able to prime the mucosal immune system following a systemic immunization, namely $1\alpha, 25(OH)_2D_3$ and cholera toxin.

mucosal adjuvants / pattern recognition receptors / dendritic cells / domestic animals / systemic immunization

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1. INTRODUCTION

It is generally accepted that initiation of a specific immune response requires activation of a specific defense mechanisms resulting in a pro-inflammatory response. The released cytokines and chemokines assist in activating and directing the adaptive immune response. As a consequence, a vaccine has to induce a pro-inflammatory response to be effective. Most antigens are not immunogenic enough and need help to induce a good immune response. Adjuvants (*adjuvare* (latin) = to help) are substances added to an antigen with the purpose of enhancing its immunogenicity [77]. Whereas a very long list of adjuvants for systemic immunization exists, the number of mucosal adjuvants is far more limited since mucosal adjuvants have to stimulate the mucosa-associated lymphoid tissue underneath the mucosa without disturbing the barrier function of the mucosa.

There is currently no optimal classification system to include all adjuvants [54]. Cox and Coulter [41] described that adjuvants act in one or more of five ways: (1) immunomodulation = modulation of the cytokine response; (2) presentation = preservation of the conformational integrity; (3) induction of CD8+ cytotoxic T-lymphocyte responses; (4) targeting = the ability to deliver the antigen to immune effector cells; and (5) depot generation (Tab. I). Therefore, an interesting classification might be based on their mode of action. However, the complete working mechanism of many adjuvants is not known at the moment, impairing this classification system. It is becoming more and more clear that most non-particulated mucosal adjuvants act by

binding to a receptor, whereas the particulated adjuvants act by targeting antigens towards antigen presenting cells (Tab. I). In the present review, these properties were used to classify mucosal adjuvants.

The present review mainly deals with the non-particulated mucosal adjuvants. Several particulated adjuvants will be described in another article [70] of this special issue on "Mucosal immunology in domestic animals". Additionally, we review adjuvants reported to modulate a systemic immune response towards a mucosal one. The latter is particularly interesting for antigens for which a mucosal administration is less feasible due to rapid degradation in the gastrointestinal tract.

2. RECEPTOR-SPECIFIC MUCOSAL ADJUVANTS

Almost all data on mucosal adjuvants for veterinary species are from experimental work and most of these experiments have been performed in pigs, sheep and rabbits. Most of these data are summarized in the present review. The experimental work on horses, cattle, dogs, cats and other veterinary species is far more limited. Data from experiments on the latter species are mainly mentioned in the Tables.

In this chapter extra attention is given to the ganglioside receptor-binding toxins, since they have been most extensively tested for their mucosal adjuvanticity and this in different animal species. However, other adjuvants that have also been shown to enhance and or modulate mucosal immune responses in animals are discussed in the present

Table I. Properties of some adjuvants adapted from Cox and Coulter [41].

Adjuvant	Particle form	Immunomodulation*	Targeting	Receptor	Presentation	CTL induction	Depot
Aluminium salts	+	++++2	+	–	–	–	+
W/O emulsions	+	+1,+2	–	–	–	–	++
O/W emulsions	+	+1,+2	+	–	+++	–	–
ISCOMS	+	+++1,+2	+++	–	++++	++++	–
ISCOMATRIX		+++1,+++2	+++	–	++++	++	–
Saponin	–	+++1,+++2	–	–	–	+	–
LPS/Lipid A	–	++++1	–	TLR4, RP105	–	–	–
Muramyl dipeptide hydrophil	–	++++2	–	TLR2	–	–	–
Muramyl dipeptide lipophil	–	++++1	–	TLR2	–	–	–
CpG ODN	–	++++1	++**	TLR9	–	–	–
Carbohydrate polymers	–	++1 or 1 and 2	+++***	Mannose receptor, Dectin-1	–	–	·
Vitamin D3	–	++++2	–	Nuclear vitamin D receptor	–	–	–
CTA1-DD	–	+++2	–	Surface immunoglobulin	–	–	–
Cholera toxin	–	++++2	++++***	GM1, GD1b, ganglioside	+	++	·
LT enterotoxin	–	++++1,++++2	++++***	GM1, GD1b GM2 ganglioside†	+	++	–

*1= Th1-like response, 2=Th2-like response.
** If incorporated in DNA plasmide vaccine.
*** If conjugated.
† In addition asialo-GM1 ganglioside, polyglycosylceramides, and polylactosamine-containing glycoproteins.

review. All these non-particulated mucosal adjuvants are classified based on their receptor-specificity.

2.1. Ganglioside receptor-binding toxins

2.1.1. *Cholera toxin and* Escherichia coli *thermolabile enterotoxin*

Cholera toxin (CT) and *E. coli* thermolabile enterotoxin (LT) are highly resembling molecules that function as virulence factors in *V. cholerae* and *E. coli* infections, respectively. Both toxins are composed of an A and B subunit of which the B subunit forms a homopentamer structure that binds to epithelial cells. The B subunit of CT and LT binds with high affinity to the glycosphingolipid, GM1-ganglioside (Gal(1-3)GalNAc(1-4)(NeuAc(2-3)Gal(1-4)Glc(1-1)ceramide) [71] and with a lower affinity to GD1b-ganglioside [88]. In addition, LT-B also binds with low affinity to polyglycosylceramides, asialo-GM1, GM2 and polylactosamine-containing glycoproteins [66, 89]. GM1 is present on virtually all cells including enterocytes, dendritic cells (DC), macrophages (MØ), B and T lymphocytes. The A subunit is composed of a globular A1 domain and an A2 domain that interacts with the B subunit. The ADPribosyltransferase activity is facilitated following proteolytic cleavage of the trypsin-sensitive loop between the two domains and reduction of the disulfide bond [71]. Then, the A1 fragment enters the cytosol, enzymatically ribosylates the Gs protein of adenylate cyclase, leading to an increased cAMP production.

CT as well as LT are potent immunogens and induce antigen-specific sIgA and serum IgG antibody responses [56, 184]. In addition, both toxins can act as adjuvants for the enhancement of mucosal and serum antibody responses to a mucosal co-administered antigen, resulting in a long-term memory to this antigen [35, 105, 202]. The immune response that is occurring against these toxins is not an advantage. High levels of toxin-specific IgA at the inductive site reduce the adjuvant effect. However, these toxin-specific antibodies do not completely inhibit the mucosal adjuvanticity [192, 193].

Following intestinal administration of LT and CT, both toxins bind to intestinal epithelial cells, which subsequently secrete IL-1, IL-6, IL-10 and IL-1Ra [21, 138]. In addition, CT is reported to increase both the intestinal permeability [131] and the protein uptake [213]. Verma et al. [213] suggested a change in tight junctional permeability following LT administration, which may be due to a change in the cytoskeletal microfilaments. However, the influence of LT and CT on intestinal permeability to macromolecules is controversial since some reports argue against it [98, 152]. What is not controversial is that both toxins can be transported by M cells into the Peyer's patches and are subsequently present within monomorphonuclear cells in the lamina propria [116].

The toxins passing the mucosa will subsequently reach cells of the immune system. The interaction of LT and CT with leukocytes is thought to be of major importance for mediating their adjuvant effects. Effects on DC, MØ, T and B cells will be described. Especially DC seem to be the principal cell type by which LT and CT mediate their adjuvant effect in vivo. Luminal CT attracts DC to the intestinal epithelial layer, where they seem to take up luminal antigens [167]. Furthermore, the toxins induce phenotypic and functional maturation of DC, so upregulating the expression of MHCII, B7.1 and B7.2, downregulating the expression of CD40 and ICAM-1 and increasing the secretion of IL-1β [9, 55, 133, 158]. In addition, a cAMP-dependent upregulation of the chemokine receptors CXCR4 and CCR7 occurs [67], enabling the migration of DC to lymph nodes. Here they can interact with naïve T cells [97, 179] in an enhanced way since their ability to present protein antigen is improved [158]. In vitro, CT-matured DC are able to prime naive CD4$^+$/CD45RA$^+$ T

cells and to direct them towards the Th2 phenotype. Inhibition of the expression of the Th1-response promoting cytokine IL-12 has been implicated as the mechanism by which CT mediates this polarization [9, 19, 48]. The ability of CT to activate DC seems to dependent on its specific interaction with GM1 ganglioside [110].

CT and LT exert several effects on MØ, which they also exert on DC, such as enhanced expression of B7.2 [37, 133, 174, 231, 232], enhanced secretion of IL-1 [20, 64] and reduced secretion of IL-12 [19, 174]. In addition, both toxins induce the secretion of IL-10 by MØ, which also promotes a Th2 response [61, 174]. CT also suppresses TNF-α production in response to LPS [24, 29, 38] and as a consequence the NO production is reduced [38].

The effects of CT on T cells also promote a Th2 response. Indeed, the initial event induced by CT in CD4$^+$ T cells involves the upregulation of IL-4 [154, 202, 232]. This results in the secretion of IL-5, IL-6 and IL-10, a typical Th2 response, providing helper signals for the induction of antigen-specific sIgA as well as serum IgG1, IgA and IgE responses in mouse models [91, 230]. In addition CT selectively inhibits proliferation and IFN-γ synthesis of Th1 clones [149, 231] and abrogates IL-12R expression by T cells [19]. In contrast to CT, LT induces both Th1- and Th2-responses with subsequent mucosal sIgA as well as serum IgG1, IgG2a and IgA responses. The LT-induced Th2-response is largely IL-4 independent [232]. This difference in CT and LT adjuvanticity is suggested to rely on differences in either the A [18, 174] or the B subunit [16].

LT and CT have been reported to induce selective apoptosis of CD8$^+$ T cells, naïve cells being more sensitive than activated cells [57, 151, 175].

Binding of CT and LT to B cells leads to the upregulated expression of MHCII, B7.1 and B7.2, CD40, ICAM-1 and IL-2Rα [6, 17, 65, 133]. This activation of B cells enhances their role as MHC II-restricted antigen presenting cells and favors the induction of Th2-dominated responses. In vitro studies indicate that CT facilitates B cell switching to IgA through the action of TGF-β1 and increases the effects of IL-4 and IL-5 on IgG1 and IgA synthesis in lipopolysaccharide (LPS)-triggered spleen B cells [111, 130]. This IgA induction is independent of the A subunit [111].

The induced mucosal immune response is best at the mucosal site directly exposed to the antigen and the adjuvant [159]. This is probably due to an increased expression of homing receptors on endothelial cells [126].

During the last five years, CT was used in an important number of studies in animals as the mucosal model antigen or as the mucosal adjuvant (Tab. II). The most frequently used route is the intranasal one. This route has been used in horses, pigs, rabbits, cattle and sheep and resulted in the first three species in a clear nasal IgA response which was not so in sheep.

Oral immunization with CT as the adjuvant was tested in pigs, chickens and rabbits and mostly induced antigen-specific intestinal IgA responses (Tab. II). The oral use of CT and LT in humans is hampered by their enterotoxicity. As little as 5 µg CT can induce significant diarrhea and 25 µg elicits 20-litre watery diarrhea. However, in all tested animals CT seems to be less toxic. For example in 3- to 4-week-old piglets, a dose of 1 mg CT induced only a pasty to semiliquid diarrhea for 2 h [40], whereas a dose of 50 and 100 µg did not cause clinical signs at all, but still showed a significant adjuvant effect [63, 210]. In pigs, LT loses its enterotoxicity very rapidly with increasing age and 14 times the dose of LT provoking severe watery diarrhea with dehydration in neonatal pigs induces only a pasty to semiliquid diarrhea for 24 h in 3- to 4-week-old pigs [40]. So, there seems to be less need for detoxified variants of both enterotoxins in pigs. Evaluation of the adjuvanticity of different dosages of CT in pigs revealed that 10 µg already significantly enhanced the mucosal IgA and the serum

Table II. CT as mucosal adjuvant in animals.

Species	Route	Dose (µg)	Antigen	Measured antigen-specific response	References
Pig	IN	25	*Ascaris* 16 kDa protein (rAs16) - CT conjugate	Increased serum IgG, nasal IgA, IL-4, IL-10 and protection	[199]
	O	100	CT-B-A2-PRRSV nucleocapsid protein peptide	Increased intestinal IgA, serum IgG and IgA, no vaginal response	[94]
		50	F4 fimbriae-HSA conjugate, HSA-HSA	Increased serum IgA and IgG and salivary IgA	[211]
		50	rFaeG (adhesin of F4 fimbriae)	Increased serum IgA and	[210]
		10, 100	CTB, KLH, OVA or OVA-CTB	Increased IgA in intestine (not for KLH, OVA)	[63]
Sheep	IN	100	PLG + *Toxoplasma gondii* tachyzoite antigen	No clear effect	[187]
	R	25	Keyhole limpet hemocyanin (KLH)	Increased IgG1 and IgA ASC in blood	[161]
		10	*Hemonchus contortus* L3 larvae surface antigen	Very low (total) serum antibody response	[99]
Cattle	IN	100	Limulus hemocyanin (LH)	Increased serum IgA	[166]
Horse	IN	0.2	100 µg CTB, two aerosol boosts with 1 mg CT	Induction of nasal and serum IgA, IgGb and IgGT and serum IgGa	[183]
		1000	Equine influenza virus hemagglutinin (HA) DNA	Increased nasal IgA response	[183]
Rabbit	IN	20	Extract of *Pasteurella multocida* (*P. mult.*)	Induction of serum IgG and nasal IgA	[189]
		200	*P. mult.* OMP	Induction of serum IgG and nasal IgA	[36]
	O	200	Extract of *P. mult.* in alignate microparticles	Increased serum IgG and induction of nasal IgA	[189]
Cat	R, IN/R	10	FIV-peptides or fixed whole FIV	Induction of serum IgG and IgA	[62]
Chicken	O	50	*Eimeria tenella* and r1PE1 of *E. tenella*	Only with recombinant increased IgA and IgG in intestine, serum.	[72]
		4, 20, 100	Inactivated infectious bursal disease virus	No effect	[93]
	IC	50	r Ea1A of *Eimeria acervulina*, CT-rEa1A conjugate	Increased serum antibody response slightly better with the conjugate	[215]
	O, R	10	Mycotoxin aflatoxin B1 (AFB)	No enhanced serum IgG, no fecal IgA response	[226]

R: rectal; O: oral; IN: intranasal; IC: intra-caecal; OMP: outer membrane proteints; FIV: feline immunodeficiency virus.

IgA and IgG responses against coadministered CT-B. However, when using keyhole limpet hemocyanin or ovalbumin as antigen, even 50 µg CT could not induce an antibody response, whereas when added to OVA-CT-B conjugate, a clear response was observed [63]. This indicates that not only the adjuvant and its dose but also the antigen are important in mucosal immunization. In experiments we performed, 50 µg CT did enhance the IgG and IgA response following oral immunization of pigs with a human serum albumin (HSA)-HSA conjugate (HSA-HSA conjugate) or a HSA-F4 conjugate (purified F4 fimbriae of enterotoxigenic *E. coli* (ETEC)) [211]. However the most pronounced effect was observed for the latter conjugate. A good adjuvant effect was also seen following oral immunization with the recombinant adhesin of F4 [210]. These observations seem to indicate that CT is especially suitable for antigens that bind to (target) the mucosa.

2.1.2. CT-B and LT-B

An important strategy for utilizing the immune-stimulatory properties of LT and CT has been the use of the non-toxic B subunits. However, the results obtained with LT-B and CT-B alone as mucosal adjuvants are highly inconsistent. Studies have shown that neither LT-B nor CT-B enhance immune responses to mucosal co-administered protein antigens when given orally [165, 231], whereas some other reports have suggested that LT-B and CT-B could display mucosal adjuvant activity when intranasally administered (large doses) in combination with proteins (large doses) [46, 47, 52, 165]. Antibody responses can be observed when LT-B or CT-B are directly conjugated to the antigen itself [165] and are given either orally or intranasally (Tab. III). However, it is interesting to note that the holotoxin stimulates stronger responses on a dose-by-dose basis following intranasal delivery compared to the B subunit [52, 108].

On the contrary, there are studies reporting the use of recombinant LT-B and CT-B

subunits to induce tolerance following oral delivery, but only in the complete absence of holotoxin [227]. Hereto, the B-subunits need to be directly coupled to the antigen so that they can function as a carrier. Following GM1-receptor-mediated uptake, the antigen reaches immature antigen presenting cells, resulting in the induction of TGF-ß-secreting regulatory T cells. In contrast to CT, rCT-B and the catalytically inactive holotoxin do not cause significant maturation of human DC [179]. As for CT, CT-B suppresses the production of TNF-α in response to LPS, but in contrast to CT it may also suppress production of other pro-inflammatory cytokines [29].

2.1.3. CT and LT mutants

Another attempt to dissociate the enterotoxicity of LT and CT from their adjuvanticity has been the construction of mutations in the enzymatically active A subunit. This subunit consists of two chains, A1 and A2, joined by a proteolytically sensitive peptide (Arg192). Well characterized mutations in the A subunit are lysine for serine at position 63 (LT(S63K)) and arginine for alanine at position 72 (LT(A72R)). Both mutations differ in their residual enzymatic activity and this activity is positively correlated with their adjuvanticity with LT(A72R) having the highest activity [10, 132]. Both mutants are active in domestic animals when applied intranasally (Tab. IV). Dickinson and Clements [49] constructed LT(R129G) with a mutation of arginine to lysine in the proteolytic cleavage site. This mutant has a reduced enterotoxicity, shows in vitro absence of ADP-ribosyltransferase activity, but still has its mucosal adjuvanticity when given intranasally, orally or rectally [31, 155, 237]. Yuan et al. [237] used this adjuvant intranasally in gnotobiotic pigs at a dose of 5 µg and although the adjuvant enhanced the IgA ASC response, it did not induce a protective intestinal immunity. The dose of 5 µg was chosen since 10 µg produced diarrhea in 85% of neonatal piglets. We used this adjuvant for oral immunization of conventional weaned piglets

Table III. CT-B and LT-B as mucosal adjuvants in animals.

Adjuvant	Species	Route	Dose (µg)	Antigen	Measured antigen-specific response	References
CT-B	Pig	O	1000	CT (50 µg) and KLH	No KLH specific antibody response	[63]
				PRRSV N peptide and CT (100µg)	No N-peptide antibody response in serum, intestine, vagina	[94]
				CT-B-A2-N-myc or CT-B-myc9 and CT (100µg)	Induction of anti-N-peptide serum IgG, IgA or anti-myc serum IgG	[94]
	Cattle	SC/IN	10/15	Bovine herpesvirus-1 glycoprotein D	No positive effect of CT-B adjuvant	[241]
	Rabbit	IN		Influenza HA	High Serum IgG, nasal IgG	[73]
	Fox	IN	100	OVA	No OVA-specific antibody response	[170]
	Chicken	O	200	CT-B-BSA glutaraldehyde-conjugated	Induction of BSA specific serum IgG	[135]
				CT-B and BSA	Increased BSA-specific serum IgG, IgM, IgA	[136]
		IN	20	Inactivated Newcastle disease virus	Induced IgA and neutralizing antibody titers in nasal washes and enhanced protection.	[190]
CT-B-A2	Horse	IN	300-5000	CT-B-A2-SeMF3(peptide) of *Str. equi*	Induction of serum IgA, Igb, IgGa and IgG(T) and nasal IgA, IgGb	[177]
LT-B	Chicken	O, R	100	AFB-BSA-rLT-B conjugate or AFB-BSA mixed with rLTB	No enhanced serum IgG, no faecal IgA response	[226]
	Rabbit	O	± 1000	LT-B-*E. coli* STa carbodiimide-conjugated	Induction of ST- and LT-B- specific serum IgG and mucosal IgA antibody responses	[114]

O: Oral; SC: subcutaneous; IN: intranasal; R: rectal; KLH: keyhole limpet hemocyanin; PRRSV: porcine reproductive and respiratory syndrome virus; OVA: ovalbumin; BSA: bovine serum albumin; Str.: *Streptococcus*; AFB: Mycotoxin aflatoxin B1; STa: thermostable enterotoxin a.

with F18 fimbriae in a dose of 25 µg and could only see a weak immune response and no clinical signs (unpublished results, Tab. IV). Another mutant that lacked the nick site in the A subunit by deleting a tripeptide (Arg192-Thr193-Ile194), was insensitive to activation by trypsin. This mutant has no ADP-ribosyltransferase activity, but has a strong adjuvant activity (LT triple-aa deletion mutant) [235] and enhanced nasal and serum antibody responses in pigs and cattle following intranasal immunization (Tab. IV).

2.2. Surface immunoglobulin-binding CTA1-DD

Another approach to detoxify CT is linking the enzymatically active A subunit domain of the toxin to a molecule with another cell-binding specificity than the natural B subunit. Agren et al. [2] did this by genetically fusing the gene of the ADP-ribosyltransferase active A1 subunit of CT to a gene encoding a synthetic analogue of the *Staphylococcus aureus* protein A. The latter is called D and the fusion protein, CTA1-DD, containing a dimer of D. CTA1-DD targets the ADPribosyltransferase towards B cells. The fusion protein binds to naïve and memory B cells of all isotypes, with adjuvant effects similar to CT [3]. This protein is non-toxic to mice even when given at 1000-fold the toxic dose of CT. Both CT and CTA1-DD have been observed to enhance antibody and cell-mediated immune responses, the fusion protein exerting these effects primarily via B cells [4, 180]. CTA1-DD mostly functions when applied nasally, but not when given orally. However, the authors used CTA1-DD as an adjuvant in 5-week-old pigs intranasally immunized with F18 fimbriae and only observed weak priming of the antibody response (unpublished results, Tab. IV).

2.3. Pattern Recognition Receptor binding adjuvants

Several molecular structures of microorganisms are recognized by so called pattern recognition receptors (PRR). These PRR are among others present on MØ and DC. Some PRR are mainly involved in opsonization and thus enhance phagocytosis of microorganisms such as the mannose receptor, scavenger receptors and Dectin-1. Others, such as Toll-like receptors (TLR) seem to function exclusively as signaling receptors resulting in the following: (1) activation of innate immune responses, (2) expression of co-stimulatory molecules and (3) via activation of DC, an enhanced T cell stimulatory potential as well as (4) production of immunomodulatory cytokines, essential for T cell priming and effector T cell differentiation [157, 185].

It is therefore not surprising that several of the molecules of microbial origin shown in the past to have adjuvant activity, have more recently been identified to act via these PRR.

2.3.1. TLR4- and radioprotective 105 (RP105)-binding

In 1956, Johnson et al. [103] demonstrated the adjuvant activity of lipopolysaccharide (LPS) from Gram-negative bacteria. LPS exerts its activity mainly via TLR4 on antigen-presenting cells [139, 178]. In fact it is a multi step mechanism. LPS first binds in the blood or extracellular fluid to the soluble LPS binding protein (LBP) with its lipid A moiety [197]. LBP is a lipid transferase that catalyzes the LPS' transfer from the outer membrane of the bacteria to CD14, another LPS binding molecule. CD14 is expressed on the myelomonocytic cells as a glycosylphosphatidylinositol-anchored (GPI-anchored) membrane molecule (mCD14) or is present as a soluble molecule in the circulation (sCD14) [228]. mCD14 enables LPS to get close to TLR4-MD-2, suggesting that LPS needs to be transferred from CD14 to TLR4-MD-2 [44, 150]. MD-2 is an extracellular molecule associated with the extracellular leucine-rich-domain of TLR4. Association of TLR4 with MD-2, is required for the expression of TLR4. As most other

Table IV. LT and CT mutants as mucosal adjuvants in animals.

Adjuvant	Species	Route	Dose (µg)	Antigen	Measured antigen-specific response	References
LT triple-aa deletion mutant	Pig	IN	40, 200, 1000	*Erysipelothrix rhusiopathiae* SPA 46.5 kDa or *Bordetella bronchiseptica* sialic acid-binding HA	Increased nasal IgA and serum IgG; 200 µg the highest IgA responses and best protection	[235]
	Cattle	IN	1000	Intimin (*E. coli* O157:H7) C terminal 64 kDa	Increased nasal and salivary IgA, serum and colostral IgG1	[235]
LT(R192G)	Pig	IN	5	Rotavirus-like particles (VLP) containing VP2 and VP6	Rotavirus-specific IgA (memory) B cells in intestinal tissues, but no protection	[237]
		O/IN or IN/O or IN	5	Attenuated rotavirus oral/VLP intranasal with LT mutant	One oral followed by two IN immunizations induces strongest B-cell responses in GALT, high protection	[238]
		O	25	F18 fimbriae of *Escherichia coli*	Weak IgM response, no protection	*
	Cat	IN	10	P24Gag of feline immunodeficiency virus	Increased IgG in serum and IgA in saliva and vaginal washes	[122]
LTR72	Rabbit	IN	50	Influenza/A HA in esterified hyaluronic acid (HYAFF) microspheres or soluble HA	Increased serum IgG	[181]
LTK63	Micro-pig	IN	100	Influenza/A HA in esterified hyaluronic acid (HYAFF) microspheres or soluble HA	Increased nasal IgA, serum IgG for the HYAFF formulation	[181]
	Rabbit	IN	25	Influenza/A HA in esterified hyaluronic acid (HYAFF) microspheres	Increased serum IgG	[181]
CTA1-DD	pig	IN	100	F18 fimbriae	Slightly increased serum F18-specific Ig	*

O: oral; IN: intranasal; HA: hemagglutinin; SPA: surface protective antigen; * unpublished results.

TLR, TLR4 is a type I transmembrane protein with a conserved cytoplasmic domain called the Toll/interleukin (IL)-1 receptor (TIR) domain [156]. This domain is essential for signaling. Stimulation of TLR results in dimerization and conformational changes, which initiate the signaling via TIR and leads to activation of NF-κB. The subsequent signaling pathway was reviewed recently [191].

LPS is also capable of activating B cells. This does not occur via TLR4-MD-2, but via a structurally related cell surface complex, radioprotective 105 (RP105), which associates with MD-1 [142, 143]. This complex does not signal via TIR. Another transmembrane protein, CD19, was shown to be important for delivering the RP105 signal [233].

Stimulation with LPS results in production of pro-inflammatory cytokines such as IL-1 and TNF-α but also in the release of colony stimulating factors (CSF) and increase in MHC class II expression. Ligation of RP105-MD-1 on B cells induces resistance against apoptosis, upregulation of co-stimulatory molecules and proliferation [27]. LPS itself has only experimentally been used as an adjuvant due to its high toxicity. Several studies have been conducted with chemically modified forms of lipid A. One of the best studied is monophosphoryl lipid A (MPL). MPL is derived from the LPS of *Salmonella minnesota*. Like LPS, MPL is thought to act via TLR4 on antigen-presenting cells and has been shown to induce the release of pro-inflammatory cytokines, but also of IL-2 and IFN-γ. It has been used in vaccines against melanomas and breast cancers, against several infections such as hepatitis B virus infection and against allergies. For the latter it has been approved in Europe [30, 225]. MPL is most effective when used in liposomes.

2.3.2. TLR2-binding

Muramyl dipeptide (MDP) (N-acetyl-muramyl-L-alanyl-D-isoglutamine) is derived from the cell wall of mycobacteria and is one of the active components in the Freund complete adjuvant. MDP acts on TLR2 and was used for intravaginal, oral, intragastric or intraduodenal immunizations of mice, giving an enhanced mucosal immune response [101, 195].

There exists several analogues of MDP. One analogue, adamantylamide dipeptide, constructed by linking amantadine to MDP, has been shown to induce in rabbits a serum IgG and IgA response as well as fecal IgA when given orally in a dose of 100 mg together with 5 mg ovalbumin. The response was observed 10 days after a booster immunization [12]. Recently, a new analogue was constructed, with changes in both the sugar and the peptide parts of the molecule. This analogue shows an enhanced adjuvant activity and suppressed adverse side effects following subcutaneous administration. The introduction of lipophilic residues has improved its incorporation into liposomes. However, evaluation of this MDP analogue in mucosal immunizations has not yet been performed [201].

2.3.3. Mannose receptor-binding

Mannan, a polymannose, can be derived from the cell wall of yeasts. Oxidatively coupled to recombinant protein antigens and given intranasally, but not intraperitoneally, to mice it markedly enhanced the production of IgA, IgG1 and IgG2a in serum and IgA locally in the lung and at remote mucosal sites, including tears, vaginal and salivary secretions. The response was better with CT as the adjuvant. It is thought that the enhanced immune response results from binding to the mannose receptor on phagocytes [186].

2.3.4. Dectin-1-binding

ß 1,3/1,6 glucans are also present in the cell wall of yeast, but in addition, they can be derived form the cell wall of fungi, some bacteria, algae, seaweed and corns [203].

Many studies in mammalians demonstrate that soluble and particulate ß 1,3/1,6 glucans are immunological response modifiers and can be used in the therapy of neoplasia, infectious diseases and immunosuppression. Their main direct target cells appear to be DC, monocytes/MØ, neutrophils and natural killer cells. Glucan treatment of monocyte/ MØ induces the production of TNF-α, IL-1, platelet-activating factor and arachidonic acid metabolites, such as PGE_2 and LTB_4 [1, 50, 58]. The direct interaction of ß-glucans with their target cells is mediated via PRR. Until now, five different PRR have been identified which bind ß-glucans or are involved in the interaction with the glucan: Dectin-1 [22], the lectin-binding domain of the ß chain (CD11b) of the complement receptor 3 [173], the lactosylceramide receptor (CDw17) [219], some scavenger receptors [171] and TLR2/6 [68]. Dectin-1 is thought to synergize with TLR2 to induce TNF-α and IL-12 production and very recently it was demonstrated that Dectin-1 activation of DC even results in production of IL-10 and IL-2 [169].

Several studies have demonstrated the adjuvant effect of glucans when co-administered with either bacterial, fungal, protozoa or viral antigens [13, 144, 168]. In all these studies, ß-glucans were administered via the parental route. Experiments in our laboratory with ß-glucans in pigs have shown that their addition to the feed for a period of two weeks immediately after weaning drastically reduced the excretion of ETEC upon challenge. Supplementation of glucans to piglet's feed can also have a modulating adjuvant effect on simultaneously administered antigens. Different glucans can have distinct immunomodulating effects. One glucan shifted the systemic antibody response against intramuscularly administered bovine thyroglobulin towards IgA while the other suppressed the antigen-specific proliferation of peripheral blood leukocytes [78]. Furthermore, the glucan administration resulted in an enhanced priming of an F4-specific intestinal immune response in piglets when given together with an oral immu-

nization with F4 fimbriae[1]. In dogs, the effect of oral supplementation of glucans on a systemic immunization seems to be different from this in pigs in that an increased antigen-specific IgM response was observed whereas the IgA response should have had a tendency to be lower[2].

2.3.5. TLR9-binding

Cytidine-phosphate-Guanosines (CpG) are unmethylated dinucleotides present at a frequency of approximately 1 on 16 nucleotides in bacterial DNA, whereas they are underrepresented (1/50 to 1/60) and methylated in the vertebrate (mammalian) genomes [25, 160]. Because of these differences, a nonself pattern recognition mechanism has evolved in the vertebrate immune system using PRR enabling them to encounter invading pathogens via their unmethylated CpG-dinucleotides [119]. Hemmi et al. [86] demonstrated that TLR-9 is required for the immune activation by CpG-dinucleotides. This receptor is highly expressed on plasmacytoid precursor DC, B cells, and cells of the monocytes/MØ lineage [53, 104, 239]. Whereas a lot of studies demonstrated an intracellular presence, more recent studies also show cell surface expression. Upon IFNγ treatment, both TLR9 mRNA expression and responsiveness to CpG DNA are upregulated in PBMC, whereas LPS upregulates cell surface expression [53].

The biological activity of these CpG-dinucleotides can be mimicked by chemically synthesized CpG-oligodeoxynucleotides (CpG-ODN). CpG-ODN are chemically synthesized single stranded DNA sequences that are able to stimulate MØ, NK cells, DC and B cells. They were originally synthesized in a specific motif in which the CpG-dinucleotide is flanked preferentially by two purines, adenine (A) or guanine (G) at the 5'-end, and two pyrimidines, cytosine (C) or thymine (T) at the 3'-end,

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[1] Cox et al., unpublished results.
[2] Stuyven et al., unpublished results.

making for example AG<u>CpG</u>TT. The experimental use and success of some DNA-vaccination trials is partly due to the presence of CpG-motifs in the DNA expression vectors [8, 200, 212].

The immunostimulating effects of CpG-ODN also depend on the sequence of the nucleotides flanking the CpG-dinucleotide as well as the target species. Optimal CpG-ODN motifs have been reported for several animal species (Tab. V). Comparing these motifs, it was obvious that recognition of a GT<u>CG</u>TT motif is highly conserved. A GA<u>CG</u>TT motif however, was optimal for inbred strains of mice and rabbits [164].

Upon contact with CpG-ODN, DC and MØ produce IL-12. This in turn activates NK cells, which subsequently produce IFN-γ. IFN-γ suppresses Th2 cells but induces MØ to produce more IL-12. All this results in a Th1 microenvironment. Besides IL-12 and IFN-γ, there is also increased secretion of IL-18, which also modulates towards Th1 [112, 113, 127, 234]. In this Th1-environment, CpG activated NK cells have an increased lytic activity [223]. The effects on APC function seem to differ for DC and MØ. In vitro CpG-ODN cause a decrease in the synthesis of MHC II molecules by peritoneal MØ resulting in down regulation of antigen presentation [32]. However treatment of DC with CpG-ODN induces maturation, increased expression of both MHC II and co-stimulatory molecules and a transient increase in antigen processing followed by a decline [100]. Stimulation of B cells with CpG-ODN results in proliferation [117], IL-6, IL-10 and IgM secretion [234], enhanced expression of activation markers including CD69, CD86, IL-2R (CD25) and IFN-γR [134], and of MHCII in pigs [208]. Proliferation has been observed in mice [117], humans [83], cattle [23] and pigs [208].

Not all CpG-ODN have immunostimulatory properties and some may even have neutralizing effects when co-administered with stimulating CpG-ODN. This is the case with CpG-dinucleotide sequences in the genome of adenoviruses in which the CpG-dinucleotide is preceded by a C and/or followed by a G [118].

Horner et al. [90] demonstrated in mice that CpG given intranasally together with ß-galactosidase was as good as CT for induction of antigen-specific mucosal IgA responses (Th2-responses), whereas a systemic Th1-response was obtained. Several other studies using other antigens confirmed the adjuvant effect following intranasal administration leading to a Th1-biased immune response, but did not always demonstrate the strong IgA response seen by Horner et al. [137]. More recently, the adjuvant effect was also demonstrated for oral immunization [69] and intravaginal immunization [7]. One of the problems with the oral immunization is that the CpG-ODN are rapidly degraded in the gastrointestinal tract. Therefore Wang et al. [220] synthesized oligonucleotides consisting of a novel 3'-3'-linked structure and synthetic stimulatory motifs and called these second-generation immunomodulatory oligonucleotides or IMO. These IMO were more stable in the murine gastrointestinal tract resulting in a stronger immune response, making them a potentially interesting intestinal adjuvant. Table V gives an overview of CpG-ODN with immunostimulatory properties for domestic animals.

2.4. Cytokine or chemokine receptor binding adjuvants

Several cytokines and chemokines have been tested in mice as mucosal adjuvants such as IL-1, IL-5, IL-6, IL-12, IL-15, RANTES, lymphotactin, MCP-1 (monocyte chemoattractant protein 1), MIP-1α, (macrophage inflammatory protein 1α), MIP-1ß, MIP-2, HNP-1 (human neutrophil peptide 1), HNP-2, and HNP-3 [188]. They can be administered as a soluble protein or as a gene encoded by a DNA vaccine. Limitation of the protein form is that high concentrations are needed due to their short half-life time. This problem can be overcome by administering it as a DNA vaccine. DNA vaccination will result in the production of

Table V. Immunostimulatory CpG-ODN motifs for several animal species.

Species	Name	ODN sequence 5'-3'	Reference
Sheep	2007	tcgtcgttttgtcgttttgtcgtt	[164]
	2135	tcgtcgtttgtcgttttgtcgtt	[164]
	2216	ggGGGACGATCGTCgggggG	[140]
	Immunomer 6	5'-Tctgargttct-l-tcttgragtct-5'	[107]
	Immunomer 7	5'-tctgtrgttct-l-tcttgrtgtct-5'	[107]
Goat	2135	tcgtcgtttgtcgttttgtcgtt	[164]
	Immunomer 6	5'-Tctgargttct-l-tcttgragtct-5'	[107]
	Immunomer 7	5'-tctgtrgttct-l-tcttgrtgtct-5'	[107]
	Oligo 4	ctatctgtcgttctctgt	[107]
Horse	2135	tcgtcgtttgtcgttttgtcgtt	[164]
Pig	2007	tcgtcgttgtcgttttgtcgtt	[164, 236]
	D19	ggTGCATCGATGCAGggggg	[106]
	D25	ggtgcatcgatgcagggggg	[106]
	D32	ggTGCGTCGACGCAGggggg	[106]
	A2	gctagacgttagcgt	[112, 204]
	H	Ttttcaattcgaagatgaat	[51]
	I	attcatcttcgaattgaaaa	[51]
	2216	ggGGGACGATCGTCgggggG	[51]
	PCV-2/2	acttcggcagcggcagcacc	[84]
	PCV-2/3	accctgtaacgtttgtcaga	[8]
	PCV-2/4	ctgtgtgatcgatcgatatccatt	[84]
	PCV-2/5	gttttcgaacgcagcgccga	[84]
	Immunomer 6	5'-Tctgargttct-l-tcttgragtct-5'	[107]
	Immunomer 7	5'-tctgtrgttct-l-tcttgrtgtct-5'	[107]
Dog	No. 2	GGtgcatcgatgcagGGGGG	[121]
	1968	tcgtcgctgttgtcgtttctt	[224]
	2005	tcgtcgttgtcgttgtcgtt	[224]
	2006	tcgtcgttttgtcgttttgtcgtt	[224]
	2007	tcgtcgttgtcgttttgtcgtt	[224]
	2012	tgtcgtttgtcgtttgtcgtt	[224]
	2014	tgtcgttgtcgttgtcgtt	[224]
Cat	n.i.	gttcttcggggcgttcttttttaagaacgcccc	[125]
	n.i.	gaagaacgttttccaatgatttttcattggaaaac	[125]
	1968	tcgtcgctgttgtcgtttctt	[224]
	2005	tcgtcgttgtcgttgtcgtt	[224]
	2006	tcgtcgttttgtcgttttgtcgtt	[224]
	2007	tcgtcgttgtcgttttgtcgtt	[224]
	2012	tgtcgtttgtcgtttgtcgtt	[224]
	2014	tgtcgttgtcgttgtcgtt	[224]
Cattle	2006	tcgtcgttttgtcgttttgtcgtt	[162]
	2007	tcgtcgttgtcgttttgtcgtt	[162]
	2059	tcgtcgttttgtcgtttgtcgtt	[240]
	2135	tcgtcgtttgtcgttttgtcgtt	[162]
	2216	ggGGGACGATCGTCgggggG	[140]
Chicken	2006	tcgtcgttttgtcgttttgtcgtt	[229]
	2007	tcgtcgttgtcgttttgtcgtt	[79]
	#1	tcgatcgacgttgaggggggg	[85]
	#17	gtcgttgtcgttgtcgtt	[85]
	n.i.	gctagacgttagcgt	[218]
	n.i.	tccatgacgttcctgacgtt	[221]
Rabbit	2000	tccatgacgttcctgcagttcctgacgtt	[164]
	2007	tcgtcgttgtcgttttgtcgtt	[95]
	ISS	tgactgtgaacgttcgagatga	[196]

l = glycerol linker in immunomers; r = 1-(2'-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine; n.i. = not indicated; small letters = phosphorothionate binding; CAPITALS = phosphodiester binding.

the cytokine/chemokine over a period of weeks to months. However, mucosal application of DNA vaccines needs further optimization in large animals in order to be a feasible method. Due to these limitations, the number of cytokines/chemokines tested as mucosal adjuvants in veterinary species is still very limited. One of the rare examples is the use of IL-1 in rabbits. IL-1 enhances APC activity and IgG and IgA production by B cells. In rabbits, IL-1 was shown to increase the immune response against *Streptococcus sobrinus* when given together at the palatine tonsils [115].

3. ANTIGEN-PRESENTING CELL TARGETING MUCOSAL ADJUVANTS

Besides the non-particulated mucosal adjuvants that mainly exert their effect via binding to a receptor, there are several particulated adjuvants, which have as an important property that they transport the antigen through the mucosal barrier towards AGP cells. Most of these adjuvants are reviewed in the article on mucosal delivery of vaccines [70]. Here, we only highlight the saponin-based mucosal adjuvants used in domestic animals.

The rough extract derived from the bark of a Chilean tree *Quillaja saponaria Molina* containing a mixture of triterpenoid glycosides is called saponins. This extract has adjuvant effects, but its toxicity is too high. Quil A is a part of this rough extract with less toxicity but still containing a mixture of saponins with strong adjuvant activity [42]. Quil A has been used in rectal immunizations of cats with FIV antigens [62] and aerosol immunization of pigs with killed *Actinobacillus pleuropneumoniae* [128]. Whereas the aerosol immunization in pigs induced a marked IgA response in serum, broncheoalveolar and nasal fluids, the rectal immunization in cats induced a weak antibody response but a strong lymphocyte proliferation. Fractionation of Quil A resulted in 28 fractions of which QS21 and ISCO-

PREP 703 have been extensively analysed [110]. QS21, one of the best-tested fractions, has been shown to induce IL-2 and IFN-γ, both Th1 cytokines [15, 176].

Several saponin fractions have been used to prepare ISCOM [147]. ISCOM are 40 nm large particles made up of saponins (Quil A), lipids and antigen held together by hydrophobic interactions between these three components. The compulsory elements to form the ISCOM structure are cholesterol and saponin. Cholesterol is the ligand that binds to saponin forming 12 nm rings. These rings are fixed together by lipids (phosphatidylcholine) to form the spherical nanoparticles. Hydrophobic or amphiphatic antigens can be easily incorporated into this complex. The amphiphatic nature of the antigen is necessary for the interaction with the Quil A/cholesterol matrix. Using phosphatidylcholine enables incorporating a greater variety of antigens. So, ISCOM are a versatile and flexible delivery system in which all molecules/components are exchangeable apart from cholesterol and one saponin. The first ISCOM used Quil A as saponins [147]. Later on, more purified fractions of Quil A, such as ISCOPREP™, were used to form these complexes [172]. ISCOM are preferentially targeting DC [182]. However, all antigen-presenting cells take up antigen from ISCOM more efficiently [216]. Following contact with the ISCOM, the expression of MHC class II molecules is upregulated [14] and pro-inflammatory cytokines and IL-12 are released enhancing Th1-like responses [217]. However, Th2 cytokines can also be induced [87]. Besides CD4+ T cells, also CD8+ cytotoxic T cells become activated [194]. Nevertheless, the immunological properties of ISCOM can easily be varied since a large variety of saponins with different properties exists [172] and since other hydrophobic or amphiphatic molecules with immunomodulatory properties can be integrated into the ISCOM.

Whereas most studies use the systemic route for administering ISCOM, it has been demonstrated that mucosal applications can

be effective. Studies with CT as the antigen have demonstrated the potential to use ISCOM for oral and nasal administration. This was confirmed for the nasal route using influenza [129], RSV [92] and envelope proteins of herpes simplex [146] and for the oral route using rabies virus ISCOM [34] or ovalbumin [145]. Today, mucosal administration in domestic animals is still limited. Intranasal immunization has been performed in pigs against rotavirus [80], in sheep against influenza hemagglutinin [39] and in dogs against *Echinococcus granulosus* surface antigens or ovalbumin [26]. In all three studies, an enhanced mucosal IgA response was observed. However, protection was either not obtained [80] or not evaluated [26]. Oral administration has been analyzed in pigs [96, 153] and sheep [209] with rotavirus as the antigen and it resulted in good IgA responses with partial protection.

ISCOPREP™ is also present in the ISCOMATRIX® adjuvant [148]. This is an adjuvant that is identical to the ISCOM except that it does not contain antigen. This adjuvant can be mixed with antigens and when applied at a mucosa should have some of the advantages of ISCOM such as the preferential targeting of antigen presenting cells. The ISCOMATRIX has been analyzed in oral [209] and intranasal immunization in sheep [39] and in oral immunization in pigs [96] and it induced good mucosal IgA responses. However, the response obtained in sheep differed from that of an ISCOM vaccination in that the ISCOMATRIX induced a Th2-like response, whereas the ISCOM vaccine induced a mixed Th1-/Th2-like response.

4. MODULATION OF A SYSTEMIC IMMUNE RESPONSE TOWARDS A MUCOSAL ONE

Parenteral immunization with an antigen generally induces a systemic and not a mucosal immune response. However, it has been demonstrated that a mucosal immune response can be induced by a systemic immunization using an appropriate immunomodulator as adjuvant. Two adjuvants for which priming of the mucosal immune system has been observed when applied systemically are the steroid hormone 1α, 25(OH)$_2$D$_3$ (Vitamin D3) and CT.

4.1. Vitamin D3 (Vit D3)

It was first demonstrated in mice that an intramuscular, subcutaneous [45, 59] or intradermal [60] immunization with a microbial antigen and aluminium hydroxide as the adjuvant induces a mucosal immune response when 1α,25(OH)$_2$D$_3$ (Vit D3, calcitriol), the active metabolite of vitamin D, is added as an additional immunomodulator. Mice show enhanced antigen-specific IgG and IgA in serum and all tested mucosal secretions (tears, oral, vaginal and colorectal secretions) [45] and increased numbers of IgA and IgG secreting cells (ASC) in systemic (the local draining lymph node and spleen) and mucosa-associated lymphoid tissues (Peyer's patches, mesenteric lymph nodes). Vit D3 modulated the production of cytokines in the local draining lymph node. Indeed, when cells from the local draining lymph node of a mouse treated with Vit D3 were stimulated in vitro with anti-CD3ε, an enhanced production of IL-4, IL-5 and IL-10 (Th2-cytokines) and a reduced production of IL-2 and IFN-γ (Th1-cytokines) was seen [45]. So, in the local draining lymph node, a switch towards a Th2-cytokine profile occurred, as is necessary for an IgA response. Furthermore, migration of antigen-pulsed dendritic cells from the local draining lymph nodes towards the Peyer's patches was seen, where the activation and differentiation of antigen-specific B cells for a mucosal immune response is initiated [59, 60].

Vit D3 exerts its effects on the immune system by binding to a nuclear receptor (nVDR), which is present in activated Th, cytotoxic T cells (CTL) as well as in activated B cells [11, 141, 163]. Via this receptor, Vit D3 modulates the production of

cytokines [123] by reducing the transcription and secretion of among others IFN-γ [33], IL-2 [5], IL-8 [82], IL-12 [43] and GM-CSF [198]. IL-12 is the most important cytokine for promoting differentiation of Th0 cells towards Th1 cells. Furthermore, Vit D3 favours the induction of CD4+CD25+ regulatory T cells and enhances the production of Th2 cytokines such as IL-4 and IL-10, which in turn inhibits Th1 responses [45]. Vit D3 is also known to stimulate TGF-ß [223] that is involved in mucosal immunity, isotype-switching towards IgA as well as IgG2b [124]. So the steroid hormone can be classified as a Th2-modulating adjuvant.

In pigs, intramuscular injection of human serum albumin (HSA) in incomplete Freund adjuvant (IFA) with 2 μg Vit D3 transiently enhanced the antigen-specific serum IgA and IgM responses, the IgA titres in saliva, feces and nasal secretions and the antigen-specific IgA and IgG ASC in the local draining lymph nodes [204]. Furthermore, slightly higher numbers of HSA-specific IgA ASC appeared in the Peyer's patches (PP) of the Vit D3 injected pigs, indicating priming of the gut-associated lymphoid tissue [207]. Thus, when suckling piglets were intramuscularly immunized with fimbriae (0.1 mg) of F4+ ETEC strains in IFA supplemented with Vit D3, these piglets showed a secondary F4-specific serum IgA response and a reduced F4+-ETEC fecal excretion upon challenge 4 days post-weaning [206].

In turkeys, IM immunization with the recombinant major outer membrane protein (rMOMP) of *Chlamydophila psittaci* in IFA with 2 μg Vit D3 also resulted in an enhanced serum IgA response, but not in an increased protection against a challenge infection [214]. In humans, no enhanced IgA response could be observed after IM injection of 1μg Vit D3 co-administered with an influenza vaccine [120].

4.2. Cholera toxin

Transcutaneous immunization by topical administration of CT to the skin has been demonstrated to induce a systemic antibody response against both itself and co-administered proteins in mice [74], humans [76], sheep, cats and dogs [81]. In mice [75] and humans [76] it was shown that this immunization route also induces a mucosal immune response. In mice, even comparable IgA titers were obtained as following an oral immunization [102]. In sheep transcutaneous immunization could induce a systemic immune response but had little or no effect on the mucosa-associated lymphoid tissue [28]. Indeed, the serum IgG1, IgG2 and IgA responses against a co-administered antigen as well as the IgG1 and IgG2 responses in lung wash fluid were lower and the IgA response was absent in comparison with the response following intramuscular injection. Antibodies against the adjuvant were also only detected in serum (IgG1, IgG2, IgA) and lung wash fluid (IgG1), but not in other mucosal secretions. So, extended research is needed to determine the usefulness of the transcutaneous route for priming of the mucosal immune system in domestic animals.

5. GENERAL CONCLUSIONS

The advantage of mucosal vaccines for prevention of numerous infectious diseases is indisputable. Nevertheless, the number of available mucosal vaccines is still very limited. This is due to important difficulties that are encountered by using the mucosal route: (1) the antigen should pass the mucosal barrier in sufficient amount, (2) mucosal tolerance mechanisms should be overcome, (3) protective immune mechanisms should be activated and (4) this should ideally occur with minimal/or no influence on mucosal functionality. Mucosal adjuvants are crucial in reaching these goals. Recent insights that probably all non-particulated mucosal adjuvants activate/modulate mucosal immunity via receptor-mediated mechanisms in which activation of innate immunity is an important step will help in developing new vaccination strategies. In most studies only one adjuvant is used. An ideal mucosal

adjuvant should carry the antigen through the epithelial layer without disturbing this layer, conserve the native conformation of the antigen, attract antigen presenting cells allowing the antigen to be taken up in optimal circumstances, enhance its presentation and activate immune mechanisms resulting in a protective IgA response. This ideal adjuvant does not exist, but it will be one of the challenges to come close to this ideal picture. Hereto, combining adjuvants acting via different mechanisms will be inevitable. So, increased efforts are needed to elucidate the mechanisms of action of adjuvants and to find new mucosal adjuvants differing in action mechanism and effects.

Another challenge for veterinary medicine is the different animal species. Results in mice are often not extrapolatable to other species and this also accounts for data obtained in one domestic animal species. But not only species differences but also the antigen can influence the effect of the adjuvant. Therefore, adjuvanticity (and adverse effects) remains to be analyzed in the target species using the intended antigen(s) and the intended route of administration. As can be learned from this review, most data on veterinary mucosal adjuvants have been obtained from experiments in swine and sheep, but even in these species information is too limited. There is an urgent need for more studies in the different domestic animal species.

REFERENCES

[1] Abel G., Czop J.K., Stimulation of human monocyte beta-glucan receptors by glucan particles induces production of TNF-alpha and IL-1, Int. J. Immunopharmacol. 14 (1992) 1363–1373.

[2] Agren L.C., Ekman L., Lowenadler B., Lycke N.Y., Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit, J. Immunol. 158 (1997) 3936–3946.

[3] Agren L.C., Ekman L., Lowenadler B., Nedrud J.G., Lycke N.Y., Adjuvanticity of the cholera toxin A1-based fusion protein, CTA1-DD, is critically dependent on the ADP-ribosyltransferase and Ig-binding activity, J. Immunol. 162 (1999) 2432–2440.

[4] Agren L., Sverremark E., Ekman L., Schon K., Lowenadler B., Fernandez C., Lycke N.Y., The ADP-ribosylating CTA1-DD adjuvant enhances T-cell dependent and independent responses by direct action on B cells involving anti-apoptotic Bcl-2- and germinal center-promoting effects, J. Immunol. 164 (2000) 6276–6286.

[5] Alroy I., Towers T.L., Freedman L.P., Transcriptional repression of the interleukin-2 gene by vitamin D3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor, Mol. Cell. Biol. 15 (1995) 5789–5799.

[6] Anastassiou E.D., Yamada H., Francis M.L., Mond J.J., Tsokos G.C., Effects of cholera toxin on human B cells. Cholera toxin induces B cell surface DR expression while it inhibits anti-mu antibody-induced cell proliferation, J. Immunol. 145 (1990) 2375–2380.

[7] Ashkar A.A., Bauer S., Mitchell W.J., Vieira J., Rosenthal K.L., Local delivery of CpG oligodeoxynucleotides induces rapid changes in the genital mucosa and inhibits replication, but not entry, of herpes simplex virus type 2, J. Virol. 77 (2003) 8948–8956.

[8] Babiuk L.A., van Drunen Littel-van den Hurk S., Loehr B.I., Uwiera R., Veterinary applications of DNA vaccines, Dev. Biol. 104 (2000) 73–81.

[9] Bagley K.C., Abdelwahab S.F., Tuskan R.G., Fouts T.R., Lewis G.K., Cholera toxin and heat-labile enterotoxin activate human monocyte-derived dendritic cells and dominantly inhibit cytokine production through a cyclic AMP-dependent pathway, Infect. Immun. 70 (2002) 5533–5539.

[10] Barackman J.D., Ott G., Pine S., O'Hagan D.T., Oral administration of influenza vaccine in combination with the adjuvants LT-K63 and LT-R72 induces potent immune responses comparable to or stronger than traditional intramuscular immunization, Clin. Diagn. Lab. Immunol. 8 (2001) 652–657.

[11] Baran D.T., Sorensen A.M., Rapid actions of 1 alpha-25-dihydroxyvitamin D3 physiologic role, Proc. Soc. Exp. Biol. Med. 207 (1994) 175–179.

[12] Becker P.D., Corral R.S., Guzman C.A., Grinstein S., Adamantylamide dipeptide as effective immunoadjuvant in rabbits and mice, Vaccine 19 (2001) 4603–4609.

[13] Benach J.L., Habicht G.S., Holbrook T.W., Cook J.A., Glucans as an adjuvant for a

murine *Babesea microti* immunization trail, Infect. Immun. 35 (1982) 947–951.

[14] Bergström-Mollaoglu M., Lövgren K., Akerblom L., Fossum C., Morein B., Antigen-specific increases in the number of splenocytes expressing MHC class II molecules following restimulation with antigen in various physical forms, Scand. J. Immunol. 36 (1992) 565–574.

[15] Boyaka P.N., Marinaro M., Jackson R.J., et al., Oral QS-21 requires early IL-4 help for the induction of mucosal and systemic immunity, J. Immunol. 166 (2001) 2283–2290.

[16] Boyaka P.N., Ohmura M., Fujihashi K., Koga T., Yamamoto M., Kweon M.N., Takeda Y., Jackson R.J., Kiyono H., Yuki Y., McGhee J.R., Chimeras of labile toxin one and cholera toxin retain mucosal adjuvanticity and direct Th cell subsets via their B subunit, J. Immunol. 170 (2003) 454–462.

[17] Bone H., Eckholdt S., Williams N.A., Modulation of B lymphocyte signalling by the B subunit of *Escherichia coli* heat-labile enterotoxin, Int. Immunol. 14 (2002) 647–658.

[18] Bowman C.C., Clements J.D., Differential biological and adjuvant activities of cholera toxin and *Escherichia coli* heat-labile enterotoxin hybrids, Infect. Immun. 69 (2001) 1528–1535.

[19] Braun M.C., He J., Wu C.Y., Kelsall B.L., Cholera toxin suppresses interleukin (IL)-12 production and IL-12 receptor beta1 and beta2 chain espresso, J. Exp. Med. 189 (1999) 541–552.

[20] Bromander A., Holmgren J., Lycke N., Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages in vitro, J. Immunol. 146 (1991) 2908–2914.

[21] Bromander A.K., Kjerrulf M., Holmgren J., Lycke N., Cholera toxin enhances alloantigen presentation by cultured intestinal epithelial cells, Scand. J. Immunol. 37 (1993) 452–458.

[22] Brown G.D., Gordon S., Immune recognition: a new receptor for beta-glucans, Nature 413 (2001) 36–37.

[23] Brown W.C., Estes D.M., Chantler S.E., Keggerreis K.A., Suarez C.E., DNA and CpG oligonucleotide derived from *Babesia bovis* are mitogenic for bovine B cells, Infect. Immun. 66 (1998) 5423–5432.

[24] Burkart V., Kim Y.E., Hartmann B., Ghiea I., Syldath U., Kauer M., Fingberg W., Hanifi-Moghaddam P., Muller S., Kolb H., Cholera toxin B pretreatment of macrophages and monocytes diminishes their proinflammatory responsiveness to lipopolysaccharide, J. Immunol. 168 (2002) 1730–1737.

[25] Cardon L.R., Burge C., Claydon D.A., Karlin S., Pervasive CpG suppression in animal mitochondrial genomes, Proc. Natl. Acad. Sci. USA 91 (1994) 3799–3803.

[26] Carol H., Nieto A., A mucosal IgA response, but no systemic antibody response is evoked by intranasal immunization of dogs with *Echinococcus granulosus* surface antigens ISCOMs, Vet. Immunol. Immunopathol. 65 (1998) 29–41.

[27] Chan V.W., Mecklenbrauker I., Su I., Texido G., Leitges M., Carsetti R., Lowell C.A., Rajewsky K., Miyake K., Tarakhovsky A., The molecular mechanism of B cell activation by Toll-like receptor protein RP-105, J. Exp. Med. 188 (1998) 93–101.

[28] Chen D., Colditz I.G., Glenn G.M., Tsonis C.G., Effect of transcutaneous immunization with co-administered antigen and cholera toxin on systemic and mucosal antibody responses in sheep, Vet. Immunol. Immunopathol. 86 (2002) 177–182.

[29] Chen P., Li J., Barnes J., Kokkonen G.C., Lee J.C., Liu Y., Restraint of proinflammatory cytokine biosynthesis by mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages, J. Immunol. 169 (2002) 6408–6416.

[30] Childers N.K., Miller K.L., Tong G., Llarena J.C., Greenway T., Ulrich J.T., Michalek S.M., Adjuvant activity of monophosphoryl lipid A for nasal and oral immunization with soluble or liposome-associated antigen, Infect. Immun. 68 (2000) 5509–5516.

[31] Chong C., Friberg M., Clements J.D., LT(R192G), a non-toxic mutant of the heat-labile enterotoxin of *Escherichia coli*, elicits enhanced humoral and cellular immune responses associated with protection against lethal oral challenge with *Salmonella* spp., Vaccine 16 (1998) 732–740.

[32] Chu R.S., Askew D., Noss E.H., Tobian A., Krieg A.M., Harding C.V., CpG oligodeoxynucleotides down-regulate macrophage class II MHC antigen processing, J. Immunol. 163 (1999) 1188–1194.

[33] Cippitelli M., Santoni A., Vitamin D3: a transcriptional modulator of the interferon-gamma gene, Eur. J. Immunol. 28 (1998) 3017–3030.

[34] Claassen I.J., Osterhaus A.D., Poelen M., Van Rooijen N., Claassen E., Antigen detection in vivo after immunization with different presentation forms of rabies virus antigen, II Cellular, but not humoral, systemic immune

responses against rabies virus immune-stimulating complexes are macrophage dependent, Immunology 94 (1998) 455–460.

[35] Clements J.D., Hartzog N.M., Lyon F.L., Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens, Vaccine 6 (1988) 269–277.

[36] Confer A.W., Suckow M.A., Montelongo M., Dabo S.M., Miloscio L.J., Gillespie A.J., Meredith GL Intranasal vaccination of rabbits with *Pasteurella multocida* A:3 outer membranes that express iron-regulated proteins, Am. J. Vet. Res. 62 (2001) 697–703.

[37] Cong Y., Weaver C.T., Elson C.O., The mucosal adjuvanticity of cholera toxin involves enhancement of costimulatory activity by selective up-regulation of B7.2 expression, J. Immunol. 159 (1997) 5301–5308.

[38] Cong Y., Oliver A.O., Elson C.O., Effects of cholera toxin on macrophage production of co-stimulatory cytokines, Eur. J. Immunol. 31 (2001) 64–71.

[39] Coulter A., Harris R., Davis R., Drane D., Cox J., Ryan D., Sutton P., Rockman S., Pearse M., Intranasal vaccination with ISCOMATRIX adjuvanted influenza vaccine, Vaccine 21 (2003) 946–949.

[40] Cox E., Houvenaghel A., Cools V., Schrauwen E., Experimental induction of diarrhea in newly-weaned piglets, J. Vet. Med. B 38 (1991) 418–426.

[41] Cox J.C., Coulter A.R., Adjuvants – a classification and review of their modes of action, Vaccine 15 (1997) 248–256.

[42] Dalsgaard K., Saponin adjuvants. III Isolation of a substance from *Quillaja saponaria* Molina with adjuvant activity in foot-and-mouth disease vaccines, Arch. Gesamte Virusforsch. 44 (1974) 243–254.

[43] D'Ambrosio D., Cippitelli M., Cocciolo M.G., Mazzeo D., Di Lucia P., Lang R., Sinigaglia F., Panina-Bordignon P., Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NFκB downregulation in transcriptional repression of the p40 gene, J. Clin. Invest. 101 (1998) 252–262.

[44] Da Silva Correia J., Soldau K., Christen U., Tobias P.S., Ulevitch R.J., Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. Transfer From CD14 to TLR4 and MD-2, J. Biol. Chem. 276 (2001) 21129–21135.

[45] Daynes R.A., Enioutina E.Y., Butler S., Mu H.H., McGee Z.A., Araneo B.A., Induction of common mucosal immunity by hormonally immunomodulated peripheral immunization, Infect. Immun. 64 (1996) 1100–1109.

[46] De Haan L., Feil I.K., Verweij W.R., Holtrop M., Hol W.G., Agsteribbe E., Wilschut J., Mutational analysis of the role of ADP-ribosylation activity and GM1-binding activity in the adjuvant properties of the *Escherichia coli* heat-labile enterotoxin towards intranasally administered keyhole limpet hemocyanin, Eur. J. Immunol. 28 (1998) 1243–1250.

[47] De Haan L., Verweij W.R., Feil I.K., Holtrop M., Hol W.G., Agsteribbe E., Wilschut J., Role of GM1 binding in the mucosal immunogenicity and adjuvant activity of the *Escherichia coli* heat-labile enterotoxin and its B subunit, Immunology 94 (1998) 424–430.

[48] De Jong E.C., Vieira P.L., Kalinski P., Schuitemaker J.H., Tanaka Y., Wierenga E.A., Yazdanbakhsh M., Kapsenberg M.L., Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse Th cell-polarizing signals, J. Immunol. 168 (2002) 1704–1709.

[49] Dickinson B.L., Clements J.D., Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity, Infect. Immun. 63 (1995) 1617–1623.

[50] Doita M., Rasmussen L.T., Seljelid R., Lipsky P.E., Effect of soluble aminated beta-1,3-D-polyglucose on human monocytes: stimulation of cytokine and prostaglandin E2 production but not antigen-presenting function, J. Leukoc. Biol. 49 (1991) 342–351.

[51] Domeika K., Magnusson M., Eloranta M.L., Fuxler L., Alm G.V., Fossum C., Characteristics of oligodeoxyribonucleotides that induce interferon (IFN)-alpha in the pig and the phenotype of the IFN-alpha producing cells, Vet. Immunol. Immunopathol. 101 (2004) 87–102.

[52] Douce G., Fontana M., Pizza M., Rappuoli R., Dougan G., Intranasal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin, Infect. Immun. 65 (1997) 2821–2828.

[53] Eaton-Bassiri A., Dillon S.B., Cunningham M., Rycyzyn M.A., Mills J., Sarisky R.T., Mbow M.L., Toll-Like Receptor 9 can be expressed at the cell surface of distinct populations of tonsils and human peripheral blood mononuclear cells, Infect. Immun. 72 (2004) 7202–7211.

[54] Edelman R., Tacket C.O., Adjuvants, Int. Rev. Immunol. 7 (1990) 51–66.

[55] Eriksson K., Fredriksson M., Nordstrom I., Holmgren J., Cholera toxin and its B subunit promote dendritic cell vaccination with different influences on Th1 and Th2 development, Infect. Immun. 71 (2003) 1740–1747.

[56] Elson C.O., Ealding W., Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin, J. Immunol. 132 (1984) 2736–2741.

[57] Elson C.O., Holland S.P., Dertzbaugh M.T., Cuff C.F., Anderson A.O., Morphologic and functional alterations of mucosal T cells by cholera toxin and its B subunit, J. Immunol. 154 (1995) 1032–1040.

[58] Elstad M.R., Cowley P.F.S., Wilcox L.A., McIntyre T.M., Prescott S.M., Zimmerman G.A., CD11b/CD18 integrin and a ß-glucan receptor act in concert to induce the synthesis of platelet-activating factor by monocytes, J. Immunol. 152 (1994) 220–230.

[59] Enioutina E.Y., Visic D., McGee Z.A., Daynes R.A., The induction of systemic and mucosal immune responses following the subcutaneous immunisation of mature adult mice: characterization of the antibodies in mucosal secretions of animals immunized with antigen formulations containing a vitamin D3 adjuvant, Vaccine 17 (1999) 3050–3064.

[60] Enioutina E.Y., Visic D., Daynes R.A., The induction of systemic and mucosal immune responses to antigen-adjuvant compositions administered into the skin: alterations in the migratory properties of dendritic cells appears to be important for stimulating mucosal immunity, Vaccine 18 (2000) 2753–2767.

[61] Feng W., Wang Y., Zhang J., Wang X., Li C., Chang Z., Effects of CTx and 8-bromo-cAMP on LPS-induced gene expression of cytokines in murine peritoneal macrophages, Biochem. Biophys. Res. Commun. 269 (2000) 570–573.

[62] Finerty S., Stokes C.R., Gruffydd-Jones T.J., Hillman T.J., Reeves N.A., Whiting C.V., Schaaper W.M., Dalsgaard K., Harbour D.A., Mucosal immunization with experimental feline immunodeficiency virus (FIV) vaccines induces both antibody and T cell responses but does not protect against rectal FIV challenge, Vaccine 18 (2000) 3254–3265.

[63] Foss D.L., Murtaugh M.P., Mucosal immunogenicity and adjuvanticity of cholera toxin in swine, Vaccine 17 (1999) 788–801.

[64] Foss D.L., Zilliox M.J., Murtaugh M.P., Differential regulation of macrophage interleukin-1 (IL-1), IL-12 and CD80-CD86 by two bacterial toxins, Infect. Immun. 67 (1999) 5275–5281.

[65] Francis M.L., Ryan J., Jobling M.G., Holmes R.K., Moss J., Mond J.J., Cyclic AMP-independent effects of cholera toxin on B cell activation: II. Binding of ganglioside GM1 induces B cell activation, J. Immunol. 148 (1992) 1999–2005.

[66] Fukuta S., Magnani J.L., Twiddy E.M., Holmes R.K., Ginsburg V., Comparison of the carbohydrate-binding specificities of cholera toxin and *Escherichia coli* heat-labile enterotoxins LTh-I, LT-IIa, and LT-Iib, Infect. Immun. 56 (1988) 1748–1753.

[67] Gagliardi M.C., De Magistris M.T., Maturation of human dendritic cells induced by the adjuvant cholera toxin: role of cAMP on chemokine receptor expression, Vaccine 21 (2003) 856–861.

[68] Gantner B.N., Simmons R.M., Canavera S.J., Akira S., Underhill D.M., Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2, J. Exp. Med. 197 (2003) 1107–1117.

[69] Gerber S., Lane C., Brown D.M., Lord E., DiLorenzo M., Clements J.D., Rybicki E., Williamson A.L., Rose R.C., Human papillomavirus virus-like particles are efficient oral immunogens when coadministered with *Escherichia coli* heat-labile enterotoxin mutant R192G or CpG DNA, J. Virol. 75 (2001) 4752–4760.

[70] Gerdts W., Mutwiri G.K., Tikoo S.K., Babiuk L.A., Mucosal delivery of vaccines in domestic animals, Vet. Res. 37 (2006) 487–510.

[71] Gill D.M., Rappaport R.S., Origin of the enzymatically active A1 fragment of cholera toxin, J. Infect. Dis. 139 (1979) 674–680.

[72] Girard F., Pery P., Naciri M., Quere P., Adjuvant effect of cholera toxin on systemic and mucosal immune responses in chickens infected with *E. tenella* or given recombinant parasitic antigen per os, Vaccine 17 (1999) 1516–1524.

[73] Gizurarson S., Tamura S., Aizawa C., Kurata T., Stimulation of the transepithelial flux of influenza HA vaccine by cholera toxin B subunit, Vaccine 10 (1992) 101–106.

[74] Glenn G.M., Rao M., Matyas G.R., Alving C.R., Cholera toxin opens up skin immunization route, Nature 391 (1998) 851.

[75] Glenn G.M., Scharton-Kersten T., Vassell R., Mallett C.P., Hale T.L., Alving C.R., Transcutaneous immunization with cholera toxin protects mice against lethal mucosal challenge, J. Immunol. 161 (1998) 3211–3214.

[76] Glenn G.M., Taylor D.N., Frakel S., Montermarano A., Alving C.R., Transcutaneous immunization: a human vaccine delivery strategy using a patch, Nat. Med. 6 (2000) 1403–1406.

[77] Glenny A.T., Pope C.G., Waddington H., Wallace U., Immunological notes, J. Pathol. Bacteriol. 29 (1926) 31–40.

[78] Goddeeris B.M., Boersma W.J.A., Cox E., Mast J., Van der Stede Y., Koenen M.E.,

Vancaeneghem S., Mast J., Van den Broeck W., The porcine and avian intestinal immune system and its nutritional modulation, in: Blok M.C., Vahl H.A., de Lange L., van de Braak A.E., Hemke G., Hessing M. (Eds.), Nutrition and health of the gastrointestinal tract, Wageningen Academic publishers, 2002, pp. 97–134.

[79] Gomis S., Babiuk L., Godson D.L., Allan B., Thrush T., Townsend H., Willson P., Waters E., Hecker R., Potter A., Protection of chickens against *Escherichia coli* infections by DNA containing CpG motifs, Infect. Immun. 71 (2003) 857–863.

[80] Gonzalez A.M., Nguyen T.V., Azevedo M.S., Jeong K., Agarib F., Iosef C., Chang K., Lovgren-Bengtsson K., Morein B., Saif L.J., Antibody responses to human rotavirus (HRV) in gnotobiotic pigs following a new prime/boost vaccine strategy using oral attenuated HRV priming and intranasal VP2/6 rotavirus-like particle (VLP) boosting with ISCOM, Clin. Exp. Immunol. 135 (2004) 361–372.

[81] Hammond S.A., Tsonis C., Sellins K., Rushlow K., Scharton-Kersten T., Colditz I., Glenn G.M., Transcutaneous immunization of domestic animals: opportunities and challenges, Adv. Drug Deliv. Rev. 43 (2000) 45–55.

[82] Harant H., Andrew P.J., Reddy G.S., Foglar E., Lindley I.J., 1alpha,25-dihydroxyvitamin D3 and a variety of its natural metabolites transcriptionally repress nuclear-factor-kappaB-mediated interleukin-8 gene expression, Eur. J. Biochem. 250 (1997) 63–71.

[83] Hartmann G., Krieg A.M., Mechanism and function of a newly identified CpG-DNA motif in human primary B-cells, J. Immunol. 164 (2000) 944–953.

[84] Hasslung F.C., Berg M., Allan G.M., Meehan B.M., McNeilly F., Fossum C., Identification of a sequence from the genome of porcine circovirus type 2 with an inhibitory effect on IFN-alpha production by porcine PBMCs, J. Gen. Virol. 84 (2003) 2937–2945.

[85] He H., Lowry V.K., Swaggerty C.L., Ferro P.J., Kogut M.H., In vitro activation of chicken leukocytes and in vivo protection against *Salmonella enteritidis* organ invasion and peritoneal S. enteritidis infection-induced mortality in neonatal chickens by immunostimulatory CpG oligodeoxynucleotide, FEMS Immunol. Med. Microbiol. 43 (2005) 81–89.

[86] Hemmi H., Takeuchi O., Kawai T., Kaiso T., Sato S., Sanjo H., Matsumoto M., Hoshino K., Wagner H., Takeda K., Akira S., A Toll-like receptor recognizes bacterial DNA, Nature 408 (2000) 740–745.

[87] Höglund S., Dalsgaard K., Lövgren K., Sundquist B., Osterhaus A., Morein B., ISCOMs and immunostimulation with viral antigens, Subcell. Biochem. 15 (1989) 39–68.

[88] Holmgren J., Lonnroth I., Svennerholm L., Tissue receptor for cholera exotoxin: postulated structure from studies with GM1 ganglioside and related glycolipids, Infect. Immun. 8 (1973) 208–214.

[89] Holmgren J., Fredman P., Lindblad M., Svennerholm A.M., Svennerholm L., Rabbit intestinal glycoprotein receptor for *Escherichia coli* heat-labile enterotoxin lacking affinity for cholera toxin, Infect. Immun. 38 (1982) 424–433.

[90] Horner A.A., Ronaghy A., Cheng P.M., Nguyen M.D., Cho H.J., Broide D., Raz E., Immunostimulatory DNA is a potent mucosal adjuvant, Cell. Immunol. 190 (1998) 77–82.

[91] Hornquist E., Lycke N., Cholera toxin adjuvant greatly promotes antigen priming of T cells, Eur. J. Immunol. 23 (1993) 2136–2143.

[92] Hu K.F., Ekstrom J., Merza M., Lovgren-Bengtsson K., Morein B., Induction of antibodies in the common mucosal immune system by respiratory syncytial virus immunostimulating complexes, Med. Microbiol. Immunol. (Berl.) 187 (1999) 191–198.

[93] Hoshi S., Nakamura T., Nunoya T., Ueda S., Induction of protective immunity in chickens orally immunized with inactivated infectious bursal disease virus, Vaccine 13 (1995) 245–252.

[94] Hyland K., Foss D.L., Johnson C.R., Murtaugh M.P., Oral immunization induces local and distant mucosal immunity in swine, Vet. Immunol. Immunopathol. 102 (2004) 329–338.

[95] Ioannou X.P., Gomis S.M., Karvonen B., Hecker R., Babiuk L.A., van Drunen Littel-van den Hurk S., CpG-containing oligodeoxynucleotides, in combination with conventional adjuvants, enhance the magnitude and change the bias of the immune responses to a herpesvirus glycoprotein, Vaccine 21 (2002) 127–137.

[96] Iosef C., Van Nguyen T., Jeong K., Bengtsson K., Morein B., Kim Y., Chang K.O., Azevedo M.S., Yuan L., Nielsen P., Saif L.J., Systemic and intestinal antibody secreting cell responses and protection in gnotobiotic pigs immunized orally with attenuated Wa human rotavirus and Wa 2/6-rotavirus-like-particles associated with immunostimulating complexes, Vaccine 20 (2002) 1741–1753.

[97] Iwasaki A., Kelsall B.L., Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine, J. Exp. Med. 191 (2000) 1381–1394.

[98] Jackson R.J., Fujihashi K., Xu-Amano J., Kiyono H., Elson C.O., McGhee J.R., Optimizing oral vaccines: induction of systemic and mucosal B-cell and antibody responses to tetanus toxoid by use of cholera toxin as an adjuvant, Infect. Immun. 61 (1993) 4272–4279.

[99] Jacobs H.J., Wiltshire C., Ashman K., Meeusen E.N., Vaccination against the gastrointestinal nematode, *Haemonchus contortus*, using a purified larval surface antigen, Vaccine 17 (1999) 362–368.

[100] Jakob T., Walker P.S., Krieg A.M., Udey M.C., Vogel J.C., Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA, J. Immunol. 161 (1998) 3042–3049.

[101] Jeurissen S.H., Sminia T., Beuvery E.C., Induction of mucosal immunoglobulin A immune response by preparations of *Neisseria gonorrhoea* porin proteins, Infect. Immun. 55 (1987) 253–257.

[102] John M., Bridges E.A., Miller A.O., Calderwood S.B., Ryan E.T., Comparison of mucosal and systemic humoral immune responses after transcutaneous and oral immunization strategies, Vaccine 20 (2002) 2720–2726.

[103] Johnson A.J., Gaines S., Landy M., Studies on the O antigen of Salmonella typhosa. V. Enhancement of the antibody response to protein antigens by the purified lipopolysaccharide, J. Exp. Med. 103 (1956) 225–233.

[104] Kadowaki N., Ho S., Antonenko S., Malefyt R.W., Kastelein R.A., Bazan F., Liu Y.J., Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens, J. Exp. Med. 194 (2001) 863–869.

[105] Kamiya N., Asano Y., Yoshino J., Sasaki K., Honma Y., Kawase H., Yokochi T., Shiraki K., Tsuji T., Long-term persistence of cellular immunity to Oka vaccine virus induced by pernasal co-administration with *Escherichia coli* enterotoxin in mice, Vaccine 19 (2001) 3131–3136.

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

[107] Kandimalla E.R., Bhagat L., Zhu F.G., Yu D., Cong Y.P., Wang D., Tang J.X., Tang J.Y., Knetter C.F., Lien E., Agrawal S., A dinucleotide motif in oligonucleotides shows potent immunomodulatory activity and overrides species-specific recognition observed with CpG motif, Proc. Natl. Acad. Sci. USA 100 (2003) 14303–14308.

[108] Kang S.M., Yao Q., Guo L., Compans R.W., Mucosal immunization with virus-like particles of simian immunodeficiency virus conjugated with cholera toxin subunit B, J. Virol. 77 (2003) 9823–9830.

[109] Kawamura Y.I., Kawashima R., Shirai Y., Kato R., Hamabata T., Yamamoto M., Furukawa K., Fujihashi K., McGhee J.R., Hayashi H., Dohi T., Cholera toxin activates dendritic cells through dependence on GM1-ganglioside which is mediated by NF- B translocation, Eur. J. Immunol. 33 (2003) 3205–3212.

[110] Kensil C.R., Patel U., Lennick M., Marciani D., Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex, J. Immunol. 146 (1991) 431–437.

[111] Kim P.H., Eckmann L., Lee W.J., Han W., Kagnoff M.F., Cholera toxin and cholera toxin B subunit induce IgA switching through the action of TGF-ß1, J. Immunol. 160 (1998) 1198–1203.

[112] Klinman D.M., Yi A.K., Beaucage S.L., Conover J., Krieg A.M., CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma, Proc. Natl. Acad. Sci. USA 93 (1996) 2879–2883.

[113] Klinman D.M., Yamshichikov G., Ishigatsubo Y., Contribution of CpG motifs to the immunogenicity of DNA-vaccines, J. Immunol. 158 (1997) 3635–3639.

[114] Klipstein F.A., Engert R.F., Houghten R.A., Protection in rabbits immunized with a vaccine of *Escherichia coli* heat-stable toxin cross-linked to the heat-labile toxin B subunit, Infect. Immun. 40 (1983) 888–893.

[115] Kokuryo S., Inoue H., Fukuizumi T., Tsujisawa T., Tominaga K., Fukuda J., Evaluation of interleukin 1 as a mucosal adjuvant in immunization with *Streptococcus sobrinus* cells by tonsillar application in rabbits, Oral Microbiol. Immunol. 17 (2002) 163–171.

[116] Kraehenbuhl J.P., Neutra M.R., Epithelial M cells: differentiation and function, Annu. Rev. Cell Dev. Biol. 16 (2000) 301–332.

[117] Krieg A.M., Yi A.-K., Matson S., Waldschmidt T.J., Bishop G.A., Teasdale R., Koretzky G.A., Klinman D.M., CpG motifs in bacterial DNA trigger direct B-cell activation, Nature 374 (1995) 546–549.

[118] Krieg A.M., Wu T., Weeratna R., Efler S.M., Love-Homan L., Yang L., Yi A.-K., Short D., Davis H.L., Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG-motifs, Proc. Natl. Acad. Sci. USA 95 (1998) 12631–12636.

[119] Krieg A.M., Immune effects and mechanisms of action of CpG motifs, Vaccine 19 (2001) 618–622.

[120] Kriesel J.D., Spruance J., Calcitriol (1,25-dihydroxy-vitamin D3 coadministered with influenza vaccine does not enhance humoral immunity in human volunteers, Vaccine 17 (1999) 1883–1888.

[121] Kurata K., Iwata A., Masuda K., Sakaguchi M., Ohno K., Tsujimoto H., Identification of CpG oligodeoxynucleotide sequences that induce IFN-gamma production in canine peripheral blood mononuclear cells, Vet. Immunol. Immunopathol. 102 (2004) 441–450.

[122] Leavell S., Wright B., Scappino L., Sirriyah J., Chen C., Clements J.D., Burkhard M.J., Induction of serum and mucosal FIV-specific immune responses by intranasal immunization with p24Gag, Vaccine 23 (2005) 1471–1478.

[123] Lemire J.M., Adams J.S., Sakai R., Jordan S.C., 1 alpha,25-dihydroxyvitamin D3 suppresses proliferation and immunoglobulin production by normal human peripheral blood monomorphonuclear cells, J. Clin. Invest. 74 (1984) 657–661.

[124] Letterio J.J., Roberts A.B., Regulation of immune responses by TGF-ß, Annu. Rev. Immunol. 16 (1998) 137–161.

[125] Leutenegger C.M., Boretti F.S., Mislin C.N., Flynn J.N., Schroff M., Habel A., Junghans C., Koenig-Merediz S.A., Sigrist B., Aubert A., Pedersen N.C., Wittig B., Lutz H., Immunization of cats against feline immunodeficiency virus (FIV) infection by using minimalistic immunogenic defined gene expression vector vaccines expressing FIV gp140 alone or with feline interleukin-12 (IL-12), IL-16, or a CpG motif, J. Virol. 74 (2000) 10447–10457.

[126] Lindholm C., Naylor A., Johansson E.L., Quiding-Jarbrink M., Mucosal vaccination increases endothelial expression of mucosal addressin cell adhesion molecule 1 in the human gastrointestinal tract, Infect. Immun. 2 (2004) 1004–1009.

[127] Lipford G.B., Bauer M., Blank C., Reiter R., Wagner H., Heeg K., CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants, Eur. J. Immunol. 27 (1997) 2340–2344.

[128] Loftager M.K., Eriksen L., Aasted B., Nielsen R., Protective immunity following immunisation of pigs with aerosol of *Actinobacillus pleuropneumoniae* serotype 2, Res. Vet. Sci. 55 (1993) 281–286.

[129] Lövgren K., Kaberg H., Morein B., An experimental influenza subunit vaccine (ISCOM): induction of protective immunity to challenge infection in mice after intranasal or subcutaneous administration, Clin. Exp. Immunol. 82 (1990) 435–439.

[130] Lycke N., Severinson E., Strober W., Cholera toxin acts synergistically with IL-4 to promote IgG1 switch differentiation, J. Immunol. 145 (1990) 3316–3324.

[131] Lycke N., Karlsson U., Sjolander A., Magnusson K.E., The adjuvant action of cholera toxin is associated with an increased intestinal permeability for luminal antigens, Scand. J. Immunol. 33 (1991) 691–698.

[132] Magagnoli C., Manetti R., Fontana M.R., Giannelli V., Giuliani M.M., Rappuoli R., Pizza M., Mutations in the A subunit affect yield, stability, and protease sensitivity on non-toxic derivatives of heat-labile enterotoxin, Infect. Immun. 64 (1996) 5434–5438.

[133] Martin M., Sharpe A., Clements J.D., Michalek S.M., Role of B7 costimulatory molecules in the adjuvant activity of the heat-labile enterotoxin of *Escherichia coli*, J. Immunol. 169 (2002) 1744–1752.

[134] Martin-Orozco E., Kobayashi H., Van Uden J., Nguyen M.D., Kornbluth R.S., Raz E., Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences, Int. Immunol. 11 (1999) 1111–1118.

[135] Mayo S., Royo F., Hau J., Correlation between adjuvanticity and immunogenicity of cholera toxin B subunit in orally immunised young chickens, APMIS 113 (2005) 284–287.

[136] Mayo S.L., Persdotter-Hedlund G., Tufvesson M., Hau J., Systemic immune response of young chickens orally immunized with bovine serum albumin, In vivo 17 (2003) 261–268.

[137] McCluskie M., Davis H.L., CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice, J. Immunol. 161 (1998) 4463–4466.

[138] McGee D.W., Elson C.O., McGhee J.R., Enhancing effect of cholera toxin on interleukin-6 secretion by IEC-6 intestinal epithelial cells: mode of action and augmenting effect of inflammatory cytokines, Infect. Immun. 61 (1993) 4637–4644.

[139] Medzhitov R., Preston-Hurlburt P., Janeway C.A. Jr., A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity, Nature 388 (1997) 394–397.

[140] Mena A., Nichani A.K., Popowych Y., Ioannou X.P., Godson D.L., Mutwiri G.K., Hecker R., Babiuk L.A., Griebel P., Bovine and ovine blood mononuclear leukocytes differ markedly in innate immune responses induced by Class A and Class B CpG-oligodeoxynucleotide, Oligonucleotides 13 (2003) 245–259.

[141] Minghetti P.P., Norman A.W., 1,25(OH)2-vitamin D3 receptors: gene regulation and genetic circuitry, FASEB J. 2 (1988) 3043–3053.

[142] Miyake K., Yamashita Y., Ogata M., Sudo T., Kimoto M., RP105, a novel B cell surface molecule implicated in B cell activation, is a member of the leucine-rich repeat protein family, J. Immunol. 154 (1995) 3333–3340.

[143] Miyake K., Shimazu R., Kondo J., Niki T., Akashi S., Ogata H., Yamashita Y., Miura Y., Kimoto M., Mouse MD-1, a molecule that is physically associated with RP105 and positively regulates its expression, J. Immunol. 161 (1998) 1348–1353.

[144] Mohagheghpour N., Dawson M., Hobbs P., Judd A., Winant R., Dousman L., Waldeck N., Hokama L., Tusé D., Kos F., Benike C., Engleman E., Glucans as immunological adjuvant, Adv. Exp. Med. Biol. 383 (1995) 13–22.

[145] Mowat A.M., Maloy K.J., Donachie A.M., Immune-stimulating complexes as vectors for oral immunization with protein antigens, Immunology 80 (1993) 527–534.

[146] Morein B., Merza M., Vaccination against herpesvirus, fiction or reality? Scand. J. Infect. Dis. (Suppl.) 80 (1991) 110–118.

[147] Morein B., Sundquist B., Höglund S., Dalsgaard K., Osterhaus A., ISCOM, a novel structure for antigenic presentation of membrane proteins from enveloped viruses, Nature 308 (1984) 457–460.

[148] Morein B., Hu K.-F., Abusugra I., Current status and potential application of ISCOMs in veterinary Medicine, Adv. Drug Deliv. Rev. 56 (2004) 1367–1382.

[149] Munoz E., Zubiaga A.M., Merrow M., Sauter N.P., Huber B.T., Cholera toxin discriminates between T helper 1 and 2 cells in T cell receptor-mediated activation: role of cAMP in T cell proliferation, J. Exp. Med. 172 (1990) 95–103.

[150] Muroi M., Ohnishi T., Tanamoto K., Regions of the mouse CD14 molecule required for toll-like receptor 2- and 4-mediated activation of NF-kappa B, J. Biol. Chem. 277 (2002) 42372–42379.

[151] Nashar T.O., Webb H.M., Eaglestone S., Williams N.A., Hirst T.R., Potent immunogenicity of the B subunits of *Escherichia coli* heat-labile enterotoxin: receptor binding is essential and induces differential modulation of lymphocytes subsets, Proc. Natl. Acad. Sci. USA 93 (1996) 226–230.

[152] Nedrud J.G., Sigmund N., Cholera toxin as a mucosal adjuvant: III. Antibody responses to nontarget dietary antigens are not increased, Reg. Immunol. 3 (1990–91) 217–222.

[153] Nguyen T.V., Iosef C., Jeong K., Kim Y., Chang K.O., Lovgren-Bengtsson K., Morein B., Azevedo M.S., Lewis P., Nielsen P., Yuan L., Saif L.J., Protection and antibody responses to oral priming by attenuated human rotavirus followed by oral boosting with 2/6-rotavirus-like particles with immunostimulating complexes in gnotobiotic pigs, Vaccine 21 (2003) 4059–4070.

[154] Okahashi N., Yamamoto M., Vancott J.L., Chatfield S.N., Roberts M., Bluethmann H., Hiroi T., Kiyono H., McGhee J.R., Oral immunization of interleukin-4 (IL-4) knock-out mice with a recombinant *Salmonella* strain or cholera toxin reveals that CD4+ Th2 cells producing IL-6 and IL-10 are associated with mucosal immunoglobulin A responses, Infect. Immun. 64 (1996) 1516–1525.

[155] O'Neal C.M., Clements J.D., Estes M.K., Conner M.E., Rotavirus 2/6 viruslike particles administered intranasally with cholera toxin *Escherichia coli* heat-labile toxin (LT), and LT-R192G induce protection from rotavirus challenge, J. Virol. 72 (1998) 3390–3393.

[156] O'Neill L.A., Dinarello C.A., The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defence, Immunol. Today 21 (2000) 206–209.

[157] Pasare C., Medzhitov R., Toll-dependent control mechanisms of CD4 T cell activation, Immunity 21 (2004) 733–741.

[158] Petrovska L., Lopes L., Simmons C.P., Pizza M., Dougan G., Chain B.M., Modulation of dendritic cell endocytosis and antigen processing pathways by *Escherichia coli* heat-labile enterotoxin and mutant derivatives, Vaccine 21 (2003) 1445–1454.

[159] Pierce N.F., Cray W.C. Jr., Determinants of the localization, magnitude, and duration of a specific mucosal IgA plasma cell response in enterically immunized rats, J. Immunol. 128 (1982) 1311–1315.

[160] Pisetsky D.S., The immunologic properties of DNA, J. Immunol. 156 (1996) 421–423.

[161] Premier R.R., Jacobs H.J., Lofthouse S.A., Sedgmen B.J., Meeusen E.N., Antibody isotype profiles in serum and circulating antibody-secreting cells following mucosal and peripheral immunisations of sheep, Vet. Immunol. Immunopathol. 98 (2004) 77–84.

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

[163] Provvedini D.M., Rulot C.M., Sobol R.E., Tsoukas C.D., Manolagas S.C., 1 alpha,25-Dihydroxyvitamin D3 receptors in human thymic and tonsillar lymphocytes, J. Bone Miner. Res. 2 (1987) 239–247.

[164] Rankin R., Pontarollo R., Ioannou X., Krieg A.M., Hecker R., Babiuk L.A., Van drunen Little-Van den Hurk S., CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved, Antisense Nucleic Acid Drug Dev. 11 (2001) 333–340.

[165] Rappuoli R., Pizza M., Douce G., Dougan G., Structure and mucosal adjuvanticity of cholera and Escherichia coli heat-labile enterotoxins, Immunol. Today 20 (1999) 493–500.

[166] Rebelatto M.C., Siger L., Hogenesch H., Kinetics and type of immune response following intranasal and subcutaneous immunisation of calves, Res. Vet. Sci. 71 (2001) 9–15.

[167] Rescigno M., Urbano M., Valzasina B., Francolini M., Rotta G., Bonasio R., Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria, Nat. Immunol. 2 (2001) 361–367.

[168] Reynolds J.A., Kastello M.D., Harrington D.G., Crabbs C.L., Peters C.J., Jemski J.V., Scott G.H., Diluzio N.R., Glucan-induced enhancement of host-resistance to selected infectious diseases, Infect. Immun. 30 (1980) 51–57.

[169] Rogers N.C., Slack E.C., Edwards A.D., Nolte M.A., Schulz O., Schweighoffer E., Williams D.L., Gordon S., Tybulewics V.L., Brown G.D., Reis e Sousa C., Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins, Immunity 22 (2005) 507–517.

[170] Rolland-Turner M., Farre G., Muller D., Rouet N., Boue F., Immunological tools for the assessment of both humoral and cellular immune responses in Foxes (Vulpes vulpes) using ovalbumin and cholera toxin B as an antigenic model, Vaccine 22 (2004) 4163–4172.

[171] Rice P.J., Kelley J.L., Kogan G., Ensley H.E., Kalbfleisch J.H., Browder I.W., Williams D.L., Human monocyte scavenger receptors are pattern recognition receptors for $(1{\rightarrow}3)$-beta-D-glucans, J. Leukoc. Biol. 72 (2002) 140–146.

[172] Ronnberg B., Fekadu M., Morein B., Adjuvant activity of non-toxic Quillaja saponaria Molina components for use in ISCOM matrix, Vaccine (1995) 1375–1382.

[173] Ross G.D., Vetvicka V., Yan J., Xia Y., Vetvickova J., Therapeutic intervention with complement and beta-glucan in cancer, Immunopharmacology 42 (1999) 61–74.

[174] Ryan E.J., McNeela E., Pizza M., Rappuoli R., O'Neill L., Mills K.H., Modulation of innate and acquired immune responses by Escherichia coli heat-labile toxin: distinct pro- and anti-inflammatory effects of the nontoxic AB complex and the enzyme activity, J. Immunol. 165 (2000) 5750–5759.

[175] Salmond R.J., Williams R., Hirst T.R., Williams N.A., Selective induction of CD8+CD4- thymocyte apoptosis mediated by the B-subunit of Escherichia coli heat-labile enterotoxin, Immunol. Lett. 88 (2003) 43–46.

[176] Sasaki S., Sumino K., Hamajima K., Fukushima J., Ishii N., Kawamoto S., Mohri H., Kensil C.R., Okuda K., Induction of systemic and mucosal immune responses to human immunodeficiency virus type 1 by a DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes, J. Virol. 72 (1998) 4931–4939.

[177] Sheoran A.S., Artiushin S., Timoney J.F., Nasal mucosal immunogenicity for the horse of a SeM peptide of Streptococcus equi genetically coupled to cholera toxin, Vaccine 20 (2002) 1653–1659.

[178] Shimazu R., Akashi S., Ogata H., Nagai Y., Fukudome K., Miyake K., Kimoto M., MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4, J. Exp. Med. 189 (1999)1777–1782.

[179] Shreedhar V.K., Kelsall B.L., Neutra M.R., Cholera toxin induces migration of dendritic cells from the subepithelial dome region to T- and B-cell areas of Peyer's patches, Infect. Immun. 71 (2003) 504–509.

[180] Simmons C.P., Mastroeni P., Fowler R., Ghaem-maghami M., Lycke N., Pizza M., Rappuoli R., Dougan G., MHC class I-restricted cytotoxic lymphocyte responses induced by enterotoxin-based mucosal adjuvant, J. Immunol. 163 (1999) 6502–6510.

[181] Singh M., Briones M., O'Hagan D.T., A novel bioadhesive intranasal delivery system for inactivated influenza vaccines, J. Control. Release 70 (2001) 267–276.

[182] Smith R.E., Donarchie A.M., Grdic D., Lycke N., Mowat A.M., Immune-stimulating complexes induce an IL-12-dependent cascade of innate immune responses, J. Immunol. 162 (1999) 5536–5546.

[183] Soboll G., Nelson K.M., Leuthner E.S., Clark R.J., Drape R., Macklin M.D., Swain W.F., Olsen C.W., Lunn D.P., Mucosal co-administration of cholera toxin and influenza virus hemagglutinin-DNA in ponies generates a local IgA response, Vaccine 21 (2003) 3081–3092.

[184] Spangler B.D., Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin, Microbiol. Rev. 56 (1992) 622–647.

[185] Spörri R., Reis e Sousa C., Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function, Nat. Immunol. 6 (2005) 163–170.

[186] Stambas J., Pietersz G., McKenzie I., Nagabhushanam V., Cheers C., Oxidised mannan-listeriolysin O conjugates induce Th1/Th2 cytokine responses after intranasal immunisation, Vaccine 20 (2002) 1877–1886.

[187] Stanley A.C., Buxton D., Innes E.A., Huntley J.F., Intranasal immunisation with *Toxoplasma gondii* tachyzoite antigen encapsulated into PLG microspheres induces humoral and cell-mediated immunity in sheep, Vaccine 22 (2004) 3929–3941.

[188] Stevceva L., Ferrari M.G., Mucosal adjuvants, Curr. Pharm. Des. 11 (2005) 801–811.

[189] Suckow A., Jarvinend L.S., HogenEsch H., Park K., Bowersock T.L., Immunization of rabbits against a bacterial pathogen with an alginate microparticle vaccine, J. Control. Release 85 (2002) 227–235.

[190] Takada A., Kida H., Protective immune response of chickens against Newcastle disease, induced by the intranasal vaccination with inactivated virus, Vet. Microbiol. 50 (1996) 17–25.

[191] Takeda K., Akira S., TLR signalling pathways, Semin. Immunol. 16 (2004) 3–9.

[192] Tamura S., Funato H., Nagamine T., Aizawa C., Kurata T., Effectiveness of cholera toxin B subunit as an adjuvant for nasal influenza vaccination despite pre-existing immunity to CTB, Vaccine 7 (1989) 503–505.

[193] Tamura S., Yajima A., Hatori E., Tamura S., Asanuma H., Suzuki Y., Aizawa C., Kurata T., Effects of frequent intranasal administration of adjuvant-combined influenza vaccine

on the protection against virus infection, Vaccine 15 (1997) 1784–1790.

[194] Takahashi H., Takeshita T., Morein B., Putney S., Germain R.N., Berzofsky J.A., Induction of CD8+ cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMS, Nature 344 (1990) 873–875.

[195] Thapar M.A., Parr E.L., Parr M.B., The effect of adjuvants on antibody titers in mouse vaginal fluid after intravaginal immunization, J. Reprod. Immunol. 17 (1990) 207–216.

[196] Tighe H., Takabayashi K., Schwartz D., Van Nest G., Tuck S., Eiden J.J., Kagey-Sobotka A., Creticos P.S., Lichtenstein L.M., Spiegelberg H.L., Raz E., Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity, J. Allergy Clin. Immunol. 106 (2000) 124–134.

[197] Tobias P.S., Soldau K., Gegner J.A., Mintz D., Ulevitch R.J., Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14, J. Biol. Chem. 270 (1995) 10482–10488.

[198] Towers T.L., Freedman L.P., Granulocyte-macrophage colony stimulating factor gene transcription is directly repressed by the vitamin D3 receptor, J. Biol. Chem. 273 (1998) 8483–8491.

[199] Tsuji N., Miyoshi T., Islam M.K., Isobe T., Yoshihara S., Arakawa T., Matsumoto Y., Yokomizo Y., Recombinant Ascaris 16-Kilodalton protein-induced protection against *Ascaris suum* larval migration after intranasal vaccination in pigs, J. Infect. Dis. 190 (2004) 1812–1820.

[200] Tudor D., Dubuquoy C., Gaboriau V., Lefevre F., Charley B., Riffault S., TLR9 pathway is involved in adjuvant effects of plasmid DNA-based vaccines, Vaccine 23 (2005) 1258–1264.

[201] Turanek J., Ledvina M., Kasna A., Vacek A., Hrbalova V., Krejc B., Miller A.D., Liposomal preparations of muramyl glycopeptides as immunomodulators and adjuvants, Vaccine (2006) in press.

[202] Vajdy M., Lycke N., Presence of antigen-specific long-term memory cells in systemic lymphoid tissues as well as locally in the gut lamina propria following oral immunization with cholera toxin adjuvant, Adv. Exp. Med. Biol. 371B (1995) 1495–1500.

[203] Vancaeneghem S., Cox E., Deprez P., Arnouts S., Goddeeris B.M., ß-glucanen als immunostimulantia en als adjuvantia, Flemish Vet. J. 69 (2000) 412–421.

[204] Van der Stede Y., Cox E., Van den Broeck W., Goddeeris B.M., Enhanced induction of the IgA response in pigs by calcitriol after intramuscular immunization, Vaccine 19 (2001) 1870–1878.

[205] Van der Stede Y., Verdonck F., Verfaillie T., Cox E., Goddeeris B., CpG-ODN as an effective adjuvant in pigs for intramuscular immunisations, Vet. Immunol. Immunopathol. 86 (2002) 31–41.

[206] Van der Stede Y., Cox E., Verdonck F., Vancaeneghem S., Goddeeris B.M., Reduced faecal excretion of F4+-*E coli* by the intramuscular immunisation of suckling piglets by the addition of 1alpha,25-dihydroxyvitamin D3 or CpG-oligodeoxynucleotides, Vaccine 21 (2003) 1023–1032.

[207] Van Der Stede Y., Verfaillie T., Cox E., Verdonck F., Goddeeris B.M., 1alpha,25-dihydroxyvitamin D3 increases IgA serum antibody responses and IgA antibody-secreting cell numbers in the Peyer's patches of pigs after intramuscular immunization, Clin. Exp. Immunol. 135 (2004) 380–390.

[208] Van der Stede Y., Verdonck F., Verfaillie T., Goddeeris B.M., Cox E., Porcine-specific CpG-oligodeoxynucleotide activates B-cells and increases their expression of MHC-II molecules, Vet. Immunol. Immunopathol. 105 (2005) 115–124.

[209] van Pinxteren L.A., Bruce M.G., Campbell I., Wood A., Clarke C.J., Bellman A., Morein B., Snodgrass D.R., Effect of oral rotavirus/ISCOM vaccines on immune responses in gnotobiotic lambs, Vet. Immunol. Immunopathol. 71 (1999) 53–67.

[210] Verdonck F., Snoeck V., Goddeeris B.M., Cox E., Cholera toxin improves the F4(K88)-specific immune response following oral immunization of pigs with recombinant FaeG, Vet. Immunol. Immunopathol. 103 (2005) 21–29.

[211] Verdonck F., De Hauwere V., Bouckaert J., Goddeeris B., Cox E., Fimbriae of enterotoxigenic *Escherichia coli* function as a mucosal carrier for a coupled heterologous antigen, J. Control. Release 104 (2005) 243–258.

[212] Verfaillie T., Cox E., Goddeeris B.M., Immunostimulatory capacity of DNA vaccine vectors in porcine PBMC: a specific role for CpG-motifs, Vet. Immunol. Immunopathol. 103 (2005) 141–151.

[213] Verma M., Majumdar S., Ganguly N.K., Walia B.N., Effect of *Escherichia coli* enterotoxins on macromolecular absorption, Gut 35 (1994) 1613–1616.

[214] Verminnen K., Van Loock M., Cox E., Goddeeris B., Vanrompay D., Protection of turkeys against *Chlamydophila psittaci* challenge by DNA and rMOMP vaccination and evaluation of the immunomodulating effect of 1α,25-dihydroxyvitamin D3, Vaccine 23 (2005) 4509–4516.

[215] Vervelde L., Janse E.M., Vermeulen A.N., Jeurissen S.H., Induction of a local and systemic immune response using cholera toxin as vehicle to deliver antigen in the lamina propria of the chicken intestine, Vet. Immunol. Immunopathol. 62 (1998) 261–272.

[216] Villacres-Eriksson M., Antigen-presentation by naive macrophages, dendritic cells and B cells to primed T lymphocytes and their cytokine production following exposure to immunostimulating complexes, Clin. Exp. Immunol. 102 (1995) 46–52.

[217] Villacres-Eriksson M., Behboudi S., Morgan A.J., Trinchieri G., Morein B., Immunomodulation by Quillaja saponaria adjuvant formulations: in vivo stimulation of interleukin 12 and its effects on the antibody response, Cytokine 9 (1997) 73–82.

[218] Vleugels B., Ververken C., Goddeeris B.M., Stimulatory effect of CpG sequences on humoral response in chickens, Poult. Sci. 81 (2002) 1317–1321.

[219] Wakshull E., Brunke-Reese D., Lindermuth J., Fisette L., Nathans R.S., Crowley J.J., Tufts J.C., Zimmerman J., Mackin W., Adams D.S., PGG-glucan, a soluble beta-(1,3)-glucan, enhances the oxidative burst response, microbicidal activity, and activates a NF-kappa B-like factor in human PMN: evidence for a glycosfingolipid beta-(1,3)-glucan receptor, Immunopharmacology 41 (1999) 89–107.

[220] Wang D., Kandimalla E.R., Yu D., Tang J.X., Agrawal S., Oral administration of second-generation immunomodulatory oligonucleotides induces mucosal Th1 immune responses and adjuvant activity, Vaccine 23 (2005) 2614–2622.

[221] Wang X., Jiang P., Deen S., Wu J., Liu X., Xu J., Efficacy of DNA vaccines against infectious bursal disease virus in chickens enhanced by coadministration with CpG oligodeoxynucleotide, Avian Dis. 47 (2003) 1305–1312.

[222] Weiner G.J., CpG DNA in cancer immunotherapy, Curr. Top. Microbiol. Immunol. 247 (2000) 157–170.

[223] Weinreich T., Landolt M., Booy C., Wuthrich R., Binswanger U., 1,25-dihydroxyvitamin D3 stimulates transforming growth factor-beta1 synthesis by mouse renal proximal tubular cells, Kidney Blood Press. Res. 22 (1999) 99–105.

[224] Wernette C.M., Smith B.F., Barksdale Z.L., Hecker R., Baker H.J., CpG oligodeoxynucleotides stimulate canine and feline immune cell proliferation, Vet. Immunol. Immunopathol. 84 (2002) 223–236.

[225] Wheeler A.W., Marshall J.S., Ulrich J.T., A Th1-inducing adjuvant, MPL, enhances antibody profiles in experimental animals suggesting it has the potential to improve the efficacy of allergy vaccines, Int. Arch. Allergy Immunol. 126 (2001) 135–139.

[226] Wilkinson J., Rood D., Minior D., Guillard K., Darre M., Silbart L.K., Immune response to a mucosally administered aflatoxin B1 vaccine, Poult. Sci. 82 (2003) 1565–1572.

[227] Williams N.A., Hirst T.R., Nashar T.O., Immune modulation by the cholera-like enterotoxins: from adjuvant to therapeutic, Immunol. Today 20 (1999) 95–101.

[228] Wright S.D., Ramos R.A., Tobias P.S., Ulevitch R.J., Mathison J.C., CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein, Science 249 (1990) 1431–1433.

[229] Xie H., Raybourne R.B., Babu U.S., Lillehoj H.S., Heckert R.A., CpG-induced immunomodulation and intracellular bacterial killing in a chicken macrophage cell line, Dev. Comp. Immunol. 27 (2003) 823–834.

[230] Xu-Amano J., Kiyono H., Jackson R.J., Staats H.F., Fujihashi K., Burrows P.D., Elson C.O., Pillai S., McGhee J.R., Helper T cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues, J. Exp. Med. 178 (1993) 1309–1320.

[231] Yamamoto M., Kiyono H., Yamamoto S., Batanero E., Kweon M.N., Azuma M., Takeda Y., McGhee J.R., Direct effects on antigen-presenting cells and T lymphocytes explain the adjuvanticity of a nontoxic cholera toxin mutant, J. Immunol. 162 (1999) 7015–7021.

[232] Yamamoto M., Kiyono H., Kweon M.N., Yamamoto S., Fujihashi K., Kurazono H., Imaoka K., Bluethmann H., Takahashi I., Takeda Y., Azuma M., McGhee J.R., Enterotoxin adjuvants have direct effects on T cells and antigen-presenting cells that result in either interleukin-4-dependent or -independent immune responses, J. Infect. Dis. 182 (2000) 180–190.

[233] Yazawa N., Fujimoto M., Sato S., Miyake K., Asano N., Nagai Y., et al., CD19 regulates innate immunity by the Toll-like receptor RP105 signaling in B lymphocytes, Blood 102 (2003) 1374–1380.

[234] Yi A.-K., Chace J.H., Cowdery J.S., Krieg A.M., IFN-gamma promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides, J. Immunol. 156 (1996) 558–564.

[235] Yokomizo Y., Watanabe F., Imada Y., Inumaru S., Yanaka T., Tsuji T., Mucosal immunoadjuvant activity of the low toxic recombinant *Escherichia coli* heat-labile enterotoxin produced by *Bacillus brevis* for the bacterial subunit or component vaccine in pigs and cattle, Vet. Immunol. Immunopathol. 87 (2002) 291–300.

[236] Yu H., Babiuk L.A., van Drunen Littel-van den Hurk S., Priming with CpG-enriched plasmid and boosting with protein formulated with CpG oligodeoxynucleotides and Quil A induces strong cellular and humoral immune responses to hepatitis C virus NS3, J. Gen. Virol. 85 (2004) 1533–1543.

[237] Yuan L., Geyer A., Hodgins D.C., Fan Z., Qian Y., Chang K.O., Crawford S.E., Parreno V., Ward L.A., Estes M.K., Conner M.E., Saif L.J., Intranasal administration of 2/6-rotavirus-like particles with mutant *Escherichia coli* heat-labile toxin (LT-R192G) induces antibody-secreting cell responses but not protective immunity in gnotobiotic pigs, J. Virol. 74 (2000) 8843–8853.

[238] Yuan L., Iosef C., Azevedo M.S., Kim Y., Qian Y., Geyer A., Nguyen T.V., Chang K.O., Saif L.J., Protective immunity and antibody-secreting cell responses elicited by combined oral attenuated Wa human rotavirus and intranasal Wa 2/6-VLPs with mutant *Escherichia coli* heat-labile toxin in gnotobiotic pigs, J. Virol. 75 (2001) 9229–9238.

[239] Zarember K.A., Godowski P.J., 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 (2002) 554–561.

[240] Zhang Y., Shoda L.K., Brayton K.A., Estes D.M., Palmer G.H., Brown W.C., Induction of interleukin-6 and interleukin-12 in bovine B lymphocytes, monocytes, and macrophages by a CpG oligodeoxynucleotide (ODN 2059) containing the GTCGTT motif, J. Interferon Cytokine Res. 21 (2001) 871–881.

[241] Zhu X., Letchworth G.J. III, Mucosal and systemic immunity to bovine herpesvirus-1 glycoprotein D confer resistance to viral replication and latency in cattle, Vaccine 14 (1996) 61–69.