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APN-targeted β-glucan microparticles for oral immunisation

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Promotoren

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Ambition is the path to success

Persistence is the vehicle you arrive in

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ABBREVIATIONS

Ab	antibody
AFM	atomic force microscope
APC	antigen-presenting cell
APN	aminopeptidase N
BMDC	bone marrow-derived dendritic cells
BSA	bovine serum albumin
CARD	caspase recruit domain
CCP	complement control protein
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CLR	C-type lectin receptor
Cq	quantification cycle
CR3	complement receptor 3
CRD	carbohydrate recognition domain
CSR	class-switch recombination
CT	cholera toxin
CTL	cytotoxic T lymphocyte
DC	dendritic cell
dmLT	double mutant heat-labile enterotoxin
DMP	dimethyl pimelimidate
DMSO	dimethylsulfoxide
DP	degree of polymerisation
ECL	enhanced chemiluminescence
EDC	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
EGF	epidermal growth factor
ERK	extracellular signal regulated kinase
ETEC	enterotoxigenic *Escherichia coli*
FAE	follicle-associated epithelium
FAK	focal adhesion kinase
FCS	fetal calf serum
FDC	follicular dendritic cell
GALT	gut-associated lymphoid tissue
GC	germinal center
GIT	gastrointestinal tract
GM-CSF	granulocyte macrophage colony-stimulating factor
GP	β-glucan microparticle
HBSS	Hanks' balanced salt solution
ICAM	intercellular adhesion molecule-1
IFN	interferon
Ig	immunoglobulin
IKK	inhibitor of κB kinase
IL	interleukin
IPEC	intestinal porcine epithelial cells
ISCOM	immune stimulating complex

ITAM	immunoreceptor tyrosine-based activation motif
LacCer	lactosylceramide
LAL	limulus Amebocyte Lysate
LP	lamina propria
LPS	lipopolysaccharide
LT	heat-labile enterotoxin
M cell	microfold/ membranous or membrane-like cell
mAb	monoclonal antibody
MAM	meprin A5 antigen and receptor protein tyrosine phosphatase μ
MAMP	microbe-associated molecular pattern
MDM	monocyte-derived macrophages
MES	2-(N-morpholino)ethanesulfonic acid
MHC	major histocompatibility complex
MLN	mesenteric lymph nodes
MoDC	monocyte-derived dendritic cell
MW	molecular weight
NFAT	nuclear factor of activated T cells
NF-κβ	nuclear factor kappa B
NHS	N-hydroxysuccinimide
NIK	NF-κβ-inducing kinase
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PEG	polyethylene glycol
PEG-NH_2	O-(2-aminoethyl)polyethylene glycol
PEI	polyethylenimine
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
pIgR	polymeric Ig receptor
PKC	protein kinase C
PLCγ2	phospholipase-Cγ2
PLGA	poly(lactic-co-glycolic acid)
PMA	phorbol 12-myristate 13-acetate
PP	Peyer's patches
PRR	pattern recognition receptor
PTKs	protein tyrosine kinases
PVDF	polyvinylidene difluoride
PWD	post-weaning diarrhoea
Pyk2	proline-rich tyrosine kinase
qPCR	quantitative polymerase chain reaction
RLU	relative light units
RNA	ribonucleic acid
ROS	reactive oxygen species
Rq	relative expression level
SCARF1	scavenger receptor class F, member 1
SCFA	short-chain fatty acids

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	src homology 2
SHP2	tyrosine phosphatase containing Src homology 2 (SH2) domain
SIgA	secretory IgA
siRNA	small interfering RNA
Src	sarcoma
SSC	side scatter
ST	heat-stable enterotoxin
STEC	shiga-toxin producing *Escherichia coli*
syk	spleen tyrosine kinase
TEDS	transepithelial dendrites
Th	T helper cell
TLR	toll-like receptor
Tm	melting temperature
TM	transmembrane region
TMB	3,3’,5,5’-tetramethylbenzidine
TNF	tumor necrosis factor
Treg	regulatory T cell
tRNA	transfer RNA
VLP	virus-like particle

PREFACE

Enterotoxigenic *Escherichia coli* (ETEC) infections are a major cause of morbidity and mortality among both humans and pigs. Human ETEC strains affect mainly children and are also a source of traveler's diarrhoea in regions where ETEC is endemic. Porcine ETEC cause diarrhoea in neonatal and newly weaned piglets.[1-3] Pathogenicity of these bacteria is associated with fimbrial adhesins that mediate their colonisation to the microvilli of the intestinal epithelial cells in the small intestine. Porcine ETEC strains express five different fimbriae, namely the F4, F5, F6, F18 and F41 fimbriae, of which F18 and F4 fimbriae are most frequently associated with post-weaning disease in pigs.[4] Once colonised, ETEC strains will secrete heat-labile enterotoxins (LT) or heat-stable enterotoxins (STa or STb). These enterotoxins will disrupt the water and electrolyte balance in the intestine which will cause severe watery diarrhoea.[5]

In neonatal and recently weaned piglets, ETEC infections results in severe economic losses due to growth retardation, increased drug use and elevated mortality.[6] Most neonatal infections can be prevented by a passive lactogenic immunity obtained by vaccination of the sow. These maternal vaccines are mainly applied parenterally in the pregnant sow and contain inactivated ETEC bacteria with fimbriae or purified fimbriae with or without LT.[7] However, this passive protection disappears at the moment of weaning.[8] For the control of post-weaning diarrhoea (PWD), antibiotics are commonly used.[9, 10] Besides their prophylactic usage, antibiotics were also applied for improving growth and production. The long term and extensive use of antibiotics has resulted in the development of antibiotic resistance. Therefore, since 1 January 2006 all commonly-used antimicrobial growth promoters have been banned in the EU member states. However, this caused a reduced performance and increased morbidity in post-weaning pigs and consequently, the development of alternative strategies is required.[11]

A wide variety of immunomodulating substances used as in-feed additives have been proposed to help post-weaning piglets to cope with feed transition and stress during this period.[12, 13] The aim of these dietary substances is to help piglets develop an 'appropriate' innate and acquired immunity at the intestinal mucosal surface to support a microenvironment for protection against enteric infections, including ETEC.[11] Among these substances, a variety of non-digestible carbohydrates are extensively studied, such as β-glucans.[14] The beneficial effects of dietary β-glucans have already been demonstrated. Indeed, these polysaccharides display immunomodulatory effects upon oral administration and are also known to improve growth and general performance of the individual.[15-20] Although numerous articles have tried to unravel these immunostimulatory effects, there is no consensus about their mechanism of action. Understanding β-glucan-mediated effects by elucidating

the main β-glucan receptor and its signalling pathway in immune cells is important to use these powerful modulating properties in the protection of newly-weaned piglets against enteropathogens.

Another strategy to prevent ETEC infections in post-weaning piglets consist of inducing an active mucosal immune response by oral vaccination of piglets. Thereby, it would be interesting to use the immunostimulatory potential of β-glucans. Interestingly, β-glucan microparticles (GPs) were recently described as promising antigen vehicle systems with inherent adjuvant capacity owing to their β-glucans. Moreover, these particles are known for their high antigen encapsulation, efficiency and safety.[21-32] Unfortunately, developing oral subunit vaccines has been challenging due to numerous potential obstacles, such as the hostile environment of the gastro-intestinal tract, oral tolerance and the epithelial barrier.[33-37] Many approaches have been described to overcome these limitations, including enteric coating, encapsulation in immune-stimulating antigen delivery systems and targeting to endocytotic receptors, located at the apical surface of intestinal epithelial cells.[38]

Chapter one will provide background information about β-glucans, their receptor usage and signalling, while the second chapter is focused on oral vaccination strategies and the potential role of β-glucans as both mucosal adjuvants and antigen vehicle system. Different methods for targeting particles to endocytotic receptors are discussed in the second chapter as well.

PART I

Review of the literature

CHAPTER 1: β-glucans

1.1 Molecular structure of β-glucans

β-glucans are carbohydrate polymers consisting of long linear chains of glucose and are classified by their intrachain linkages (α- or β-linked),[39] their stereoisomer of glucose (D- or L-glucose), different types of glycosidic bonds (β-1,3/1,4- or -1,6-glucans) and their constitution (homoglucans or heteroglucans).[40]

These glucose polymers can be derived from several sources, such as fungi, yeast, some bacteria, seaweeds, algae and cereals (Table 1).[41] In seaweeds and algae, β-glucans serve as storage carbohydrates, while in bacteria, fungi, yeast and cereals, they play a role as structural frame and define cellular shape and rigidity.[42] The molecular structure of β-glucans, such as linkages, ramification and molecular weights, depends not only on the cited source, but also on the isolation and purification procedure. β-glucans purified from yeast, fungi, seaweed and some algae consist of a β-1,3-linked glucose backbone with small numbers of β-1,6-linked branches, while β-glucans from bacterial origin and some algae are unbranched β-1,3-linked glucose residues (Figure 1A).[43, 44] In contrast, β-glucans derived from cereals contain single β-1,3-linkages of cellotriosyl and cellotetraosyl units together with regions that have a more cellulose-like structure, with two to four consecutive β-1,4-linked glucose residues (Figure 1B).[45]

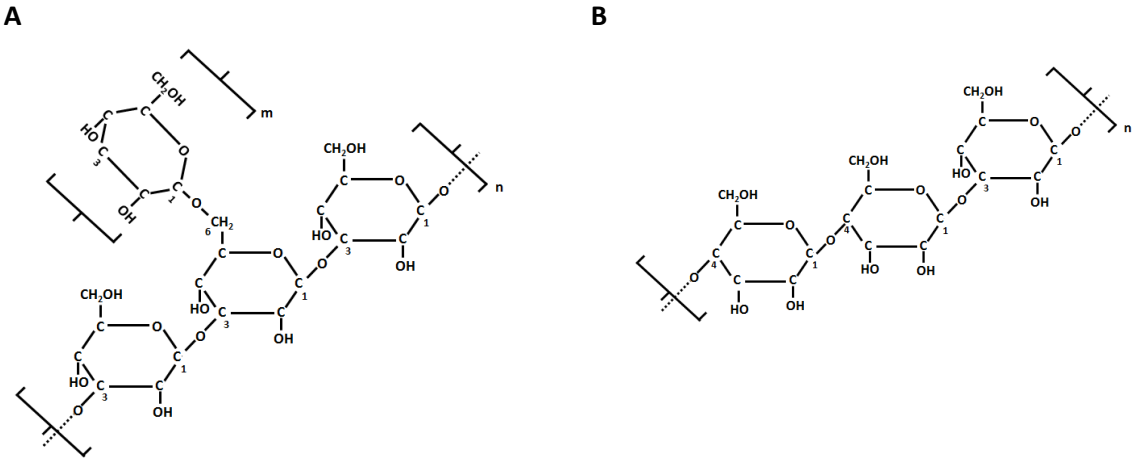


Figure 1. Basic structure of β-1,3/1,6-glucans and β-1,3/1,4-glucans.
(A) The main chain consists of 1,3-linked β-D-glucose units along with 1,6-linked β-D-glucose side chains. **(B)** β-glucans with semi-random alternating β-1,3- and β-1,4-linkages.

Table 1. Overview of the origin, solubility, structure and molecular weight (MW) of β-glucans derived from different sources.

Origin	β-glucan name	Solubility	Structure	MW	References
SEAWEED					
Laminaria digitata	Laminarin	Soluble	β-1,3 with some β-1,6-branching (30:1). The β-1,6 side chains are composed of 2 glucose units	7.7	46-48
YEAST					
Saccharomyces cerevisiae	Macrogard®	Particulate	β-1,3/1,6-branched (10:1 or 20:1). The β-1,6 side chains are composed of 2 or 3 glucose-units	unknown	20, 49
	Zymosan	Particulate	Crude extract with β-glucans, mannan and proteins; non-uniform branches and backbone units. β-1,3/1,6-branched	<200	50-52
	Glucans from *Saccharomyces cerevisiae*	Particulate	β-1,3/1,6-branched (30:1)	200	53
ALGAE					
Euglena gracilis	Glucans from *Euglena gracilis*	Particulate	β-1,3-unbranched	500	53, 54
FUNGI					
Sclerotium rolfsii	Scleroglucan	Soluble	β-1,3/1,6-branched (6:1). The β-1,6 side chains are composed of 2 glucose units	1020	53, 54
CEREALS					
Avena sativa	Glucans from oat	Soluble	β-1,3-branched, mixed with two to four β-1,4-glucose units	50-3000	45
BACTERIA					
Alcaligenes faecalis	Curdlan	Particulate	β-1,3-unbranched	100	55, 56

Most of the β-1,3-linked-glucans exist in a stiff, triple stranded helical structure, where hydrogen bonds hold the individual polymer chains together.[57] When the strength of these bonds decreases below a critical limit, insoluble polysaccharides can be denatured into single helixes or random coil structures.[58-63] Denaturation of β-1,3-D-glucan triplexes occurs by increasing the temperature above the triple-helix melting temperature (Tm = 135°C), by dissolving the molecules in strongly alkaline solutions (1 M NaOH) or in dimethylsulfoxide (DMSO; water weight fraction W_H < 0.13).[57] When exposed to high temperatures, the thermal energy of the β-glucan strands leads to helix destabilisation. Molecular separation by alkaline solutions occurs by repulsion between the ionised groups, and leads to disruption of the ordered structure and causes dissolution, while dissolving β-glucans in DMSO destabilises the polysaccharide hydroxyl groups by the formation of hydrogen bonds. Interestingly, β-1,3-glucans with a high degree of polymerisation are insoluble in water, as their conformation allows stronger cooperative interactions and associations between chains than between the chains and water molecules.[64] However, the degree of branching influences the solubility as well;[65] many β-1,6-glucans side chains render the glucans more soluble.[66] Thus, the physical organisation and structure influences the solubility of β-glucans. Moreover, these factors

contribute to the biological activity of β-glucans as well. Indeed, studies revealed that primary structure, solubility, degree of branching (DB) and molecular weight (MW), as well as the charge of their polymers and structure in aqueous media, are important parameters in the biological activity of β-glucans.[67] The relation between the β-glucan structure and acitivity is already extensively reviewed by Soltanian et al.[68] The most active β-glucans are particulate with a triple helical configuration and consist of many branches, a large main chain and have a high molecular weight.[64, 69] For example, scleroglucan has a linear triple helical arrangement and triggers cytokine secretion by monocytes, however, denaturation abolished this ability.[58]

Although the structure and immune-modulating character of β-glucans are well known, how these polysaccharides exhibit their biological activity is largely unclear. Numerous discrepancies are found in the literature concerning the effect of β-glucans on innate and adaptive immunity, since different β-glucan preparations were used during studies, and this is often inappropriately extrapolated to all β-glucans. These inconsistencies stems from the use of β-glucans which vary in origin, purity and molecular structure (particulated vs soluble) as well as the use of different cell types and model systems to study these β-glucans.

1.2 β-glucan receptors and signalling

As β-glucans are not found in animals, they are considered to be classic microbe-associated molecular patterns (MAMPs).[70] These polysaccharides are recognised as MAMPs by immune cells via several pattern recognition receptors (PRRs), including dectin-1,[71] complement receptor 3 (CR3),[72] scavenger receptors[73] and lactosylceramide.[74] As cells can express several β-glucan receptors and engagement of these receptors results in different biological outcomes, it is interesting to know which intracellular pathways are activated upon engagement of the different β-glucan receptors.

1.2.1 Dectin-1

Dectin-1 has been recognised as the most important receptor for β-glucans in mice and consequently most studies have focused on the function of dectin-1 in the immunomodulating effect of β-glucans.[71, 75] Dectin-1 is highly specific for β-glucans with a pure β-1,3-linked backbone structure with β-1,6-branches.[76] This receptor was originally thought to be a dendritic cell (DC)-specific receptor, hence its name 'dendritic-cell-associated C-type lectin-1'.[77] However, dectin-1 is also expressed by neutrophils, monocytes, macrophages and human intestinal and pulmonary epithelial cells.[78-85]

Dectin-1 consists of an extracellular, transmembrane and cytoplasmic domain. The extracellular domain contains a stalk regions and one c-type lectin domain (carbohydrate binding domain). The cytoplasmic region contains an immunoreceptor tyrosine-based activation motif (ITAM)-like signalling motif.[86] The full-length dectin-1 is encoded by the C-type lectin domain family 7 member A (CLEC7A) gene,[87] which contains of six exons (Figure 2). Exon 1 encodes the N-terminal cytoplasmic tail (Cyto). This tail varies in length and commonly contains internalisation motifs or binding sites for cytosolic proteins. The transmembrane region (TM) is encoded by exon 2. The extracellular variable length-neck (stalk) region and C-terminal carbohydrate recognition domain (CRD) are encoded by exon 3 and exons 4-6, respectively.[77, 81]

Interestingly, alternative splicing of dectin-1 generates species-specific transcripts. In mice, at least two major isoforms exist. The first isoform is the full-length dectin-1A, containing all six exons, while the second dectin-1B isoform lacks the stalk region between the CRD and transmembrane region.[88] Human dectin-1 is alternatively spliced into two major (A-B) and six minor isoforms (C-H), however, only the two major isoforms are able to bind β-glucans.[81, 82, 89] The minor isoforms consist of variations of the major isoforms, some with deletions in the CRD-encoding (isoform C and D) or the transmembrane and stalk region (isoform E and F), while two isoforms (G and H) possess small insertions.[81] In pigs, two major isoforms, which differ by the presence of the stalk region, and one

minor isoform were identified. The minor isoform is a variation of the major isoform with a deletion in the transmembrane and stalk region (Figure 2).[83]

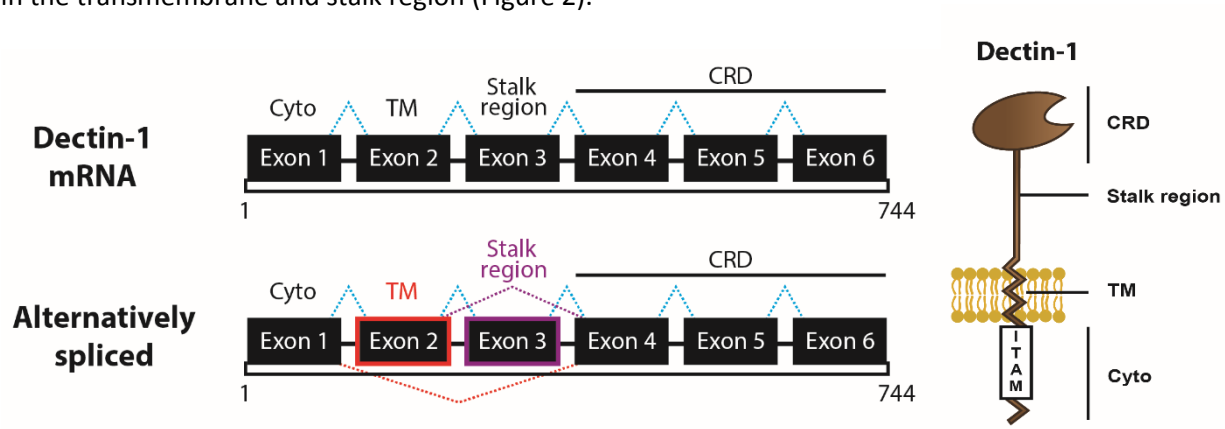


Figure 2. Schematic illustration of the dectin-1 gene, splice variants and structure of dectin-1.
The N-terminal cytoplasmic tail (Cyto) is encoded by exon 1, the transmembrane region (TM; exon 2), stalk region (exon 3), and the carbohydrate recognition domain (CRD; exon 4–6) are illustrated. In pigs, dectin-1 is alternatively spliced in two major isoforms, with (blue) or without (purple) the stalk region, and one minor isoform (red) with a deletion in the TM and stalk region.

Activation of dectin-1 results in numerous cellular responses including phagocytosis, autophagy, respiratory burst and the production of inflammatory lipids, cytokines and chemokines.[90] Different signalling pathways triggered by dectin-1, which result in these cellular responses, will be outlined below.

After ligand engagement, the intracellular hemi-ITAM domain of dectin-1 is phosphorylated (P) by sarcoma (Src) family kinases on its tyrosine (Y) residue, which serves as docking site for spleen tyrosine kinase (Syk).[91] Tyrosine phosphatase containing Src homology 2 domain (SHP2) is required to facilitate the recruitment of Syk to dectin-1.[92] The immunoreceptor tyrosine-based activation motif (ITAM) consists of two tyrosine (YxxL/I) motifs (Y =tyrosine, x = amino acid residues, L = leucine or I= isoleucine) separated by a defined interval of 6-8 amino acids.[93-95] In contrast, the hemi-ITAMs have only a single tyrosine-based motif (YxxL), suggesting that activation of dectin-1 requires receptor clustering into a phagocytic synapse to properly activate its signalling molecule Syk. Indeed, Syk possess tandem SH2 (Src Homology 2) domains and each of these domains can bind to a phosphorylated tyrosine residue (YxxL) by bridging two dectin-1 molecules (Figure 3).[96-100] Recruitment of Syk further activates phospholipase-Cγ2 (PLCγ2), resulting in the engagement of the caspase recruit domain (CARD)-containing protein (CARD9), which together with Bcl10 and MALT1, signals for activation of NF-κβ (canonical pathway). NF-κβ is a master transcription factor, regulating the expression of many inflammatory genes. In mammals, the NF-κβ family is composed of five related transcription factors: p50, p52, RelA (p65), c-Rel and RelB.[101] The canonical pathway activates NF-κβ dimers comprising RelA, c-Rel, RelB and p50. The non-canonical pathway however activates p52/RelB complexes.[101, 102] Dectin-1 is the first PRR for which activation of NF-κβ via the non-

canonical pathway utilising NF-κβ-inducing kinase (NIK) through Syk activation has been described (Figure 3).[103] There is also evidence of another Syk-dependent, CARD9-independent signalling pathway. Ligation of dectin-1 can activate the transcription factor nuclear factor of activated T cells (NFAT) through Syk activation of PLCγ2 (Figure 3).[103] The functions of NFAT in myeloid cells include modulation of cytokine production, survival, induction of cell fusion and differentiation into osteoclasts.[104]

Besides these Syk-dependent pathways, dectin-1 ligation triggers a Syk-independent pathway as well (Figure 3). Indeed, dectin-1 ligation also activates the serine-threonine kinase Raf-1, which promotes the activation of canonical NF-κβ.[90, 97] This dectin-1/Raf-1 pathway activated by β-glucans also triggers trained innate immunity by epigenetic reprogramming of monocytes.[90, 105] Thus, dectin-1 activates two independent signalling pathways, one through Syk and one through Raf-1, to trigger immunity.[106] Which signalling pathway is activated by dectin-1 depends on the cell type, state of activation and encountered ligand.[106]

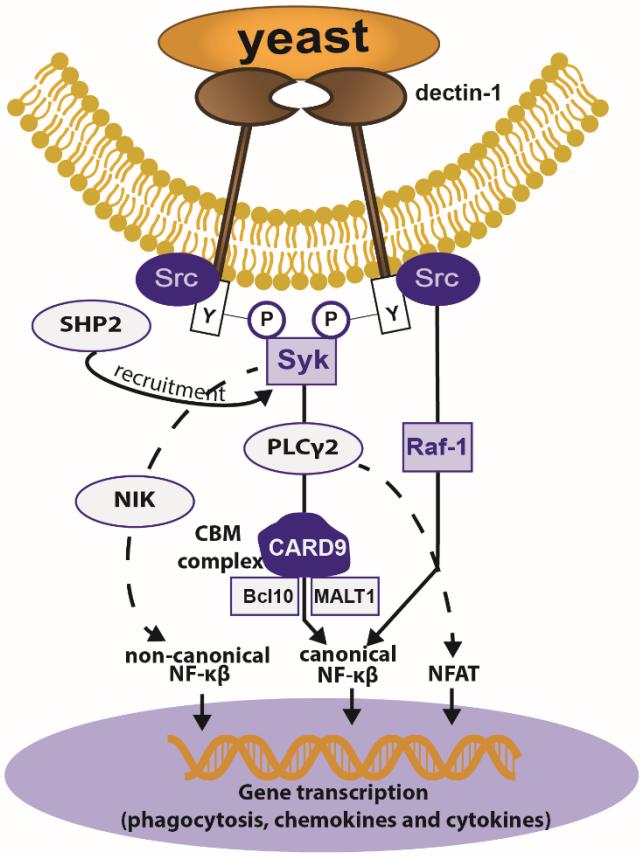


Figure 3. Schematic illustration of dectin-1 signalling.
Yeast cells can bind dectin-1 into a phagocytic synapse, which results in the phosphorylation of hemi-ITAMs by Src kinases. Syk is recruited to these phosphorylated ITAMs, facilitated by SHP2, resulting in the activation of PLCγ2, which in turn will activate the CBM complex (CARD9, Bcl10 and MALT1). This complex then activates canonical NF-κβ. Dectin-1 can also activate NFAT and non-canonical NF-κβ in a Syk-dependent, CARD-9-independent manner. Dectin-1 ligation triggers a Syk-independent pathway by activation of Raf-1, which results in the activation of canonical NF-κβ. Figure adapted from Marakalala et al. (2011).[91]

1.2.2 Complement receptor 3

Complement receptor 3 (CR3, CD11b/CD18, otM/32, Mac-l, Mol) is typically known as a mediator of leukocyte adhesion to activated endothelium and as the phagocytic receptor for iC3b opsonised particles.[107, 108] However, CR3 has also been reported to recognise β-glucans.[72, 109-112] β-glucan recognition by CR3 can induce anti-tumor effects by priming the complement receptor 3 to clear iC3b-opsonised tumor cells.[113-118]

Since complement function is associated with immunity, CR3 is highly expressed on leukocytes. Complement receptor 3 belongs to the β2-integrin family, consisting of an α-chain (CD11b) noncovalently associated with the β-chain (CD18).[119] The CD11b subunit has a unique structure consisting of two distinct domains: the I- or A-domain is an essential binding site for a diverse array of ligands, such as ICAM-1 and iC3b,[119, 120] and the spatially separated carbohydrate-binding domain, located C-terminal to the I-domain. This lectin domain binds with highest affinity to β-1,3-glucans with β-1,6-linked branches.[72, 110, 119, 121-123]

In pigs, complement receptor 3 is expressed on immune cells as well, including granulocytes and peripheral blood mononuclear cells. The α subunit (CD11b) of CR3 is not yet identified, but two candidate molecules, namely CD11R1 and CD11R3, have been described. Both CD11R1 and CD11R3 dimerise with the β-chain CD18, but their role in complement or β-glucan recognition is still unclear. CD11R1 and CD11R3 differ in their expression pattern and molecular weight. CD11R1 is present on approximately 50% of all porcine neutrophils, whereas CD11R3 is expressed on all neutrophils and monocytes. Although the expression pattern of CD11R3 correlates with human CD11b, CD11R3 is not recognised by human CD11b monoclonal antibodies (mAbs). In contrast, human CD11b mAbs cross-react with CD11R1, which has the same molecular weight as its human counterpart.[124]

In the absence of exposure to inflammatory stimuli, such as chemokines and cytokines, CR3 binds poorly to ligands. Exposure to these stimuli increases the avidity of CR3 through inside-out signalling (Figure 4A).[122] This tight regulation serves to avoid systemic inflammation. Indeed, only after binding of specific cytokine or chemokine receptors, CR3 undergoes a conformational change from a non-adhesive to an adhesive state, which recognises CR3 ligands (such as iC3b and the components of the extracellular matrix) (Figure 4B).[122] Intriguingly, binding of β-glucans to the lectin domain directly induces conformational changes of the A-domain and activates CD11b, without the need for inside-out signalling.[118, 122]

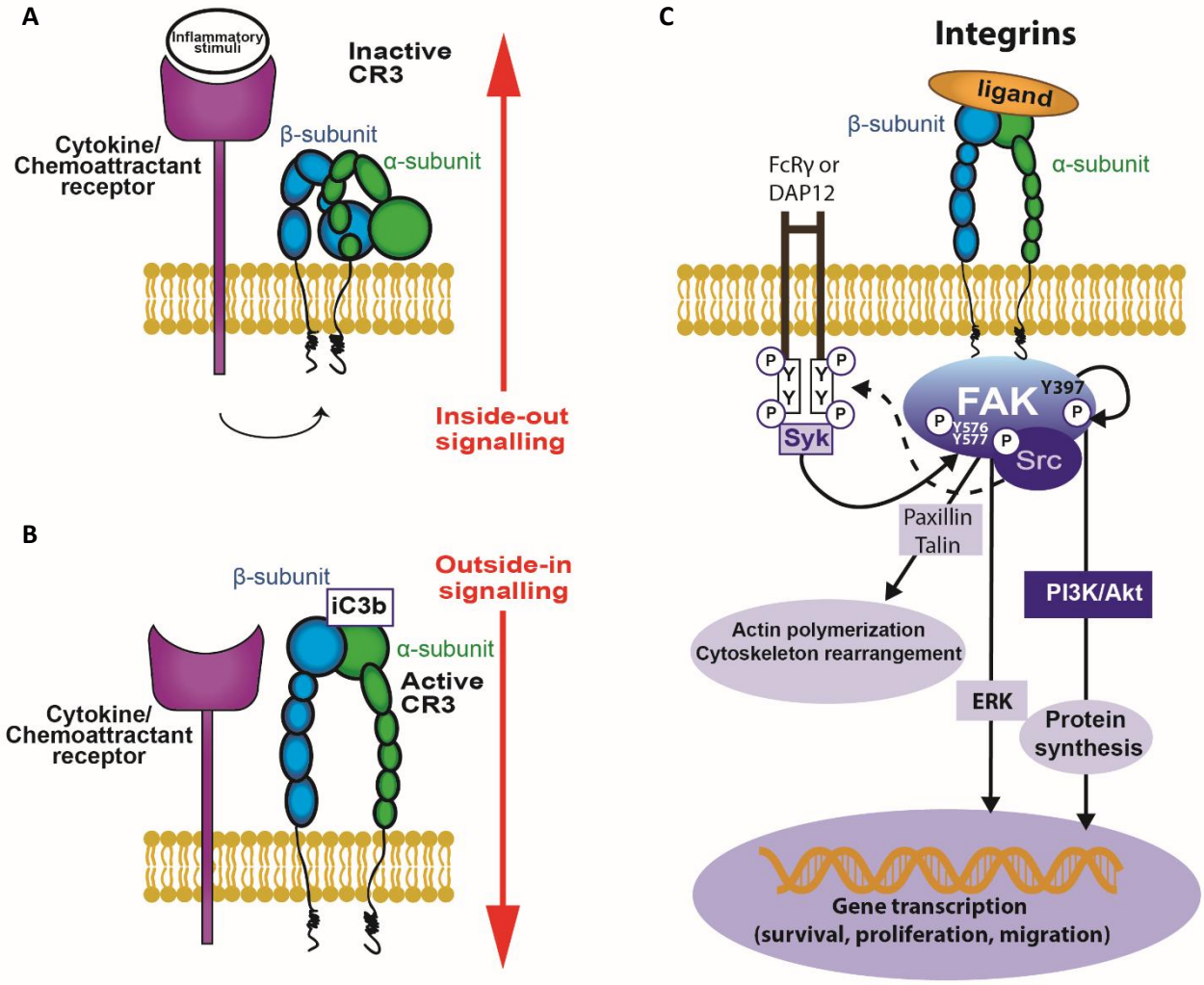


Figure 4. Schematic illustration of inside-out signalling and outside-in signalling of CR3 and integrin signalling. (A) Inside-out signalling: inflammatory stimuli can occupy specific cytokine/chemokine receptors on immune cells, which results in the conformational changes of the A-domain of CD11b. **(B) Outside-in signalling:** CR3, in its active state, can bind specific integrin ligands such as iC3b. This leads to an intracellular signalling pathway (Figure adapted from Li et al. (2011)[125]). **(C)** Clustering of integrins activates focal adhesion kinase (FAK), which will autophosphorylate its Y397 residue. This leads to the FAK-Src kinase complex, which further phosphorylates FAK at additional sites (Y576, Y577). Interaction of Paxillin and Talin with FAK results in actin polymerisation and cytoskeleton rearrangements. Furthermore, the mutually activated FAK/Src complex triggers several signalling pathways (e.g. ERK and PI3K/Akt) that result in cell survival, proliferation and migration. Ligation of β2-integrins can also induce phosphorylation of ITAM-containing adaptor molecules, such as DAP12 and FcRγ, by Src kinases. This results in docking sites for Syk kinases, which will activate FAK as well. Figure adapted from Bolos et al. (2010) and Pentassuglia et al. (2013).[126, 127]

The signalling pathways of complement receptor 3 have not yet been fully identified, only the involvement of the Src kinases Hck, Fgr and Lyn have been reported to be essential for CD11b/CD18-mediated cellular responses, such as the production of reactive oxygen species (ROS).[122, 128] On the contrary, much more is known about the signalling pathway triggered by β_2-integrins (Figure 4C).

Two nonreceptor intracellular tyrosine kinases are associated with integrin activation, namely focal adhesion kinase (FAK) and sarcoma kinase (Src). FAK associates to the cytoplasmic tail of the integrin and autophosphorylates its tyrosine residue at position 397 (Y397).[126] This phosphorylated tyrosine

provides a docking site for the SH2 domains of Src family kinases, which leads to the creation of a transient FAK-Src kinase signalling complex. This complex further phosphorylates FAK on additional sites (resulting in further increased activity of FAK), which amplifies the recruitment and activation of multiple downstream signalling proteins, including Paxillin, Talin, PI3K and ERK, that eventually leads to different cellular responses (cell survival, proliferation and migration) (Figure 4C).[126, 129]

Interestingly, β_2-integrins can associate with adaptor proteins, such as DAP12 or FcRγ, in order to initiate its signalling pathway. Typically, DAP12 and FcRγ are coupled to immunoreceptors through charged amino acid interactions within the transmembrane region of each protein. Although integrins lack such charged residues in their transmembrane segment, after integrin ligation the ITAM-bearing adaptor molecules DAP12 and FcRγ become phosphorylated by Src family kinases. This creates docking sites for Syk,[130-132] which also contributes to activation of FAK (Figure 4C).[130]

1.2.3 Other putative β-glucan receptors

1.2.3.1 Scavenger receptors

Scavenger receptors (SR) have been reported as potential receptors for β-glucans.[73] These receptors constitute a diverse family of pattern recognition receptors (PRRs) and have a range of cellular functions, being involved in the pathogenesis of chronic inflammatory conditions, such as atherosclerosis and Alzheimer's disease, and in the host response to some bacterial pathogens.[133, 134] Scavenger receptors are integral membrane proteins consisting of at least eight different subclasses (Class A to Class H),[135] which bind to a range of ligands, including modified lipoproteins, selected polyanionic molecules and a number of microbial structures.[133, 136] Most scavenger receptors are expressed on myeloid and certain endothelial cells (Class E and F), although some scavenger receptors are also present on epithelial cells.[133, 134]

Several studies have reported that these receptors are implicated in β-glucan recognition.[73, 134, 137-139] The basic β-(1,3)-glucan structure is probably recognised by these receptors, however, the affinity of these interactions can be influenced by the polymer charge (polyanionic) as well as other structural characteristics that remain undefined.[137] Until today, only the scavenger receptors class A, B, C and F have been associated with β-glucan recognition (Figure 5). For example, the scavenger receptor collectin placenta 1 (CL-P1) (belonging to class A) is involved in nonopsonic zymosan uptake in human endothelial cells.[135] Furthermore, it has been demonstrated that CD5, expressed on lymphocytes, is able to bind and aggregate several fungal species by recognition of β-glucans.[139] In addition, two conserved members of the scavenger receptor family, SCARF1 and CD36, also recognise β-glucans and have an essential function in antifungal immunity and host defence against the pathogenic yeast

Candida neoformans.[134] Although vertebrate β-glucan recognition mechanisms differ from those of invertebrates, a *Drosophila melanogaster* scavenger receptor (dSR-CI) has been shown to recognise these carbohydrates as well, indicating a functional conservation in the evolution of these receptors.[140]

1.2.3.2 Lactosylceramide

Lactosylceramide (LacCer, CDw17 or Galβ4Glcβ1Cer) is a neutral glycosphingolipid PRR found in the plasma membrane of many cells, including neutrophils, macrophages and lung epithelial cells. This receptor consists of a hydrophobic ceramide lipid and a hydrophilic sugar moiety (Figure 5).[141-143] Lactosylceramide has been shown to bind macromolecular β-1,3-glucans[74] and this interaction has been reported to induce a number of cellular responses *in vitro*;[144-146] such as evoking inflammatory cytokine responses in alveolar epithelial cells[144, 145] and chemotactic activities for human lung neutrophils.[146]

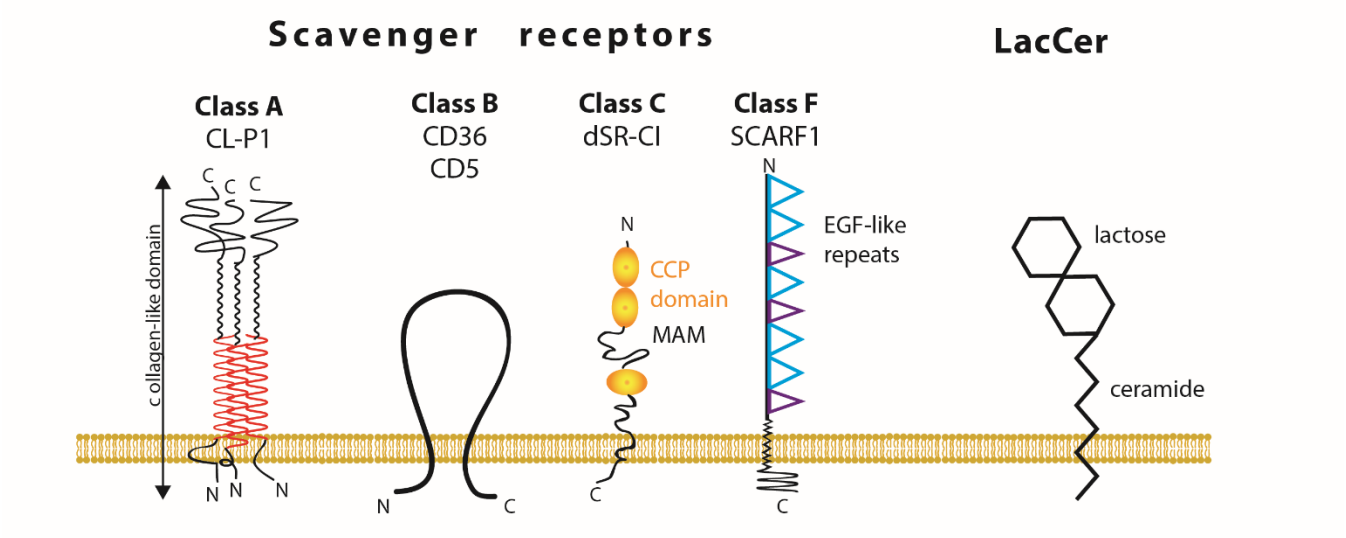


Figure 5. Overview of the other putative β-glucan cell surface receptors.
The structural diversity of the different scavenger receptors classes and lactosylceramide (LacCer) are shown. Scavenger receptor class A have a collagen-like domain. Class B scavenger receptors, such as CD36 and CD5, have an extracellular loop structure. dSR-C1 has two complement control protein (CCP) domains at the extracellular N-terminus (yellow), followed by an extracellular domain of the meprin A5 antigen and receptor protein tyrosine phosphatase μ (MAM) family (black). SCARF1 contains multiple N-terminal extracellular epidermal growth factor (EGF)-like repeats (purple and blue), a transmembrane domain and a long cytoplasmic tail. Lactosylceramide is composed of lactose (hexagon), incorporated in a ceramide. Figure adapted from Wilkinson et al. (2012) and Pluddemann et al. (2007).[133, 147]

1.2.4 Contribution of dectin-1 and CR3 in β-glucan recognition and -signalling

Since the discovery of dectin-1 as a β-glucan receptor in 2001,[71] several studies in mice have established that this receptor is the most important β-glucan receptor on immune cells,[75, 81, 125, 148, 149] while the role of CR3 in the recognition of β-glucans became neglected. However, recent articles have convincingly demonstrated that CR3 is pivotal in β-glucan recognition and -signalling, although the contribution of this receptor seems to be cell type and species-dependent.

Neutrophils are usually the first cells to arrive at infection sites early in antifungal immunity and are therefore interesting cell types to unravel the mechanism of β-glucan recognition and -signalling. In human neutrophils, it has been demonstrated that CR3 is the major receptor involved in the binding of zymosan and *S. cerevisiae* yeasts as well as the ROS production triggered by these stimuli, while dectin-1 was demonstrated to be dispensable for these responses.[150] In addition, NET (neutrophil extracellular trap) release for the response towards *Candida albicans* is solely dependent on β-glucan recognition by complement receptor 3 in human neutrophils.[151] NETosis is a process where neutrophils release chromatin fibrils (NETs) outside the cells to kill invading pathogens.[152] During this process, neutrophils lose their viability by destruction of the intracellular and plasma membranes.[151, 152]

In contrast to human studies, dectin-1 seems to play a more important role in mice. The absence of dectin-1 resulted in loss of β-glucan (zymosan) recognition in murine neutrophils.[148] Moreover, dectin-1 is important for the recruitment of neutrophils after intraperitoneal injection of zymosan in mice.[153] Interestingly, it has been demonstrated in mice that the cooperation of dectin-1 and CR3 is necessary for neutrophil responses towards zymosan and β-glucans. This mechanism requires the inside-out activation of CR3 by dectin-1-mediated recognition of β-glucans. Indeed, engagement of dectin-1 by β-glucans leads to phosphorylation of its ITAMs by Src kinases and recruitment of Syk. This results in vav activation and Ca^{2+} release, which induce a conformational change of CR3, enabling CR3 to bind β-glucans. As such, complement receptor 3 contributes to the signalling pathways initiated by dectin-1, resulting in the activation of downstream targets including Pyk2, PAK and ERK (Figure 6).[125] These results are however in contrast with previous observations,[118, 154] where it was shown that CR3 did not need inside-out signalling for β-glucan recognition in neutrophils. A possible explanation for these discrepancies is the use of different species. Indeed, experiments were conducted with murine[125] or human neutrophils,[118, 154] indicating a species-dependent receptor usage for β-glucan recognition in neutrophils.

Interestingly, in both human and mice, complement receptor 3 seems to play a major role in recognising β-glucans, however, only after opsonisation with complement. Indeed, Bose et al. (2013) demonstrated that antibodies blocking CD11b and CD18 significantly inhibited binding of soluble

serum-opsonised β-glucans to human neutrophils, establishing CR3 as the key receptor recognising soluble β-glucans through serum iC3b.[155] In mice as well, a minor role was observed for dectin-1 in the response towards opsonised β-glucans rather than non-opsonised β-glucans.[148, 153]

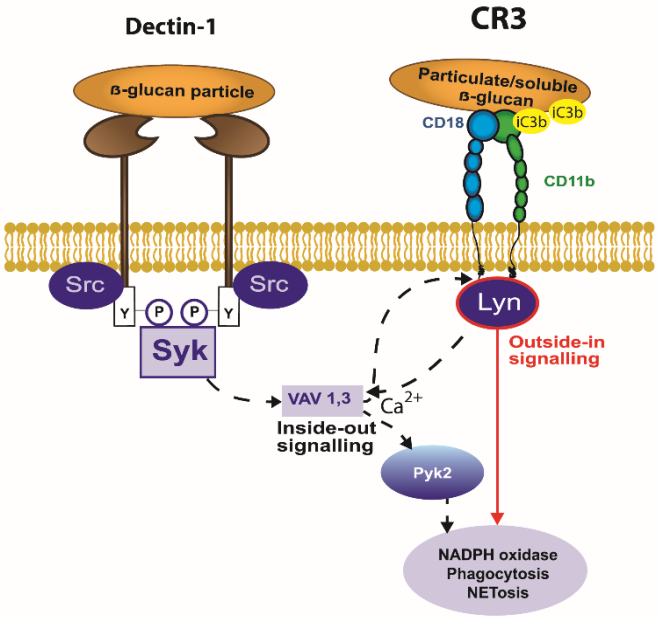


Figure 6. Species-dependent β-glucan receptor usage in neutrophils.
In humans (red), particulate or soluble β-glucans bind CR3, which results in phosphorylation of the Src kinases Lyn, followed by ROS production, phagocytosis of β-glucans and/or NETosis. In mice (black), binding of β-glucans to dectin-1 stimulates CR3 to recognise β-glucans via vav-proteins (inside-out signalling). These steps result in CR3 activation, which is now able to bind β-glucans. β-glucan-bound CR3 will phosphorylate Pyk2, which results in the phagocytosis of β-glucans and the β-glucan-mediated radical oxygen production. Serum-opsonised β-glucans will bind complement receptor 3 through iC3b.

Many controversies are found in the literature about the contribution of dectin-1 and CR3 in β-glucan recognition and -signalling by monocytes/macrophages. Different studies indicate a structure-dependent β-glucan recognition in murine and human monocytes. It has been demonstrated in human monocytes that the responses to soluble β-glucans requires CR3-mediated detection,[155, 156] while β-glucan particles require dectin-1-mediated recognition.[156] Likewise, the results from the Marakalala's and Goodridge's studies suggest that productive intracellular signalling from dectin-1 can only occur following recognition of large β-glucan complexes, which cluster the receptor in synapse-like structures, indicating that soluble β-glucans are unable to activate dectin-1.[157, 158] Furthermore, binding of particulate β-glucans to dectin-1 on monocytes/macrophages induces Syk-dependent and -independent pathways (see above),[75, 98, 105, 156, 159, 160] while binding of soluble β-glucans to complement receptor 3 phosphorylates FAK/Pyk2. This results in the activation of Src, Syk, PI3K/Akt, PLC/PKC and MAPK kinases (Figure 7).[156]

Besides the demonstration of dectin-1 as the most important receptor for (particulate) β-glucans in mice and man,[75, 125, 158, 161-163] several studies showed a redundant role for dectin-1 in particulate β-glucan-mediated signalling.[23, 157, 164] Huang and coworkers demonstrated in mice that phagocytosis of yeast-derived β-glucans by peritoneal macrophages was comparable in wild-type and dectin-1$^{-/-}$ mice.[165] Another group could not detect an involvement of dectin-1 in inducing inflammatory responses towards zymosan in mice.[157] Data from a recent study have demonstrated that blocking dectin-1 or the downstream Syk/Raf-1 pathways in human mononuclear cells only marginally reduced particulate β-glucan-induced IL-1 receptor antagonist (IL-1Ra) production.[164]

Furthermore, different studies highlight that other receptors than dectin-1 are involved in promoting β-glucan-mediated intracellular signalling and that they may contribute to β-glucan recognition in monocytes/macrophages lacking dectin-1.[148, 157, 161, 164] Indeed, the importance of heterogeneity of receptor expression across myeloid cell subsets in protective immune responses towards fungal particles was recently demonstrated.[153] Inflammatory monocyte recruitment seems to be dependent on dectin-1 when low doses of zymosan are administered to mice, while this effect was lost in the higher dose studies, indicating that other receptors could substitute for dectin-1.[153] In addition, no role for dectin-1 or CR3 was detected in the recognition of soluble β-glucans by murine macrophages[161] and in the particulate β-glucan-induced IL-1Ra production,[164] indicating the presence of a diverse receptor array in eliciting protective immunity towards fungal particles.

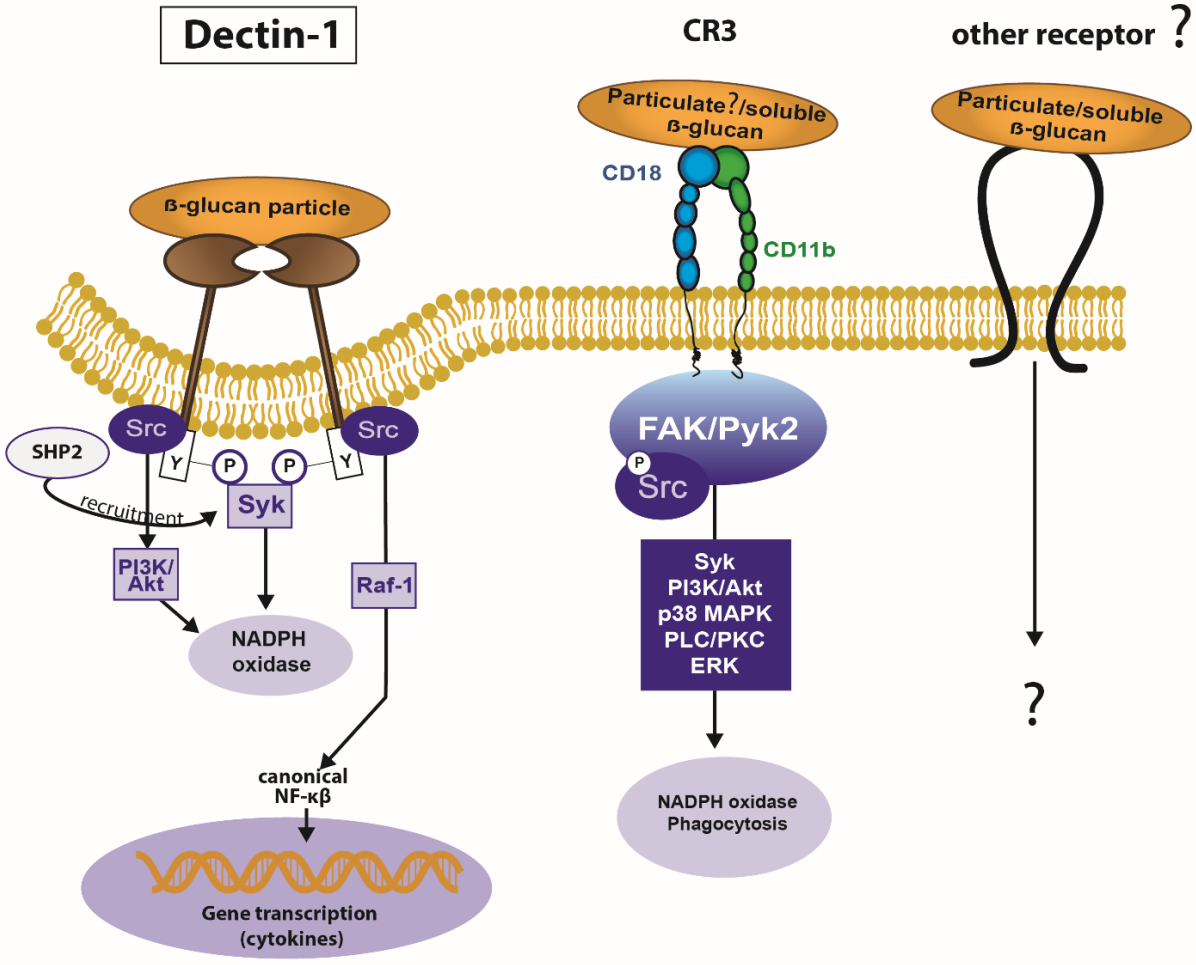


Figure 7. Contribution of dectin-1 and CR3 in β-glucan recognition and signalling in monocytes/macrophages. Particulate β-glucans activate the Src-family kinases (Src) by binding dectin-1. Syk is recruited to these phosphorylated ITAMs, facilitated by SHP2, which results in ROS production. This ROS production can be induced in a Syk-independent pathway as well by activating PI3K/Akt. On the other hand, β-glucans will bind dectin-1, which lead to the activation of the Raf-1 pathway, resulting in canonical NF-kβ activation. Soluble β-glucans get recognised by complement receptor 3 (CR3), which induces FAK/Pyk2 phosphorylation. Activation of FAK results in activation of Src, Syk, PI3K/Akt, PLC/PKC and MAPK kinases. CR3 or other receptors can bind particulate and/or soluble β-glucans in the absence of dectin-1, which induces the activation of different signalling pathways.

Taken together, there is no consensus on the cell-type and species-specific contribution of dectin-1 or CR3 to recognise β-glucans. In human neutrophils, CR3 seems to play a major role in β-glucan recognition, however, dectin-1 is more important in murine neutrophils. In monocytes/macrophages, the β-glucan structure seems to be important for receptor binding. Particulate β-glucans are more likely to engage dectin-1, while soluble β-glucans preferentially bind to complement receptor 3. However, it is feasible that multiple receptors are used for β-glucan recognition in monocytes, where one receptor can dominate over the other. In addition, overlapping signalling pathways between the different β-glucan receptors and species-specific differences limit the identification of one major receptor in this cell type.

1.3 Biological activities of β-glucans

1.3.1 Interaction with the immune system

The first indication for biological activity of β-glucans appeared when zymosan was observed to generate hyperplasia and hyperfunctionality in macrophages.[166] Compositional analyses revealed that zymosan preparations contained β-glucans, mannans, proteins, and fat in addition to small amounts of glucosamine, phosphorus, and magnesium.[167] Subsequently, β-glucans were identified as the immunostimulating component[168] and classified as biological response modifiers.[169] Since this discovery, β-glucans have been recognised to be capable of modulating the immune system of various living organisms from insects[170-173] to mammals[43, 64, 174-179] through specific interactions with different immune cells.[180] Interaction of β-glucans with receptors located on innate immune cells is crucial for their activity. Binding to these β-glucan receptors will induce phagocytosis, permitting their destruction by lytic enzymes in the acidic environment of the phagolysosome.[181] The phagosome is also the site of assembly of the phagocyte oxidase complex, which is responsible for the production of reactive oxygen species (ROS) upon detection and internalisation of β-glucans.[98, 100, 182] In addition, internalisation of β-glucans by antigen-presenting cells (dendritic cells, macrophages) can stimulate secretion of pro-inflammatory cytokines[64, 183-187] and chemokines,[187] which in turn can activate adaptive immunity. It has been demonstrated that large amounts of IL-23 are secreted after stimulation of dendritic cells with purified particulate β-glucans, which direct the subsequent polarisation of naïve $CD4^+$ T cells to Th17 effector cells.[32, 181, 188, 189] However, TNFα and IL12 is also secreted by β-glucan-stimulated murine macrophages skewing towards a Th1 immune response.[189-191] It is hypothesised that IL-17 is important for early steps in immunity involving the recruitment of efficient numbers of neutrophils to deal with the β-glucan bearing pathogen, and that subsequent IFN-γ produced by Th1 cells is important for activation of these neutrophils and other phagocytes to kill the pathogen.[181]

Besides activating macrophages, β-glucans are able to epigenetically reprogram monocytes, which can create innate immune memory.[192] Although establishing immunological memory is often attributed to T and B cell function, it was recently proposed that the enhanced and sustained innate immune responses following initial infection or vaccination may also afford protection against reinfection/revaccination.[193] This phenomenon has been named “trained immunity”.[192] Quintin and coworkers have shown that mice lacking functional T and B lymphocytes are protected against reinfection with *Candida albicans* in a monocyte-dependent manner.[193] The molecular mechanism of this protection is mediated by the dectin-1/Raf-1 pathway activated by β-glucans on the cell wall of *C. albicans*.[192-194] Reprogramming of these monocytes was maintained for at least a week and is

associated with specific trimethylation of histones, which suggests the involvement of epigenetic mechanisms in this phenomenon.[193, 195]

1.3.2 Therapeutic application of β-glucans

β-glucans, derived from yeast, could have numerous applications in curing patients by improving their immune system. The most attractive therapeutic properties of β-glucans are their anti-tumor effect and cholesterol-lowering potential.[196]

1.3.2.1 Anti-tumor effect

Since 1980, many studies have pointed out the positive effects of β-glucans in tumor therapy.[196-199] Under normal conditions, the immune system is able to overcome the invasion of cancer cells, however, unfavourable factors, such as stress, UV-radiation and an unbalanced diet may, impair the immune functions of the body. Thus, when the immune system is compromised, β-glucans as immunomodulators can compensate for such factors and help the immune cells. β-glucans can bind macrophages and natural killer (NK) cells and trigger their activation. As a result, activated killer cells circulate in the body and destroy their targets.[196, 200] Indeed, experiments in mice demonstrated that intravenous injection of soluble and particulate β-1,3-glucans from yeast inhibits tumor growth of the syngenic anaplastic mammary carcinoma and melanoma B-16.[201] This study indicated that β-glucans, if used therapeutically, could inhibit either hepatic metastases or primary tumor growth and result in enhanced survival.[64]

In addition, the effectiveness of β-glucans in combination with antitumor monoclonal antibodies has been demonstrated in several studies. β-1,3/1,6-glucans in combination with anti-tumor mAbs improved tumor regression and long-term survival to a greater degree than monoclonal antibodies alone. This β-glucan action is mediated via complement receptor 3 (CR3), which is found on NK-cells, neutrophils, monocytes and lymphocytes. First antitumor monoclonal antibodies will activate the complement system, by which tumor cells will be opsonised with the complement fragment iC3b. β-glucans will then prime the granulocyte receptor CR3 to clear the iC3b-opsonised tumor cells.[113-118]

1.3.2.2 Cholesterol-lowering effect

Oral administration of specific particulate β-glucans is well-tolerated and has received the Generally Recognised as Safe status (GRAS).[167] Additionally, β-glucans from various sources have effective

cholesterol and lipid lowering properties. Worldwide studies have demonstrated that uptake of β-glucan is a safe, powerful and inexpensive way to lower blood cholesterol and high lipid levels in humans and laboratory animals with high cholesterol.[196, 202, 203] Clinical trials proved the benefical effect of dietary pure β-glucans and cereals on hypercholesterolemia.[204] The hypocholesterolemic mechanisms of β-glucans include (a) reducing the intestinal absorption of cholesterol and bile acids by sequestration to β-glucans, (b) shifting the liver from cholesterol synthesis to bile acid production, (c) fermentation of β-glucans by intestinal bacteria to short-chain fatty acids, which are absorbed and inhibit hepatic cholesterol synthesis.[205]

1.3.3 Health-promoting and immunostimulatory effects upon oral administration

The health-promoting and immunomodulatory effects of β-glucans can be exerted upon parenteral and enteral administration.[206] The strongest immunomodulatory β-glucans are particulate, since they can directly activate leukocytes, triggering phagocytosis and antimicrobial activities.[43] However, due to the particulate nature of the glucan preparation, it is difficult to administer these via a parenteral route. Moreover, intravenous administration of particulate β-glucans results in a number of side effects, such as development of pulmonary granulomatous vasculitis, splenomegaly and inflammation. These adverse side effects have made particulate β-glucans useless in clinical medicine when administrated parenterally.[64] In contrast, oral administration of particulate β-glucans are well-tolerated and are approved as novel food ingredients by the European Food Safety Authority.[167]

Besides their cholesterol-lowering effect, dietary β-glucans have multiple other beneficial effects on the health and well-being of the individual attributed to the interaction with the microbiota and/or the immune system.[207] Indeed, since vertebrates lack the appropriate glucanases that are necessary to metabolise the β-glycosidic bonds of these polysaccharides, complex β-glucans will reach the terminal gut, where they interact with the intestinal microbiota (prebiotic effect).[208] The intestinal microbial flora is known to ferment β-glucans, derived from oat and barley, into short-chain fatty acids (SCFA),[209-211] with the strongest relative effect on butyrate.[212] Butyrate plays an important role in promoting and maintaining colonic health.[213, 214] Furthermore, SCFA are known to regulate several leukocyte functions including production of cytokines and chemokines.[215] β-glucans are also known to alter the composition of microbiota as shown by the higher abundance of *Lactobacillus* and *Bifidobacterium*, together with the lower abundance of the *Bacteroides fragilis* group in the caecum.[214] These β-glucan-mediated effects make them attractive functional feed ingredients.[216]

Besides these prebiotic effects, β-glucans can directly bind to specific receptors on immune cells, resulting in a microbial independent immunomodulatory effect.[207] Indeed, orally administered yeast β-1,3-glucans can be taken up by murine mononuclear phagocytes via β-glucan receptors and are

subsequently transported to the spleen, lymph nodes, and bone marrow.[113] However, although numerous articles have tried to unravel β-glucan receptor binding and their signalling pathways *in vitro*, their mechanism of action upon oral administration is still unknown. Nevertheless, because of their known health-promoting and immunomodulatory effects, β-glucans have been proposed as alternative for antibiotics to improve health, growth and general performance of post-weaning piglets and protect them against enteric infections.[180] Indeed, β-glucans can balance their pro-inflammatory response by inducing IL-1 receptor antagonist production, resulting in higher feed intake and growth performance.[217, 218] Other reports have shown the health-promoting and immunomodulatory effects of β-glucans after oral administration as well. In human studies, orally administered β-1,3/1,6-glucans derived from yeast significantly reduced the incidence of upper respiratory tract infections in persons susceptible to upper respiratory tract infections.[167, 219] In pigs, oral administration of β-glucans derived from *Saccharomyces cerevisiae* reduced the replication of swine influenza virus.[15] Furthermore, our lab demonstrated the priming effect of β-glucans on the immune system in pigs and dogs. In weaned piglets, oral administration of Macrogard or *Sclerotium rolfsii* β-glucans as feed additive resulted in a lower susceptibility to $F4^+$ ETEC infections in weaned piglets, evidenced by a reduction in the faecal excretion of the bacteria as well as a reduced F4-specific serum antibody response.[20] In dogs, oral administration of Macrogard influenced the systemic humoral immune response by temporarily decreasing the total IgA response, while the total IgM response increased.[220] Significant systemic immunomodulating effects in terms of humoral and cellular immune responses were demonstrated in rats as well.[208] In conclusion, orally delivered β-glucans appear to elicit diverse immunomodulatory effects in different species.

In summary, based on animal experiments, orally administered particulate β-glucans enter the proximal small intestine rapidly where β-glucans can be taken up by immune cells or will transit further to the lower intestinal tract to be fermented by the intestinal microflora. As a result, β-glucans improve the health status of the individual and boost mucosal immunity. Thereby, it would be interesting to use this immunostimulatory potential of β-glucans in oral vaccine design.

CHAPTER 2: Oral vaccination for protection against enteropathogens

2.1 Introduction

Since the development of the first vaccine against smallpox in the 18th century by Edward Jenner, vaccines have become crucial in the control of many human and veterinary diseases.[38] The majority of vaccines used today are parenteral vaccines, which can be administered intradermally, subcutaneously or intramuscularly. However, most of the pathogens initiate their infections at mucosal surfaces and parenteral vaccines only induce humoral immune protection without pathogen-specific mucosal immunity. Although experiments conducted in ruminants have demonstrated mucosal immunity upon parenteral immunisation.[221] Therefore, mucosal vaccination is highly advantageous for infectious diseases that are inhaled, ingested or sexually transmitted.[222] Mucosal vaccination can be achieved by oral, intranasal, sublingual, pulmonary, rectal or vaginal administration.[223] To prevent gastrointestinal infections, the oral route seems to be the most favourable way, since it induces an immune response at the site of pathogen entry and it obviates the need for needles and trained medical personnel. Furthermore, for veterinary purposes, the oral route is also the most practical.[224-226]

Enteropathogens, such as pathogenic *E. coli*, *Vibrio cholera* and rotavirus, can cause infectious diarrhoea, which is a significant global health challenge. For example, the global disease burden of enterotoxigenic *Escherichia coli* (ETEC) is estimated at over 210 million human cases and 380 000 deaths annually.[38] Moreover, in neonatal and recently weaned piglets this infection causes huge economic losses due to growth retardation, increased drug use and elevated mortality.[6] Therefore, the development of effective oral vaccines for these pathogens is very desirable.[38] However, only a very limited number of oral vaccines is approved for human use (Table 2), such as vaccines against polio (OPV), *Salmonella*, *Vibrio cholerae* and rotavirus.[33, 38, 227, 228] In pigs, oral vaccines against *Lawsonia intracellularis*, rotavirus, *Erysipelothrix rhusiopathiae* and *Salmonellla* have been commercialised (Table 2). However, all these vaccines are composed of live-attenuated or whole killed bacteria, which are often genetically unstable or not immunogenic enough.[229] Recombinant subunit vaccines use only a part of the pathogen to provoke an immune response and thus are much safer. Until today, although recombinant strategies are dominating the development of modern vaccines, recombinant non-living oral vaccines are not available.[230] Designing an oral subunit vaccine platform remains a major challenge as oral vaccines face many hurdles posed by the gastro-intestinal tract. Vaccine antigens have to survive the harsh conditions in the gastro-intestinal tract, cross the epithelial barrier and avoid the induction of tolerance. Indeed, oral administration of antigens generally results in a state of immunological hyporesponsiveness or oral tolerance.[231, 232] When

developing an oral subunit vaccine platform, all these limiting factors have to be taken into consideration in order to combat these challenges. Therefore, it is necessary to design antigen delivery systems which protect the pathogen antigens and aid them in the induction of an effective mucosal immune response. Before designing an oral vaccine, understanding the complexity of the intestinal immune system is essential.

Table 2. Overview of the globally licensed oral vaccines in humans and pigs. Adapted from Davitt et al. (2015).[38]

Pathogen	Species	Commercial name oral vaccine (manufacturer)	Components (dosing)
Poliovirus	Human	OPV (many)	Trivalent, bivalent and monovalent **live-attenuated** poliovirus strains (3 doses)
Rotavirus	Human	RotaTeq (Merck)	Pentavalent **live-attenuated** rotavirus (3 doses)
	Human	Rotarix (GSK)	**Live-attenuated** human rotavirus RIX4414 strain (2 doses)
	Pig	ProSystem® Rota (Merck)	Bivalent **live-attenuated** rotavirus (1 oral dose and 1 IM dose)
Salmonella	Human	Vivotif (Crucell)	**Live-attenuated** *S. typhi* Ty21a (3-4 doses)
	Pig	Argus® SC/ST with Diluent (Intervet)	**Live-attenuated** *S. choleraesuis* (single dose)
	Pig	Nitro-Sal FD (Arko Laboratories)	**Live-attenuated** *S. choleraesuis* (single dose)
	Pig	Enterisol SC-54 (Boehringer Ingelheim Vetmedica)	**Live-attenuated** *S. choleraesuis* (single dose, oral or intranasal)
	Pig	Salmo Shield Live (Novartis Animal Health US)	**Live-attenuated** *S. choleraesuis* (single dose, oral or intranasal)
	Pig	Salmoporc (IDT Biologika)	**Live-attenuated** *S.choleraesuis* (single dose)
Vibrio cholerae	Human	Dukoral (Crucell)	Whole killed *V.cholerae* (O1 strains) and recombinant CTB (2-3 doses)
	Human	Orochol (Crucell)	Bivalent **live recombinant** V. *cholera* (O1 and O139 strains) (mutant lacking CTA) (single dose)
	Human	mORC-Vax (VaBiotech)	Whole killed *V.cholerae* (O1 and O139 strains) (2-3 doses)
	Human	Shanchol (Shantha biotechnics)	Whole killed *V.cholerae* (O1 and O139 strains) (two doses)
Enterotoxigenic *E.coli*	Pig	Coliprotec F4 (Prevtec microbial)	**Live** non-pathogenic (not attenuated) *E.coli* O8:K87 (single dose)
	Pig	Entero Vac (Arko Laboratories)	**Live-attenuated** (K88 *E.coli*, single dose)
	Pig	Edema Vac (Arko Laboratories)	**Live-attenuated** (F18 *E.coli*, single dose)
	Pig	Porcine Ecolizer 3+C (Novartis Animal health)	Antiserum against K88, K99 and 987P *E. Coli* and *C. Perfringens* type C.
Lawsonia intracellularis	Pig	Enterisol Ileitis (Boehringer Ingelheim Vetmedica)	**Live-attenuated** *Lawsonia intracellularis* (single dose)
Erysipelothrix rhusiopathiae	Pig	Ery Vac (Arko Laboratories)	**Live-attenuated** *Erysipelothrix rhusiopathiae* (single dose)
	Pig	Nitro-Ery ALC (Arko Laboratories)	**Live-attenuated** *Erysipelothrix rhusiopathiae* (single dose)
	Pig	Ingelvac ERY-ALC (Boehringer Ingelheim Vetmedica)	**Live-attenuated** *Erysipelothrix rhusiopathiae* (single dose)
	Pig	Suvaxyn E-Oral (Zoetis)	**Live-attenuated** *Erysipelothrix rhusiopathiae* (2 doses)
Adenovirus	Human (military personnel)	Oral adenovirus vaccine (Barr Pharmaceuticals)	**Live** (not attenuated) adenovirus type 4 and type 7 (single dose, two tablets)
Clostridium perfringens	Pig	Clostratox (Novartis Animal health)	*Clostridium Perfringens* Type C antitoxin (single dose)

2.2 The gastrointestinal mucosal immune system

2.2.1 Structure of the small intestine and gut-associated lymphoid tissue

The gastrointestinal tract consists of the mucosa, submucosa, muscularis externa and serosa (Figure 8A). The submucosa, muscularis externa and serosa consist mainly of muscular layers and connective tissue with large blood vessels and lymphatics in order to supply immune cells to the mucosa. The mucosa is the inner layer surrounding the lumen, consisting of the epithelium, the lamina propria and the muscularis mucosae. The epithelium is constituted of a monolayer of epithelial cells, which are continuously renewed from stem cells present at the crypt base. These multipotent stem cells give rise to four major epithelial cells: enterocytes, paneth cells, enteroendocrine cells and goblet cells (Figure 8A). The enterocytes, goblet and enteroendocrine cells migrate upwards to the villous tip, while paneth cells differentiate during a downward migration to the crypt base. The enterocytes form the basis of the integrity of the epithelium and are held together by tight junctions. These tight junctions form a seal against the external environment and thus regulate the paracellular transport of antigens.[233] The paneth cells establish a protective environment as well by secreting large dense core granules from the apical membrane, which are rich in antimicrobial proteins and peptides (defensins, lysozyme) as well as potent proinflammatory mediators.[233-235] At the crypt base, paneth cells also synthesize and secrete factors that are capable of influencing proliferation and migration of intestinal stem cells, including epidermal growth factor (EGF).[236] Enteroendocrine cells are essential for normal life. They are sensory epithelial cells that coordinate nutrient sensing with metabolic and behavioural functions, like insulin secretion and the regulation of food intake. Such fine coordination is achieved through the secretion of a broad range of neuropeptides.[237, 238] The last epithelial cell type, the goblet cells, are specialised in the synthesis and secretion of mucus.[239] The mucus of the small intestine is a complex viscous matrix and can be divided in two different layers: the loosely attached external (mucus layer) and internal (glycocalyx) layer. The mucus provides an effective chemical and physical barrier to foreign materials, such as bacteria.[240, 241] The major component of the mucus are the mucins, which are carbohydrate-modified proteins. There are two different types of mucins: the classical gel-forming mucins, which form extremely large polymers (mucus layer) and the transmembrane mucins, which cover the apical surface of the enterocytes (glycocalyx).[242]

The glycocalyx is much thinner at certain places, particularly above the gut-associated lymphoid tissue (GALT). The GALT is located in the intestinal mucosa and is a component of the mucosa-associated lymphoid tissue (MALT), in which approximately 70 percent of the body's immune cells are found and is therefore described as the largest and most complex part of the immune system.[243, 244] The lymphoid tissue of the small intestine, the Peyer's patches (PPs), is composed of a specialised follicle associated epithelium (FAE), a subepithelial dome (SED) containing numerous immune cells, B

cell follicles containing germinal centers (GCs) and intrafollicular regions (IFR) containing mostly T cells (Figure 8B).[226, 243] The FAE differs from the villus epithelium because of the presence of Microfold cells (M cells), while goblet and enteroendocrine cells are largely absent.[245] M cells are characterised by irregular shaped microvilli, the absence of an extensive mucus layer, a thin glycocalyx and a reduced enzymatic activity. In addition, the basolateral membrane is invaginated, forming an intraepithelial pocket, which contains lymphocytes and antigen presenting cells (APCs) (Figure 9B).[246] Hence, M cells can transport macromolecules and bacteria through the epithelial barrier to deliver them to antigen presenting cells (APCs) in the subepithelial dome (SED) (Figure 8b).[245]

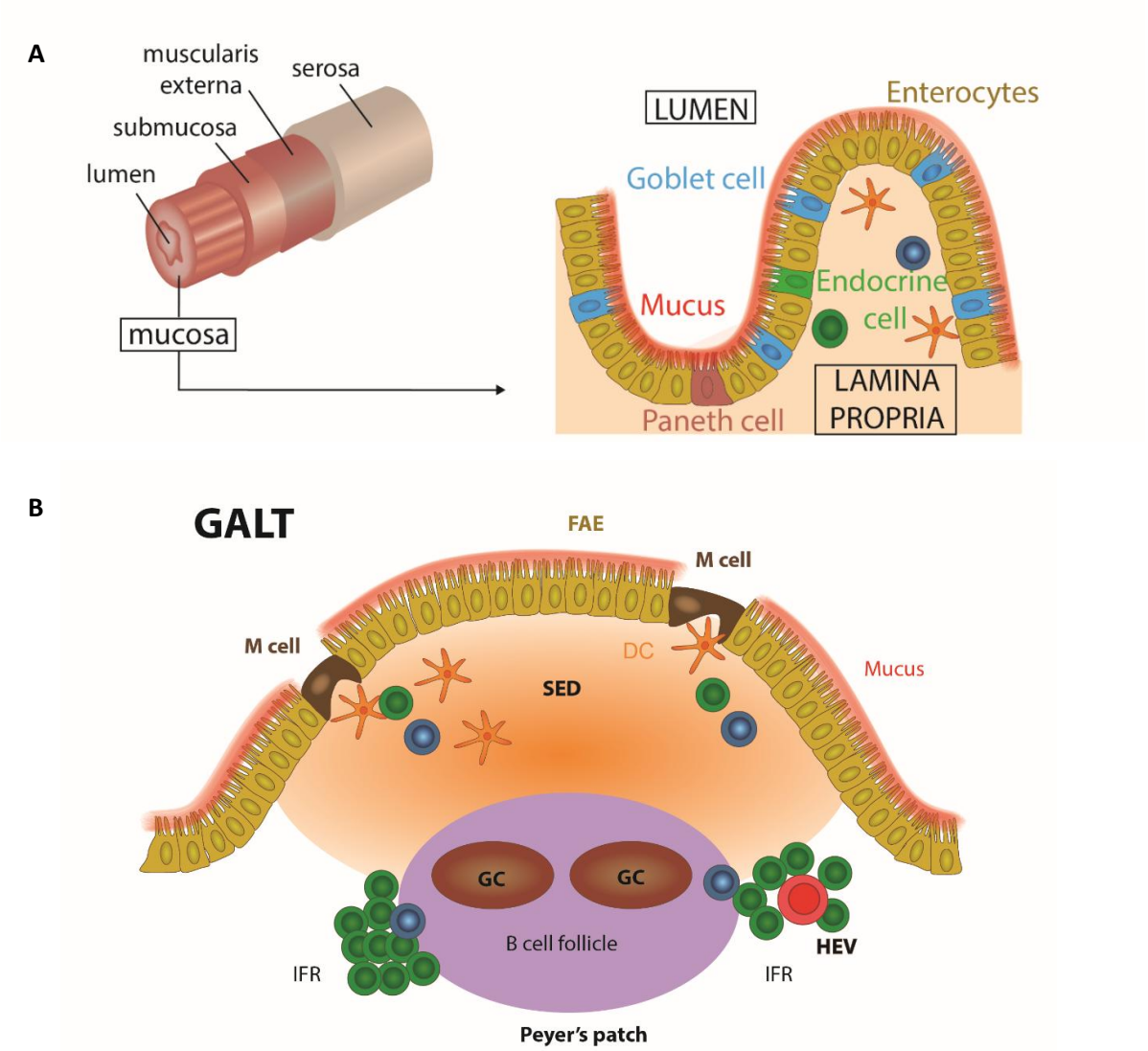


Figure 8. Schematic representation of the small intestine and the intestinal mucosa.
(A) Schematic illustration of the intestine. The intestine is composed of the mucosa, submucosa, muscularis externa and serosa. The mucosa consists of the epithelium and the lamina propria. The epithelium is constituted by enterocytes, goblet cells, paneth cells and enteroendocrine cells and is covered by mucus. The lamina propria contains immune cells, such as dendritic cells and lymphocytes. **(B)** Schematic illustration of the GALT. The FAE consists of enterocytes and M cells. This epithelium overlies the SED, which contains immune cells, such as dendritic cells (DCs). The Peyer's patches also contain B-cell follicles with germinal centers (GCs) and intrafollicular regions (IFR) containing mostly T cells. Figures adapted from Fagarasan et al. (2003).[247]

M cells are present in varying proportions in the FAE of a wide variety of species such as laboratory, domestic and farm animals and man.[204, 248-250] Interestingly, the occurrence of other GALT-like structures is species-dependent, such as lymphocyte-filled villi (rat and human), cryptopatches (mouse) and lympho-glandular complexes (pig).[251] In pigs, Peyer's patches are found sporadically in the jejunum (JPP) and continuously in the ileum (IPP). Intriguingly, porcine IPP are not primary lymphoid organs[252] as they are neither required to maintain the systemic B cell pool nor are they a site of B cell lymphogenesis.[253] Similar to dolphins, elephants, rhinoceros, hippopotamus and warthog, porcine peripheral and mucosa-associated lymph nodes are inverted:[254] the lymph nodes are preferentially composed of cortical areas and paracortex and lack a larger medullary area.[255, 256]

2.2.2 Antigen uptake in the small intestine

Understanding the mechanisms of antigen uptake in the intestine is important to develop oral vaccines, since transcytosis of antigens is crucial for inducing intestinal immune responses. Uptake of antigens in the small intestine can occur by four main mechanisms.

The first mechanism involves M cells. These cells located in the Peyer's patches play a decisive role in the uptake and transcytosis of both soluble and particulate matter to the underlying lymphoid follicles.[245] Antigens can also be taken up by lysozyme expressing antigen presenting cells (APCs) present in the M-cell pocket. These cells can extend transcellular dendrites through pores in the M cell membrane to sample the lumen and take up bacteria.[257] M cells are generally considered as the preferred route to trigger intestinal immune reactions, however, the importance of villous enterocytes should not be disregarded.[258, 259]

Receptor-mediated endocytosis is another mechanism for antigen sampling in the intestinal lumen. At the villous epithelium, luminal antigens are shuttled through the epithelial layer to the intestinal tissues via epithelial transcytotic receptors, such as FcRn, dectin-1 and aminopeptidase N.[260-262] FcRn binds the Fc domain of IgG in a pH-dependent manner and is capable of bidirectional transport of immunoglobulin G (IgG) across the intestinal barrier.[263] Immunoglobulin G (IgG) binds the receptor at the basolateral side, which is then transported into the lumen across the epithelial barrier, where the IgG can bind cognate antigens. Subsequently, FcRn recycles the IgG-antigen complex back across the intestinal barrier into the lamina propria for processing by immune cells.[263] Moreover, recent data indicate that FcRn mediates uptake of Fc-conjugated particles as well.[264] FcRn is expressed by adult enterocytes in humans[265] and in pigs.[266] Furthermore, It has been demonstrated that intestinal secretory IgA (SIgA) antibodies in complex with its antigens are taken up by M cells via dectin-1.[261] Carbohydrate residues on the antibodies are involved in this M-cell-mediated transcytosis. Since SIgA-antigen complexes can be taken back up by the intestinal epithelium, binding to dectin-1 can

promote the uptake and delivery of antigens from the intestinal lumen to the underlying immune cells.[261] Besides immunoglobulin-mediated transcytosis of antigens across the epithelial barrier, antigens can bind different transcytotic receptors located at the apical surface of enterocytes. For example, porcine aminopeptidase N has been identified as an endocytotic receptor for F4 fimbriae in the epithelial barrier, the adhesin of $F4^+$ enterotoxigenic *Escherischia coli* (ETEC).[260]

A third route of antigen uptake in the lamina propria of the small intestine involves phagocytes. It seems that $CX3CR1^+$ mononuclear phagocytes extend transepithelial dendrites (TEDS) to sample luminal content.[267, 268 269, 270] Therefore, they extend their dendrites through the intestinal epithelium in the gut lumen.[247, 268] The integrity of the epithelial barrier is not disturbed as the immune cells express tight junction proteins, such as occludin, claudin and zonula occludens.[268] After antigen uptake, $CX3CR1^+$ cells transfer their antigens to $CD103^+$ DCs, which will migrate to the mesenteric lymph nodes (MLN) and present the bacterial antigens to T cells and B cells, which can induce IgA class-switching and differentiation.[247, 269, 270]

Finally, recent data indicate that goblet cells can transport small soluble molecules to underlying tolerogenic APCs via goblet cell-associated antigen passages (GAPs).[271]

How each uptake mechanism contributes and interplays in tolerance or protective immunity is however still a matter of debate, although targeting to apical receptors to enhance bioavailability at subepithelial tissues might be necessary to design an efficient oral vaccine.

2.2.3 Intestinal immune reaction against enteric pathogens

Mucosal surfaces are continuously exposed to the external environment and therefore represent the largest lymphoid organ of the body.[226] The key element in intestinal mucosal immunity is the local production of pathogen-specific secretory IgA (SIgA) molecules at the infection site.[272]

The intestinal immune system can be subdivided into distinct functional compartments. These are classified as the inductive site, considered as sites of initiation of lymphocyte education, and the effector site, where lymphocytes neutralise foreign antigens.[273] Luminal antigens are taken up by the different mechanisms described above into the immune inductive site, resulting in the activation of antigen presenting cells (APCs). Once activated by foreign antigens, the antigen-loaded cells migrate into the mesenteric lymph nodes, where they can present the antigens to naïve $CD4^+$ T cells. It is believed that differentiation of naïve $CD4^+$ T cells continues within the mesenteric lymph node (MLNs) that drain the gastrointestinal mucosal tissue.[273] Alternatively, antigen-loaded APCs can migrate through the lymph vessels towards the draining mesenteric lymph nodes where they stimulate $CD4^+$ T cells.[274] Primed T cells will activate B cells, which will then migrate into B cell follicles

to form a germinal center together with follicular dendritic cells (FDC).[275] These germinal centers (GC) play a crucial role in the development of class-switched antibodies and the formation of B cell memory.[247, 276, 277] GCs contain mainly rapidly dividing B cells (dark zone) and resting B cells (light zone). B cells expand in the dark zone, mutate their immunoglobulin genes (somatic hypermutation), and those that acquire mutations that maintain or improve the affinity of the B cell receptor (BCR) for the foreign antigen are rescued from programmed cell death.[278] B cells, which have acquired affinity-increasing mutations, will be able to interact with germinal center T cells (CD40/CD40 ligand interaction) and follicular dendritic cells (FDCs) in the light zone. Selected germinal center B cells undergo repeated rounds of proliferation, mutation and selection[278] and will further undergo IgA class-switch recombination (CSR).[273] These activated IgA-producing B lymphocytes migrate to mesenteric lymph nodes and pass into the blood stream via efferent vessels (ductus thoracicus). Subsequently, the IgA effector B cells travel to their mucosal effector sites (lamina propria or epithelium)[251, 277] where they differentiate into plasma cells. Finally, at their effector sites, dimeric IgA is secreted and transported across the epithelium by binding to the polymeric Ig receptor (pIgR) expressed on the basolateral surface of mucosal epithelial cells.[247] The secretory IgA (SIgA) in the intestinal lumen is composed of dimeric IgA molecules and the secretory component (SC), which is derived from the pIgR.[279] These secretory IgA (SIgA) complexes play multiple protective roles.[280] Indeed, SIgA forms a critical part of the intestinal immune system both in protection from harmful pathogens and in homeostasis[38] by promoting immune exclusion via entrapping dietary antigens and microorganisms in the mucus and by downmodulating the expression of proinflammatory bacterial epitopes on commensal bacteria.[280] On the other hand, as described above, SIgA-antigen complexes can also facilitate antigen sampling by binding to M cells, leading to inflammatory responses.[261] Furthermore, IgA dimers, locally released by plasma cells, remove microorganisms that passed the epithelial barrier in two ways: by transporting them back into the lumen through the pIgR and by promoting their clearance via FcαRI (also known as CD89), an IgA receptor expressed by dendritic cells (DCs), neutrophils, and other phagocytes (Figure 9).[280]

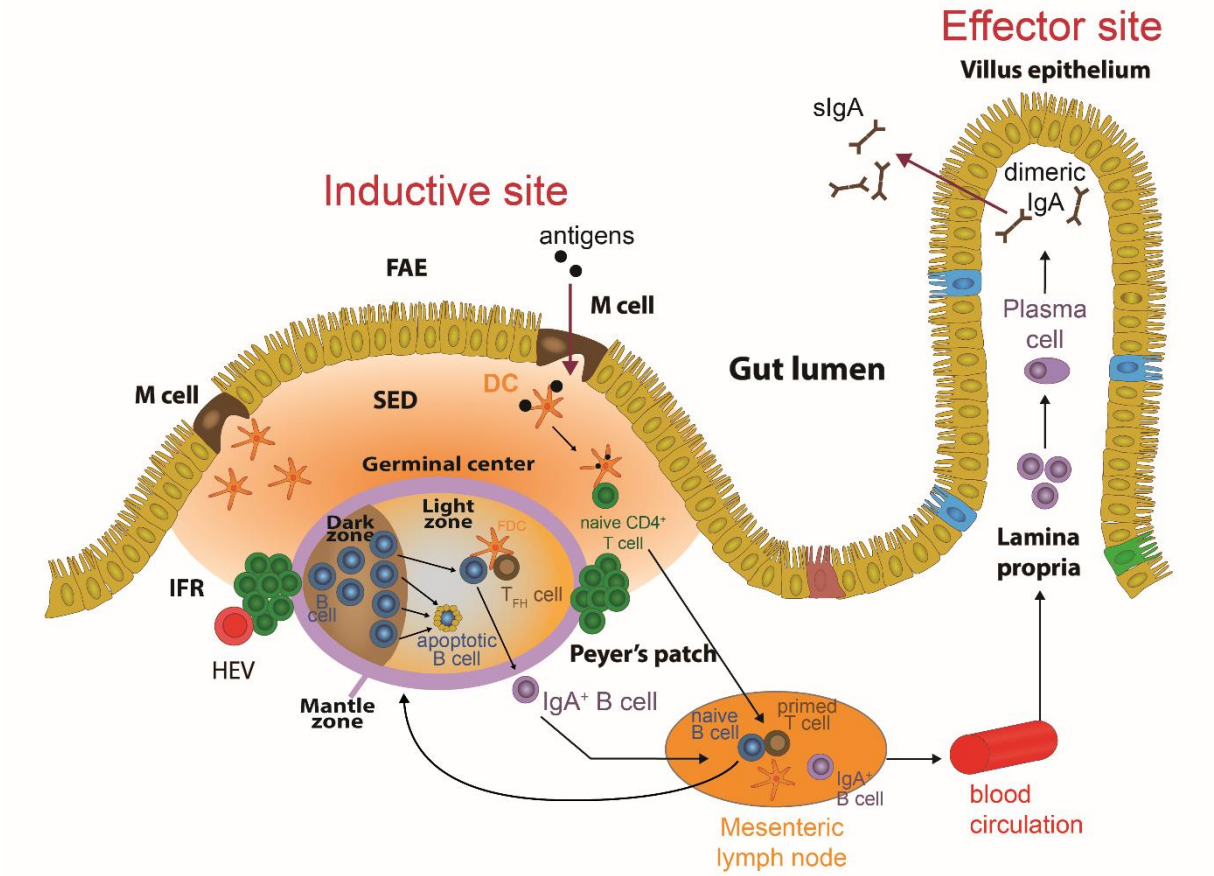


Figure 9. Schematic representation of the intestinal mucosal immune system.
Foreign antigens are taken up from the lumen to the subepithelial dome (SED) by M cells located in the follicle associated epithelium (FAE) of the GALT. The SED contains antigen-presenting cells, such as dendritic cells (DCs), which capture the antigens and present them to naïve $CD4^+$ T cells. These primed T cells within the mesenteric lymph node (MLNs) will activate B cells, which will then migrate into B cell follicles to form a germinal center. After clonal expansion and somatic hypermutation in the dark zone, B cells with acquired affinity-increasing mutations are selected and will interact with primed $CD4^+$ T cells and follicular DCs (FDC) in the light zone. Here, class switching into IgA-producing cells occur. IgA^+ B cells leave the PP through efferent lymph and migrate first to the mesenteric lymph node and then to the blood. These cells migrate to effector sites such as the intestinal lamina propria and differentiate into plasma cells. Plasma cells secrete dimeric IgA antibodies that are transcytosed by polymeric Ig receptors (pIgRs) located in the mucosal epithelial cells. Figure adapted from Lamichhane et al. (2014).[226]

2.3 Oral vaccine design strategies

2.3.1 Challenges of oral subunit vaccines

The current licensed oral vaccines are composed of inactivated or live-attenuated pathogens (Table 2). Unfortunately, inactivated vaccines are often not immunogenic, while the use of live-attenuated micro-organisms could have devastating consequences when transmitted to immunocompromised individuals or infants, who lack a mature immune system, and as such are less able to cope with the attenuated pathogens.[229] Indeed, the previously available oral live rotavirus vaccine (RotaShield) was withdrawn after a short time on the market because of potential serious adverse reactions (intussusception).[281, 282] Due to the safety risks associated with live-attenuated vaccines there is a shift toward the development of safer subunit vaccines that are composed of purified native or recombinant proteins of the target pathogen.[283]

Unfortunately, oral delivery of soluble recombinant proteins have to overcome at least three challenges to induce efficient mucosal immune responses. First, the luminal environment in the gastrointestinal tract is hostile to antigens, degrading most antigenic epitopes delivered in soluble form.[284] Second, the epithelial barrier with its mucus secreting epithelium limits the antigen absorption from mucosal surfaces. Third, mucosal tolerance protects against unwanted immune responses to dietary and commensal bacterial antigens.[133, 285, 286] The mechanism of this oral tolerance has not been fully elucidated. The combination of commensals, regulatory T cells and $CD103^+$ DCs are probably involved in the creation of a tolerogenic environment in the gut.[286-288]

In this regard, designing oral vaccines is rather a difficult task. To overcome these challenges, several solutions are available such as an enteric coating for protecting the antigens against the harsh environment of the gastro-intestinal tract. Furthermore, potent mucosal adjuvants and/or special delivery systems are often required for successful oral vaccination as well (Figure 10).[282]

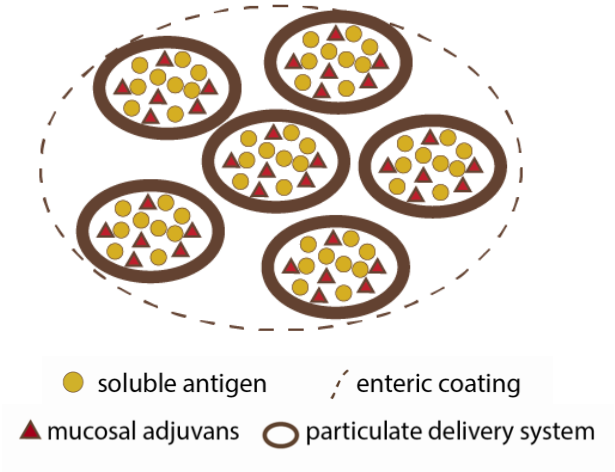


Figure 10. Schematic representation of the different strategies for oral vaccine subunit design.
Soluble antigens can be protected from physical elimination and enzymatic digestion via an enteric coating. A mucosal adjuvant can be added to soluble antigens to generate long lasting immunological memory. Finally, soluble antigens can be encapsulated in nano- or microparticles. The combination of all three strategies is illustrated.

2.3.2 Enteric coating

Enteric coating protects soluble antigen-based vaccines against the harsh, acidic condition of the stomach[289] by using pH-sensitive polymers, (e.g. methacrylic acid, hydroxypropyl methylcellulose phthalate).[290] These polymers remain impermeable at the low gastric pH but dissolve in the slightly basic pH in the intestinal fluid.[291] Eudragit is the brand name for a diverse range of poly(methacrylic acid-co-ethylacrylate) copolymers and are commonly used to coat tablets and to prepare controlled release formulations.[292, 293] Several types of Eudragits are available, which dissolve at different pHs. Eudragit L100-55 contains an anionic copolymer, which dissolves when the pH is higher than 5.5.[294] Combinations of Eudragit L100-55 with other polymers have been shown to improve the oral bioavailability and the controlled release of drugs.[290] Our lab used Eudragit L30 D-55 to coat F4ac fimbriae pellets to be used as solid formulation, mixable with creep feed. This enteric coating protected the F4 fimbriae against the acid environment in the stomach as well as against neutralisation by maternal milk. Oral administration of these pellets in pigs followed by virulent $F4^+$ enterotoxigenic *Escherichia coli* (ETEC) challenge resulted in a significant reduction in $F4^+$ *Escherichia coli* excretion compared to F4 fimbriae in solution.[295]

2.3.3 Mucosal adjuvants

Mucosal adjuvants are used to prevent oral tolerance and to induce a protective immune response. These adjuvants can be divided into two different groups: the immunopotentiators and the antigen delivery systems (i.e. nano- and microparticles). For oral subunit vaccines, the combination of immunopotentiators and delivery systems is probably required to elicit optimal immune responses.[296]

Mucosal immunopotentiators can improve the effectiveness of soluble antigen-based vaccines by enhancing the immunogenicity of the vaccine.[296] It is important that these mucosal adjuvants induce protective immune responses both in the mucosal and systemic immune compartments, stimulate local antibody production (SIgA), trigger $CD4^+$/$CD8^+$ T cell responses and long-term B and T cell memory.[223, 297] Classical adjuvants, e.g. aluminium salts and water-in-oil emulsions, are unable to enhance cell-mediated responses.[298] Therefore novel mucosal adjuvant formulations, such as bacterial toxins, cytokines, TLR ligands and β-glucans have been developed.

The most powerful mucosal adjuvants are the enterotoxins secreted by *V. cholerae* and *E. coli*, cholera toxin (CT) and *E. coli* heat-labile (LT) enterotoxin, respectively.[299-301] These enterotoxins are structure-related proteins: both toxins are composed of an A subunit and a pentameric B subunit.

The A subunit possesses strong ADP-ribosyltransferase activity, while the B subunit binds to its receptor, GM1 gangliosides.[302, 303] However, these mucosal adjuvants have a high level of toxicity limiting their use for human vaccine formulations. Therefore, several genetically modified forms have been engineered to detoxify these enterotoxines by mutagenesis of the A subunit and are currently under clinical evaluation.[300, 301, 304, 305] For example, administration of a single oral dose of 100 µg of the double mutant heat-labile enterotoxin (dmLT) was found to be safe, well tolerated and reasonably immunogenic in a clinical trial.[304]

Furthermore, immunomodulatory cytokines, Toll-like receptor (TLR) agoinsts and β-glucans can be used as mucosal adjuvants as well. Cytokines such as IL-12 and granuloyte/macrophage colony-stimulating factor (GM-CSF) have shown adjuvant activity when administered mucosally together with soluble antigens.[300, 306] TLR ligands including CpG-containing oligonucleotide, flagellin and bacterial porins elicit the secretion of proinflammatory cytokines and chemokines.[300, 307-309] β-glucans, co-administered with bacterial, fungal, protozoal or viral antigens, display adjuvant effects when administered parenterally[310-312] and orally.[20, 32] In addition, as described above, β-glucans are used as immunoadjuvant therapy for cancer.[199]

2.3.4 Encapsulation in nano- or microparticles

2.3.4.1 Particle design

The encapsulation of soluble antigens in particles effectively protects them against degradation in the gastrointestinal tract, enhances the immunogenicity of the antigens, can have a dose-sparing effect and mimics pathogen dimensions.[313-315] Indeed, soluble antigens preferential induce oral tolerance in the absence of adjuvants, while particulate antigens are more effective in stimulating protective immunity and cross-presentation by antigen presenting cells (APCs).[241, 290, 316] When designing these oral particle systems, many factors should be taken into consideration such as particle size, hydrophobicity and surface charge.

Determining the optimal particle size seems to be very important for their uptake in the gastro-intestinal tract.[317] M cells, located in Peyer's patches, have been mostly implicated in the process of particle uptake[318], however, nanoparticles can gain access to the mucosa through normal villous epithelial cells as well.[319, 320] Particulate vaccines within the 0.1-10 µm range are efficiently internalised by M cells and antigen presenting cells (APCs), probably due to resembling the dimensions of common bacteria and viruses.[290] In contrast, the particle size for receptor-mediated uptake by villous enterocytes is much smaller, since the mucin network forms an exclusion filter for

larger particles.[321] Particles up to 200 nm are able to migrate through the physical mucin pore, however, at certain circumstances native mucin fibres can create larger pores allowing larger particles to transit.[240, 241] The size of a particle also plays a critical role in determining antigen and adjuvant loading and release: nanoparticles exhibit a large surface area, which accelerates carrier dissolution and antigen release, while microparticles allow higher antigen encapsulation and slower antigen release.[290]

Hydrophobicity and charge of the particles is important for designing oral vaccines as well, as the mucus is negatively charged and has hydrophobic domains.[321] To increase the bioavailability of oral vaccine delivery systems, it is important that they rapidly penetrate mucus to avoid being shed.[322] Mucus-penetrating particles possess near neutrally charged surfaces and lack of hydrophobic regions, in order to reduce the hydrophobic or electrostatic interaction with mucus.[322, 323] To achieve such mucus-penetrating surface properties, PEGylation of particles has been used as a strategy, since polyethylene glycol (PEG) is a hydrophilic, uncharged and biocompatible polymer. Coating particles with a dense layer of low MW PEG (between 2-5 kDa) effectively reduces hydrophobic interaction and hydrogen bonding with the mucus.[321, 322]

2.3.4.2 Oral particulate delivery systems

A wide variety of delivery systems for the oral route has been developed, including lipid based delivery systems (micelles, liposomes and immune stimulating complexes (ISCOMs)), virus-like particles and synthetic or natural particle-based strategies (for example poly(lactic-co-glycolic acid) (PLGA) and β-glucan microparticles).[26, 38, 324-328]

Micelles are composed of lipid monolayers, while liposomes are characterised by a single or collection of multiple phospholipid bilayers. The natural hydrophobicity of these delivery systems, especially when they are positively charged, allows them to fuse with cell membranes. As such, antigens are directly delivered into the cytosol and will traffic to the MHC class I presentation machinery. However, their use in oral vaccines is more limited, since micelles and liposomes are susceptible to degradation in the gastrointestinal tract (GIT) through acidic pH, lipases and bile salts. Bile salts are secreted into the GIT to digest lipids making it unlikely that lipid based delivery systems would retain their structural integrity in such an environment, thus compromising their ability to protect antigens.[38] In addition, liposomes typically have a poor antigen encapsulation efficiency for hydrophilic molecules.[326] Immune-stimulating complexes (ISCOMs) are related to liposomes, but form icosahedral pentagons due to the inclusion of cholesterol and saponins in the membrane.

Despite the fact that the incorporation of these additional components confers intrinsic adjuvant properties, oral vaccines based on ISCOMs probably require the addition of an immune stimulatory component to potentiate their oral efficacy.[38, 329] The incorporation of antigens into ISCOMs occurs via hydrophobic interactions, which potentially limits the utility of this particle system for soluble protein antigens.[330] Another major drawback of these ISCOMs is the inability to incorporate negatively charged antigens. The development of PLUSCOMS and Posintro™ has been successful in altering the surface charge of the particle, making it possible to load positively charged antigens as well.[38] Moreover, these modifications allow the loading of higher amounts of unmodified protein, thus retaining the natural conformation and immunogenicity of the antigen, while ISCOMs have poor incorporation of either native or derivitized proteins.[331] However, the production process of these cationic ISCOMs is very complex and requires at least a 3-day reaction.[332]

Virus-like particles (VLPs) and virosomes are multiprotein structures that mimic the organisation and 3D conformation of authentic native viruses but lack the viral genome, potentially yielding safer vaccine candidates.[333] VLPs are able to incorporate vaccine antigen, either produced by recombination (genetically inserting genes into the recombinant viral genome) or chemically coupling antigens to the VLP.[334] As such, virus-like particles can also be engineered to incorporate proteins with a variety of beneficial functions, including targeting and immunostimulatory activity. The advantages of VLPs and virosomes for oral delivery are their small size and the variable composition of their surface chemistry. However, virus-like particles and virosomes both suffer from challenges in formulation and scaling up. The production process of these particles is also very extensive, which adds to manufacturing costs. In addition, VLPs require the addition of adjuvants in order to elicit enhanced immune responses.[38] For example, oral administration of non-replicating rotavirus-like particles, expressing VP2 and VP6 proteins, are only effective as booster vaccine, after priming the pigs with live-attenuated viruses.[335]

Synthetic or natural-based particles are one of the most versatile oral delivery systems. Vaccine antigens can be admixed together with the particles, encapsulated in or conjugated to particles. Their size can be controlled, ranging from 30 nm up to mm.[38] Both synthetic and naturally occurring polymeric particulate delivery systems can be engineered to have a specific particle size, surface chemistry and 3D architecture in order to protect the payload from degradation, overcome mucosal barriers, interact with specific cell types and exert desired immunomodulatory functions.[38] Synthetic nanoparticles using the copolymer poly(lactic-co-glycolic acid) (PLGA) are most widely used as a results of its biodegradability and biocompatibility.[334] In addition, PLGA has been approved for human use in sutures, bone implants, and screws as well as in implants for sustained drug delivery by

the FDA.[334] Despite these attractive features, application of PLGA-based oral vaccines has many challenges, such as the relatively high cost of producing these delivery systems, the unknown toxicity of the used organic solvents and reagents, low antigen encapsulation efficiency and the use of rigorous processes to develop these particles which may be damaging to the recombinant antigens.[336] Apart from PLGA, other polymers have also been used for vaccination purposes, such as β-glucan microparticles (GPs), alginate and chitosan. In this thesis, the use of β-glucan microparticles is extensively discussed.

β-glucan microparticles (GPs) are emerging as an oral delivery system. These particles display a high antigen encapsulation efficiency (> 90%) and an intrinsic immunomodulatory capacity owing to the β-glucan cell wall.[21-32] Furthermore, GPs have been generally recognised as safe by the Food and Drug Administration.[38] These promising antigen-carriers are highly purified, hollow and porous biomimetic 3-4 µm particles derived from the cell wall of *Saccharomyces cerevisiae* (Baker's yeast) and are composed of >85% β-1,3-D-glucan polymers (β-glucans), ~2% chitin and <1% lipids and proteins, with the rest being mostly ash and moisture.[26] β-glucan particles were initially engineered for DNA delivery. Encapsulation of DNA in these microparticulate delivery systems was based on the in situ layer-by-layer synthesis. Polyethyleneimine (PEI) and carrier tRNA were sequentially adsorped inside the particles in order to obtain a positively charged gel matrix within the hollow β-glucan particles, whereafter DNA was captured inside the particles via electrostatic interactions.[26] Huang and coworkers were the first to demonstrate the capture of soluble antigens inside these β-glucan particles.[25] This technique used hydration and lyophilisation steps to load the antigen inside the particles, thus eliminating the use of the cytotoxic PEI,[25] rendering them more suitable for oral immunisation.

β-glucans possess immunomodulating capacities and are also recognised by specific β-glucan receptors on immune cells, indicating that these particles have a great potential to be exploited as an antigen delivery vehicle. Indeed, many reports have demonstrated the immunostimulatory activity of these DNA or protein loaded β-glucan particles *in vitro* and in preclinical animal models.[21, 25, 26, 29-31, 165]

It has been demonstrated that incubation of mouse bone marrow derived dendritic cells (BMDCs) with ovalbumin (OVA) loaded GPs resulted in phagocytosis, upregulation of maturation markers, rapid proteolysis of ovalbumin and proliferation of OVA-reactive transgenic $CD8^+$ OT-I and $CD4^+$ OT-II T cells.[25] Furthermore, subcutaneous immunisation of GP-OVA induced strong humoral and Th1- and Th17-biased $CD4^+$ T cell responses in mice.[25] Oral administration of silencing RNA (siRNA)-loaded particles in mice was investigated as well. siRNA-mediated gene silencing occurred by internalisation of the particles by GALT mononuclear phagocytes. These cells then migrated to spleen, liver and lung.[21]

Only two articles have evaluated the capacity of these β-glucan microparticles as particulate antigen vehicle for oral vaccination.[28, 32] The first study by Berner et al. (2008) demonstrated that BSA-conjugated GPs were able to initiate an enhanced systemic IgG response against BSA after intradermal and oral immunisation in mice.[28] However, BSA was conjugated to the particles surface, losing the advantage of antigen protection. De Smet and coworkers, on the other hand, have loaded ovalbumin inside the GPs using the same technique as described by Huang et al. (2010). Oral administration of these GP-OVA particles in mice resulted in a Th17-biased response and the production of OVA-specific IgA, secretory IgA (SIgA) and secretory component antibodies.[32] This local immune response is obtained via the transepithelial transport of GPs by Peyer's patch M cells,[22, 32] resulting in the accumulation of GPs in $CD11c^+$ mononuclear phagocytes situated in the Peyer's patch sub-epithelial dome (SED) regions.[22] However, because of the small number of M cells present in the intestinal tract, a huge amount of particles is probably necessary to induce protective immune responses against intestinal pathogens. Since villous enterocytes outnumber M cells and possess a transcytotic capability for macromolecules and inert particles, specific targeting of particles to enterocytes could enhance particle absorption in the gastro-intestinal tract.[337]

Taken together, a suitable delivery system in combination with powerful mucosal adjuvants and an enteric coating have been demonstrated to greatly enhance the efficacy and efficiency of oral vaccines, however, many oral route-specific challenges that must be overcome remain. Targeting these immunostimulating particulate vehicle systems to epithelial transcytotic receptors is a promising technological adaptation that can be made in order to enhance the efficacy of the oral vaccine.

2.4 Targeting

2.4.1 Bioadhesion and delivery

Although encapsulation of antigens in nano- or microparticles is a promising approach for oral vaccination, the uptake of particles by epithelial cells remains poor due to the rapid transit time in the intestinal tract and the limited availability of M cells.[241] As a consequence, high and multiple oral doses of these antigen carriers are often required to elicit sufficient immune responses against the antigen of interest. Decorating these particles with bioadhesive molecules can increase the bioavailability of the particles by increasing the residence time of the vaccines within the gut.[338] The pivotal role played by M cells in the uptake of intestinal antigens has made them a key target for oral vaccine delivery. However, directing vaccine towards mucosal DCs or enterocytes should not be disregarded.[38] A combination of antigen encapsulation in nano- or microparticles and the functionalisation of these particles with bioadhesive molecules is an advanced concept for oral vaccine design and holds much promise (Figure 11). The most common used bioadhesins are lectins, bacterial adhesins or specific antibodies targeting transcytotic receptors.

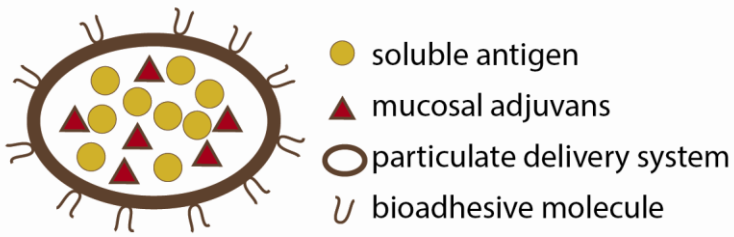


Figure 11. Schematic representation of a promising functionalised particulate oral antigen delivery vehicle. Soluble antigens together with a mucosal adjuvant can be encapsulated in nano- or microparticles. These particles can be coated with bioadhesive molecules in order to increase the residence time in the gastrointestinal tract.

Lectins are naturally occurring proteins, which bind specifically to carbohydrates.[339] These lectins can bind to membrane anchored glycoconjugates present on intestinal epithelial cells.[241] Since these intestinal epithelial cells exhibit regional and even cell-type specific differences in their carbohydrate composition, the use of lectins may permit targeting to specific locations within the intestinal tract.[337, 340] The most investigated lectin is *Ulex europaeus* agglutinin 1 (UEA-1), specific for α-L-fucose residues expressed on the apical surface of M cells, goblet and Paneth cells in mice.[337, 341] Other lectins that have demonstrated an ability to both target and enhance Peyer's patches uptake when associated to nanocarriers are: wheat germ agglutinin, peanut agglutinin, asparagus pea lectin and *Aleuria aurantia* lectin.[241] However, once coated on the surface of particles, these plant lectins are susceptible to proteolytic degradation in the gastrointestinal tract.[342] Moreover, lectins as targeting ligand for oral vaccine delivery are toxic, resulting in anti-nutritional properties.[343] To overcome these

limitations, recombinant lectins or peptides/molecules which mimic the function of lectins can be produced. However, using lectins in vaccine delivery should be restricted since the glycosylation pattern in the intestine differs between enterocytes and M cells at different intestinal locations (presumably influenced by the local microflora) with age and between species.[337, 338, 340] Moreover, the interaction of these lectins with the mucus layer could lead to immobilised particles in the mucus layer. As a consequence, when the mucus is renewed, these lectin-conjugated particles will be cleared.[241, 344]

The use of microbial adhesins to functionalise particles is an interesting approach, since these adhesins are relatively resistant to intestinal degradation, may exhibit mucus-permeating properties and can have an important effect as immunomodulators (adjuvants). In this biomimetic approach different ligands have been proposed including the use of flagellin, LPS, F4 fimbriae or FimH.[241, 345] Several enterotoxigenic *E. coli* (ETEC) strains express fimbriae on their surface allowing adhesion to the intestinal epithelium. Conjugation of these fimbriae to antigens has been shown to enhance mucosal antigen-specific antibody responses upon oral administration.[346] However, although bacterial adhesins are potent immunogens, immune responses directed against the adhesin itself could be generated, thereby decreasing the endocytosis of the vaccine delivery systems and the efficient induction of immune responses.[337]

Due to the drawbacks that lectins and microbial adhesins entail, research has recently focused on fusing particles to antibodies to target antigen sampling routes at the mucosal surfaces.[320, 337] Mimicking the invasive strategies from enteric pathogens, mediated by specific pathogen-host interactions, is a promising approach to increase the endocytosis of particulate vaccine antigens. Indeed, it has already been demonstrated that targeting of antigen-loaded microspheres to ligands of pathogenic adhesins can enhance oral vaccine delivery.[337] For example, antibody-mediated targeting to porcine aminopeptidase N (APN or ANPEP), expressed on intestinal epithelial cells, results in a strong IgA, IgG and IgM immune response.[260] Porcine aminopeptidase N (APN) serves as an uptake receptor for bacteria and viruses, such as group I coronaviruses,[347] transmissible gastroenteritis virus (TGEV)[348] and $F4^+$ enterotoxigenic *E. coli*.[260] Our lab has demonstrated that F4 fimbriae interact with APN in a sialic acid-dependent manner and this interaction results in F4 endocytosis.[260] Moreover, APN serves as a transcytotic receptor for antibodies as well, indicating that APN may represent a promising target for oral delivery of antigens across the epithelial barrier.[260] Aminopeptidase N or CD13[349] is expressed by many tissues, including on myeloid cells of the intestine.[350, 351] It has been demonstrated that crosslinking CD13 on immune cells positively modulates phagocytosis,[352] making APN an attractive target for both transcytosis and immune stimulation.

2.4.2 Strategies to conjugate antibodies to particles

A great challenge in the development of oral delivery systems is providing target specificity to the particles.[353] Targeting oral delivery systems to intestinal transcytotic receptors allows the particles to cross epithelial particles in order to maximally reach the mucosal immune cells, thereby reducing vaccination doses and any potential side effects.[264, 354] One classical approach for active targeting is mediated by antibodies specific for the target.[355] Conjugation of antibodies to particles offers great opportunities: they have the ability to bind to their target with high affinity and can improve cell penetration.[353, 356] Decoration of particles with antibodies has already been demonstrated as a feasible approach for an increased internalisation of particles by intestinal epithelial cells[264, 354] and by M cells.[357-359] Antibody conjugation methods for the formation of targeted particles should be efficient, reproducible, stable and non-toxic, yielding an oriented antibody binding. Here, we describe different strategies to conjugate antibodies (Abs) to nano- or microparticles.

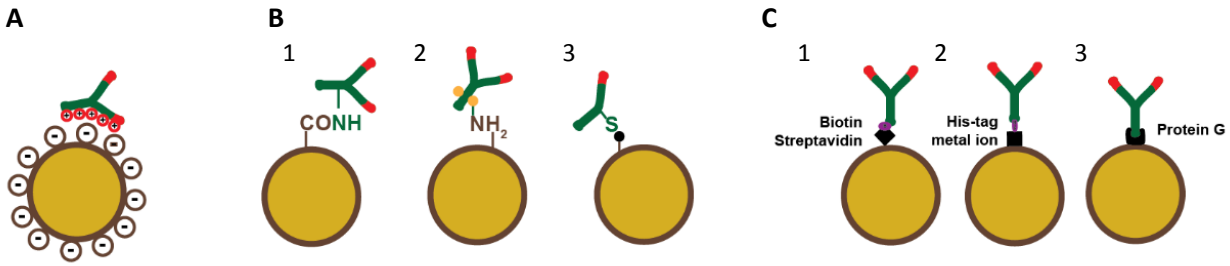


Figure 12. Schematic representation of different strategies for functionalisation of particles with antibodies. **A)** Electrostatic adsorption of antibodies to negatively charged particles. **B1)** Carbodiimide coupling method: covalent binding via primary amines on the antibodies and carboxylgroup on the particles. **B2)** AminoLink method: covalent binding via carbohydrate groups on the antibodies. **B3)** Thiolation of antibodies: reduction of disulfide groups in the hinge region of the antibodies. These sulfhydryl groups will react with thiol reactive groups on the particles. **C)** Use of adapter biomolecules: streptavidin-biotin (1), his-tag (2) and protein G (3).

The first method to conjugate antibodies to particles is by physical adsorption. Physical adsorption is generally based on hydrophobic, electrostatic, hydrogen bonding and van der Waals attractive forces between the antibody and the surface of the particle (Figure 12A). However, decoration of antibodies to particles by physical adsorption results in a weak, unstable and/or pH-dependent attachment.[355, 360]

Attachment of antibodies via covalent coupling is more attractive than physical adsorption due to its strong binding. However, this coupling method is more complex, since the conjugation requires the introduction of functional groups on the particle surface, use of chemical linkers and/or chemical modification of the antibodies.[355] Most covalent coupling methods use one of the following targets: 1) primary amines (NH_2), 2) carbohydrates or 3) sulfhydryl groups (-SH) on the antibody.

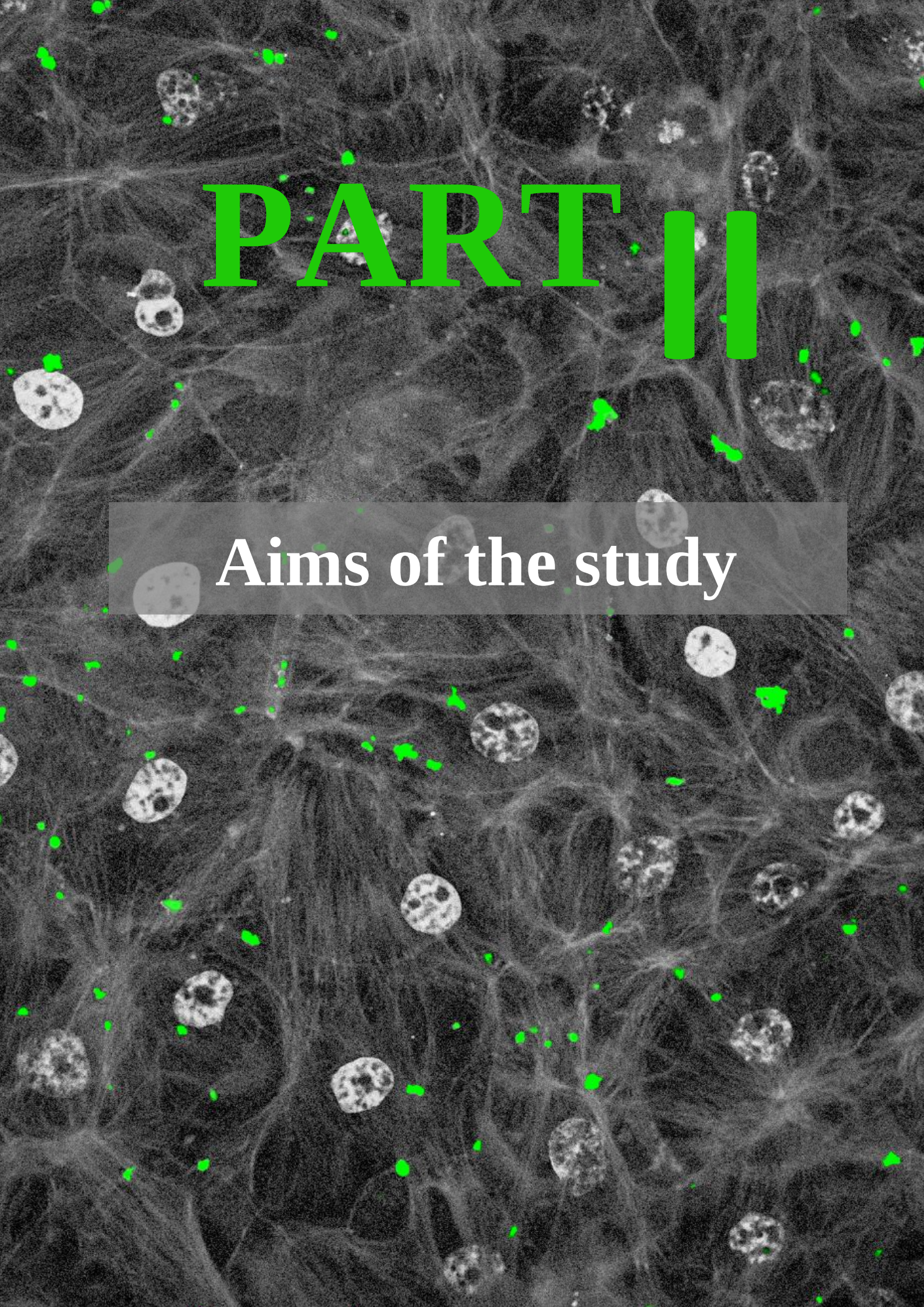
The first techniques (carbodiimide method) allows the direct reaction of the particles (containing amino-reactive groups) with primary amines (NH_2) available on the antibody surface (Figure 12B1). However, this coupling method results in randomly oriented Abs on the particle surface. Moreover, the high reactivity of all the aminebinding linkers makes them very unstable at alkaline pH values, thus the coupling reaction must be carried out under mild pH values. The most reactive amino group on the antibody under mild pH conditions is the terminal amino group, which is near to the antigen recognition place. Consequently, most of the Ab molecules would adopt "head-on" and "sideways-on" spatial orientations. This causes the loss of antigen binding capacity due to direct binding of the antigen-binding site or steric hindrance by the particle surface.[355, 361]

Using the carbohydrates of the Abs to covalently conjugate them to particles results in an oriented immobilisation (Figure 12B2). Therefore, the carbohydrate chains of the antibodies (located in the Fc fragment) are mildly oxidated using sodium periodate to create reactive aldehydes for coupling. Then, these aldehyde groups form stable secondary amine bonds with primary amines ($-NH_2$) present on the particles, followed by stabilisation of these bonds with a mild reductant.[355] However, some antibodies are not glycosylated (like hybridoma's) or are glycosylated near the antigen binding area.[361] In addition, the highly toxic sodium cyanoborohydride is routinely used for the reduction of the Schiff's base to a covalent bond, making this method unfavourable as a standard process for targeted oral vaccines.

Another common reactive group on antibodies are the thiol residues (carbon-bonded sulhydryl (-SH) group, derived from the amino acid cystine and cysteine) (Figure 12B3). Unfortunately, in antibodies these sulfhydryl groups (-SH) are oxidised as disulfide bonds (S-S), which covalently connect the heavy and light chains and the two antibody halves at the hinge region.[355] Since only free sulfhydryls (-SH) can be conjugated directly with thiol-reactive groups, these groups have to be created (thiolation reagent system) or generated (reduction of disulfide groups). Reduction of these disulfide bonds with a reagent such as dithiothreitol (DTT) or 2-mercaptoethylamine is a common method to generate free thiol groups. Disulfides in the hinge region are the most susceptible to reduction, which will split the chain linkage of these antibodies into two monovalent immunoglobulin fragments without altering the 3D structure and antigen-binding site.[355, 362] Now, the antibody fragments can be crosslinked to particles with thiol reactive groups on their surface (such as maleimide and iodoacetyl).[361]

Conjugation of antibodies to particles can also be accomplished using adapter biomolecules. These molecules are directly coupled to the particle surface. There are several options described in the literature: biotin binding proteins (avidin, streptavidin), genetically engineered Abs with terminal linker-peptide residues, proteins that specifically bind to the Fc region (Protein A or G) and nucleic

acid-mediated hybridisation.[355] For oral administration, conjugation of antibodies to particles via nucleic acids is unsuitable, since high levels of DNase I are present in the digestive tissue.[363, 364] When using the adaptor molecules avidin or streptavidin, the antibodies first need to be modified with biotin and the surface of the particles need to be functionalised with (strept)avidin (Figure 12C1). This technique is among the most rapid and strongest non-covalent interactions known, making the (strept)avidin-biotin complex very resistant to a wide range of pH values, elevated temperatures, and harsh chemical conditions. However, biotinylation of antibodies is required, which only can be obtained by covalent coupling.[355] Likewise, modification of antibodies is also required when using terminal linker-peptide residues, such as his-tags (Figure 12C2). The antibodies first have to be genetically engineered with these additional peptide residues, before conjugation to microspheres.[355] In contrast, the antibodies do not require any modification when using Fc-binding proteins to conjugate antibodies to particles. Only the particles need to be modified with Fc-binding proteins (Figure 12C2). Since these proteins contain several IgG binding sites per molecule, randomly covalent binding of Fc-binding proteins to the particles does not represent a problem.[355] Protein A and G are bacterial cell wall proteins that specifically bind the Fc region of the immunoglobulin and therefore provide proper orientation of the bound antibody.[355, 365] These proteins bind exclusively the IgG class of the antibodies, but differ in IgG subclasses specificity and are species-dependent. Protein A is generally preferred for rabbit, pig, dog and cat IgG, while protein G shows a broader binding activity to Abs of different species as well as to different isotypes.[355] Nowadays, recombinant forms of these proteins, which lack the albumin and cell surface binding domains, which are present in their native forms, are commonly used.[355]



PART II

Aims of the study

AIMS OF THE STUDY

Enterotoxigenic *E. coli* (ETEC) are an important cause of neonatal and post-weaning diarrhoea in piglets, resulting in severe economic losses in swine husbandry.[3] Antibiotics are commonly used to control these diseases instead of prevention with immunomodulators or vaccination.[9, 10] The ongoing debate on the use of antibiotics has increased the search for alternatives, especially natural bioactive materials capable of maintaining animal health and improving growth performance.[180] One of the promising alternatives for antibotics are β-glucans. Despite the fact that orally administered β-glucans are already used as immunomodulatory agents in humans and animals, their mechanism of action is still unclear.[14] Recognition of β-glucans is mediated by specific cell surface receptors, however, many discrepancies are found in the literature about the most important β-glucan receptor and signalling molecules involved in their biological activities. In this dissertation, we examined the involvement of the β-glucan receptors dectin-1 and complement receptor 3 (CR3) and their signalling pathways in the β-glucan-mediated activation of porcine innate immune cells.

Besides the use of immunomodulators, active oral vaccination against these ETEC infections is an appealing approach to prevent an outbreak. Commercial oral vaccines mainly use live-attenuated microorganisms, however, the identification of adverse risks associated with the use of these vaccines has intensified the search for recombinant oral subunit vaccines. Unfortunately, these types of vaccines have to combat a number of obstacles to induce immunity after oral delivery, such as the harsh environment in the gastrointestinal tract, oral tolerance and the epithelial barrier. Encapsulation of antigens in immune-stimulating particles combined with targeting to apical endocytotic receptors located on intestinal epithelial cells seems to be an attractive approach to successfully design oral vaccines. The aim of this part of the thesis was to investigate if β-glucan particles could be used as an antigen delivery vehicle, without impeding their immunomodulating character. In addition, we determined if APN could be used as target for delivery of antigens to the gut-associated immune system.

The following questions were addressed:

1. Which is the most important β-glucan receptor in porcine neutrophils and macrophages? Which signalling molecules are involved in β-glucan-mediated ROS production and cytokine production?

2. Could the adhesin of F18 fimbriae (FedF) be loaded efficiently and intact inside the β-glucan microparticles? Do these loaded particles still possess immunostimulatory effects? Which receptor is involved in the particle-mediated ROS production in porcine neutrophils?

3. Could β-glucan microparticles be efficiently decorated with monoclonal antibodies? Does APN-targeting result in an increased transcytosis through the epithelial barrier? Does APN-targeting trigger an elevated immune response in porcine dendritic cells and in pigs *in vivo*?

PART III

Experimental studies

CHAPTER 3:

Cell type-specific differences in β-glucan recognition and signalling in porcine innate immune cells

Baert K., Sonck E., Goddeeris B.M., Devriendt B., Cox E.
Dev and Comp Immunol 48 (2015): 192-203.

3.1 Abstract

β-glucans exert receptor-mediated immunomodulating activities, including oxidative burst activity and cytokine secretion. The role of the β-glucan receptors dectin-1 and complement receptor 3 (CR3) in the response of immune cells towards β-glucans is still unresolved. Dectin-1 is considered as the main β-glucan receptor in mice, while recent studies in man show that CR3 is more important in β-glucan-mediated responses. This incited us to elucidate which receptor contributes to the response of innate immune cells towards particulate β-glucans in pigs as the latter might serve as a better model for man. Our results show an important role of CR3 in β-glucan recognition, as blocking this receptor strongly reduced the phagocytosis of β-glucans and the β-glucan-induced ROS production by porcine neutrophils. Conversely, dectin-1 does not seem to play a major role in β-glucan recognition in neutrophils. However, recognition of β-glucans appeared cell type-specific as both dectin-1 and CR3 are involved in the β-glucan-mediated responses in pig macrophages. Moreover, CR3 signalling through focal adhesion kinase (FAK) was indispensable for β-glucan-mediated ROS production and cytokine production in neutrophils and macrophages, while the Syk-dependent pathway was only partly involved in these responses. We may conclude that CR3 plays a cardinal role in β-glucan signalling in porcine neutrophils, while macrophages use a more diverse receptor array to detect and respond towards β-glucans. Nonetheless, FAK acts as a master switch that regulates β-glucan-mediated responses in neutrophils as well as macrophages.

3.2 Introduction

β-glucans exert immunomodulatory effects upon oral and i.v. administration.[366, 367] These glucose polymers can be derived from several sources, such as fungi, yeast, some bacteria, seaweeds and cereals.[41] In seaweeds, they serve as storage carbohydrates, while in bacteria, fungi, yeast and cereals, such as oat and barley, they play a role as structural frame and define cellular shape and rigidity.[42] The oat and barley cell walls contain unbranched β-glucans with 1,3- and 1,4-β-linked glucopyranosyl residues, whereas β-glucans from bacterial origin and some algae are unbranched 1,3-β-linked glucopyranosyl residues.[43, 368] In contrast, cell wall β-glucans of yeast, fungi and β-glucans produced by some algae consist of 1,3-β-linked glucopyranosyl residues with small numbers of 1,6-β-linked branches. Both the frequency and length of these branches differ depending on the source.

β-glucans are recognised as microorganism-associated molecular patterns (MAMPs) by immune cells via several pattern recognition receptors, including dectin-1, complement receptor 3 (CR3), lactosylceramide and scavenger receptors.[369] Receptor activation results in an enhanced production

of pro-inflammatory cytokines, chemokines and reactive oxygen species by innate immune cells in several species.[74, 75, 137, 183, 187, 370] Dectin-1 has been recognised as the most important receptor for β-glucans in mice and consequently most studies have focused on the function of dectin-1 in the immunomodulating effect of β-glucans.[75] This receptor is expressed by dendritic cells (DCs), neutrophils, monocytes and macrophages.[79-82, 371] The intracellular domain of this receptor contains a hemi-immunoreceptor tyrosine-based activation motif (hemITAM), probably indicating the need for receptor dimerisation to properly activate signalling pathways. Dually phoshorylated ITAMs serve as docking site for spleen tyrosine kinase (Syk), a key player in the activation of immune cells.[96-98] Recently, several studies have questioned the role of dectin-1 in β-glucan recognition.[157, 161] Indeed, dectin-1 was not involved in triggering inflammatory responses to zymosan in mice.[157] Likewise, although neutrophils in man express dectin-1, phagocytosis of β-glucans and β-glucan-mediated ROS production is here completely dependent on CR3.[372] Interestingly, Bose et al.[156] demonstrated that human monocytes use distinct receptors (dectin-1 and CR3) for oxidative burst in response to different physical forms of β-glucans. CR3, a β2-integrin, is usually associated with complement function, but recognises an array of various ligands. The ability of CR3 to bind diverse ligands is mainly attributed to a consensus binding site within its CD11b subunit.[373] The carbohydrate binding domain is however spatially separated from this consensus binding domain.[374-377] Upon ligand recognition, the cytoplasmic domain of CR3 interacts with kinases, such as focal adhesion kinase (FAK), regulating the interaction between cells and the extracellular matrix and controlling several integrin-dependent processes.[129, 378, 379] In pigs, dectin-1 and CR3 are expressed on immune cells, including neutrophils, monocytes, macrophages and DCs.[83] The α subunit (CD11b) of CR3 is not yet identified, but two candidate molecules, namely CD11R1 and CD11R3, have been described.[124] Both CD11R1 and CD11R3 dimerise with the β2 chain CD18, but their role in complement or β-glucan recognition is still unclear.[124]

The involvement of dectin-1 and CR3 in mediating the biological activities of particulate β-glucans remains largely unresolved, but seems to be cell type-specific, structure-related and species-dependent.[76, 156, 161, 165, 372] Here, we investigate the role of these receptors in immunomodulating innate immune cells by different particulate β-glucans in order to elucidate the mechanism of β-glucan recognition and signalling in pigs.

3.3 Materials and Methods

3.3.1 β-glucans

Six different β-glucans were used to stimulate porcine innate immune cells (Table 1). Curdlan, zymosan and β-glucans purified from *Saccharomyces cerevisiae* and *Euglena gracilis* were purchased from Sigma (Diegem, Belgium). Scleroglucan and Macrogard were kindly provided by INVE (Dendermonde, Belgium) and Biotec Pharmacon ASA (Tromsø, Norway), respectively. A description and comparison of the carbohydrate structures as well as the preparation and storage of these β-glucans was previously published.[380] The endotoxin concentration present in each β-glucan preparation was determined by the Chromogenic Limulus Amebocyte Lysate (LAL) test (Cambrex Bio Science Walkersville, Inc., Wiesbaden, Germany) and with exception of curdlan (47 endotoxin units/µg β-glucan), these levels were consistently lower than 0.5 endotoxin units/µg β-glucan. Laminarin was kindly provided by INVE (Dendermonde, Belgium). Depleted zymosan was purchased from Invivogen (Toulouse, France).

Table 1. Overview of the origin, solubility, structure and molecular weight (MW) of different β-glucans.

Origin	β-glucan name	Solubility	Structure	MW	References
Laminaria digitata	Laminarin	Soluble	β-1,3 with some β-1,6-branching (30:1). The β-1,6 side chains are composed of 2 glucose units	7.7	46-48
Saccharomyces cerevisiae	Macrogard®	Particulate	β-1,3/1,6-branched (10:1 or 20:1). The β-1,6 side chains are composed of 2 or 3 glucose-units	unknown	20, 49
	Zymosan	Particulate	Crude extract with β-glucans, mannan and proteins; non-uniform branches and backbone units. β-1,3/1,6-branched	<200	50-52
	Glucans from *Saccharomyces cerevisiae*	Particulate	β-1,3/1,6-branched (30:1)	200	53
Euglena gracilis	Glucans from *Euglena gracilis*	Particulate	β-1,3-unbranched	500	53, 54
Sclerotium rolfsii	Scleroglucan	Soluble	β-1,3/1,6-branched (6:1). The β-1,6 side chains are composed of 2 glucose units	1020	53, 54
Alcaligenes faecalis	Curdlan	Particulate	β-1,3-unbranched	100	55, 56

3.3.2 Isolation of porcine PBMCs and neutrophils

Pigs (between 4 and 23 weeks old) were housed under standard conditions as blood donors. All animal experiments were approved by the animal care and ethics committee of the Faculty of

Veterinary Medicine, Ghent University (grant nr: EC2008/79). Peripheral blood (10 ml) was collected on heparin (100 µl) from the jugular vein. Subsequently, peripheral blood monomorphonuclear cells (PBMCs) and neutrophils were isolated as previously described by density gradient centrifugation on Lymphoprep (NYCOMED Pharma AS, Life Technologies, Merelbeke, Belgium) or a discontinuous Percoll gradient (68% and 75%) (GE healthcare, Diegem, Belgium), respectively.[380]

3.3.3 Generation of monocyte-derived macrophages

Monocyte-derived macrophages (MDM) were generated from blood-derived monocytes, which were enriched from the PBMC fraction to a purity of >95% by positive immunomagnetic bead selection (MACS; Miltenyi Biotec, Leiden, The Netherlands) using the CD172a-specific monoclonal antibody (clone 74-12-15a). These cells were seeded in a 96-well plate at a density of 2 x 10^5 cells/well in MDM medium (DMEM (Gibco, Merelbeke, Belgium) supplemented with 10% (v/v) fetal calf serum (FCS, Greiner, Wemmel, Belgium), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and recombinant human M-CSF (3 ng/ml; R&D systems, Abingdon, UK). After three days incubation (37°C, 90% humidity, 5% CO_2) fresh MDM medium was added to obtain monocyte-derived macrophages at day 7-8.

3.3.4 β-glucan-induced PBMC proliferation

To examine if dectin-1 is involved in the β-glucan-induced PBMC proliferation in pigs, PBMCs were seeded in a 96-well plate at 5.0 x 10^5 cells/well (100 µl/well) in leukocyte medium (RPMI-1640 containing FCS (10%), non-essential amino acids (1%), sodium-pyruvate (100 µg/ml), L-glutamine (292 µg/ml), penicillin (100 IU/ml), streptomycin (100 µg/ml) and kanamycin (100 µg/ml)) supplemented with β-mercapto-ethanol (50 µM). To block dectin-1, laminarin (1 mg/ml) was added to the cells (1h). Subsequently, different concentrations of the six β-glucans (800, 200, 50 and 5 µg/ml) were added. As a positive control the cells were treated with 1 µg/ml concanavalin A (ConA, Sigma-Aldrich, Diegem, Belgium), while medium served as a negative control. The cells were incubated for 72h at 37°C in a humidified atmosphere with 5% CO_2. Next, the cells were pulse-labeled with 1 µCi of [H^3]-methyl-thymidine (Amersham ICN, Bucks, UK) per well for 18h at 37°C and 5% CO_2, harvested onto glass fiber filters and measured incorporated radioactivity with a β scintillation counter (PerkinElmer, Life Science, Merelbeke, Belgium). Each stimulation was performed in duplicate and the results are presented as counts per minute (cpm).

3.3.5 β-glucan receptor inhibitors

To inhibit dectin-1 or CR3, cells were pre-incubated with the inhibitors in the CO_2 incubator at 37°C before exposure to the β-glucans. To inhibit dectin-1, laminarin was added to the cells. To inhibit CR3-mediated responses, monoclonal antibodies (mAbs; mouse IgG_1 isotypes) against CD18, CD11R1 and CD11R3 (AbD Serotec, Kidlington, UK) were added. These antibodies were dialysed against PBS to remove sodium azide. An isotype-matched mAb control (IgG_1) was added to the cells to measure background inhibition.

3.3.6 Signalling pathway inhibitors

To inhibit the signalling pathway of dectin-1 or CR3, cells were pre-incubated with the inhibitors before exposure to the β-glucans. Dectin-1-mediated responses (Syk) were blocked with R406 (Selleckchem, Huissen, The Netherlands), whereas PF 573228 (Tocris Biosciences, Bristol, UK) should disrupt the CR3 signalling pathway (FAK). The efficacy of the Syk signalling pathway inhibitor (R406) in pigs was investigated by stimulating neutrophils with plate-bound IgG to activate FcγR signalling. Briefly, F4 fimbriae (5 µg/ml) were coated on a 96-well plate (2h at 37°C), unbound sites were blocked by 3% BSA in PBS and F4 fimbriae-specific porcine IgG antibodies (10 µg/ml) were added to the F4 fimbriae for 1h at 37°C. Subsequently, neutrophils (2 x 10^5 cells/well) pre-incubated with different concentrations of R406 (0, 0.1, 1, 5, 10, 50, 100 or 200 µM) were added to this plate-bound IgG, and the production of reactive oxygen species (ROS) was measured.

3.3.7 Oxidative burst assay

To analyse the role of dectin-1 and CR3 in the β-glucan-induced oxidative burst by porcine neutrophils, monocytes or monocyte-derived macrophages, ROS was measured using a chemiluminescence assay as described by Donne et al.[381] with some modifications. Neutrophils or monocyte-derived macrophages were seeded in a 96-well plate at 2.0 x 10^5 cells/well, while PBMCs were seeded at a concentration of 2.0 x 10^6 cells/well. The plates were incubated at 37°C for 2h in a humidified atmosphere with 5% CO_2 to allow the cells to adhere to the plastic surface. Then, the cells were pre-treated with the β-glucan receptor inhibitors (3.3.5) or signalling pathway inhibitors (3.3.6) for 1 hour at 37°C. Subsequently, luminol was added to the cells (0.5 mM diluted in HBSS + Ca^{2+} Mg^{2+}) and, after 5 minutes of background measurement at 37°C, 25 µl of the β-glucan preparations were added. Stimulation of the cells with phorbol myristate acetate (PMA; 50 µg/ml) was used as a

positive control. ROS production was then measured during 120 min in the integration mode. Each stimulation was performed in duplicate. ROS production is expressed as relative light units (RLU).

3.3.8 Phagocytosis assay

Neutrophils or monocyte-derived macrophages were seeded in a 96-well plate or 24-well plate (2.0 x 10^5 cells/well), respectively, and incubated at 37°C in a humidified atmosphere with 5% CO_2. To investigate which receptor plays a role in the phagocytosis of β-glucans by neutrophils or macrophages, the cells were pre-treated with the β-glucan receptor inhibitors (3.3.5) for 30 minutes at 37°C to allow binding of laminarin or the mAbs to their specific receptor. Subsequently, Alexa Fluor 488-conjugated zymosan particles (Invitrogen, Gent, Belgium) were added for 1 hour to the cells. Excess particles were removed by repeated washing in cold PBS. Adherent cells were recovered by treatment with Accutase (Invitrogen) for 15 min at 37°C. The number of cells demonstrating green fluorescence was determined on a FACSCanto (BD Biosciences, Erembodegem, Belgium) and FACS analysis was performed on 10,000 events with FACSDiva software 6.1.3 (doublet discrimination).

3.3.9 Isolation of CD11R1 positive and negative populations

In pigs, CD18 dimerises with either CD11R1 or CD11R3, which differ in their expression pattern and molecular weight.[124] CD11R1 is present on approximately 50% of all porcine neutrophils and not on monocytes and macrophages, whereas CD11R3 is expressed on all neutrophils, monocytes and macrophages. Although the expression pattern of CD11R3 correlates with the human CD11b, CD11R3 is not recognised by human CD11b mAbs. In contrast, human CD11b mAbs cross-react with CD11R1, which has the same molecular weight as CD11b.[124, 382] To investigate whether CD11R1 is important in β-glucan recognition by neutrophils, and thus serves as the α subunit of CR3, $CD11R1^+$ and $CD11R1^-$ cells were FACS-sorted (FACSAria III Cell sorter; BD Biosciences; purity $CD11R1^{+:}$ = 99.3% ± 0.5 and $CD11R1^-$ = 98.7% ± 1.07) and stimulated with β-glucans for ROS production.

3.3.10 Cytokine ELISA

Neutrophils or monocyte-derived macrophages were seeded in a 96-well plate (2.0 x 10^5 cells/well) in leukocyte or MDM medium, respectively, and incubated at 37°C in a humidified atmosphere with 5% CO_2. First, different β-glucan receptor inhibitors (3.3.5) or signalling pathway inhibitors (3.3.6) at the indicated concentrations were added to the cells for 1h, followed by stimulation with 200 µg/ml

zymosan, Macrogard or *Euglena gracilis* for another 16h. Thereafter, culture supernatant was collected and the TNFα, IL-8 and IL-1β cytokine concentrations were determined in porcine-specific ELISAs (R&D systems). The viability of the cells was examined by propidium iodide (PI) staining and measured by flow cytometry (FACSCanto).

3.3.11 siRNA-mediated dectin-1 silencing in monocyte-derived macrophages

Monocyte-derived macrophages were seeded in a 96-well plate (2 x 10^5 cells/well) and incubated at 37°C. The culture medium was replaced 60 minutes before transfection. Dectin-1 was knocked down by a mix of two siRNAs (Sigma); the sequence of the target site for siRNA1 is 5'-GCATGTGTCTTCCCAACCT-3' and for siRNA3 5'-CTAACTGGATCACACATGA-3' (Figure 1). Macrophages were transfected with siRNA (50 nM) using the GenMute siRNA transfection Reagent for Primary Macrophages (Signagen, Kampenhout, Belgium) according to the manufacturer's instructions. Gene silencing was measured 48 hours post transfection by qPCR. Briefly, total RNA was isolated from the cells using TRIzol (Invitrogen) and was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Biorad, Temse, Belgium). The resulting cDNA served as a template for the qPCR assay (StepOnePlus™ system, Applied Biosystems). The primers (IDT) used for detection of dectin-1 (accession number: FJ386383) were 5'-TGCTCTCAACTGGGTTCTTATC-3' (sense) and 5'-CTTCAGTCTGGCTGTGAGAAA-3' (antisense). The qPCR reaction mix consisted of the SYBR Green PCR Master Mix (Applied Biosystems), 0.5 µM of each sense and antisense primer and cDNA. The cycle conditions were 1 cycle of 10 min at 95°C and 40 cycles of 15s at 95°C, 30s at the annealing temperature (60°C), and 30s at 72°C. Relative expression levels (Rq) were determined from the quantification cycle (Cq) values applying the qBase+ software from Biogazelle. Eight reference genes (ACTB, GAPDH, RPL19, CYPA, B2M, HPRT1, YWHAZ and SDHA; Table 2) were investigated on 12 samples of porcine macrophages. Based on geNorm analysis, two most stable reference genes (CYPA and RPL19) were identified and used for normalisation of qPCR data. Subsequently, the transfected cells were stimulated with β-glucans and after 16h the cytokine secretion (TNFα and IL-1β) was determined by porcine-specific ELISAs (R&D systems). The viability of the cells and CR3 expression was examined by flow cytometry (FACSCanto).

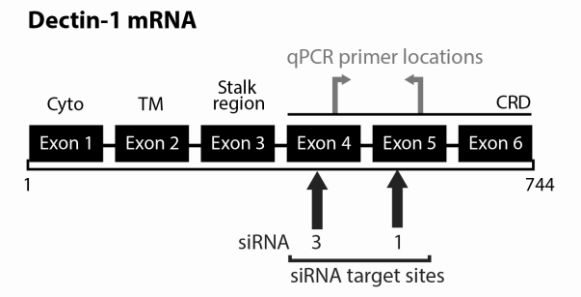


Figure 1: Schematic illustration of the dectin-1 gene. Dectin-1 consist of an N-terminal cytoplasmatic tail (Cyto), encoded by exon 1, a transmembrane region (TM; exon 2), a stalk region (exon 3), and the carbohydrate recognition domain (CRD; exon 4–6). Dectin-1 is silenced by adding siRNA 1 and 3 to the cells. Silencing efficiency (60.74% ± 6.03 dectin-1 reduction) was determined by qPCR. The primers locations are indicated.

Table 2. Primer sequences, amplicons length, annealing temperature, concentration and reference GenBank accession numbers of investigated porcine genes.

Gene symbol	Gene name	Primer sequence (5' 3')	Amplicon length (bp)	Annealing tempera-ture (°C)	Concen-tration (µM)	GenBank: accession number	Refe-rence
ACTB	**β-actin**	F: TCA TCA CCA TCG GCA ACG R: TTC CTG ATG TCC ACG TCG C	133	60	0.3	DQ178122	383
GAPDH	**Glyceraldehyde-3-phosphate dehydrogenase**	F: GGG CAT GAA CCA TGA GAA GT R: AAG CAG GGA TGA TGT TCT GG	230	60	0.2	DQ178124	384
RPL19	**Ribosomal protein L19**	F: AAC TCC CGT CAG CAG ATC C R: AGT ACC CTT CCG CTT ACC G	147	60	0.2	XM_003131509	385
CYPA	**Cyclophilin A**	F: TAA CCC CAC CGT CTT CTT R: TGC CAT CCA ACC ACT CAG	368	57	0.2	JX523419	384
B2M	**β-microglobulin**	F: AAA CGG AAA GCC AAA TTA CC R: ATC CAC AGC GTT AGG AGT GA	178	60	0.1	DQ178123	386
HPRT1	**Hypoxantine phosphoribosyl-transferase I**	F: CCG AGG ATT TGG AAA AGG T R: CTA TTT CTG TTC AGT GCT TTG ATG T	181	60	0.1	DQ178126	386
YWHAZ	**Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein**	F: ATG CAA CCA ACA CAT CCT ATC R: GCA TTA TTA GCG TGC TGT CTT	178	60	0.1	DQ178130	386
SDHA	**Succinate dehydrogenase complex, subunit A**	F: GAA CCG AAG ATG GCA AGA R: CAG GAG ATC CAA GGC AAA	191	58	0.1	DQ178128	386
Dectin-1	**Dendritic cell-associated C-type lectin 1**	F: TGC TCT CAA CTG GGT TCT TAT C R: CTT CAG TCT GGC TGT GAG AAA	127	60	0.5	FJ386383	

3.3.12 Statistics

Data are presented as the mean ± SEM. Data were analysed with GraphPad Prism 5 using a Friedman test (Post hoc: Dunn) to compare conditions with or without laminarin and each inhibitor with its isotype-matched control or control cells. Data were considered significant at $p < 0.05$.

3.4 Results

3.4.1 β-glucan-induced PBMC proliferation and ROS production is not solely dependent on dectin-1

Previously, we observed a dose-dependent β-glucan-induced PBMC proliferation.[380] Moreover, the extent of this proliferation seemed to be dependent on the β-glucan structure. In an effort to study if dectin-1 is involved in β-glucan signalling, we added six different β-glucans to PBMCs upon blocking dectin-1 via laminarin. The β-glucan-induced PBMC proliferation was not significantly different between laminarin pretreated and control cells (Figure 2). However, in the presence of laminarin the lymphocyte proliferation induced by scleroglucan, curdlan and zymosan at all assayed concentrations was decreased. In addition, at 200 µg/ml laminarin also reduced the lymphocyte proliferation upon stimulation with all β-glucan preparations. Together this implies that dectin-1 is only partially involved in the β-glucan-induced lymphocyte proliferation.

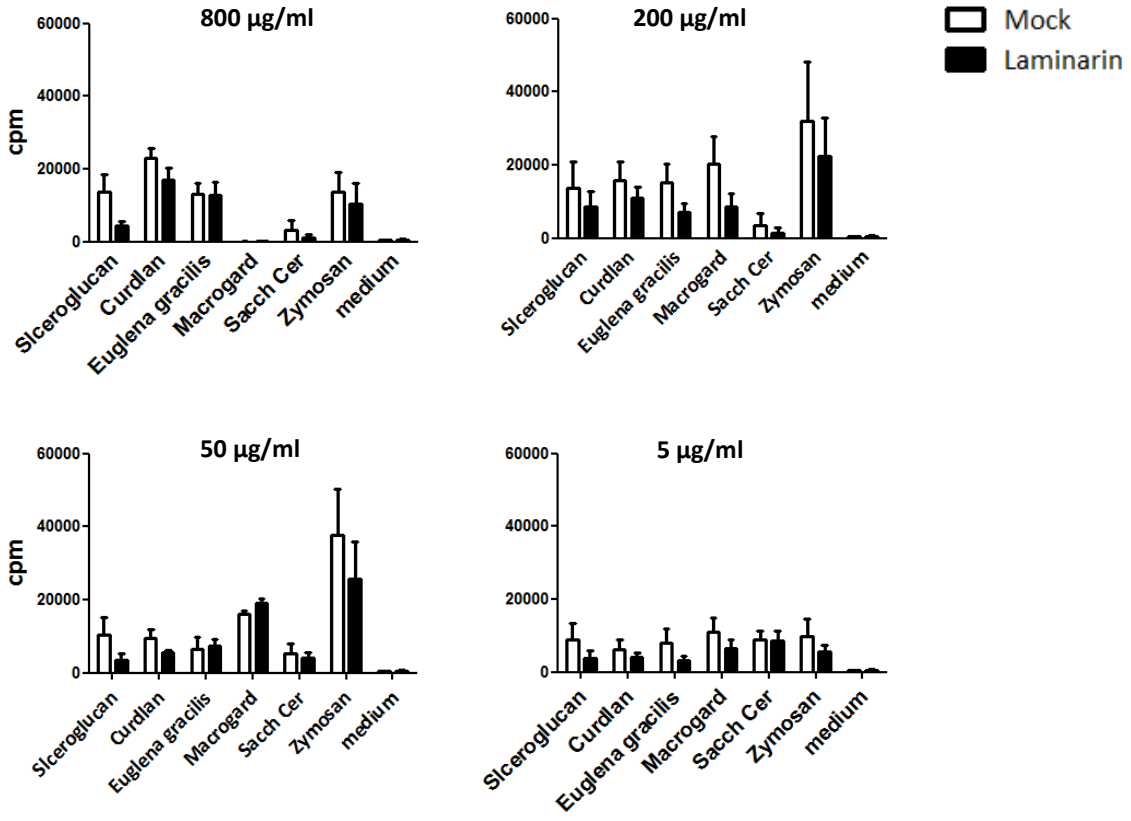


Figure 2. Dectin-1 is not involved in the β-glucan-induced PBMC proliferation.
PBMCs (5 x 10^5 cells) were incubated simultaneously with laminarin (1 mg/ml) and different concentrations of the indicated β-glucans. Data are shown as the mean counts per minute (cpm) ± SEM of four pigs.

To investigate the potential role of dectin-1 in the β-glucan-induced ROS production by porcine innate immune cells, monocytes and neutrophils were incubated with laminarin to block dectin-1

prior to β-glucans stimulation. Remarkably, scleroglucan and curdlan were unable to trigger ROS production by monocytes and neutrophils as opposed to the other β-glucans, which elicited a dose-dependent oxidative burst response (Figure 3). Dectin-1 blocking with laminarin failed to inhibit the β-glucan-induced ROS production even at a 4-fold lower β-glucan concentration, implying that dectin-1 is not involved in the respiratory burst response of neutrophils and monocytes to different β-glucans.

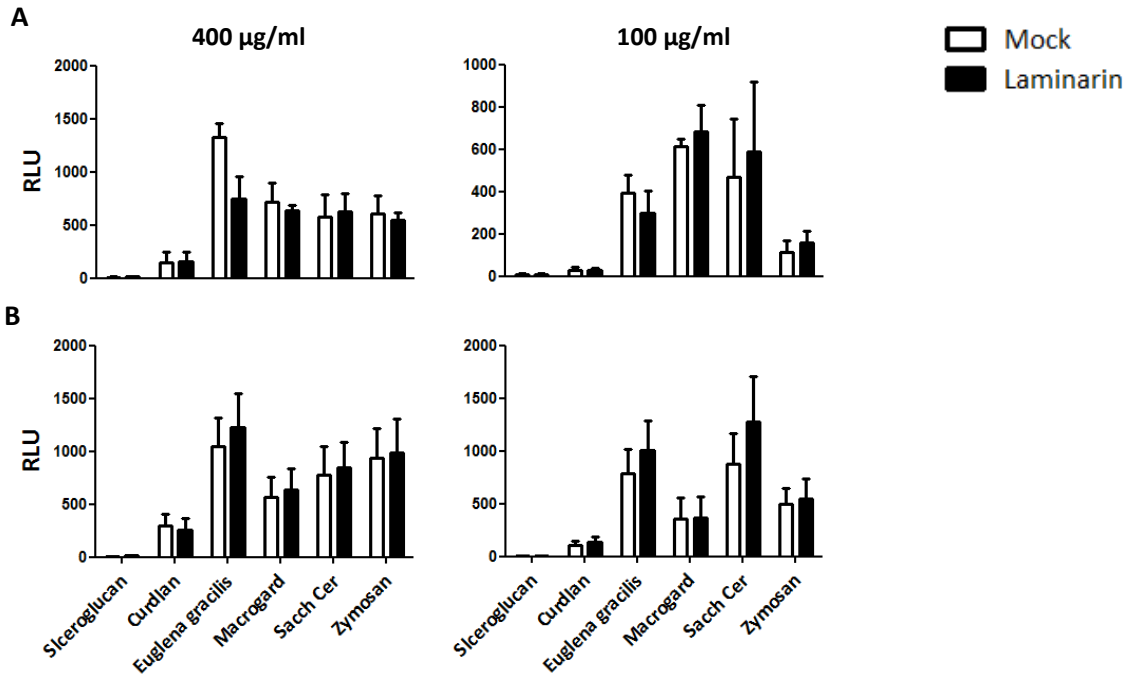


Figure 3. Dectin-1 is not involved in the β-glucan-induced ROS production by porcine neutrophils and monocytes.
Neutrophils (2 x 10^5 cells) **(A)** or monocytes (2 x 10^6 cells) **(B)** were pre-incubated with laminarin (1 mg/ml), after which the cells were stimulated with 400 and 100 µg/ml of the indicated β-glucan preparations. ROS production was determined via chemiluminescence. Data are shown as the mean relative light units (RLU) ± SEM of four pigs.

3.4.2 Receptor usage in zymosan phagocytosis is cell type-specific

Surprisingly, we could not establish a clear role for dectin-1 in the recognition of β-glucans by porcine innate immune cells. This motivated us to investigate if other β-glucan receptors, such as CR3, are involved in the phagocytosis of β-glucans by porcine monocytes and neutrophils. To examine which receptor is involved in the phagocytosis of β-glucans, dectin-1 and CR3 were blocked. In the presence of laminarin zymosan is still phagocytosed by neutrophils (Figure 4A), while the uptake of zymosan by macrophages was inhibited with 48.5% ± 11.4 by laminarin (Figure 4B). Interestingly, the phagocytosis of zymosan by neutrophils is diminished upon blocking the CR3 subunits CD18 and CD11R3, while blocking CR3 on macrophages had no influence on the phagocytosis of this β-glucan

(Figure 4). Thus, neutrophils (CR3) and macrophages (dectin-1) use different receptors to phagocytose zymosan.

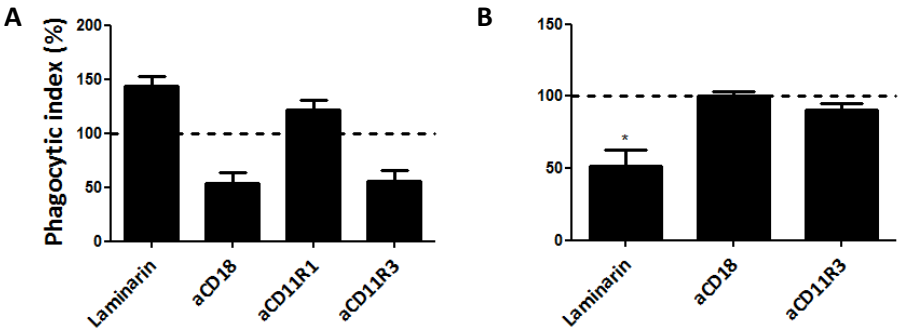


Figure 4. CR3 is involved in the phagocytosis of zymosan in porcine neutrophils, while dectin-1 is involved in the phagocytosis of zymosan in porcine macrophages.
(A) Neutrophils (2 x 10^5 cells) were incubated with 200 µg laminarin, 1.45 µg aCD18, 0.15 µg aCD11R1, 5 µg aCD11R3 or 5 µg isotype control (IgG_1). Subsequently, the cells were stimulated with fluorescent 200 µg/ml zymosan. Phagocytosis was determined via flow cytometry. Data are shown as the phagocytic index (% of mock or isotype control) of the mean fluorescence intensity ± SEM of six pigs. **(B)** Macrophages (2 x 10^5 cells) were incubated with 200 µg laminarin, 1.45 µg aCD18, 5 µg aCD11R3 or 5 µg isotype control (IgG_1). Subsequently, the cells were stimulated with fluorescent 5 µg/ml zymosan. Phagocytosis was determined via flow cytometry. Data are shown as the phagocytic index (% of mock or isotype control) of the mean fluorescence intensity ± SEM of four pigs. *: $p < 0.05$.

3.4.3 CR3 is critically involved in the β-glucan-induced ROS production by neutrophils

The results from the phagocytosis assay imply a role for CR3 in the β-glucan recognition by porcine neutrophils. Interestingly, blocking CD11R1 could not influence the phagocytosis of zymosan, while blocking CD11R3 diminished the uptake of zymosan by neutrophils (Figure 4A). This implies that CD11R3 has a carbohydrate binding domain, similar to its human CD11b counterpart, while CD11R1 is not involved in the recognition of β-glucans. To further explore this hypothesis, the role of CD11R1 in β-glucan signalling was examined. As CD11R1 is only present on 50% of porcine neutrophils, $CD11R1^+$ and $CD11R1^-$ neutrophils were FACS-purified. Subsequently, these neutrophil populations were stimulated with three different β-glucans (200 µg/ml zymosan, Macrogard and *Euglena gracilis*), since these particular β-glucans displayed high responses (Figure 2 and 3). The ROS production upon β-glucan stimulation did not differ between these neutrophil subsets (Figure 5A), confirming that CD11R1 is not involved in the response of porcine neutrophils towards β-glucans.

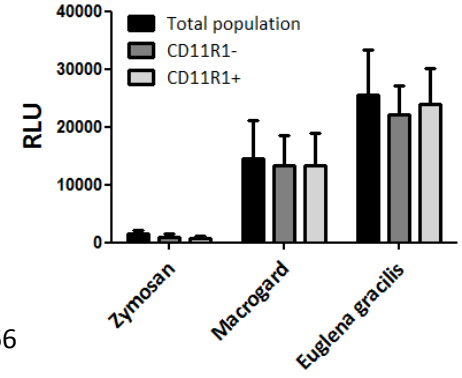


Figure 5A. CD11R1 is not involved in the recognition of β-glucans by neutrophils
Neutrophils were FACS-purified into CD11R1-positive (purity: 99.3% ± 0.5) and CD11R1-negative (purity: 98.7% ± 1.07) populations. Subsequently, the cells (2 x 10^5 cells) were stimulated with 200 µg/ml of the indicated β-glucan preparations. ROS production was determined via chemiluminescence. Data are shown as the mean relative light units (RLU) ± SEM of three pigs.

We further investigated whether CD11R3 is involved in the β-glucan-induced activation of porcine neutrophils. As shown in Figure 5B, the zymosan-induced ROS production by neutrophils was completely inhibited in the presence of anti-CD11R3 as compared to its isotype-matched control. Likewise, antibodies against CD18 decreased the ROS production, albeit not significantly. In contrast to CR3, the respiratory burst towards all three β-glucans was only slightly affected by inhibiting dectin-1, confirming our previous results. For the β-glucans Macrogard and *Euglena gracilis*, the ability of CD18-specific mAbs to block ROS production by porcine neutrophils was negligible, while blocking CD11R3 induced a reduction in this oxidative burst response.

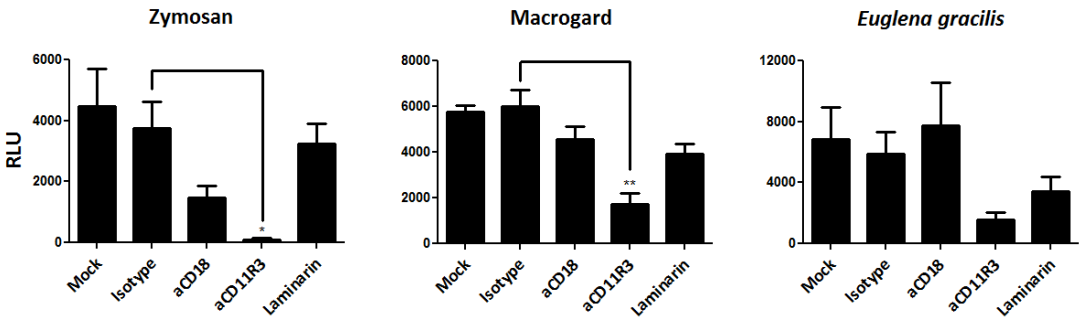


Figure 5B. CR3 is critically involved in the recognition of β-glucans by neutrophils.
Neutrophils (2 x 10^5 cells) were incubated with laminarin (1 mg/ml), aCD18 (5 µg/ml), aCD11R3 (15 µg/ml) or isotype control (IgG_1; 15 µg/ml). Subsequently, the cells were stimulated with 200 µg/ml of the indicated β-glucan preparations. ROS production was determined via chemiluminescence. Values shown are mean relative light units (RLU) ± SEM of four pigs. Values of the negative control have been subtracted from the values represented on the graph.*: $p < 0.05$; **: $p < 0.01$.

3.4.4 Role of dectin-1 and CR3 in the β-glucan-induced cytokine secretion

Besides their anti-microbial activity, innate immune cells secrete danger signals, such as pro-inflammatory cytokines, in response to pathogens to alert the adaptive immune system. Although blocking of CR3 abrogated ROS production, the secretion of TNFα, IL-1β and IL-8 by β-glucan stimulated neutrophils was unaffected by CR3 blocking (Figure 6A). In both neutrophils and macrophages, blocking of dectin-1 by laminarin failed to diminish the cytokine secretion (Figure 6A and B). However, blocking CD11R3 results in a decreased cytokine production upon stimulation with zymosan and Macrogard in porcine macrophages, indicating that CR3 is significantly involved in the β-glucan-mediated cytokine secretion (Figure 6B). The isotype control, CD11R3 mAbs and laminarin did not affect the viability of the cells during our experiments. Remarkably, the cytokine response of neutrophils and macrophages towards Macrogard was very low compared to the other β-glucans.

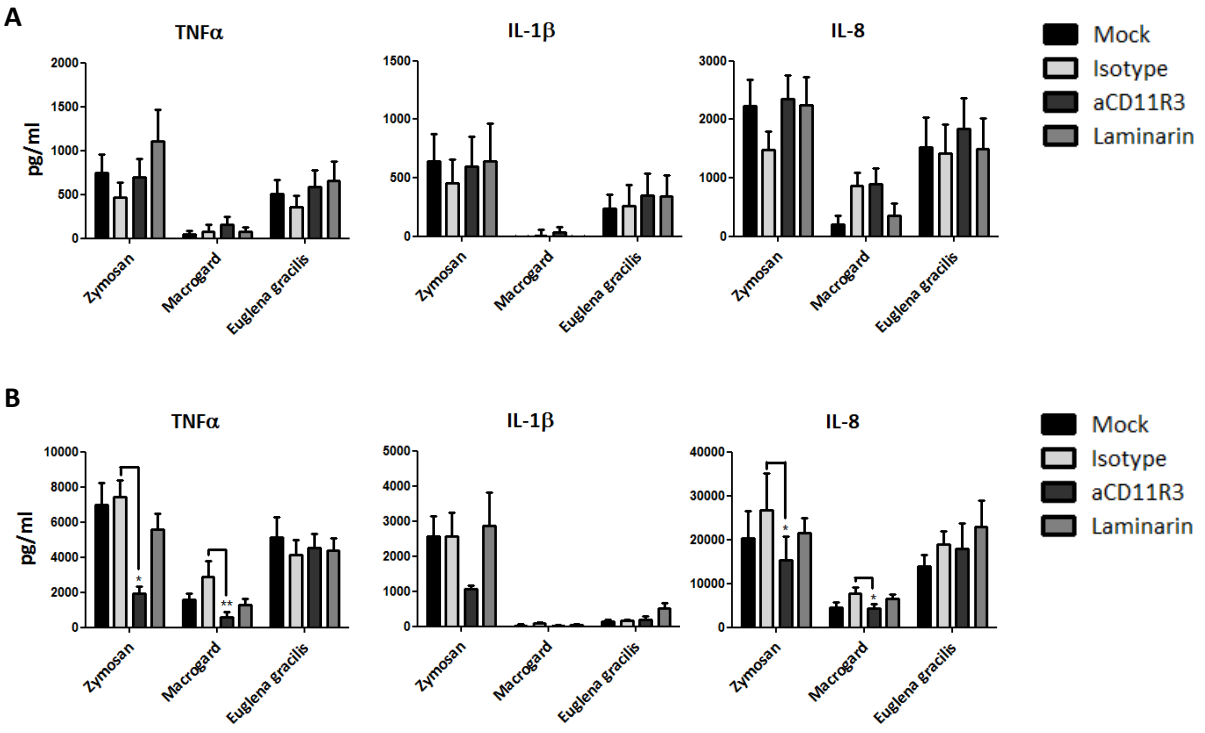


Figure 6. Role of dectin-1 and CR3 in the β-glucan-mediated cytokine response in porcine innate immune cells.
(A) Neutrophils (2 x 10^5 cells) were pre-incubated (1h) with 1 mg/ml laminarin, 3 µg/ml αCD11R3 or 3 µg/ml isotype control. β-glucans (200 µg/ml) were then added to the cells, and after 16 hours the supernatants was collected. Cytokine secretion (TNFα, IL-8 or IL-1β) was assessed by ELISA. **(B)** Monocyte-derived marcophages (2 x 10^5 cells) were pre-incubated (1h) with 1 mg/ml laminarin, 15 µg/ml αCD11R3 or 15 µg/ml isotype control. β-glucans (200 µg/ml) were then added to the cells, and after 16 hours the supernatants was collected. Cytokine secretion (IL-8, TNFα or IL-1β) was assessed by ELISA. The results illustrate the mean cytokine concentration ± SEM of four pigs. Values of the negative control have been subtracted from the values represented on the graph. Medium control values were below 22 pg/ml for TNFα, 85 pg/ml for IL-1β and 208 pg/ml for IL-8 secretion by neutrophils, while they were below 148 pg/ml for TNFα, 128 pg/ml for IL-1β and 4500 pg/ml for IL-8 secretion by macrophages. *: $p < 0.05$; **: $p < 0.01$.

3.4.5 Dectin-1 silencing in porcine macrophages does not interfere with the β-glucan-mediated cytokine secretion

To exclude the possible inability of laminarin to block porcine dectin-1, dectin-1 expression was reduced with 60.74% (± 6.03) compared to basal expression levels in primary macrophages by siRNA transfection. Furthermore, the CR3 expression (Figure 7A) and viability (Figure 7B) of the macrophages was unaffected by this siRNA transfection. However, dectin-1 knock down did not interfere with the β-glucan-induced cytokine secretion in porcine macrophages (Figure 7C), implying that other receptors may play a role in β-glucan signalling. Indeed, macrophages have several other β-glucan receptors, such as CD36 and SCARF1, which may be involved in β-glucan recognition and signalling.[134]

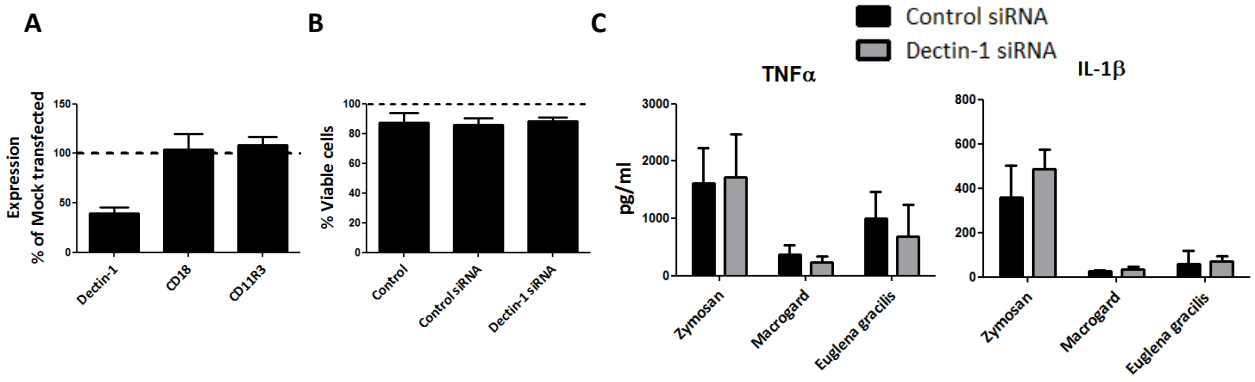


Figure 7. Dectin-1 is not involved in the β-glucan-mediated cytokine response in porcine monocyte-derived macrophages.
Monocyte-derived macrophages (2 x 10^5 cells) were transfected with 50 nM dectin-1 siRNA or control siRNA for 48h prior β-glucan stimulation (200 µg/ml). **(A)** Dectin-1 expression was silenced to 48.56% ± 5.39 as compared to basal expression levels in primary macrophages. **(B)** The viability of macrophages appeared unaffected by this siRNA transfection. **(C)** Cell supernatants were analysed for TNFα and IL-1β by ELISA and lysates were subjected to qPCR. Data represent mean cytokine concentration ± SEM of three pigs. Values of negative control have been subtracted from the values represented on the graph.

3.4.6 The FAK signalling pathway is critically involved in the β-glucan-induced signalling in neutrophils and macrophages

Engagement of integrin receptors phosphorylates FAK, while ligation of dectin-1 activates Syk.[97] In order to define the role of Syk and FAK signalling in the β-glucan-induced signalling in porcine neutrophils and monocyte-derived macrophages, these two pathways were blocked by Syk and FAK specific inhibitors. The inhibitors did not influence CR3 expression or viability of neutrophils during our experiments. Remarkably, blocking the FAK signalling pathway significantly reduced the β-glucan-induced ROS production in neutrophils and macrophages, whereas blocking the Syk signalling pathway only marginally reduced ROS production in both cell types (Figure 8A and B). The latter did not result from species-specific differences in the capacity of R406 to block Syk, as this inhibitor significantly inhibited the immune complex-mediated ROS production (Figure 8C), ruling out species-specific differences in R406's ability to inhibit Syk signalling. Furthermore, the PMA-induced ROS production in neutrophils was unaffected by R406 and PF 573228 treatment, while other FAK (FAK inhibitor 14) or Syk (piceatannol) inhibitors did interfere with the ROS production in neutrophils (data not shown).

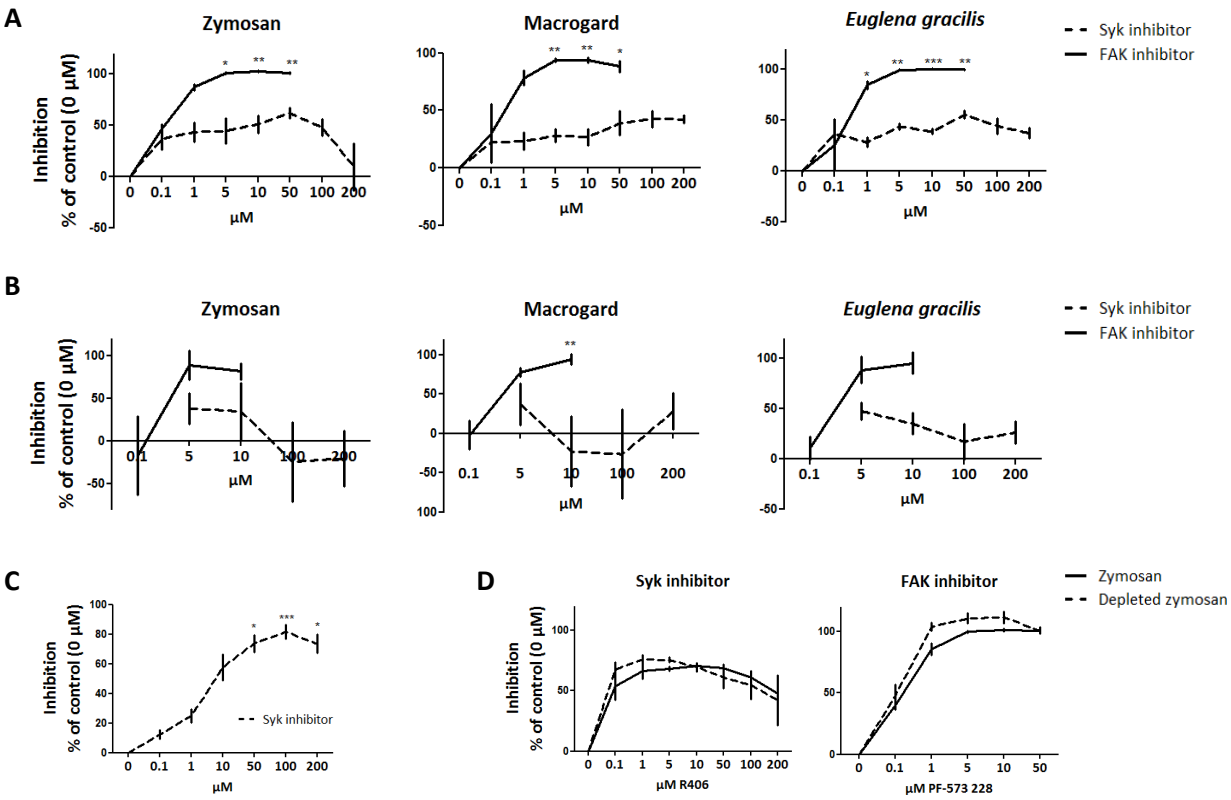


Figure 8. Focal adhesion kinase (FAK) is critically involved in the β-glucan signalling by porcine neutrophils and monocyte-derived macrophages.
Neutrophils (2 x 10^5 cells) **(A)** or monocyte-derived marcophages (2 x 10^5 cells) **(B)** were pre-incubated for 1h with Syk (R406) or FAK (PF-573 228) inhibitors at the indicated concentrations. 200 µg/ml β-glucans (Macrogard, zymosan or *Euglena gracilis*) were then added and ROS production was measured by a chemiluminescence assay. The results are presented as mean relative percentage inhibition ± SEM (% inhibition) of at least four pigs. Values of the negative control have been subtracted from the values represented on the graph. **(C)** The efficacy of the Syk inhibitor (R406) was assessed by stimulating neutrophils with immune complexes in the presence of R406. The results show the mean relative percentage inhibition ± SEM of four pigs. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. **(D)** Neutrophils (2 x 10^5 cells) were pre-incubated (1h) with the indicated concentrations of Syk (R406) or FAK (PF-573 228) inhibitors. Subsequently, the cells were stimulated with (depleted) zymosan. ROS production was measured by a chemiluminescence assay and the results are presented as mean relative percentage inhibition ± SEM (% inhibition) of four pigs.

Zymosan, a crude extract consisting of β-glucans, mannans and proteins, triggers not only β-glucan-mediated signalling pathways, but also TLR2 signalling.[71, 190, 387] As such we have evaluated the oxidative burst towards depleted zymosan, a hot alkali treated preparation of zymosan with no TLR-stimulating properties, by porcine neutrophils. Zymosan and depleted zymosan displayed an identical ROS production (Figure 7D), suggesting that only the binding of β-glucans to CR3 triggers the FAK signalling pathway in neutrophils, leading to an oxidative burst response.

3.4.7 β-glucan-induced FAK signalling controls cytokine secretion by neutrophils and macrophages

In the previous cytokine assay, we could not detect a role for CR3 or dectin-1 in the β-glucan-mediated cytokine response in neutrophils. Conversely, blocking FAK resulted in a strongly reduced secretion of IL-8 and IL-1β in neutrophils upon zymosan and *Euglena gracilis* stimulation, while this effect was less pronounced for TNFα secretion (Figure 9A). Interestingly, inhibition of Syk signalling only reduced IL-1β secretion in *Euglena gracilis* stimulated neutrophils. In macrophages a similar response was observed (Figure 9B). Both TNFα and IL-1β secretion was significantly reduced upon blocking of FAK signalling in zymosan- and *Euglena gracilis* stimulated macrophages. Similar to neutrophils, inhibition of Syk signalling also reduced IL-1β secretion by *Euglena gracilis* stimulated macrophages. These results are not due to an increased cell death, as the FAK inhibitor PF 573228 did not affect the viability of the cells during our experiments, while the Syk inhibitor R406 only reduced cell viability by 30% in neutrophils.

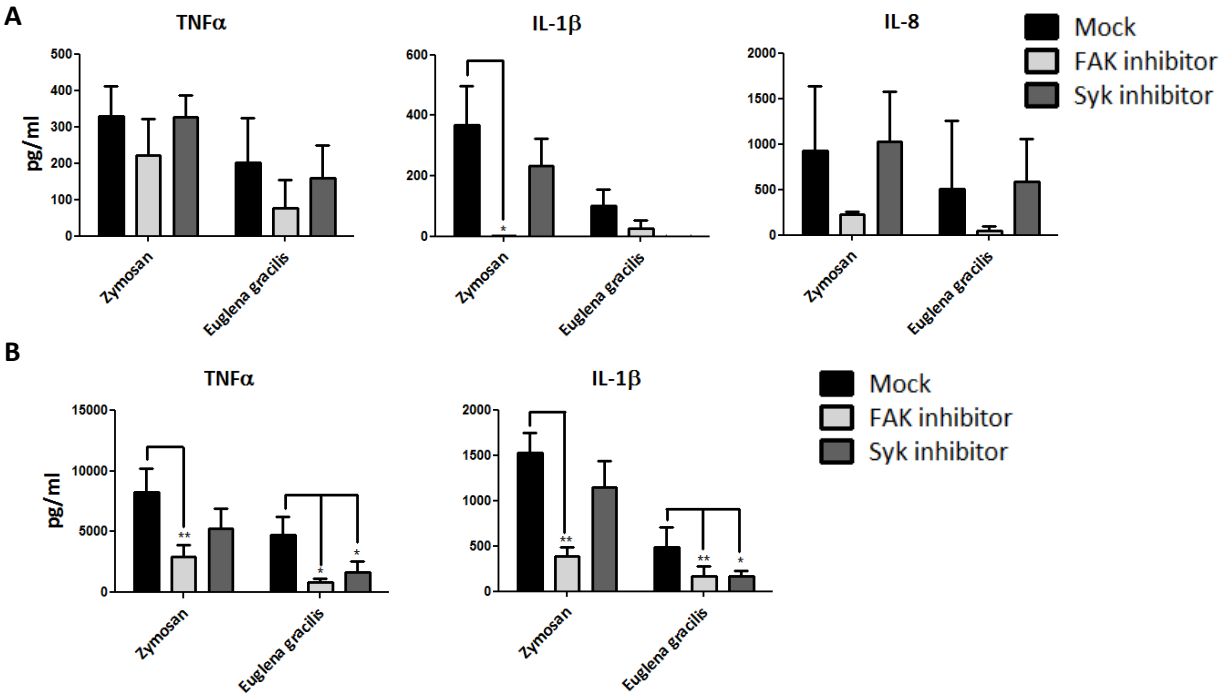


Figure 9. Focal adhesion kinase (FAK) is significantly involved in the β-glucan-mediated cytokine secretion in porcine neutrophils and monocyte-derived macrophages.
(A) Neutrophils (2 x 10^5 cells) were pre-incubated for 1h with 100 µM Syk (R406) or 5 µM FAK (PF-573 228) inhibitors. 200 µg/ml β-glucans (Macrogard, zymosan or *Euglena gracilis*) were then added and after 16h cell supernatants were analysed for TNFα, IL-1β and IL-8 by ELISA. The results are presented as mean cytokine concentration ± SEM of four pigs. Values of the negative control have been subtracted from the values represented on the graph. **(B)** Monocyte-derived macrophages (2 x 10^5 cells) were pre-incubated for 1h with 5 µM Syk (R406) or 5 µM FAK (PF-573 228) inhibitors. 200 µg/ml β-glucans (Macrogard, zymosan or *Euglena gracilis*) were then added and after 16h cell supernatants was analysed for TNFα and IL-1β by ELISA. The results are presented as mean cytokine concentration ± SEM of six pigs. Values of the negative control have been subtracted from the values represented on the graph. *: $p < 0.05$; **: $p < 0.01$.

3.5 Discussion

Different β-glucans induce oxidative burst responses and cytokine secretion in porcine innate immune cells,[380] however, little is yet known about the differential contribution of the β-glucan receptors CR3 and dectin-1 to induce these biological activities. Several studies in mice have established that dectin-1 is the most efficient β-glucan receptor on immune cells,[75, 81, 125, 148, 149] while recent studies in man have reported that CR3 is more important in these responses.[156, 372] Domestic pigs as translational model have much to offer, since recent genome wide studies indicate that pigs are immunologically far closer to man than mice.[253, 388] Here, we used pigs to investigate the specific role of two β-glucan receptors, dectin-1 and CR3, in the response of innate immune cells towards different particulate β-glucans. Recent studies in humans clearly showed that phagocytosis of and ROS production mediated by zymosan as well as whole yeast by neutrophils was completely dependent on CR3,[372] while in monocytes distinct receptors are involved in the β-glucan-mediated oxidative burst.[156] This implies that the recognition of β-glucans is cell type-specific. Indeed, similar to their human counterparts, our present study shows that the β-glucan-mediated ROS production and phagocytosis in porcine neutrophils is CR3-dependent, while in macrophages different receptors are responsible for the cellular responses towards β-glucans. In contrast with these findings in humans and pigs, Li et al. (2011) demonstrated in mice that both dectin-1 and CR3 are crucial for β-glucan-induced responses in neutrophils, whereas dectin-1 alone serves as a β-glucan receptor in macrophages. Furthermore, they demonstrated that in neutrophils dectin-1 activates CR3 in a vav protein-dependent manner to recognise and phagocytose β-glucans, while vav-proteins have no significant role in the phagocytosis of zymosan in macrophages.[125] Conversely, our results point out that CR3 alone serves as a β-glucan receptor in porcine neutrophils, while dectin-1 could assist CR3 to respond towards β-glucans by vav proteins in macrophages. The similarity between our data (pig) and human studies and the dissimilarity with those of mice studies may be due to species-differences, indicating that the pig is a more valuable model than the mouse to study the β-glucan-mediated effects on innate immunity.

We further investigated the role of dectin-1 and CR3 in β-glucan-mediated responses by inhibiting their signalling pathway. The CR3-downstream signalling kinase FAK is critically involved in both ROS and cytokine production by porcine neutrophils and macrophages stimulated with all three β-glucans, while blocking Syk (dectin-1 signalling pathway) could only partially affect these responses. This observation was not consistent with previously published results in mice,[98, 125] where it was shown that Syk is critically involved in the β-glucan-induced dectin-1 activation in murine neutrophils and a subpopulation of macrophages. Similar to our study, a recent human study demonstrated that

Syk is not involved in the antimicrobial response towards particulate β-glucans in monocytes.[156] However, Bose et al.[156] could not detect a reduced ROS production in human monocytes when blocking FAK upon stimulation with particulate β-glucans. Regardless, we report for the first time that porcine neutrophils and macrophages signal via FAK, which serves as a master switch regulating responses towards particular β-glucan in both cell types. The partial contribution of Syk in the response towards β-glucans could be explained by the activation of Syk through integrin engagement. Indeed, ligation of CR3 will activate Src kinases, which in turn can phosphorylate ITAM-containing adaptor proteins, leading to docking sites for Syk kinases.[130, 131] However, this signalling pathway is not crucial for β-glucan-mediated responses, since cytokine secretion is only slightly affected in the absence of Syk.

Taken together, we propose that β-glucans are recognised by the α_M subunit of CR3 in porcine innate immune cells. In pigs, two candidate receptors for the α_M subunit of CR3, namely CD11R1 and CD11R3, were described.[124] Here, we report that detection of β-glucans by CR3 requires only CD11R3. CD11R3 possess clearly equivalent functionality as the α_M subunit of human CR3, however, whether this receptor is involved in complement function as well has not been investigated. Furthermore, we suggest that binding of β-glucans to CD11R3 will activate Src kinases, which will induce several downstream signalling events, such as activation of FAK and Syk, the latter activating FAK as well. Besides this CR3 signalling pathway, we believe that in porcine macrophages dectin-1 and other putative β-glucan receptors, such as the scavenger receptors CD36 and SCARF1, are responsible for β-glucan recognition as well.

It is interesting to note that the minimal structural differences between zymosan, Macrogard and *Euglena gracilis* affect biological outcomes such as phagocytosis, ROS production and cytokine secretion. We detected only a marginal cytokine response by porcine neutrophils and macrophages upon stimulation with Macrogard (200 μg/ml), while zymosan and *Euglena gracilis* were able to trigger cytokine secretion in both neutrophils and macrophages. As Macrogard is able to induce cytokine secretion in innate immune cells at lower concentrations,[380] we suggest that Macrogard has a dose-dependent immunomodulatory effect. Macrogard has a β(1,3)-linked backbone with β(1,6)-linked branches, thus we suspect that the large β(1,3)(1,6)-linked glucans stereochemically interfere with each other and as a result less receptors become bound. Indeed, β-glucans derived from *Euglena gracilis* do not have branches, and zymosan has non-uniform branches, indicating that differences in branch frequencies are important for their immune stimulating properties.

3.6 Conclusions

In conclusion, porcine neutrophils detect β-glucans via CR3, while porcine macrophages use multiple receptors to detect and respond to β-glucans. However, in both cell types FAK serves as a master regulator in β-glucan-mediated responses. Furthermore, this study supports the use of pigs as a valuable model to evaluate the effects of β-glucans on innate immunity, since these results are more related to the results of human studies than mice studies. Thus, further studies in pigs can reveal the mechanism of β-glucan recognition and immune activation in pigs and humans. A better understanding of this mechanism could lead to a rationale-based decision process to improve health and as in-feed additives in livestock species and to optimise β-glucans as nutraceuticals in humans.

3.7 Acknowledgements

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CHAPTER 4:

Duality of β-glucan microparticles: antigen carrier and immunostimulants

Baert K., De Geest B., De Greve H., Devriendt B., Cox E.

4.1 Abstract

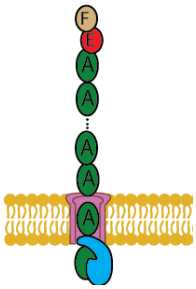
Designing efficient recombinant mucosal vaccines against enteric diseases is still a major challenge. Mucosal delivery of recombinant vaccines requires the encapsulation in potent immunostimulatory particles to induce an efficient immune response. This article evaluates the capacity of β-glucan microparticles (GPs) as antigen vehicle and characterises their immune-stimulatory effects. The relevant infectious antigen FedF was chosen to be loaded inside the microparticles. The incorporation of FedF inside the particles was highly efficient (84.53%) and occured without antigen degradation. In addition, these β-glucan microparticles have immunostimulatory effects as well, demonstrated by the strong reactive oxygen species (ROS) production by porcine neutrophils upon their recognition. Although antigen-loaded GPs still induce ROS production, antigen loading decreases this production by neutrophils for reasons yet unknown. However, these antigen-loaded β-glucan microparticles are still able to bind their specific β-glucan receptor, demonstrated by blocking complement receptor 3, which is the major β-glucan receptor on porcine neutrophils. The dual character of these particles is confirmed in a T cell proliferation assay. FedF-loaded particles induce a significantly higher FedF-specific T cell proliferation than soluble FedF. Taken together, these results show that β-glucan microparticles are efficient antigen carriers with immune-stimulatory properties.

4.2 Introduction

Mucosal vaccination is the most efficient way to protect man and animals against enteric infections. However, the development of effective mucosal subunits vaccines encounters multiple challenges, such as antigen degradation and limited immunogenicity.[33-36] The encapsulation of antigens in microparticles is a promising approach to overcome these problems, as they can protect the antigens against degradation as well as carry potent adjuvants or immune modulators to enhance the immunogenicity.[313-315] Frequently used particle system are those based on poly(lactic-co-glycolic acid) (PLGA), however, low antigen encapsulation efficiency and antigen degradation during their formation limits their use in commercial vaccines.[389] On the contrary, β-glucan microparticles (GP) are emerging microparticles known for their safety, immunogenicity and high antigen encapsulation efficiency.[21-29, 328] These promising antigen-carriers are derived from the cell wall of *Saccharomyces cerevisiae* (Baker's yeast) and are composed of >85% β-1,3-D-glucan polymers (β-glucans), ~2% chitin and <1% lipids and proteins, with the rest being mostly ash and moisture.[26] The main component of these particles, the β-glucans, is a 'microbe-associated molecular pattern' (MAMP), which is very interesting in vaccine development for their immunostimulating characteristics.[183, 187] We have previously reported that particulate β-glucans are mainly recognised by complement receptor 3

(CR3) on porcine innate immune cells and require the signalling molecule FAK to perform their immunostimulatory responses.[390] However, which receptor is involved in the immunostimulating characteristics of GPs is not yet known. Many articles have described the strong potency of these β-glucan microparticles to elicit durable immune responses in mice.[21, 22, 25, 32, 165] Incubation of mouse bone marrow derived dendritic cells (BMDCs) with ovalbumin (OVA) loaded GPs resulted in phagocytosis, upregulation of maturation markers, rapid proteolysis of ovalbumin and proliferation of OVA-reactive transgenic $CD8^+$ OT-I and $CD4^+$ OT-II T cells.[25] However, the immunological effect of these particles on porcine innate immune cells has not been investigated yet.

Moreover, besides the incorporation of model antigens, no clinically relevant antigens were incorporated in these β-glucan microparticles. We have incorporated FedF, the tipadhesin of F18 fimbriae, inside the particles, since this antigen is one of the most important virulence factors of $F18^+$ *Escherichia coli*. *E. coli* carrying the F18 fimbriae colonise the small intestine of pigs and cause post-weaning diarrhoea and edema disease in pigs, resulting in economic losses to the pig production industry.[391] One of the most important virulence factors of these infections are the fimbriae, by which the pathogen adhere to the intestinal epithelial cells and colonise the intestine. There are two closely related antigenic variants of F18, namely F18ab and F18ac. In general, post-weaning diarrhoea (ETEC infections) is caused by the F18ac variant whereas F18ab is more related to oedema disease (STEC infections).[9] Porcine ETEC strains can produce 5 fimbrial types (F4, F18, F41, F5, F6) of which F4 and F18 are most frequently associated with ETEC-induced diarrhea.[9] The F18 fimbriae are polymeric proteins composed of several subunits: FedA is the structural subunit,[392] FedB the outer membrane usher, FedC the periplasmic chaperone, FedE the minor pilin and FedF the adhesin, which is essential for fimbrial adhesion (Figure 1).[393, 394] These subunits also regulate the length of the F18 fimbriae, which are normally 1-2 µm in length.[395] The FedF subunit of F18 fimbriae is located in a dedicated single copy adhesin at the distal tip of the fimbriae.[396] Tipadhesins are usually composed of two immunoglobulin (Ig)-like domains: an N-terminal lectin or receptor binding module and a C-terminal pilin. As it needs a complementary β-strand from the FedE subunit, using donor strand complementation is necessary to achieve a stable recombinant expression of FedF.[393] To protect pigs against $F18^+$ ETEC infections, local intestinal immunity is required. However, oral immunisation with F18 fimbriae does not elicit protection in a piglet model, probably due to the presence of immunodominant epitopes in the FedA subunit[397] or release of FedF from the fimbriae.[398] We reasoned that oral immunisation of piglets with purified FedF subunit would enable induction of protective immunity, but only if FedF is encapsulated inside microparticles in order to deliver higher amounts of antigens to the gut-associated lymphoid tissue (GALT) and to induce strong immune responses by mimicking pathogen dimensions.



We have incorporated FedF inside β-glucan microparticles for oral administration to induce intestinal FedF-specific antibody responses. In this article, we investigated the capacity of these β-glucan microparticles as both antigen vehicle and immunostimulants. Porcine immune cells were used as translational model, because β-glucan recognition and signalling in porcine innate immune cells is closer related to humans than murine cells.[253, 388, 390]

Figure 1. Schematic representation of F18 fimbriae.
The main component of F18 fimbriae is FedA (green). Adhesin subunits of F18 (FedF) are in grey. Periplasmic chaperones and outer membrane ushers are in purple and blue, respectively. Structural subunits are in red.

4.3 Materials and Methods

4.3.1 Generation of FedF

FedF was generated using the method of De Kerpel et al. (2006).[399] Briefly, the *fedF*$_{15\text{-}165}$ *gene* from F18 *E. coli* 107/86 strain was cloned in the pDEST14 vector under a T7 promotor. The gene was C-terminal truncated and then transformed into *E. coli* strain C43 (DE3). The C43 (DE3) cells were grown, induced with 1 mM IPTG and incubated for 2h. Cells were subjected to osmotic shock and the periplasmic extract was loaded onto a Source 3S column (Amersham, Bucks, UK) for cation exchange in 20 mM Tris pH 7.5. Proteins were eluted at 140 mM NaCl and further purified using gel filtration on a Superdex-75HR column in 10 mM HEPES pH 7.0 and 150 mM NaCl. Finally, the protein was dialysed against 0.9% saline.

4.3.2 Development of β-glucan microparticles and protein encapsulation

Hollow β-glucan microparticles (GPs) were prepared from *Saccharomyces cerevisiae* using a series of alkaline and acidic extraction steps and loaded with FedF as previously described.[25, 26] Therefore, 10 mg of dry GPs were swollen with 5 mg FedF dissolved in 0.9% saline for 2h at 4°C, followed by lyophilisation. These dry GP-FedF preparations were reswollen with 300 µl aqua dest to maximise FedF diffusion into the GPs. Upon relyophilisation, FedF was trapped inside the GPs by adding 350 µl of 25 mg/ml tRNA (derived from torula yeast) in TEN buffer (50 mM Tris HCl with 2 mM EDTA and 0.15 M NaCl, pH 8.2) for 30 minutes at 50°C. To complete the complexation reaction, another 500 µl of 10 mg/ml tRNA was added to the particles. The suspension was centrifuged, washed four times in 0.9% saline and stored at -20°C (2.5 mg/ml).[25] To calculate the amount of FedF trapped inside the

GPs, the unbound FedF protein in the wash fractions was measured by BCA against a BSA standard (0.05-1 mg/ml).

4.3.3 Western blot

Incorporation of FedF inside the particles was confirmed using a protein gel electrophoresis after digesting the β-glucan microparticles with β-1,3-D-glucanase from *Helix pomatia* (Sigma). FedF-loaded β-glucan microparticles were first incubated in β-glucanases (1 mg/ml) diluted in 150 mM sodium acetate buffer (Sigma, Diegem, Belgium) for 24h at 37°C. Proteins, released in the supernatants, were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) consisting of a 12% separating gel and a 4% stacking gel. The gels were prepared by dissolving 30% acrylamide in Tris (pH 8.8), 10% SDS, 10% APS and TEMED. To load the samples, the proteins were mixed in equal volume ratios with loading buffer containing β-mercaptoethanol and heated at 100°C for 5 minutes. Electrophoresis was performed at 28 mA for 1h. After separating, the proteins were transferred to a 0.45 µm PVDF membrane (GE Healthcare, Diegem, Belgium) at 40 V for 2h. Next, the membrane was blocked (1h at room temperature) with 5% milk powder diluted in PBS + 0.1% Tween®20 to prevent antibodies from non-specifically binding to the membrane. Monoclonal anti-mouse FedF-specific antibodies (10 µg) were added overnight to the blot, followed by horseradish peroxidase conjugated rabbit anti-mouse IgG (1/2000; Dako, Heverlee, Belgium) for 1 hour at room temperature. The bands were visualised using ECL western blotting substrate (Pierce, Merelbeke, Belgium) and ChemiDOC™ MP imaging system (Biorad, Temse, Belgium).

4.3.4 Oxidative burst by porcine neutrophils

All animal experiments were approved by the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University (EC2013/62). Piglets (between 4 and 20 weeks old) were housed under standard conditions as blood donors. Peripheral blood was collected on heparin from the jugular vein of four pigs. Subsequently, neutrophils were isolated by density gradient centrifugation on a discontinuous Percoll gradient (68% and 75%) (GE healthcare) as described previously.[380] To examine the immunostimulating characteristics of the particles, the ability of the β-glucan microparticles to induce an oxidative burst response by porcine neutrophils was investigated. The production of reactive oxygen species (ROS) was measured by a chemiluminescence assay described by Donne et al. (2005) with some modifications.[381] Neutrophils were seeded in a 96-well plate at 2.0 x 10^5 cells/well in RPMI without phenol-red. The plates were incubated at 37°C for 2h in a

humidified atmosphere with 5% CO_2 to allow the cells to adhere to the plastic surface. Subsequently, the supernatant was replaced by 175 µl luminol (0.5 mM). After 5 minutes of background measurement at 37°C, 25 µl of the indicated β-glucan microparticles (400, 200 or 100 µg/ml) were added. Stimulation of the cells with phorbol myristate acetate (PMA; 50 µg/ml) was used as a positive control. ROS production was then measured during 120 min in the integration mode. All stimulations were performed in duplicate. The ROS production is expressed as relative light units (RLU).

4.3.5 Determination of the β-glucan receptor in porcine neutrophils

To analyse the role of dectin-1 and CR3 in the immunostimulating characteristics of these β-glucan microparticles, the production of reactive oxygen species (ROS) by neutrophils was measured using a chemiluminescence assay as described above with some modifications. Neutrophils were seeded in a 96-well plate at 2.0 x 10^5 cells/well. The plates were incubated at 37°C for 2h in a humidified atmosphere with 5% CO_2 to allow the cells to adhere to the plastic surface. Then, the cells were pre-treated with the β-glucan receptor inhibitors for 1 hour at 37°C. To inhibit dectin-1, laminarin (1 mg/ml) was added to the cells. To inhibit CR3-mediated responses, monoclonal antibodies (mAbs; mouse IgG_1 isotypes) against CD18 (5 µg/ml) and CD11R3 (15 µg/ml) (AbD Serotec, Kidlington, UK) were added. These antibodies were dialysed against PBS to remove sodium azide. An isotype-matched mAb control (IgG_1) was added to the cells to measure background inhibition. Subsequently, luminol was added to the cells (0.5 mM diluted in HBSS + Ca^{2+} Mg^{2+}) and, after 5 minutes of background measurement at 37°C, 25 µl of the β-glucan microparticles (200 µg/ml) were added. Stimulation of the cells with phorbol myristate acetate (PMA; 50 µg/ml) was used as a positive control. ROS production was then measured during 120 min in the integration mode. All stimulations were performed in duplicate. ROS production is expressed as relative light units (RLU).

4.3.6 Generation of monocyte-derived dendritic cells (MoDCs)

Peripheral blood monomorphonuclear cells (PBMCs) were isolated by density gradient centrifugation on Lymphoprep (NYCOMED Pharma AS, Life Technologies, Merelbeke, Belgium). Furthermore, monocytes were enriched from the PBMCs to a purity of >95% by positive immunomagnetic bead selection (MACS; Miltenyi Biotec, Leiden, The Netherlands) using the anti-SIRPα monoclonal antibody (clone 74-12-15a[400]). These cells were seeded in a 24-well plate at a density of 0.5 x 10^6 cells/ml in MoDC medium (DMEM supplemented with 10% FCS, 1% P/S, recombinant porcine 1/200 GM-CSF[401]

and 5 ng/ml IL-4). After three days incubation at 37°C in a humidified atmosphere at 5% CO2, we added fresh medium supplemented with GM-CSF and IL-4 at the same concentration to cells to generate monocyte-derived dendritic cells as previously described.[402]

4.3.7 Antigen presentation assay

To investigate whether incorporation of FedF inside the particles is necessary to induce FedF-specific cellular immune responses, a coculture of MoDCs and $CD6^+$ T cells was set up. Therefore, T cells isolated from pigs immunised intramuscularly with 250 µg purified FedF in incomplete Freund's adjuvant were used [35]. $CD6^+$ T cells were enriched from PBMCs to a purity of > 95% by positive immunomagnetic selection with the α-CD6 mAb (IgG1, clone a38b2; [36]) and goat anti-mouse microbeads (MACS). MoDCs were stimulated with 8 µg FedF or FedF-loaded GPs (8 µg FedF encapsulated) for 24 h or left untreated (control). These stimulated MoDCs were subsequently co-cultured at titrated numbers with 1.0 x 10^5 autologous CD6+ T cells for 5 days and the [3H]methyl-thymidine (1 µCi/well; Amersham ICN, Bucks, UK) incorporation (18h) was measured using a β-scintillation counter (Perkin-Elmer).

4.3.8 Statistics

Data are presented as the mean ± SEM. Data were analysed with GraphPad Prism 5 using the paired t-test for comparison of two data and using a repeated measures ANOVA (Post hoc: Tukey) for comparison of multiple data sets. Data were considered significant at $p < 0.05$.

4.4 Results and Discussion

4.4.1 Efficient loading of FedF inside β-glucan microparticles

To assess if loading of FedF into GPs affects the antigenicity, we performed Western blotting. We first confirmed the high loading capacity of GPs, as FedF was incorporated inside the β-glucan microparticles with a loading efficiency of 84.53% ± 2.89 (Figure 2A). Furthermore, we examined by gel electrophoresis the correct incorporation of FedF inside the particles. Therefore, we first destroyed the β-glucan particles by β-glucanases, whereafter the supernatants with the released antigens were loaded on a protein gel electrophoresis and detected by monoclonal FedF-specific antibodies (clone IMM04[398]). Figure 2B illustrates the protein FedF in lane 1 and the FedF released

from the digested GPs in lane 2. As shown, both FedF and the released FedF migrate near the expected MW of 18 kDa. No degradation is visible indicating the correct incorporation of FedF inside the particles. Based on these results we conclude that FedF is efficiently incorporated inside the particles.

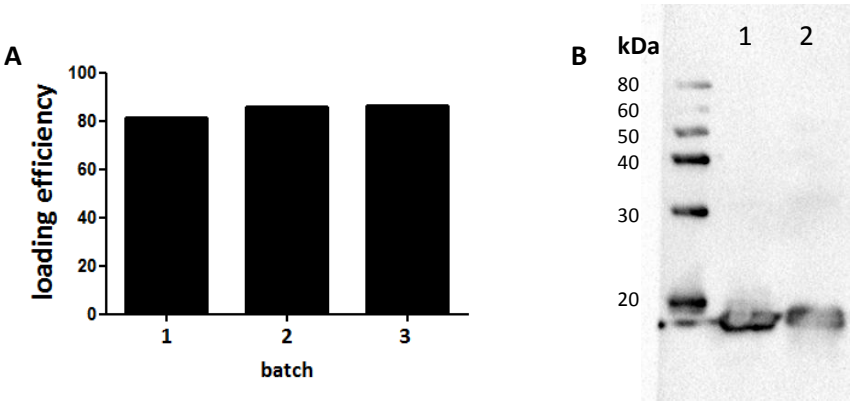


Figure 2. FedF was efficiently incorporated inside β-glucan microparticles.
(A) The loading efficiency of FedF inside GPs was determined by BCA. The unbound FedF protein in the wash fractions was measured by BCA against a BSA standard (0.05-1 mg/ml). **(B)** FedF incorporation inside the GPs was analysed by Western blotting after digesting the particles with β-glucanases. FedF was detected with specific monoclonal antibodies against FedF. Lane 1 illustrates FedF, while the FedF released from the GPs upon digestion is shown in lane 2.

4.4.2 FedF-loaded β-glucan microparticles still possess immune-stimulatory effects

To assess the effect of antigen loading on the inherent immunostimulating characteristics of the GP, we determined the oxygen radical production of porcine neutrophils after stimulation with antigen-loaded particles. Figure 3 demonstrates that FedF-loaded GPs possess immune-stimulatory effects. Interestingly, hollow β-glucan microparticles could stimulate ROS production by neutrophils more than antigen-loaded particles, indicating that antigen loading partially masks the β-glucans of the particles from recognition by their receptors, dectin-1 and complement receptor 3 (CR3). [390] However, using confocal microscopy we failed to detect any FedF associated with the surface of the particles (data not shown).

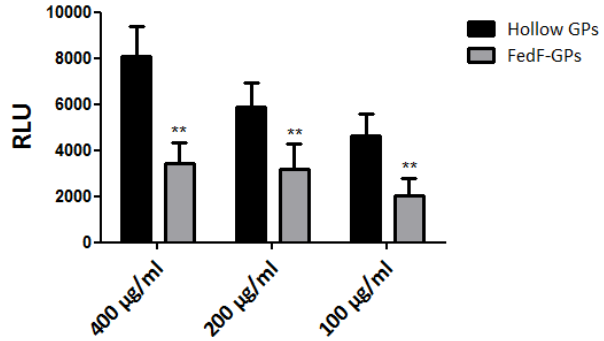


Figure 3. FedF-loaded β-glucan microparticles still possess their adjuvant function.
Neutrophils (2 x 10^5 cells) were stimulated with GPs at the indicated concentrations. ROS production was determined via chemiluminescence. Data are shown as the mean relative light units (RLU) ± SEM of four pigs. **: $p < 0.01$. Values of the negative control have been subtracted from the values represented on the graph.

4.4.3 The immune-modulatory effects of β-glucan microparticles are mainly mediated by binding to the α subunit of complement receptor 3 (CR3)

We further investigated which β-glucan receptor is involved in the particle-induced activation of porcine neutrophils. As shown in Figure 4, the β-glucan microparticles-induced ROS production by neutrophils was significantly inhibited in the presence of anti-CD11R3 as compared to its isotype-matched control. In contrast to the α subunit of CR3, the ability of CD18-specific mAbs to block ROS production by porcine neutrophils was negligible (β subunit of CR3). Interestingly, blocking dectin-1 by laminarin decreased the ROS production as well, albeit not as much as CD11R3-specific mAbs. Although incorporation of antigens inside the particles seems to influence the recognition of GPs by dectin-1, CD11R3 is still the most important receptor for the GP-induced ROS production in porcine neutrophils.

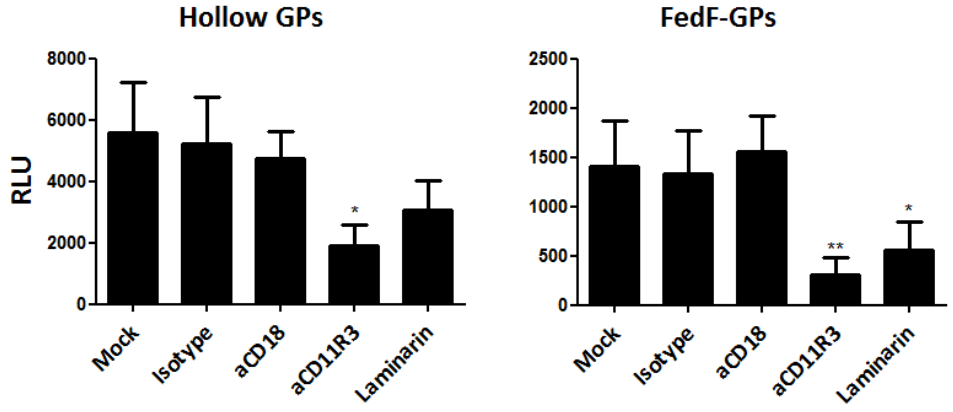


Figure 4. CR11R3 is involved in the recognition of β-glucan microparticles by neutrophils.
Neutrophils (2 x 10^5 cells) were incubated with laminarin (1 mg/ml), aCD18 (5 µg/ml), aCD11R3 (15 µg/ml) or isotype control (IgG_1; 15 µg/ml). Subsequently, the cells were stimulated with 200 µg/ml of the indicated β-glucan microparticles. ROS production was determined via chemiluminescence. Values shown are mean relative light units (RLU) ± SEM of four pigs. Values of the negative control have been subtracted from the values represented on the graph. *: $p < 0.05$; **: $p < 0.01$.

4.4.4 T cell proliferation is significantly increased when FedF is incorporated inside GPs

To assess whether β-glucan microparticles could serve as an immune stimulatory antigen delivery system, we investigated the FedF-specific T cell proliferation after stimulation of MoDCs with FedF-GPs. As illustrated in Figure 5, FedF-loaded GPs promoted the antigen presentation capacity of MoDCs to porcine $CD6^+$ T cells as compared to immature and FedF-stimulated MoDCs, clearly indicating that encapsulation of FedF inside immunostimulatory particles is necessary to induce T cell

proliferation. Interestingly, FedF stimulation of dendritic cells did not influence T cell proliferation, suggesting that FedF has an inhibitory effect on MoDCs.

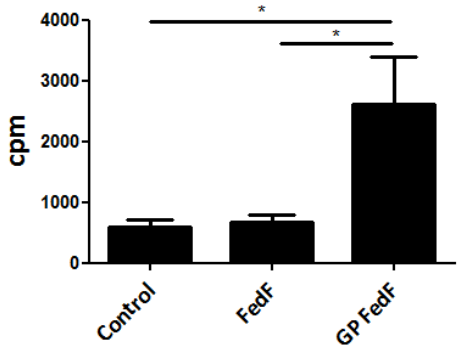


Figure 5. T cell proliferation is significantly increased when FedF is incorporated inside GPs.
Encapsulation of FedF in β-glucan microparticles promotes antigen presentation to T cells. Autologous $CD6^+$ T cells were co-cultured for 5 days with immature, FedF (8 µg) or FedF-loaded GPs (8 µg FedF encapsulated) stimulated MoDCs. The data represent the mean cpm ± SEM (n = 4). The spontaneous proliferation of the CD6+ responder cells equalled 78.25 ± 14.15 cpm, while for ConA-stimulated cells the proliferation amounted to 15203 ± 5622.9 cpm. Control: immature MoDCs. The asteriks indicates a significant difference between stimulated and immature MoDCs. *, $p < 0.05$.

4.5 Conclusions

β-glucan microparticles are emerging particles known for their high antigen loading capacity and safety. In the current study, we have evaluated the incorporation of a clinically relevant infectious disease antigen FedF, the tipadhesin of F18 fimbriae, inside these particles. The results clearly demonstrate that FedF is incorporated intact inside the particles with a high loading efficiency (84.5%). Besides antigen carrier, these particles are able to stimulate the innate immune system by binding to the α subunit of complement receptor 3 (CR3). To confirm the duality of these particles, we have demonstrated in a T cell proliferation assay that FedF inside the particles is processed by MoDCs and subsequently presented to T cells, resulting in their activation and proliferation. Taken together, FedF-loaded β-glucan microparticles are promising vaccine candidates in the protection of pigs against $F18^+$ ETEC and STEC infections and this study motivates exploring this vaccine *in vivo*.

4.6 Acknowledgements

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CHAPTER 5:

β-glucan microparticles targeted to epithelial APN as oral antigen delivery system

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5.1 Abstract

Enteric diseases still have a devastating impact on global health. Oral vaccination is crucial to prevent intestinal infections, since only vaccines delivered to the intestinal tract elicit potent immune responses at the site of pathogen entry. However, oral vaccines encounter multiple barriers, including poor uptake and tolerance mechanisms, preventing the immune system to react to innocuous environmental antigens. Antigen delivery systems combined with selective targeting seem a promising strategy to overcome these obstacles. The current study evaluates the capacity of aminopeptidase N (APN)-targeted β-glucan microparticles (GPs) as antigen delivery system. Antibodies against APN, an intestinal epithelial receptor, are conjugated oriented efficiently to GPs via the biolinker protein G. The resultant microparticles were analysed for their antigen load, adjuvanticity and interaction with enterocytes and dendritic cells (DCs). Functionalisation of GPs with antibodies neither impedes antigen load nor adjuvanticity. In addition, targeting to APN increases the uptake of microparticles by enterocytes and DCs, leading to an enhanced maturation of the latter as evidenced by an upregulation of maturation markers and a strong pro-inflammatory cytokine response. Finally, oral administration of APN-targeted antigen-loaded particles to piglets elicits higher serum antigen-specific antibody responses as compared to control particles. Taken together, these data support the use of APN-targeted GPs for oral delivery of antigens.

5.2 Introduction

Gastrointestinal infections are still the main cause of enteric morbidity and mortality in man and animals. Since parenteral vaccines are unable to elicit potent immune response at the intestinal mucosa, the oral route seems to be the most favourable way to prevent intestinal infections. However, the few current oral vaccines, consisting of live-attenuated or inactivated organisms, often do not completely prevent infection and have a high safety risk profile.[403] Therefore, several subunit vaccines with recombinant or purified antigens are currently being explored to avoid these problems. Until now no oral subunit vaccine affording protection against intestinal infections has been commercialised as their development encounters multiple challenges, such as instability and limited immunogenicity.[33-36] Antigen encapsulation in nano/microparticles is a promising approach to overcome these problems, as it can protect the antigens against degradation as well as carry potent adjuvants or immune modulators to enhance their immunogenicity.[313-315] Great advances have been made in the development of microparticle systems, of which particles based on poly(lactic-co-glycolic acid) (PLGA) are the most used one. However, low antigen encapsulation efficiency and the necessity to dissolve antigens in organic solvents limits their use in commercial vaccines.[389]

β-glucan microparticles (GPs) represent an alternative antigen particle system for oral delivery. These hollow and porous GP are known for their safety, immunogenicity and high antigen encapsulation efficiency.[21-29, 328] These promising antigen carriers are derived from the cell wall of *Saccharomyces cerevisiae* (Baker's yeast) and are mainly composed of β-1,3-D-glucans, 'microbe-associated molecular patterns' (MAMPs) with adjuvant ability.[26, 183, 187] β-glucan microparticles display a strong potency to elicit durable immune responses.[21, 22, 25, 32, 165] For instance, subcutaneous immunisation of mice with GP-OVA induced strong humoral and Th1- and Th17-biased $CD4^+$ T cell responses,[25] while oral administration of GP-OVA resulted in a Th17-biased response and the production of OVA-specific secretory IgA in intestinal fluids.[32] This local immune response is obtained following transepithelial transport of GPs by Peyer's patch M cells,[22, 32, 404] resulting in the accumulation of GPs in $CD11c^+$ phagocytes situated in the Peyer's patch sub-epithelial dome (SED) regions.[22] However, M cells represent only a minor cell population in the intestinal tract and a huge amount of particles is necessary to induce immunity. Villous enterocytes vastly outnumber M cells and possess a transcytotic capability for macromolecules and inert particles, making them interesting target cells.[337] Selective targeting of these microparticles to a transcytotic receptor present on the apical surface of both intestinal villous cells and M cells may further enhance their passage through the epithelial barrier.

Although many approaches have been reported, selective targeting is still a challenge. A classical approach for selective targeting is mediated by recognition of the target via capture molecules, such as antibodies.[337, 355] However, conventional methods to conjugate antibodies to particles often result in a loss of binding activity due to the random orientation and structural or conformational disruption of the antibodies.[365] Here, we took advantage of the antibody-binding properties of the biolinker protein G, which binds the Fc region of the antibody (IgG), to conjugate monoclonal antibodies in a proper orientation to the surface of β-glucan microparticles. To demonstrate the feasibility of this design to functionalise microparticles, aminopeptidase N (APN, CD13) was selected as a target. APN is a conserved membrane glycoprotein expressed by many cell types, including small intestinal enterocytes and dendritic cells. APN is mainly known to regulate the biological activity of various peptides by proteolysis, however, APN displays many other functions as well, such as signalling molecule and receptor for some viruses/bacteria and cholesterol.[405] Indeed, aminopeptidase N as signalling molecule is known to mediate angiogenesis and metastasis of different human tumor cells and is suggested as a suitable target for anti-cancerous therapy.[405] Furthermore, aminopeptides N (APN) is utilised as receptor for viruses and bacteria, such as group I coronaviruses[347] and transmissible gastroenteritis virus (TGEV).[348] Recently, our lab identified porcine APN as an endocytotic receptor for F4 enterotoxigenic *Escherichia coli* (ETEC) in pigs.[260] Moreover, it

has been demonstrated that intestinal APN-targeting results in a strong IgA, IgG and IgM immune response, indicating that APN may represent a promising target for oral delivery of antigens across the epithelial barrier.[260] In addition, crosslinking CD13, expressed on immune cells, positively modulates phagocytosis,[352] making APN an attractive target for both transcytosis and immune stimulation.

The present study aimed to evaluate APN-targeted GPs as candidate antigen delivery system for oral vaccination. We report on the characteristics and adjuvanticity of these microparticles and their functional surface decoration with anti-APN-specific antibodies. The interaction between APN-targeted GPs and intestinal epithelial cells was explored as well. Furthermore, we report on the intracellular uptake of the particles by porcine monocyte-derived dendritic cells (MoDCs) and their capacity to induce DC maturation. Finally, we loaded FedF, a clinically relevant antigen, inside the particles. FedF is the tipadhesin of F18 fimbriae, which are colonization factors of a porcine-specific enterotoxigenic *E. coli* strain. These strains cause diarrhea in infected animals upon FedF-mediated binding of the bacteria to small intestinal epithelial fucosylated glycosphingolipids.[396, 406] These APN-targeted FedF-loaded GPs were orally administered to piglets to investigate their capacity to trigger FedF-specific systemic immunity.

5.3 Materials and Methods

5.3.1 Reagents

Protein G, 2-(N-morpholino)ethanesulfonic acid (MES), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (sulfo-NHS), O-(2-aminoethyl)polyethylene glycol (PEG-NH_2), glycine, sodium borate, dimethyl pimelimidate (DMP), triethanolamine, ethanolamine, luminol, accutase, sheep anti-mouse IgG/FITC, N-acetyl-L-cysteine, cholera toxin (CT) and 3,3',5,5'-tetramethylbenzidine liquid substrate for membranes (TMB) were obtained from Sigma (Diegem, Belgium). Goat anti-mouse IgG-AlexaFluor647 antibody was purchased from Abd Serotec (Kidlington, UK). Percoll gradient was purchased from GE healthcare (Diegem, Belgium). Lymphoprep, latex beads, Sytox red, Phalloïdin-Texas Red, isotype-matched irrelevant antibodies and secondary fluorescent antibodies were purchased from Life Technologies (Merelbeke, Belgium). Cell culture products and reagents, unless mentioned otherwise, were purchased from GIBCO (Life Technologies). Immunomagnetic beads (MACS) were purchased from Miltenyi Biotec (Leiden, The Netherlands). Phorbol myristate acetate (PMA) was obtained from Enzo Life Sciences (Antwerpen, Belgium). Sterile biopsy foam pads were obtained from Simport (Beloeil,

Canada). Human CTLA4-muIgG2a fusion protein was purchased from Ancell (Bayport, USA). Porcine IL-4 and ELISA kits were purchased from R&D systems (Abingdon, UK). Colistin, ProMycine® Pulvis, was obtained from VMD (Arendonk, Belgium). Pariet® was purchased from Janssen-Cilag (Tilburg, The Netherlands). Anti-pig-Ig (H+L), IgA/HRP and HRP-conjugated anti-mouse IgG antibodies were obtained from Bethyl Laboratories (Antwerpen, Belgium).

5.3.2 Preparation of protein-loaded β-glucan microparticles

Hollow β-glucan microparticles (GPs) were prepared from *Saccharomyces cerevisiae* using a series of alkaline and acidic extraction steps and loaded with BSA-FITC as an antigen model as previously described.[25, 26] The resulting BSA-FITC-loaded GPs were washed four times in 0.9% saline and stored at -20°C (2.5 mg/ml). To calculate the amount of BSA-FITC trapped inside the GPs, the unbound BSA-FITC protein in the wash fractions was measured by fluorimetry against a BSA-FITC standard (3.125 – 1000 µg/ml). The incorporation of BSA-FITC into the GPs was 96.89% ± 0.31 (data not shown).

5.3.3 Functionalisation of β-glucan microparticles

β-glucan microparticles were targeted to aminopeptidase N (APN) by surface decorating the GPs with an in-house produced monoclonal antibody (mAb) against porcine APN (IMM013). An overview of all required steps to synthesize these antibody functionalised GPs is given in Figure 1. First, protein G was conjugated to the particles via carbodiimide crosslinker chemistry.[28, 407, 408] Briefly, BSA-FITC GPs (500 µg/ml) were centrifuged (500 g, 5 min) and resuspended in 1 ml 2-(N-morpholino)ethanesulfonic acid (MES) buffer (0.1 M MES; pH 6.0). To avoid aggregation, GPs were sonicated for 2 minutes (230V, 50Hz). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 1 mM) and N-hydroxysuccinimide (sulfo-NHS, 2.5 mM) were added to activate the surface carboxyl groups (15 minutes incubation, RT). Subsequently, the particles were centrifugated and resuspended in 1 ml PBS (pH 7.2). After another sonication period of 5 minutes (230V, 50Hz), 62.5 µg protein G was added to the GPs and incubated overnight on a shaker at 4°C. To block the remaining activated carboxyl groups, the particles were PEGylated by mixing with O-(2-aminoethyl)polyethylene glycol (10 mg, 3000 MW) for 30 minutes, followed by 0.1 M glycine (30 minutes).

Next, these protein G-decorated particles were functionalised with APN-specific mAbs. The particles were first washed twice and resuspended in antibody binding buffer (50 mM sodium borate, pH 8.2). After sonication for 2 minutes, mAbs against APN (250 µg/ml; IgG1) or irrelevant isotype-matched

control mAbs (250 µg/ml; IgG1) were added and gently rocked for 45 minutes at room temperature. Subsequently, the antibodies were crosslinked to the GPs by adding 20 mM dimethyl pimelimidate (DMP) dissolved in crosslinking buffer (0.2 M triethanolamine, pH 8.2). To block the remaining antibody binding spots, 0.1 M ethanolamine (pH 8.2) was added to the particles for 10 minutes. The particles were stored at -20°C in PBS at a concentration of 0.5 mg/ml.

The conjugation efficiency was determined by flow cytometry and confocal microscopy. Goat anti-mouse IgG-Alexa647 antibody (1/250) was added to the uncoated or coated β-glucan microparticles (2.5 µg) and incubated for 30 minutes. These particles were examined by flow cytometry (FACSCanto, BD Biosciences) to determine the percentage of conjugated particles and by confocal microscopy (Leica DMI6000 B inverted microscope attached to an Andor DSD2 confocal scanner) to determine the position of the antibodies on the particles. FACS analysis was performed on 20,000 events within the $FITC^+$ gate with FACSDiva software 6.1.3. Confocal images were processed with Imaris software.

5.3.4 Microparticle characterisation

The surface and internal morphology of the coated and uncoated particles was examined on an atomic force microscope (AFM) and on a FACSCanto, respectively. To investigate antigen loss during coating, the fluorescence intensity of the uncoated and coated GPs was measured by flow cytometry (20,000 events within the GP gate). In addition, we determined the concentration of BSA-FITC in the supernatant collected during the coating protocol via fluorimetry against a BSA-FITC standard (2.5 – 30 µg/ml). To determine antigen capture and the particle size, BSA-FITC-loaded, APN-targeted GPs were investigated by confocal microscopy. Image analysis was performed by Imaris software (3D visualisation) and ImageJ (diameter calculation). To calculate the mean size distribution, analysis on at least 350 particles was performed. The zeta potential of coated and uncoated particles was measured in 0.9% saline using a Malvern Zetasizer NS. Both coated and uncoated particles were neutrally charged as indicated by the low zeta-potential (-4.85 ± 3.10 mV for coated particles and -7.22 ± 4.43 mV for uncoated particles).

5.3.5 Primary cell culture

All animal experiments were approved by the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University (EC2013/62). Piglets (between 4 and 20 weeks old) were housed under standard conditions as blood donors. Peripheral blood was collected on heparin from the jugular vein of four pigs. Neutrophils were isolated by density gradient centrifugation on a

discontinuous Percoll gradient (68% and 75%) as described previously.[380] Peripheral blood monomorphonuclear cells (PBMCs) were isolated by density gradient centrifugation on Lymphoprep and monocytes were further enriched from the PBMCs by positive immunomagnetic bead selection (MACS). Monocyte-derived dendritic cells (MoDCs) were generated as previously described.[402]

5.3.6 Oxidative burst assay

The production of reactive oxygen species (ROS) was measured by a chemiluminescence assay described by Donne et al. (2005) with some modifications.[381] Neutrophils were seeded in a 96-well plate at 2.0 x 10^5 cells/well in RPMI without phenol-red. The plates were incubated at 37°C for 2 h in a humidified atmosphere with 5% CO_2 to allow the cells to adhere to the plastic surface. Subsequently, the supernatant was replaced by 175 µl luminol (0.5 mM). After 5 minutes of background measurement at 37°C, 25 µl of the indicated β-glucan microparticles (0.5 mg/ml) or 1 µm latex beads (100 µg/ml) were added. Stimulation of the cells with phorbol myristate acetate (PMA; 50 µg/ml) was used as a positive control. ROS production was then measured during 120 min in the integration mode. Each stimulation was performed in duplicate. The ROS production is expressed as relative light units (RLU).

5.3.7 Cell line

The porcine intestinal epithelial cell line IPEC-J2 was stably transfected with pAPN.[260] Cell cultures were maintained in Dulbecco's modified Eagle's (DMEM)/F12 supplemented with 10% fetal calf serum (FCS, Integro), 1% recombinant human insulin, transferrin and sodium selenite (ITS, Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin (P/S), 2% L-glutamine, 5 ng/ml epidermal growth factor (EGF; Invitrogen) and 1.5 mg/ml geneticin® (G418; Life Technologies) at 37°C and 5% CO_2.

5.3.8 Targeting effect assay by flow cytometry

IPEC-J2 cells were seeded in 24-well plates at a cell density of 1 x 10^5 cells/well and cultured for 24h until a confluent monolayer was achieved. The cells were stimulated with medium, different concentration of BSA-FITC loaded β-glucan microparticles (BSA-FITC-GP) conjugated with anti-APN or with irrelevant control monoclonal antibodies (100, 25 or 1.25 µg/ml). The particles were incubated for 3h at 37°C. After this incubation period, the cells were detached by accutase and analysed by flow cytometry (FacsCanto). Dead cells were excluded from the analysis by Sytox red staining (5 nM). Ten

thousand viable cells were analysed by FACSDiva software. To determine the expression of APN on the surface of these cells, IPEC-J2 cells (10^5 cells) were stained with an anti-APN mAb (IMM013; 1 µg) or an isotype-matched control mAb (IgG_1; 1 µg) for 20 minutes on ice, followed by the addition of sheep anti-mouse IgG/FITC (1/500) (20 minutes on ice). Cells were analysed by flow cytometry (10,000 viable cells).

5.3.9 Fluorescent confocal imaging

IPEC-J2 cells were cultured on glass coverslips in a 24 well-plate (1 x 10^5 cells/well). Upon reaching confluence, GPs (6.25, 25 or 100 µg/ml) were added to the cell monolayer for 3h. Subsequently, the cells were washed and fixed in 3% paraformaldehyde (PFA). Cells were counter-stained with 10 µg/ml Hoechst and the actin cytoskeleton was visualised by Phalloïdin-Texas Red (1/100) (1h at RT). The confocal images were taken by Leica TCS SPE confocal microscope and processed with Imaris software.

5.3.10 Porcine small intestinal explants

Fresh porcine intestinal tissue from the jejunum (with or without Peyer's patches) and the ileum (Peyer's patches) was obtained from euthanized pigs (6-10 weeks old). The serosa was removed and the intestine was first washed with ice cold PBS + 1% P/S, followed by washing with DMEM supplemented with 1% P/S and 1% kanamycine for 10 minutes at room temperature (2 times). The intestine was cut into sections by a biopsy punch (0.8 cm diameter) in a sterile hood and placed on sterile biopsy foam pads, which, in turn, were placed inside six-well plates (one section per well). Porcine intestinal explants were cultured at the air-medium interface with the apical side facing upward. Culture medium (2.25 ml per well) was poured carefully inside the wells, and was composed of DMEM with 5% FCS (Integro), 1% P/S, 20 mM HEPES (Sigma), 1% NE-AA and 1% kanamycine. The explants were then placed inside a humidified incubator at 37°C, 5% CO_2 for 1 h followed by the addition of fresh medium, supplemented with 7.5 mM N-acetyl-L-cysteine.[22] Fifteen minutes later, 40 µg antibodies (anti-APN or isotype control) or 5 µg APN-targeted GPs were added to the explants for 1h. Then, explants were washed with PBS, placed in methocel and snap-frozen in liquid nitrogen. Cryosections (14 µm) were cut by a cryotome (Leica CM3050 S), placed on APES-coated glass slides and fixed in aceton for 10 minutes at -20°C. To quantify uptake of particles by intestinal explants (5 µg GPs, 5 min incubation), cryosections (18 µm) were cut every 500 µm (duplicates per pig, three pigs

in total) and the particles within or underneath the epithelial layer were counted. The mean particle uptake of 12 different sections per explant (in duplicate) was calculated.

5.3.11 Immunohistochemistry

Cryosections were washed with PBS and blocked with 10% normal goat serum (diluted in PBS), followed by incubation with a mix of sheep anti-mouse IgG-FITC (1/500) with Phalloïdine-Texas Red (1/100) or only with Phalloïdine-Texas Red (1/100) at 37°C (60 minutes) in a humidified chamber. Slides were then washed with PBS, counterstained with Hoechst (10 µg/ml) for 30 minutes at 37°C and mounted with DABCO. Images were acquired with a confocal microscope.

5.3.12 Uptake by MoDCs

MoDCs were stimulated with medium, 100 µg/ml uncoated BSA-FITC-loaded GPs or coated with anti-APN mAb or with irrelevant control mAb. The particles were incubated for 1h at 37°C. MoDCs were detached by accutase and analysed by flow cytometry (10,000 viable cells). Dead cells were excluded from the analysis by Sytox red staining. To investigate the expression of APN on MoDCs, cells were stained with an APN-specific mAb (IMM013) or an isotype-matched control, followed by the incubation with sheep anti-mouse-IgG FITC antibodies (1/500). Sytox red was used to exclude dead cells. To confirm the uptake of the particles by MoDCs, cells were cultured on glass coverslips and visualised by confocal microscopy. Therefore, cells were first fixed in 3% PFA and then stained with anti-SIRPα mAbs, followed by the incubation with goat anti-mouse IgG AlexaFluor594 antibodies (1h at RT). Cells were counterstained with 10 µg/ml Hoechst. Confocal images were processed with Imaris software.

5.3.13 Maturation of MoDCs

The cell surface expression of DC activation markers after stimulation of MoDCs (24h, 37°C) with medium, 100 µg/ml uncoated BSA-FITC-loaded GPs or coated with anti-APN or with irrelevant control mAb was assessed by flow cytometry using mAbs against MHCII (MSA3, IgG2a[409]), CD40 (G28-5, IgG1, anti-human[410]), CD25 (K231.3B2, IgG1[411]) and a human CTLA4-muIgG2a fusion protein respectively, followed by R-phycoerythrin and AlexaFluor-647 conjugated anti-mouse isotype-specific secondary antibodies. Briefly, MoDCs were harvested with accutase, whereafter the cells were incubated with pre-titrated saturating concentrations of the primary Abs (20 min, 4°C). Cells stained

with isotype-matched irrelevant mAbs were used as negative controls. After washing, the cells were stained with the secondary Abs (20 min at 4°C). Next, the cells were washed and data were acquired and analysed as described above.

5.3.14 Cytokine secretion profile

MoDCs were stimulated with the different GPs as mentioned above. After 24h cell-free culture supernatant was collected and the porcine TNFα, IL-1β, IL-12p40, IL-8, IL-6, IL-10 and cytokine concentrations were determined with commercially available ELISA kits according to the manufacturer's instructions. Briefly, microtiter plates were coated overnight at room temperature with capture Ab specific for the analysed cytokines. The plates were washed, blocked and samples and standards were analysed in duplicate with the detection Ab and the streptavidin-HRP system. Optical densities were measured with an ELISA plate reader at 450 nm. The cytokine concentrations were calculated using DeltaSOFT JV 2.1.2 software (BioMetallics, Princeton, NJ, USA) with a 5-parameter curve-fitting algorithm applied for standard curve calculations.

5.3.15 Oral immunisation model

Piglets (3-4 weeks old) were screened for the presence of FedF-specific serum antibodies and negative animals were further tested for the presence of the F18-receptor (F18R) by detection of a polymorphism in the *FUT1* gene.[412] Twelve $F18R^+$ FedF seronegative piglets were selected and transported to isolation units with water and feed ad libitum. To prevent *E. coli* infections, animals were treated orally with colistin for four consecutive days (150 000 U/kg body weight/day; ProMycine® Pulvis) until one day before the immunisation. Experimental and animal management procedures were approved by the animal care and ethics committee of the Faculty of Veterinary Medicine (EC2014/114). Piglets received oral doses of either 50 µg cholera toxin (CT) in 0.9% saline, soluble FedF (1 mg) with CT (50 µg), FedF-GP (2.5 mg) with CT (50 µg) or anti-APN FedF-GP (2.5 mg) with CT (50 µg) in 0.9% saline. FedF was loaded inside the β-glucan microparticles with an efficiency of 84.53% ± 2.89. Booster doses were given on day 14. One day before the immunisations, 20 mg Pariet® was given and feed but not water was withheld overnight. On day 28, blood was collected to analyse total FedF-specific Ig and IgA antibody responses in sera via ELISA. Briefly, microtiter plates were coated overnight at room temperature with FedF (5 µg/ml). The plates were washed, blocked (PBS supplemented with 0.2% Tween80 and 3% BSA) and serum (1/15) was analysed in duplicate with anti-pig-Ig (H+L) or IgA/HRP (1:5000). Optical densities were measured with an ELISA plate

reader at 405 nm and data are expressed as corrected optical densities (OD_{d28} - OD_{d0}). The optical density cut-off points (corrected OD_{d28} of control group + 3 x SD) were 0.0887 for serum FedF-specific Ig and 0.0209 for serum FedF-specific IgA. At day 28, 14 days after the booster immunisation, peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood to detect circulating FedF-specific antibody secreting cells (ASC) by ELIspot as previously described.[413] Briefly, a 96-well microtiter plate (Maxisorp™ Thermo Scientific) was coated with FedF (5 µg/ml). Subsequently, the plates were blocked (RPMI + 10% FCS) and 5.0 x 10^5 PBMCs in 100 µl RPMI complete (RPMI containing 1% kanamycin, 1% P/S, 5% FCS, 1% glutamine, 1% NE-AA, 1% sodium-pyruvate) were added overnight at 37°C in a humidified 5% CO_2 atmosphere. After removing the cells, FedF-specific IgG ASC were detected with monoclonal anti-porcine IgG or IgA (1 h, 37°C) followed by an incubation for 1 h at 37°C with HRP-conjugated anti-mouse IgG antibody (1:1000). To visualise spots, 50 µl 3,3',5,5'-tetramethylbenzidine liquid substrate for membranes (TMB) was applied for 15 min at room temperature. The resulting spots were scanned with an Immuno-Spot® analyser (CTL-Europe GmbH, Germany). For each condition spots in 7 wells (0.5 × 10^6 cells/well) were counted to obtain the number of FedF-specific IgG and IgA ASCs per 3.5 × 10^6 PBMCs.

5.3.16 Statistical analysis

All statistical analyses of the experiments were conducted by GraphPad Prism using a t-test to compare two conditions or a one-way analysis of variance (ANOVA) test (post hoc: Tukey) to compare more than two conditions. A P-value of <0.05 was considered significant.

5.4 Results and Discussion

5.4.1 Oriented conjugation of monoclonal antibodies to β-glucan microparticles

To allow oriented conjugation of antibodies to BSA-FITC-loaded β-glucan microparticles (BSA*GPs), a key feature to achieve optimal targeting potential, the GPs were first conjugated with protein G. This protein is well-known for binding Fc fragments of mouse IgG_1 antibodies. Noteworthy, protein G has several Fc binding domains making oriented conjugation less critical. A carbodiimide linker was chosen to perform this conjugation, since these GPs contain carboxyl groups on their surface, due to the residual proteins present in the cell wall, available for linkage to NH_2 groups of protein G.[28] Next, the protein G-conjugated GPs were functionalised with mouse monoclonal antibodies (mAbs) against APN (IgG_1) (APN-BSA*GPs) (Figure 1). The conjugation efficiency of three independent procedures was determined by flow cytometry (Figure 2A) and confocal microscopy (Figure 2B). Figure 2A depicts a representative dotplot of the uncoated and coated particles. The mean conjugation efficiency was 96.9% ± 3, illustrating a highly efficient conjugation of the mAbs to the particles. Confocal imaging demonstrated the position of these antibodies on the particles (Figure 2B). The monoclonal antibodies (red) clearly cover the particles (green) (Figure 2B2) as compared to uncoated particles (Figure 2B1), demonstrating the equal distribution of the mAbs on the GP surface, despite its low protein content.

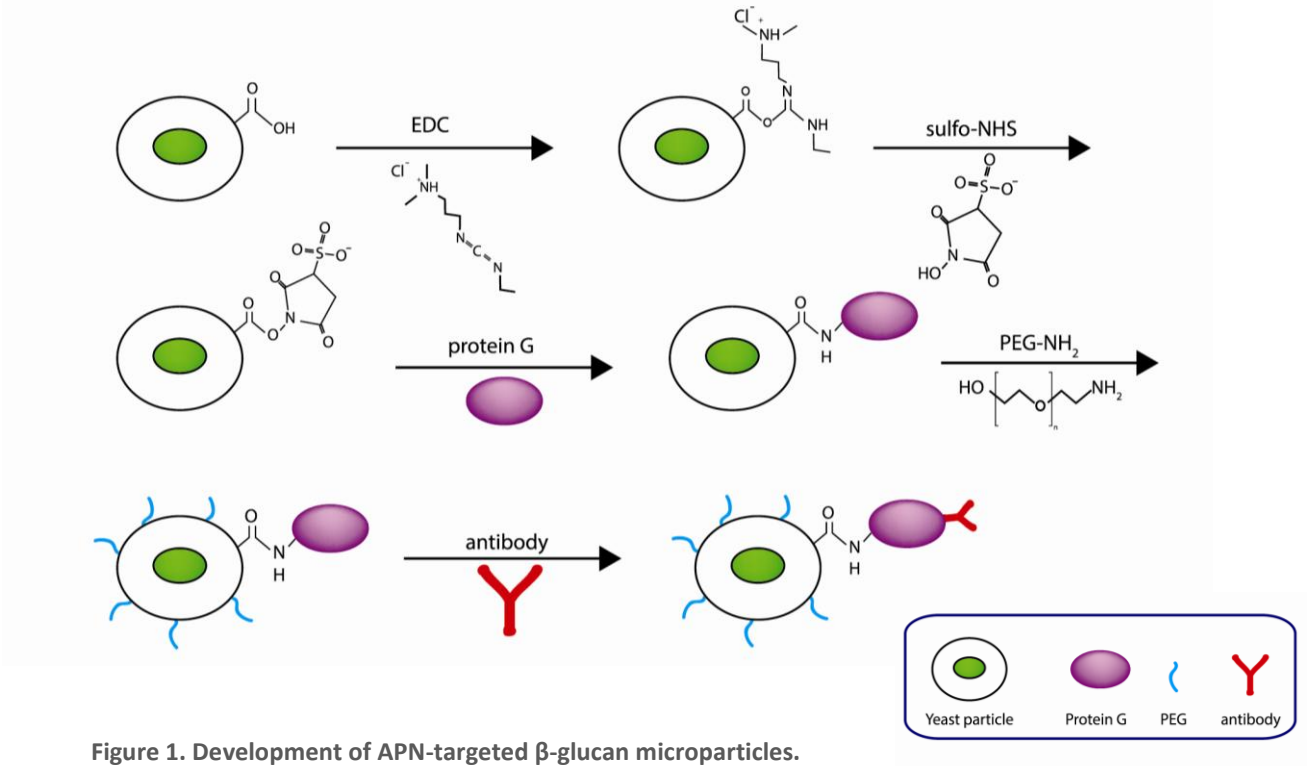


Figure 1. Development of APN-targeted β-glucan microparticles.
Schematic representation of the synthesis of antibody-conjugated β-glucan microparticles. Empty porous β-glucan microparticles were loaded with BSA-FITC (green) and coated with protein G (purple) via EDC-NHS chemistry. The unbound amino-reactive spots were blocked with PEG. Monoclonal antibodies were crosslinked to protein G.

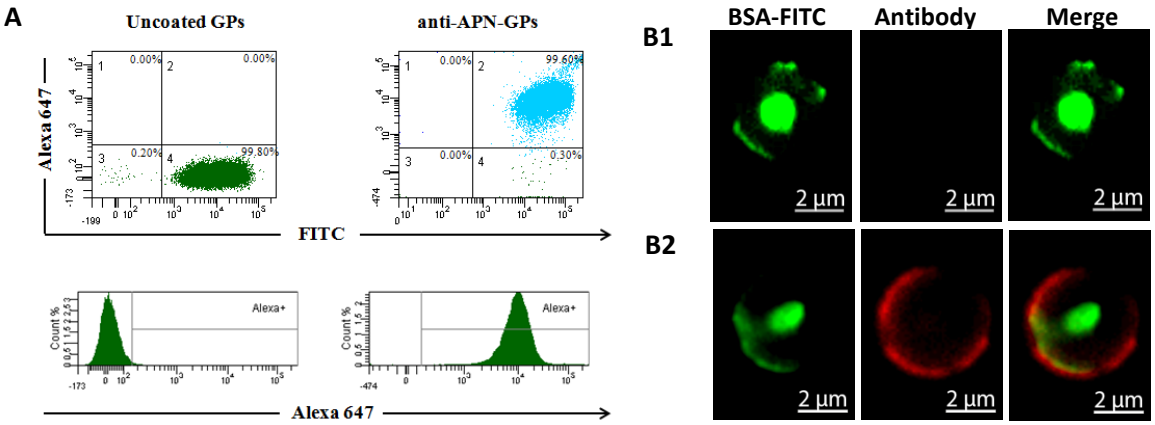


Figure 2. Coating efficiency of antibodies to β-glucan microparticles.
(A) Coating efficiency was determined by flow-cytometric quadrant statistics using a secondary anti-mouse IgG AlexaFluor 647 antibody. $FITC^+$ and $AlexaFluor647^+$ GPs were defined and compared to uncoated particles. Histogram showing the distribution of $AlexaFluor647^+$ particles. **(B)** Confocal images of uncoated BSA-FITC loaded GPs (B1) and BSA*GPs coated with anti-APN mAbs (B2). Representative data of three independent batches are shown.

5.4.2 β-glucan microparticle characteristics

When analysing the conjugation efficiency, we observed an increase in the side scatter (SSC) value of the mAb-conjugated BSA*GPs as compared to uncoated particles (Figure 3A2). This was unexpected as SSC values usually reflect the internal complexity of particles and the GP surface modification should not affect the particle's interior. To further look into this, conjugated and unconjugated particles were analysed with atomic force microscopy (AFM). As illustrated by Figure 3A1 uncoated GPs exhibit a smooth surface (Figure 3A1a), while conjugated GPs have a rougher surface (Figure 3A1b), probably due to the EDC/NHS-chemistry used to conjugate protein G. These findings also indicate that measuring SSC by flow cytometry, a high throughput technology, can inform on changes in surface morphology during microparticle fabrication. Analysis of the BSA*GPs by confocal microscopy revealed aggregation of the microparticles (Figure 3B1), a feature which could hamper its application in oral vaccination. This aggregation was reduced by the introduction of a PEGylation step during production of the mAb-conjugated BSA*GPs (Figure 3B2). To address whether conjugation affected the size of the particles, we determined the size distribution upon exclusion of aggregates. As shown in Figure 3C, the size distribution of the uncoated and coated particles was similar, just as their mean diameter (uncoated particles: 3.246 ± 0.871 µm; coated particles: 3.272 ± 0.709 µm).
Yeast-derived β-glucan microparticles combine antigen delivery and adjuvant activity.[25] Importantly, conjugation of these particles with antibodies did not interfere with these properties, as reflected by a similar antigen load and respiratory burst response by neutrophils. Indeed, we could not detect a difference in the fluorescence intensity between the uncoated GPs and anti-APN mAb-conjugated GPs (Figure 3D) and we were unable to detect BSA-FITC in the supernatant, collected during the

conjugation protocol, by a fluorimeter (data not shown), demonstrating that BSA-FITC is strongly incorporated inside the GPs. Furthermore, 3D images (Figure 3E) clearly show a similar distribution of the antigens inside the coated GPs. Besides their high encapsulation efficiency, GPs contain β-glucans, which are well-known to trigger antimicrobial activity of neutrophils.[380] To investigate whether mAb conjugation to GPs resulted in masking of β-glucans, and thus in masking their adjuvanticity, the production of reactive oxygen species (ROS) by neutrophils incubated with GPs was examined. As compared to inert latex beads (1 µm), both uncoated and coated GPs triggered ROS production by neutrophils, while mAb conjugation slightly decreased this response compared to uncoated GPs (Figure 3F). Since porcine neutrophils are APN-negative (data not shown), this ROS production results from the β-glucans, which are clearly still exposed after conjugating the particles with antibodies.

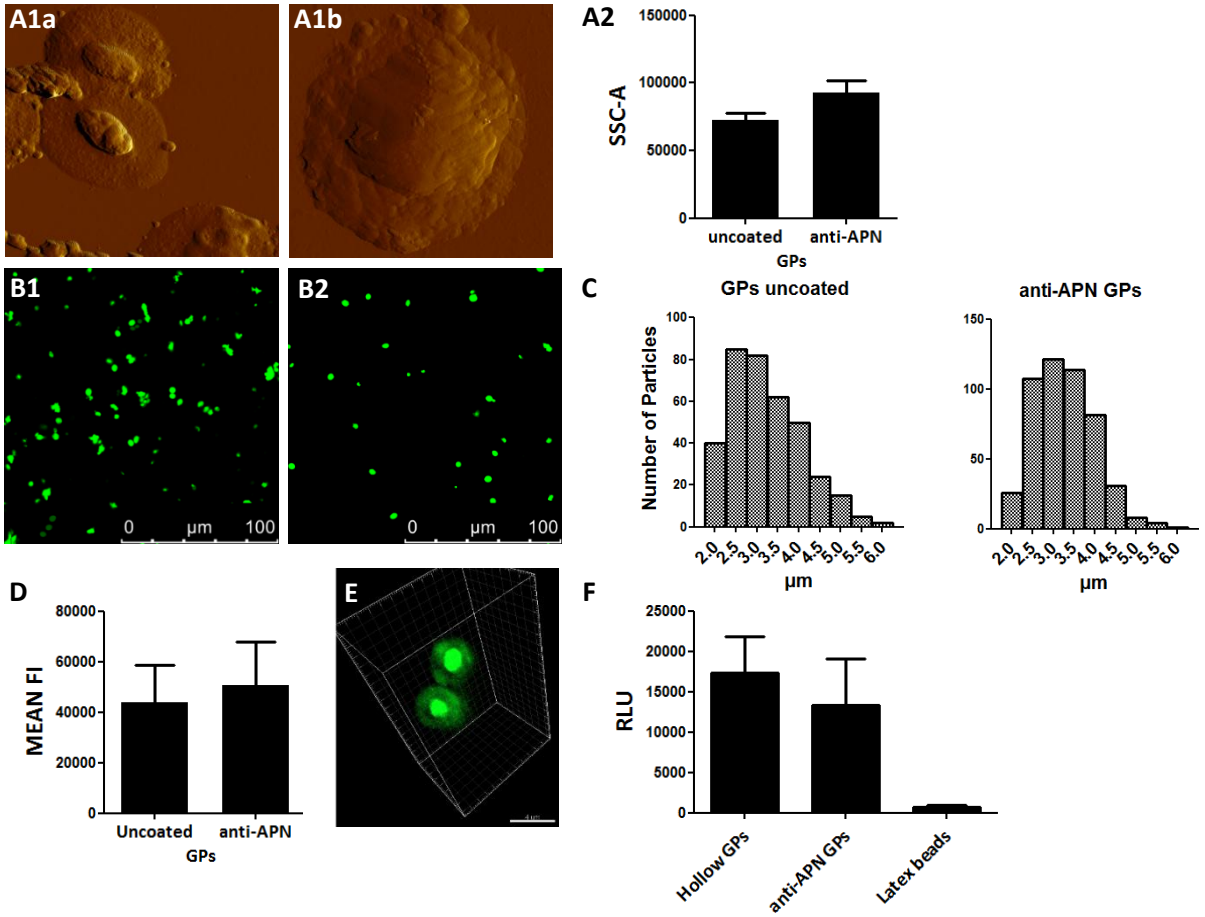


Figure 3. Characterisation of APN-targeted β-glucan microparticles.
(A) Atomic force microscopic (AFM) images of uncoated (A1a) or APN-coated (A1b) GPs. Side scatter (SSC) analysis of uncoated and coated particles. Data is presented as the mean SSC ± SEM of three batches (A2). **(B)** Confocal images of uncoated (B1) or coated (B2) BSA-FITC loaded GPs. **(C)** Size distribution histograms of uncoated and coated GPs. Aggregates were excluded from the results. **(D)** Determination of antigen (BSA-FITC) loss after coating by measuring the fluorescence intensity (FI) of uncoated and coated GPs by flow cytometry. Data are presented as the mean FI ± SD of three batches. **(E)** A 3D image of APN-BSA-FITC loaded particles. **(F)** Neutrophils (2 x 10^5 cells) were stimulated with 100 µg/ml of the indicated GPs. ROS production was determined via chemiluminescence. Data are shown as the mean relative light units (RLU) ± SEM of five pigs. Values of the negative control have been subtracted from the values represented on the graph.

5.4.3 Uptake by intestinal epithelial cells

One of the most important features of potential oral vaccine delivery systems is their efficient transcytosis through intestinal epithelial cells. Although the size of these β-glucan microparticles is ideal for internalisation by M cells in Peyer's patches, reaching villous enterocytes may promote the efficacy of oral vaccines. Targeting enterocytes as well as M cells could result in a higher bioavailability, thus lowering vaccine dosage and reducing the high cost of subunit vaccines. As an endocytotic receptor on the apical surface of enterocytes, aminopeptidase N (APN) is an appealing target to promote antigen delivery across the epithelial barrier. We recently reported that polyclonal antibody-mediated targeting to APN resulted in antigen uptake and in robust immune responses.[260] To further expand these findings to antibody-mediated uptake of microparticles, we targeted β-glucan microparticles (GP) to APN and explored GP uptake by the porcine intestinal epithelial cell line IPEC-J2[414] and by intestinal explants. This cell line was transfected to increase APN surface levels (Figure 4A). To investigate if conjugation of the anti-APN mAb to GPs resulted in an increased internalisation of GPs, we measured the endocytosis of anti-APN mAb-conjugated GPs and GPs conjugated with an irrelevant control mAb by flow cytometry. GPs targeted to APN were taken up significantly more than isotype-conjugated GPs (Figure 4B). Even at a 16-fold lower concentration, APN-targeted GPs are still ten times more internalised by IPEC-J2 cells as compared to control GPs. The viability of the cells was not reduced even at the highest GP concentration (data not shown). To discriminate between membrane-associated GPs and internalised GPs, we performed confocal microscopy. Figure 4C clearly demonstrates the presence of the APN-BSA*GPs particles inside the cells, indicating that the functionalisation of the β-glucan microparticles with APN-specific antibodies was highly efficient, resulting in an increased uptake of the targeted GPs by enterocytes.

In a next effort we evaluated internalisation of these GPs by intestinal explants. Figure 5B 1, 2 and 3 clearly show the uptake of APN-specific mAbs by intestinal epithelial cells located in the jejunum without Peyer's patches and the jejunum and ileum with Peyer's patches, respectively, while the irrelevant control mAb failed to bind to or be taken up by the intestinal epithelial cell layer in all compartments (Figure 5A). This indicates that antibodies against APN are promising ligands to target intestinal epithelial cells. Indeed, Figure 5C clearly shows that APN-targeted GPs are transcytosed through the epithelial barrier in the jejunum and ileum. Even in the jejunum without Peyer's patches, internalisation of APN-BSA*GPs was observed. Interestingly, when we quantified particles in the lamina propria (LP), APN targeting resulted in a significantly higher uptake as compared to control particles (Figure 5D). As expected, in the ileal Peyer's patches the uptake of the particles was higher than for the lamina propria (Figure 5D), however, no significant difference in uptake between the different particles was detected.

These results demonstrate the efficient functionalisation of β-glucan microparticles with anti-APN monoclonal antibodies via protein G-mediated oriented conjugation. Moreover, APN targeting clearly resulted in a higher uptake of the GPs by intestinal epithelial cells located in the jejunal lamina propria than uncoated particles. Since the lamina propria constitutes the largest surface area in the intestine, these results imply that more APN-targeted GPs may reach the intestinal mucosa upon oral administration than control particles, which may allow for a lower vaccine dosage.

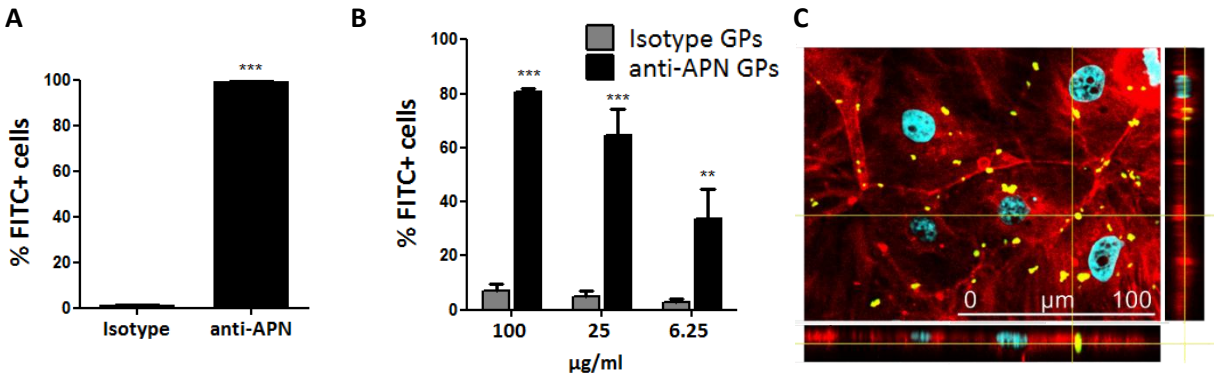
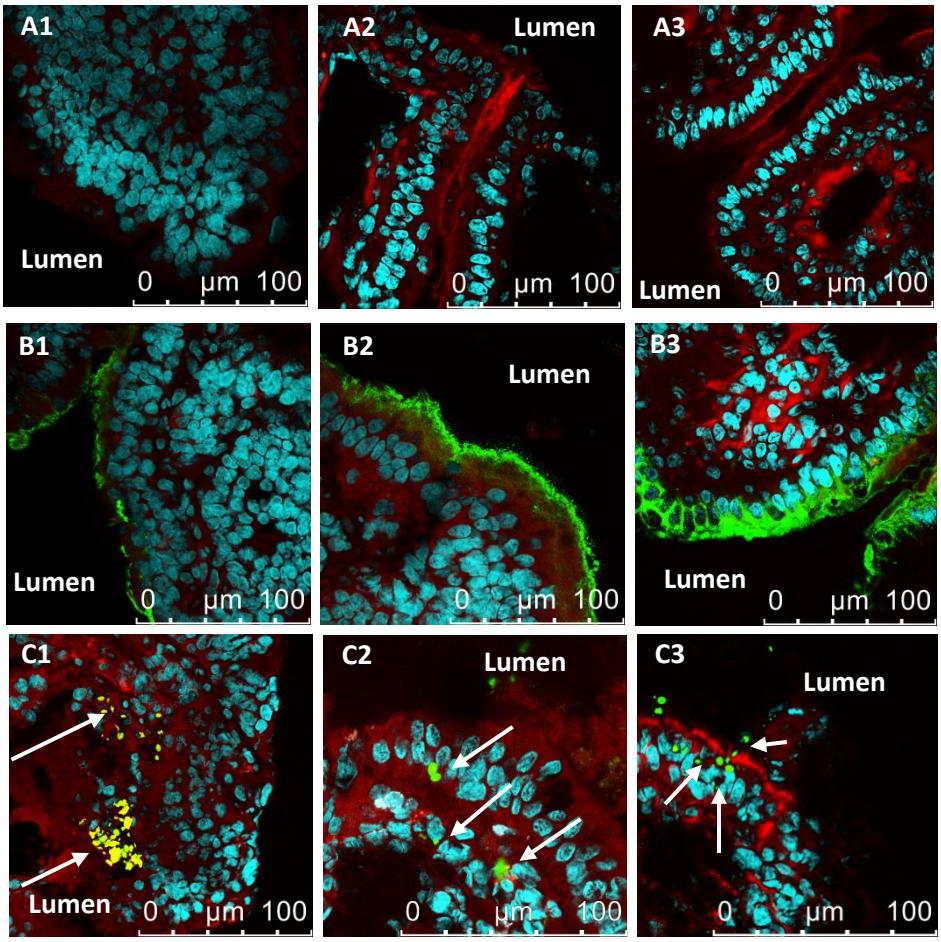


Figure 4. Endocytosis of APN-targeted GPs by IPEC-J2 cells.
(A) Binding of anti-APN to APN-IPEC-J2 cells was quantified by flow cytometry. Data are presented as the mean percentage FITC-positive cells (mean ± SD) of three independent experiments. **(B)** *In vitro* microparticle uptake by the porcine intestinal epithelial cell line APN-IPEC-J2. IPEC-J2 cells were incubated with BSA-FITC-loaded GPs coated with anti-APN or irrelevant control mAb at particle concentration of 100, 25 or 6.25 µg/ml for 3h. Data represent the percentage FITC-positive cells (mean ± SD) of three independent experiments. **: $p < 0.01$; ***: $p < 0.001$. **(C)** Confocal image of APN-IPEC-J2 cells following incubation with BSA-FITC loaded GPs coated with anti-APN mAb (green). The actin cytoskeleton was visualised with phalloidine-TR (red), while the nucleus was counterstained with Hoechst (blue).



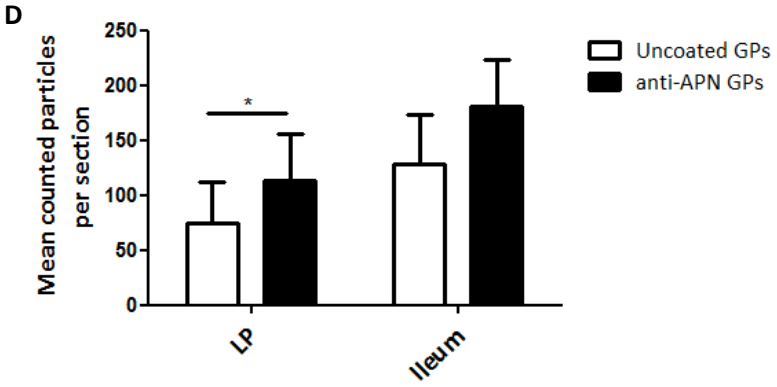


Figure 5. Endocytosis of APN-targeted GPs by intestinal explants.
Confocal images of porcine intestinal tissue following incubation (1h, 37°C) with an isotype control **(A)** or monoclonal antibodies against APN **(B)**. Binding or endocytosis of this antibody was determined in explants obtained from the jejunum without PP (LP) (1), the jejunum with PP (2) and the ileum PP (3). The monoclonal antibody was visualised with anti-mouse IgG-FITC (green), the plasma membrane with Phalloidine-TR (red) and the nucleus with Hoechst (blue). **(C)** Confocal images of porcine intestinal tissue following incubation with APN-BSA*GPs (green). Endocytosis was determined in explants obtained from the jejunum without PP (LP) (1), the jejunum with PP (2) and the ileum PP (3). The plasma membrane was visualised with Phalloidine-TR (red) and the nucleus with Hoechst (blue). The white arrows indicate engulfed particles surrounded by actin, indicative of endocytosis. **(D)** The uptake of particles by porcine intestinal explants (jejunum without PP (LP) and the ileal PP) was quantified via fluorescence microscopy. The mean particle uptake of 12 different sections per explant (in duplicate) was calculated (mean ± SEM, n = 3).*: $p<0.05$.

5.4.4 APN-targeted GPs promote DC maturation

Once the GPs are transcytosed through the epithelial barrier, they have to trigger antigen presenting cells (APCs) to activate the innate and adaptive immune system. One of the most potent APCs are dendritic cells (DCs), which bridge innate and adaptive immunity.[415] To assess whether the conjugated particles can be taken up by DCs, monocyte-derived dendritic cells (MoDCs) were cultured in the presence of GPs and their uptake was evaluated by flow cytometry and confocal microscopy. Although only a subset of porcine MoDCs (~30%) express APN (Figure 6A1), most MoDCs have taken up the particles irrespective of mAb conjugation (Figure 6A2). Interestingly, dendritic cells stimulated with APN-targeted GPs showed a significantly higher antigen load than MoDCs stimulated with uncoated particles, while the endocytosis of isotype control conjugated particles was approximately the same as the anti-APN conjugated particles (Figure 6A2). This indicates that the decoration of GPs with antibodies can promote a more efficient internalisation by DCs. Next, confocal microscopy was used to distinguish between internalised and cell membrane bound APN-targeted GPs. As shown in Figure 6A3, a huge amount of APN-targeted BSA*GPs were taken up by the MoDCs. A key feature of DCs is their maturation, a process culminating in the enhanced ability to activate T cells via the upregulated expression of membrane proteins, involved in antigen presentation and T cell interaction, and the secretion of cytokines.[415] In an effort to determine the maturation of these GP-stimulated DCs, the cell surface expression of MHCII, CD40, CD80/86 and CD25 was analysed (Figure 6B).[416] In comparison with control MoDCs, APN-targeted GPs resulted in an upregulated cell surface expression of CD40 and CD25 on the MoDCs, while MHCII and CD80/86 levels were less affected. DC maturation is accompanied by elevated cytokine secretion levels, which drive the differentiation and polarisation of T cells. To assess the effect of GPs on the MoDCs cytokine secretion profile, we analysed the secretion of TNFα, IL-1β, IL-12p40, IL-8, IL-6 and IL-10 (Figure 6C). As compared to control MoDCs, IL-10 and TNFα secretion was significantly increased for all conditions. Interestingly, APN-targeted GPs significantly triggered higher TNFα secretion levels than uncoated GPs. Moreover, APN-targeted GPs significantly upregulated the IL-1β and IL-12p40 secretion levels, while these cytokine levels were less elevated by DCs stimulated with control GPs. Intriguingly, in all these experiments no significant difference between the anti-APN and isotype control coated GPs was observed. Presumably, the Fc domains of the conjugated mAbs can still interact with the Fcγ receptors located on dendritic cells. Indeed, multiple IgG molecules within an immune complex can bind activating Fc receptors (FcγRs) present on monocyte-derived DCs.[417] Altogether, these findings demonstrate that APN-targeted GPs promote DC maturation, probably due to the synergistic effect of APN-targeting, Fc receptor engagement and β-glucan recognition. Indeed, crosslinking of APN positively influences phagocytosis by immune cells and β-glucans are

known as immune stimulators,[187, 352] suggesting that APN-targeted GPs possess a dual function: promoting the transcytosis through the epithelial barrier and DC maturation.

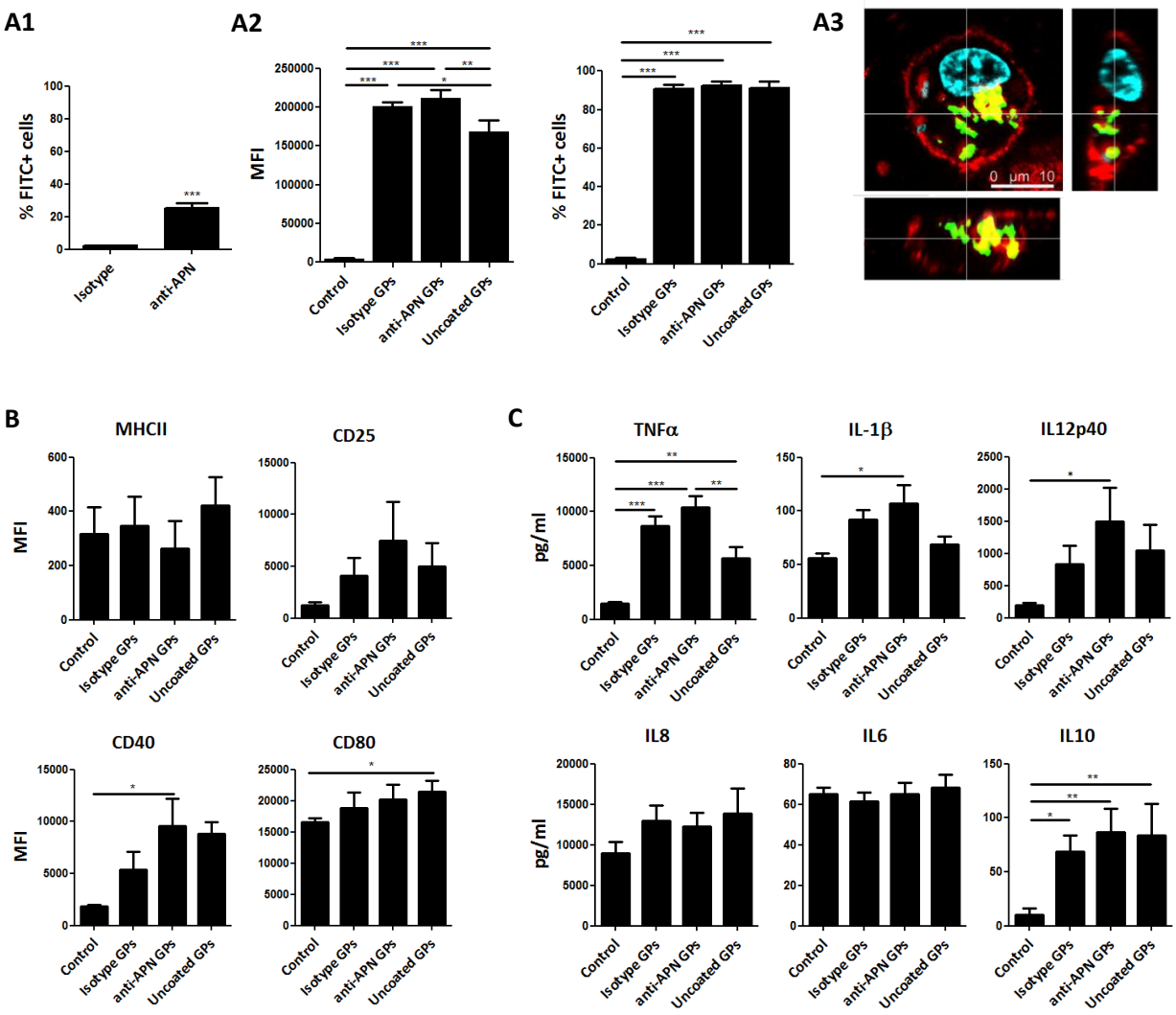


Figure 6. Immune stimulation by APN-targeted β-glucan microparticles.
(A1) Binding of anti-APN to MoDCs was quantified by flow cytometry. Data are presented as the mean percentage FITC-positive cells (mean ± SEM) of four animals in duplicate. **(A2)** MoDCs (5 x 10^5 cells) were cultured for 5 days followed by incubation with β-glucan microparticles (100 µg/ml). After 1 hour the cells were collected and analysed by flow cytometry. Data are represented as percentage FITC-positive cells (mean ± SD) or mean fluorescence intensity (MFI ± SD) of four animals in duplicate. **(A3)** Confocal image of MoDCs following incubation with APN-targeted particles (green). Nucleus was visualised with Hoechst (blue) and the plasma membrane with SIRPα-Alexa594 (red). **(B)** Analysis of DC activation marker expression. Stimulated MoDCs were analysed for the expression of the cell surface markers MHCII, CD40, CD25 and CD80/86 by flow cytometry. The data represent the mean ± SEM of four pigs. Values of the isotype control have been subtracted from the values represented on the graph. **(C)** Cytokine secretion levels of activated MoDCs. Immature MoDCs were stimulated with the indicated agents for 24h. Cell-free supernatant was collected and the concentration of TNFα, IL-1β, IL-12p40, IL-8, IL-6 and IL-10 was quantified by ELISA. Data are represented as means ± SEM (n = 5 individual blood donors).

5.4.5 Increased systemic antibody responses upon oral immunisation with APN-targeted FedF-GPs

Because of the promising *in vitro* and *ex vivo* characteristics of the APN-targeted GP delivery platform, i.e. efficient antigen encapsulation, highly efficient internalisation by porcine intestinal epithelial cells and MoDC maturation *in vitro*, we used these targeted particles in an oral immunisation experiment. We aimed to determine if oral immunisation with APN-targeted FedF-GPs could deliver encapsulated antigens (FedF) to the immune system and induce systemic antigen-specific antibody responses. As illustrated in Figure 7, piglets orally received either CT, soluble FedF and FedF-GPs coated with or without APN-specific antibodies (Figure 7A). Booster doses were given on day 14. After 28 days, the FedF-specific Ig and IgA levels in serum were not increased in the FedF-treated pigs as compared to the control group (Figure 7B). Although FedF is a weak antigen,[418] loading of FedF into GPs (FedF-GP group) triggered FedF-specific antibodies as compared to the control group, although the significance level was not reached (Figure 7B). These results are comparable with previous results where no significant antigen-specific immune responses could be detected in serum of mice orally immunised with uncoated OVA-GPs.[32] In contrast to FedF-GPs, targeting of FedF-GPs to APN elicited significantly higher FedF-specific antibody responses. In addition, FedF-specific IgA antibodies were only detected in the APN-FedF-GP group (Figure 7B). A similar result was seen for the FedF-specific IgG ASC. Only APN-targeted FedF-GP induced a significant higher number of FedF-specific IgG ASC as compared to the control group (Figure 7C). Likewise, the highest FedF-specific IgA ASC were observed in the APN-FedF-GP group (Figure 7C). We conclude that oral administration of APN-targeted FedF-GP particles to piglets triggers a higher FedF-specific systemic antibody response as compared to non-targeted particles. The production of IgA antibodies is important for protection against mucosal pathogens. Indeed, secretory IgA antibodies are known to neutralise pathogens and their enterotoxins in the lumen of the gut. Mucosal intestinal IgA responses were analysed as well, however, these data were not useful due to the high background immune responses in the control group. Further research should focus on the enteric protection triggered by oral administration of these particles.

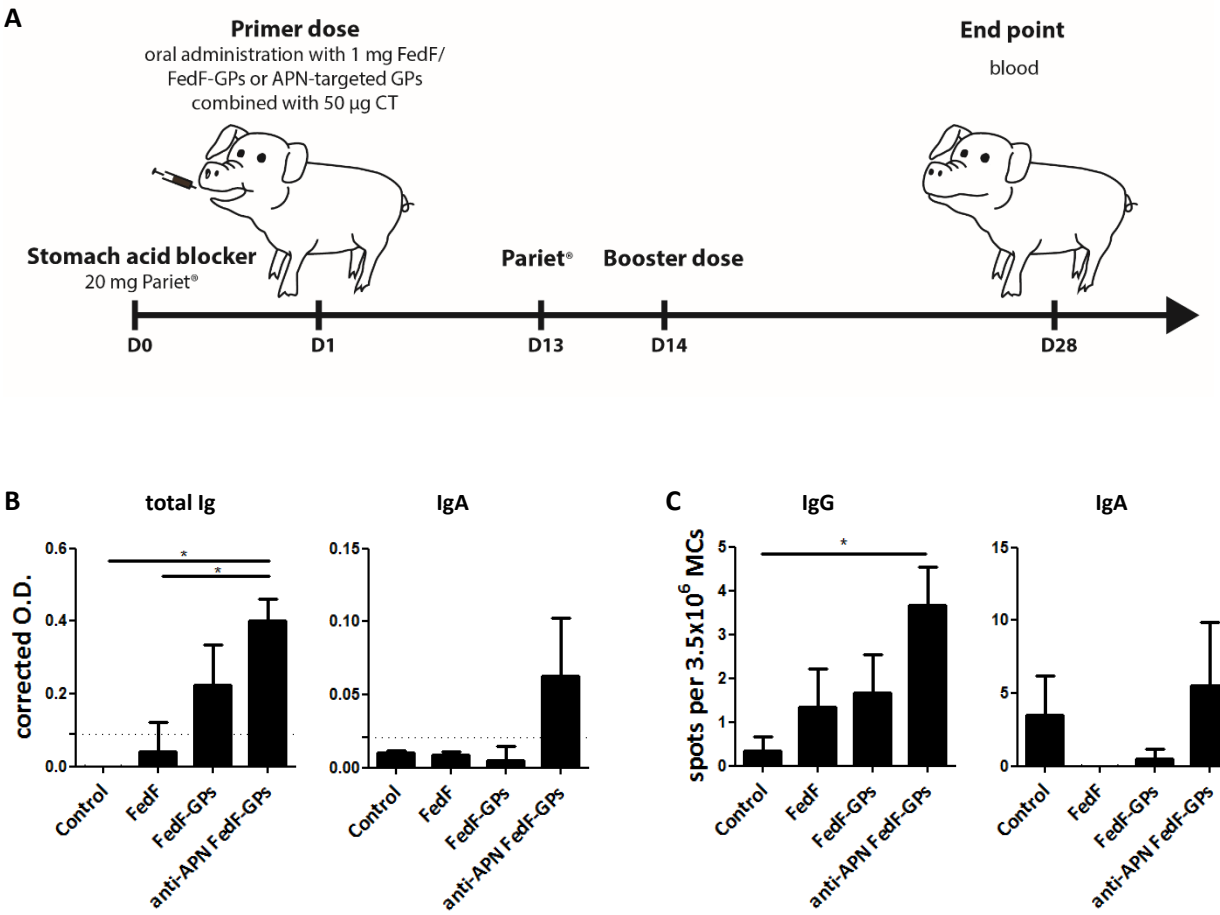


Figure 7. Systemic antibody responses upon oral immunisation of piglets.
(A) Oral administration protocol. **(B)** FedF-specific serum immunoglobulin (Ig and IgA) levels on d28 as measured by ELISA are expressed as corrected optical density (OD) (mean ± SEM, n = 3). **(C)** FedF-specific IgG and IgA ASCs on d28 as measured by ELIspot are expressed as spots per 3.5 x 10^6 cells (mean ± SEM, n = 3). *: $p < 0.05$.

5.5 Conclusions

In conclusion, by protein G conjugation we have developed a novel β-glucan microparticle (GP)-based antigen delivery platform to achieve selective targeting towards intestinal epithelial cells. Functionalisation of these β-glucan microparticles with monoclonal antibodies was highly efficient, and this conjugation did neither interfere with the antigen load nor the inherent adjuvanticity of the β-glucans. Furthermore, targeting to APN is a promising way to deliver antigens to the intestinal mucosa, since these microparticles are internalised by intestinal epithelial cells and trigger the maturation of dendritic cells, both crucial events in the induction of protective immunity. In addition, oral immunisation of piglets with APN-targeted FedF-GPs elicits systemic antigen-specific antibody responses. These findings might be crucial to assist the design of an oral subunit vaccine against enteric pathogens.

5.6 Acknowledgements

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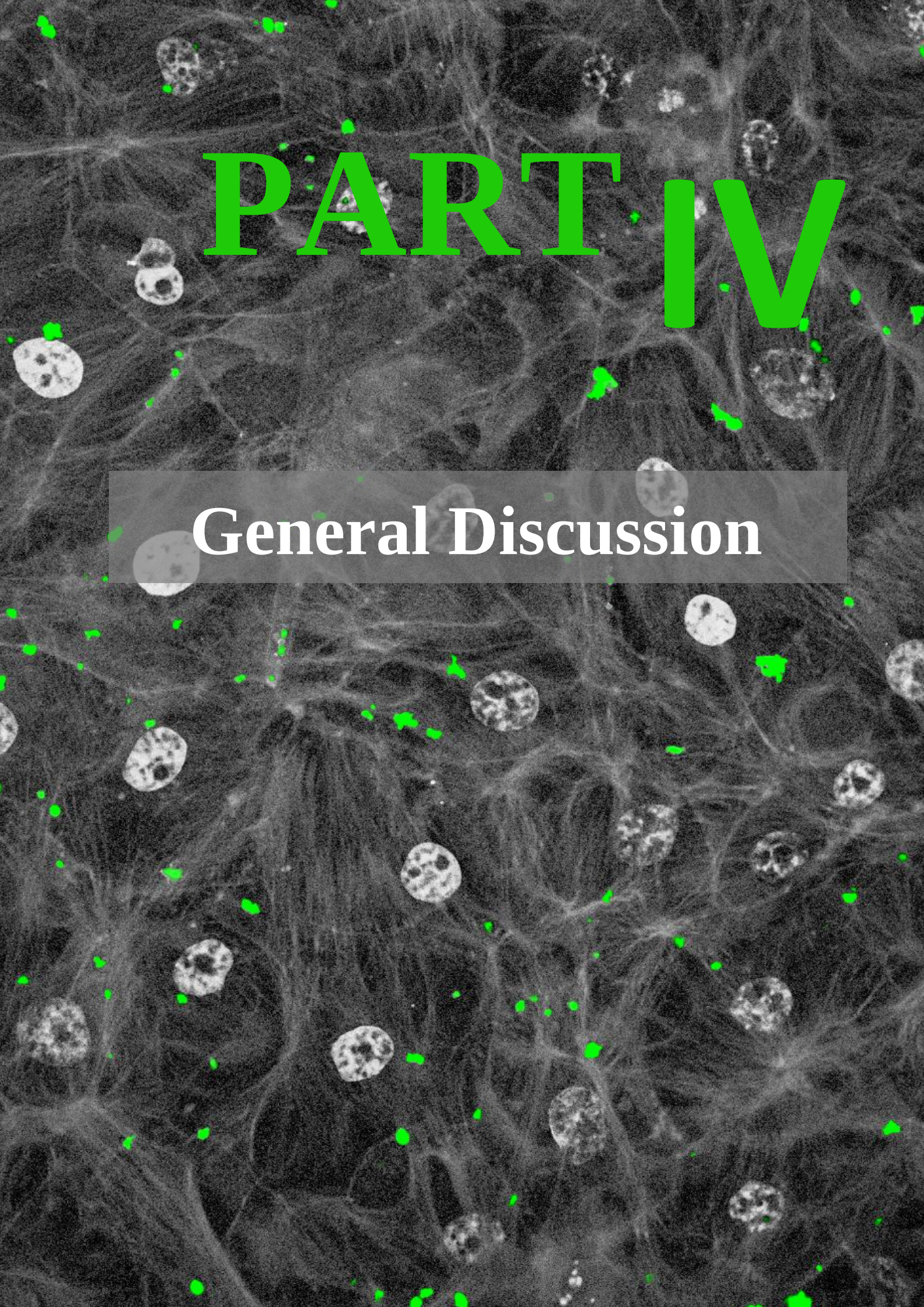


PART IV

General Discussion

CHAPTER 6: General Discussion

6.1 Introduction

To control post-weaning diarrhoea (PWD) in pigs antibiotics are commonly used.[9, 10] The long term and extensive use of antibiotics, both as therapeutic and as growth-promoting agent, has resulted in antibiotic resistance.[419] Therefore, since 2006, the use of antibiotic growth promoters for prevention of diarrhoeal diseases in piglets has been completely banned in the European Union. However, this caused a reduced performance and increased morbidity in post-weaning pigs and consequently, the development of alternative strategies, such as feed supplements or vaccination, is required to sustain animal health and performance.[11] Non-digestible carbohydrates have been tested as effective alternatives for antimicrobial growth promoters to help post-weaning piglets to cope with feed transition and stress during this period.[12, 13] Dietary β-glucans have the capacity to act as immunostimulants and are thus of particular interest as alternative nutritional additives. The immunostimulatory effects of β-glucans are well-known, however, the contribution of the different β-glucan receptors to the β-glucan mediated effects is still a matter of debate. In this dissertation, we examined the role of the β-glucan receptors dectin-1 and complement receptor 3 (CR3) and their signalling pathways in the β-glucan-mediated activation of porcine innate immune cells. Besides feed additives, oral vaccination could alleviate PWD. Oral vaccines are superior to parenteral vaccines due to their ability to trigger both systemic and local antibody responses and their socio-economic advantages. Current commercial oral vaccines mainly use live-attenuated microorganisms replicating in the gut.[38] However, live vaccines have potential risks and therefore, vaccine design also focuses on creating non-replicating oral vaccines. Thus, the development of an oral vaccine is challenging since the gastrointestinal tract is a very hostile environment, the passage through the epithelial barrier is difficult and orally administered antigens generally induce oral tolerance.[33-37] Therefore, strategies to surmount these obstacles, e.g. encapsulation, adding adjuvants and selective targeting, are required to develop oral vaccines. Here, we took advantage of the immunostimulatory potential of particulate β-glucans to create an oral vaccine. β-glucan microparticles (GPs) are already described as promising antigen vehicle systems with a high antigen encapsulation efficiency and safety.[21-32] In addition, we further improved these particles by enhancing their uptake across the epithelial barrier.

6.2 β-glucans and their receptor usage

Since no data are available on the β-glucan receptor usage and the β-glucan signalling pathway in porcine neutrophils and macrophages, **chapter 3** investigated the involvement of dectin-1 and complement receptor 3 (CR3) in the β-glucan-mediated responses by porcine innate immune cells. For this purpose, dectin-1 and CR3 were blocked and the β-glucan-mediated reactive oxygen species (ROS) production by porcine neutrophils was measured. Laminarin was used to inhibit dectin-1, since blocking monoclonal antibodies against porcine dectin-1 are not available and the human and murine dectin-1 mAbs do not crossreact with porcine dectin-1. Laminarin has been shown to be one of the most effective inhibitors of dectin-1.[420] Similar to the results in human neutrophils, dectin-1 does not seem to play a role in the ROS production by porcine neutrophils towards β-glucans, while CR3 is indispensable in these responses. Interestingly, we have strong indications that CD11R3 is the α subunit of CR3 in pigs, since this receptor is involved in the β-glucan mediated immune responses in porcine neutrophils. In contrast, CD11R1, which is recognised by cross-reactive human CD11b-specific mAbs, is not involved in these responses. Furthermore, as the FAK inhibitor is able to completely inhibit the β-glucan-mediated responses, we propose that the signal molecule FAK is indispensable for the β-glucan-mediated responses (ROS and cytokine production) in porcine neutrophils. As a role for dectin-1 is lacking, but there is a minor role for Syk in β-glucan signalling, we believe that recruitment of Syk is mainly triggered by phosphorylation of the adaptor molecules FcRγ or DAP12 by Src kinases (Figure 1a). It has already been described that integrin ligation could induce phosphorylation of the ITAM-bearing adaptor molecules FcRγ or DAP12, which creates docking sites for Syk and contributes to the activation of FAK.[130]

On the contrary, the contribution of dectin-1 and complement receptor 3 in the β-glucan-mediated responses is less clear for porcine macrophages. Dectin-1 seems to be involved in the phagocytosis of zymosan. Unlike the results obtained in porcine neutrophils, complement receptor 3 does not play a role in zymosan phagocytosis by macrophages, however, CD11R3 seems to be involved in the cytokine secretion mediated by different particulate β-glucans, including zymosan. Conversely, dectin-1 is not involved in this β-glucan-mediated response, not even for zymosan, indicating the complex mechanism of β-glucan recognition and signalling in porcine macrophages. The most interesting revelation is that FAK acts as a crucial regulator in the β-glucan-mediated responses, whereas Syk is only partially involved. Interestingly, in the *Euglena gracilis*-mediated cytokine responses Syk's role seems to be as important as FAK. Therefore, we propose that the β-glucan structure determines which receptor they bind. Indeed, complement receptor 3 (CR3) seems to play a minor role for *Euglena gracilis*-mediated responses in macrophages, whereas this receptor is significantly involved in the zymosan- and Macrogard-mediated cytokine responses. Since both

zymosan and Macrogard have a β(1,3)-linked backbone with β(1,6)-linked branches, while *Euglena gracilis* is unbranched, we suspect that branches are crucial for recognition by CR3 on porcine macrophages. Other receptors, such as scavenger receptors or lactosylceramide, are probably involved in the β-glucan-mediated responses as well (Figure 1b). We propose that macrophages can switch between different β-glucan receptors, however, one receptor will dominate over the other and will direct the immune response (Figure 1b).[421] This hypothesis correlates with the host response to infection being reliant on the cellular heterogeneity. It is likely that different receptors contribute to β-glucan recognition, and it would be interesting to determine which receptors could substitute for dectin-1 and CR3.[153] CD36 and SCARF1 are potential β-glucan receptors expressed on porcine macrophages (Supplemental Figure 1) and are probably involved in β-glucan recognition. However, experiments have to be performed to confirm this hypothesis.

Besides the difference in receptor recognition, the β-glucan structure is also responsible for their biological activity, probably attributed to the dominant β-glucan receptor binding capacity. Macrogard (200 µg/ml) could only induce marginal cytokine responses by neutrophils and macrophages, while zymosan and *Euglena gracilis* were able to trigger higher cytokine secretions. Conversely, at the same concentration, Macrogard and *Euglena gracilis* were able to induce strong ROS production by neutrophils, while this response towards zymosan was significantly lower. This indicates that differences in branches are important for their receptor recognition and subsequent immunomodulating properties. However, a cautious approach must be adopted to the interpretation of these data, since β-glucan preparations, such as zymosan, are often impure, which could influence receptor binding or cellular responses.

Altogether, the observed results in pigs correlate with the receptor usage described in human innate immune cells, while they differ with the rodent system. In porcine and human neutrophils, CR3 seems to be the most important β-glucan receptor,[150, 151] which contrasts with what has been published for murine neutrophils.[125, 148, 153] On the contrary, the importance of receptor heterogeneity in β-glucan recognition by monocytes/macrophages seems to be important in all three species. These interesting results could contribute to the implementation of β-glucans as feed additives or as alternatives for antibiotics in pigs. Indeed, knowledge about β-glucan receptor binding and signalling by porcine neutrophils could be used to steer specific immune responses *in vivo*.

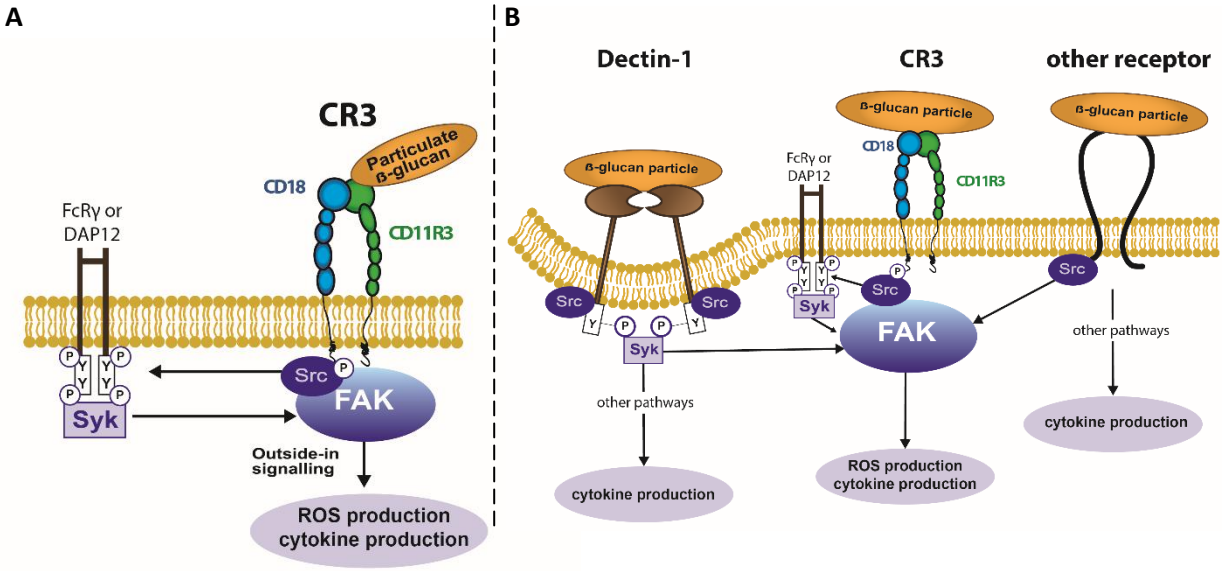


Figure 1. β-glucan recognition and signalling in porcine neutrophils and macrophages.
(A) β-glucan recognition and signalling in porcine neutrophils. Particulate β-glucans bind the α subunit of CR3 (CD11R3), which results in the activation of focal adhesion kinase (FAK) and Src kinases, followed by ROS production, phagocytosis and cytokine production. Phosphorylated Src kinases can also induce phosphorylation of ITAM-containing adaptor molecules, such as DAP12 and FcRγ. This results in docking sites for Syk kinases, which will activate FAK as well. **(B) β-glucan recognition and signalling in porcine macrophages**. Particulate β-glucans activate the Src-family kinases (Src) by binding dectin-1. Syk is recruited to these phosphorylated ITAMs, which results in cytokine production and FAK-mediated ROS production. These β-glucan-mediated responses can also be induced by binding to complement receptor 3 (CR3), which induces FAK/Src phosphorylation. Phosphorylated Src kinases can also induce phosphorylation of ITAM-containing adaptor molecules, such as DAP12 and FcRγ. This results in docking sites for Syk kinases, which will activate FAK as well. Other receptors can bind particulate β-glucans in the absence of dectin-1 and CR3, which induces the activation of different signalling pathways.

In normal homeostasis, neutrophils are present in blood. Interestingly, it has been described that orally administered β-glucan particles are found in the blood of rats,[422] indicating the possibility to be phagocytosed by blood neutrophils. However, neutrophils are also found at the intestinal mucosa and in the gut lumen. Indeed, during intestinal inflammation neutrophils are the first line of defence. Tissue-resident macrophages contribute to the recruitment of neutrophils to the lamina propria or the intestinal lumen through production of chemokines.[423] [424] Intestinal neutrophils release anti-microbial molecules, reactive oxygen intermediates and monocyte chemoattractants. Oral administration of β-glucans can modulate these neutrophil responses. Furthermore, shortly after arrival of neutrophils to the mucosa, macrophages are recruited for a second-wave inflammatory response that ensues for the next several days.[423] Although our experiments were performed on monocyte-derived macrophages from porcine blood, the results from our studies give an indication about the complex mechanism of β-glucan recognition and signalling in porcine macrophages. Different β-glucans will trigger distinct immunological responses. A better understanding of this complex interplay might provide new avenues for manipulating macrophage function. Besides the recruitment of neutrophils and macrophages during inflammation, resident macrophages are also

present in the gut. Thus, β-glucans can bind intestinal macrophages under normal homeostasis as well. Interestingly, it is well established that the epithelium works in coordination with microflora and immune cells to maintain this intestinal homeostasis.[425] Thus, the influence of β-glucans on the microflora or intestinal epithelial cells[84] could affect neutrophil or macrophage recruitment to the intestinal mucosa. Understanding the interaction of β-glucans with the microflora, intestinal epithelial cells and immune cells could result in a better decision process for the selection of β-glucans as feed additive or growth promoters.

6.3 Functionalised β-glucan microparticles as a versatile oral vaccine platform

Since particulate β-glucans are well-known for their immune-stimulating character **(chapter 3)**, using these polysaccharides as adjuvants for oral vaccination seems very interesting. In particular, β-glucans derived from the major cell wall component of yeast (zymosan and Macrogard) have DC immunostimulating properties,[53] suggesting that β-glucan-rich yeast shells have the potential to be exploited as an oral antigen delivery system. *Saccharomyces cerevisiae*-derived β-glucan microparticles (GPs) are already described as hollow and porous particles primarily composed of β-1,3-D-glucan polymers (β-glucans) with a high antigen loading efficiency.[26] The immunostimulating properties of these particles have been demonstrated as well. Indeed, β-glucan microparticles induces TNFα responses by dendritic cells (DCs) *in vitro*[31] and Th17 responses in mice after oral administration.[32]

Besides mucosal adjuvants, oral vaccines require the protection of their antigens against the hostile environment of the gastro-intestinal tract. Incorporation of antigens in microparticles is helpful to protect them against degradation in the stomach. Moreover, the encapsulation in microparticles is a promising way to deliver a sufficient amount of antigens to the intestinal mucosa in order to elicit sufficient immune responses against the orally administered soluble antigens. Interestingly, β-glucan particles have a dual function: these polysaccharides serve as both adjuvant and antigen carrier. Indeed, hollow β-glucan particles are known to be easily loaded with antigens, which can protect the antigens against enzymatic degradation in the gastrointestinal tract. The high antigen loading capacity was confirmed in our experiments. Previous studies only loaded albumin (BSA, OVA), while we successfully loaded a clinically relevant infectious antigen (FedF) inside the GPs. Antigen-loaded GPs have immunostimulatory responses, however, these responses were lower in comparison with hollow particles for reasons yet unknown. Perhaps the β-glucan structure changes after antigen loading. An extra hollow GP control, which undergoes the same protocol without antigen loading, would give us more information whether the decreased ROS production is caused by the antigen or the used protocol. However, the antigen-loaded particles still exert antimicrobial activity on porcine

neutrophils by binding to CR3, indicating the immunestimulating potential of the β-glucans in this GP-based oral vaccine.

Besides their immunostimulatory properties and their ability to carry a large amount of soluble antigens, these particles can be functionalised with targeting antibodies, making these particles very promising as oral antigen delivery vehicle. The most difficult task for conjugation of antibodies to particles is their oriented conjugation. The antigen binding site (Fab fragment) has to face outwards from the particle surface in order to detect their ligand. We used protein G as linker molecule to properly conjugate antibodies to the particles. In this thesis **(chapter 5)**, aminopeptidase N (APN) was selected as a target to increase the uptake of particles by the epithelial barrier. The correct functionalisation of the particles with APN-specific mAbs was demonstrated by the significant higher uptake of the APN-targeted GPs by APN-IPEC-J2 cells and by intestinal epithelial cells located in the jejunal lamina propria. Since the lamina propria constitutes the largest surface area in the intestine, these results suggest that the targeted particles will be transcytosed more *in vivo* than control particles. Indeed, preliminary data in ligated loops indicate that APN-targeted particles are endocytosed more than uncoated particles (Supplemental Figure 2). Interestingly, besides the efficient targeting to intestinal epithelial cells, aggregation of the APN-targeted particles was minimised due to coating with the near neutrally-charged PEG (polyethylene glycol).[322] This is another reason why we believe that targeted particles will reach the intestinal mucosa more *in vivo* than not-targeted particles. However, to serve as antigen delivery vehicle, it is also necessary that the antigens encapsulated within the microparticles are efficiently internalised and subsequently processed by antigen-presenting cells (APCs), such as dendritic cells (DCs). DCs are the most potent APCs, which orchestrate innate and adaptive immune responses and have the unique capacity to activate naïve T cells.[426] Uncoated β-glucan microparticles are highly engulfed by dendritic cells, which leads to maturation of the dendritic cells **(chapter 5)** and subsequent antigen presentation to T cells **(chapter 4)**. In **chapter 5**, we demonstrated that targeted particles are more efficiently internalised and induce increased maturation compared to uncoated particles. This observation can be explained by the synergistic effect of β-glucan recognition by different β-glucan receptors and APN targeting. Further experiments are needed to assess this proposed synergistic effect. The promising *in vitro* results incited us to investigate for the first time the efficacy of this APN-targeted FedF-loaded GP-based vaccine as oral delivery system in a large animal model. The results clearly demonstrate a higher systemic FedF-specific immune response in the group receiving APN-targeted particles than in the control group. Although these levels were only significant for total Ig in serum and IgG antibody-secreting cells (ASC) in blood, we also observed an increased IgA immune response in the APN-targeted group. These results indicate that APN-targeted FedF GPs are a promising oral antigen delivery system to combat $F18^+$ *E. coli* infection. However, whether this vaccine induces a

local immune response and could provide protection against a subsequent F18$^+$ *E. coli* infection has not been investigated yet. A schematic representation of these targeted β-glucan microparticles and their biofunctionality is given in Figure 2.

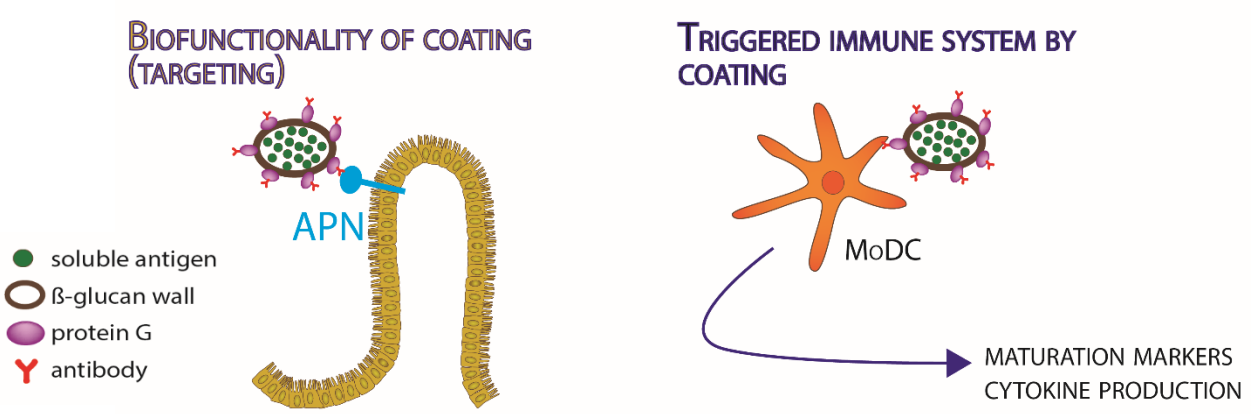


Figure 2. Schematic representation of the effectiveness of APN-targeted β-glucan microparticles.
Soluble antigens are encapsulated in β-glucan microparticles. Subsequently, these particles are coated with protein G, followed by the addition of antibodies against APN. APN-targeted β-glucan microparticles are efficiently endocytosed by intestinal epithelial cells by binding to its target protein APN. Furthermore, targeted particles induce MoDC maturation and cytokine production significantly more than uncoated particles.

6.4 Main conclusions and future perspectives

β-glucans use different receptors on innate immune cells to evoke their specific immunomodulatory responses. Interestingly, their receptor usage seems to be cell-type specific and species-dependent. Indeed, in both human and porcine neutrophils, complement receptor 3 (CR3) and its signalling molecule FAK seem to be indispensable for the β-glucan-mediated effects. On the contrary, the heterogeneity of receptors is more important to recognise β-glucans in macrophages or monocytes. The results obtained in pigs are more similar to the results in man than in mice, where dectin-1 dominates β-glucan recognition in both neutrophils as macrophages. Since the results from pigs can be extrapolated to man, we propose that pigs are a better translational model for β-glucan research than mice. Transferring this knowledge to commercial β-glucans developed as in-feed alternatives for antibiotics or as immunostimulants might protect both pigs as humans against enteric infections.

To predict the immunological outcome, the cooperation of different β-glucan receptors and their signalling pathways upon stimulation with multiple β-glucans in intestinal resident immune cells could be unravelled. Moreover, in order to select more immunostimulatory β-glucans, further research could focus on the physiology of different β-glucans and their interaction with the microbiota, intestinal epithelial cells (IEC) and the immune system in pigs. β-glucans are already known to induce secretion of the pro-inflammatory chemokines IL-8 and CCL2 by human IEC cell lines (HT-29 and SW480) via dectin-1 and Syk signalling. These findings highlight the importance of β-

glucan and IEC interactions in intestinal inflammation.[84] Investigating the interaction of β-glucans with porcine intestinal epithelial cells could be used to redirect the intestinal immune response.

Furthermore, microbiota are important for gut health and play an important role in immune enhancing effect of orally administered β-glucans. This could be attributed to 1) the effect of β-glucans on microbiota population dynamics (β-glucans have been shown to change the composition of the microbiota)[214, 427] or to 2) the fermentation of β-glucans by the microbiota into short-chain fatty acids (SCFA), such as butyrate, which has multiple beneficial effect on gut health.[212] Unfortunately, the influence of the microbiota on the fate of the β-glucans and the modulation of the immune system is not investigated yet in pigs. To address this issue, we could orally administer β-glucans to gnotobiotic pigs, localise the β-glucans and study the cytokine responses by intestinal immune cells. Furthermore, intestinal loop experiment in pigs could be used to evaluate the physicochemical behaviour (structural modification, digestibility,..) of different β-glucans. In addition, transcriptomic profiling can be assessed for cytokine production and by the pig intestinal micro-array as described earlier.[428] As such, the most effective β-glucan could be selected as feed-additive as well as for designing β-glucan-based oral subunit vaccines, where they can serve as both adjuvant and antigen vehicle.

Until today, only β-glucans derived from *Saccharomyces cerevisiae* are used as antigen vehicle system. Orally delivered yeast-derived β-glucan microparticles are already known to trigger innate and adaptive (Th1 and Th17) immune responses and have a high antigen encapsulation efficiency. However, β-glucans derived from other sources or from certain mutant strains[429] could trigger different immune responses. Multiple yeast deletion mutants, carrying a null mutation in a gene involved in cell wall synthesis, have been developed to evaluate their immunostimulatory characteristics in invertebrates.[429] For instance, the Mnn9 yeast mutant, which has less cell-wall bound mannoproteins and more glucan and chitin, seems to completely protect *Artemia* against the pathogen *Vibrio campbellii*.[429] Likewise, our preliminary results demonstrate a higher ROS production by porcine neutrophils when stimulated with Mnn9 in comparison with the wild-type yeast cells (WT) (Supplemental Figure 3).[429] However, more research has to be performed to assess the immunostimulatory capacity of these yeast mutants. Selection of the most effective β-glucan preparation for the desired purpose is important to direct the immune system.

In this thesis, we have used yeast-derived β-glucan microparticles as antigen carrier for oral administration, since its high loading efficiency and immunostimulatory characteristics were already thoroughly described. We have incorporated for the first time a clinically relevant antigen, FedF, inside these particles. Since the loading of FedF inside the particles was less efficient than for the model antigens albumin, optimalisation of the antigen concentration could lead to a higher antigen load. However, FedF-loaded GPs were able to induce FedF-specific T cell proliferation and FedF-

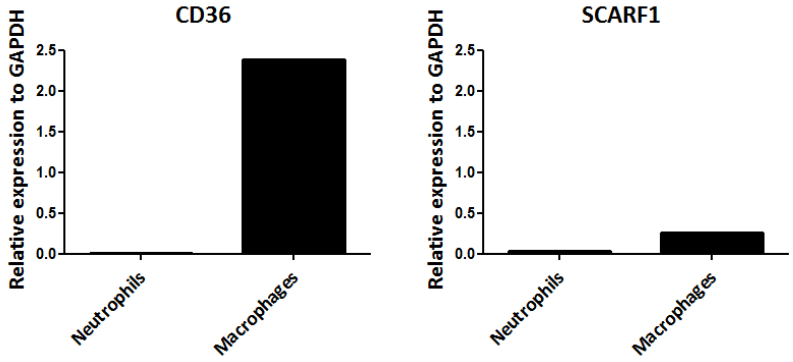
specific antibody responses after oral administration, indicating the successful incorporation of FedF inside the particles.

Furthermore, besides their role as immunostimulants and antigen vehicle, these particles can be functionalised with targeting antibodies to direct them towards specific cell types and/or help them crossing the epithelial barrier. Aminopeptidase N (APN) was selected as target for the oral GP-based vaccine, since APN targeting results in an efficient transcytosis of the particles inside intestinal epithelial cells and this protein is also expressed on immune cells. Up till now, the *in vivo* performance and fate of these APN-targeted particles upon oral administration in pigs remain unanswered. Are these particles able to cross the epithelial barrier? Although we observed transcytosis of the particles in porcine intestinal explants and intestinal loops, the kinetics and physiology of the *in vivo* uptake by enterocytes after oral administration awaits in-depth research. Furthermore, it would be interesting to investigate which immune cells phagocytose GPs upon crossing the epithelial barrier. Are these immune cells activated and what kind of immune response do they elicit? An additional loop experiment is necessary to answer these questions.

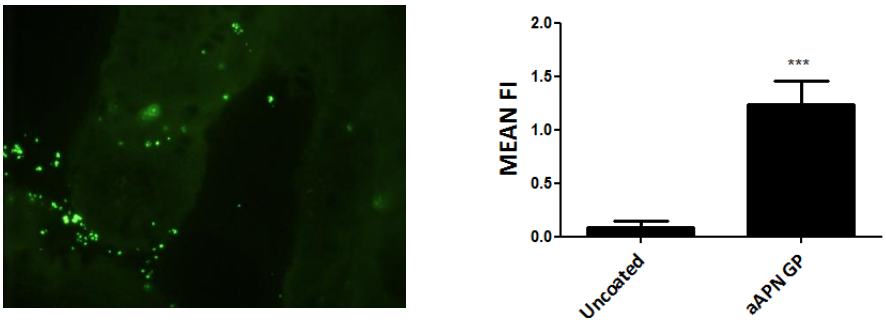
Interestingly, our oral immunisation experiment has demonstrated that APN-targeted FedF-loaded particles in combination with cholera toxin (CT) induce FedF-specific serum antibody responses in vaccinated pigs, indicating the immune-stimulating capacity of these particles *in vivo*. Despite these promising results, other *in vivo* experiments should be performed to investigate whether local FedF-specific SIgA responses are triggered and if these are adequate to protect piglets against an $F18^+$ ETEC infection. Since cholera toxin was used as adjuvant immunising piglets with APN-targeted GPs, it would be of interest to study the effect of these particles without adjuvant. De Smet et al.[32] and De Jesus et al.[22] already reported that unconjugated microspheres without adjuvant were efficiently transcytosed through M cells in the murine gut *in vivo* and are excellent inducers of both innate and T cell responses. However, whether these APN-targeted particle formulations without adjuvant are able to overcome oral tolerance in larger animals is not known yet. To address this issue, we could investigate whether intestinal APCs will trigger pro-inflammatory cytokine responses upon GP uptake. As such, we could predict whether the APN-targeted particles will induce effector T cell or regulatory T cell (Treg) responses, thus if this leads to a state of immunological hyporesponsiveness towards the administered antigen or not. If these particles are not able to induce protective immune responses, modification of the particles with other targeting molecules or adjuvants might direct the immune response towards protection. Since the conjugation of protein G creates a platform which allows easy switching of the targeting moiety, a combination of targeting ligands could be used to functionalise the particles, such as APN to get through the epithelial barrier and TLR5 to target DCs. In mice, the ability of TLR5 signalling by DCs to induce mucosal production of IL-17 and IL-22 has been demonstrated.[430] Since recent studies highlighted the contribution of IL-17 and IL-22 to defensive

reactions within the mucosa,[431-433] targeting TLR5 could differentiate lymphocytes into T helper 17 (Th17) cells, which could results in protective immune responses. In addition, TLR5 is also expressed on lamina propria DCs in pigs and stimulating these DCs results in functional maturation.[434] Furthermore, the double mutant heat-labile enterotoxin (dmLT) is an attractive adjuvant for inclusion in mucosal vaccines. Indeed, dmLT was not only found to be safe and well tolerated in a clinical trial,[304] this adjuvant also induces cytokine secretion (IL-17, IFN-γ, tumor necrosis factor (TNF)), antigen-specific mucosal IgA and cellular immune responses when mucosally administered in combination with a pathogenic antigen in mice.[435] In addition, further optimising the number of antibodies on the surface of the particles, optimising the vaccination dose and encapsulation in an enteric coating could result in an efficient immune response towards the pathogenic antigen incorporated inside the particles, and consequently, result in a lower vaccination dose.

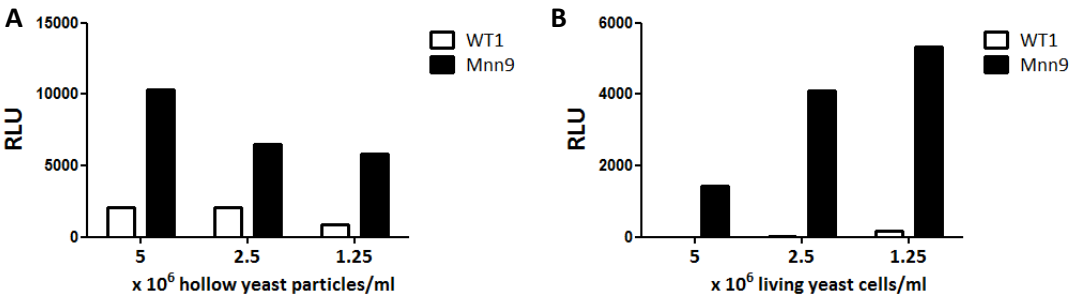
SUPPLEMENTAL FIGURES



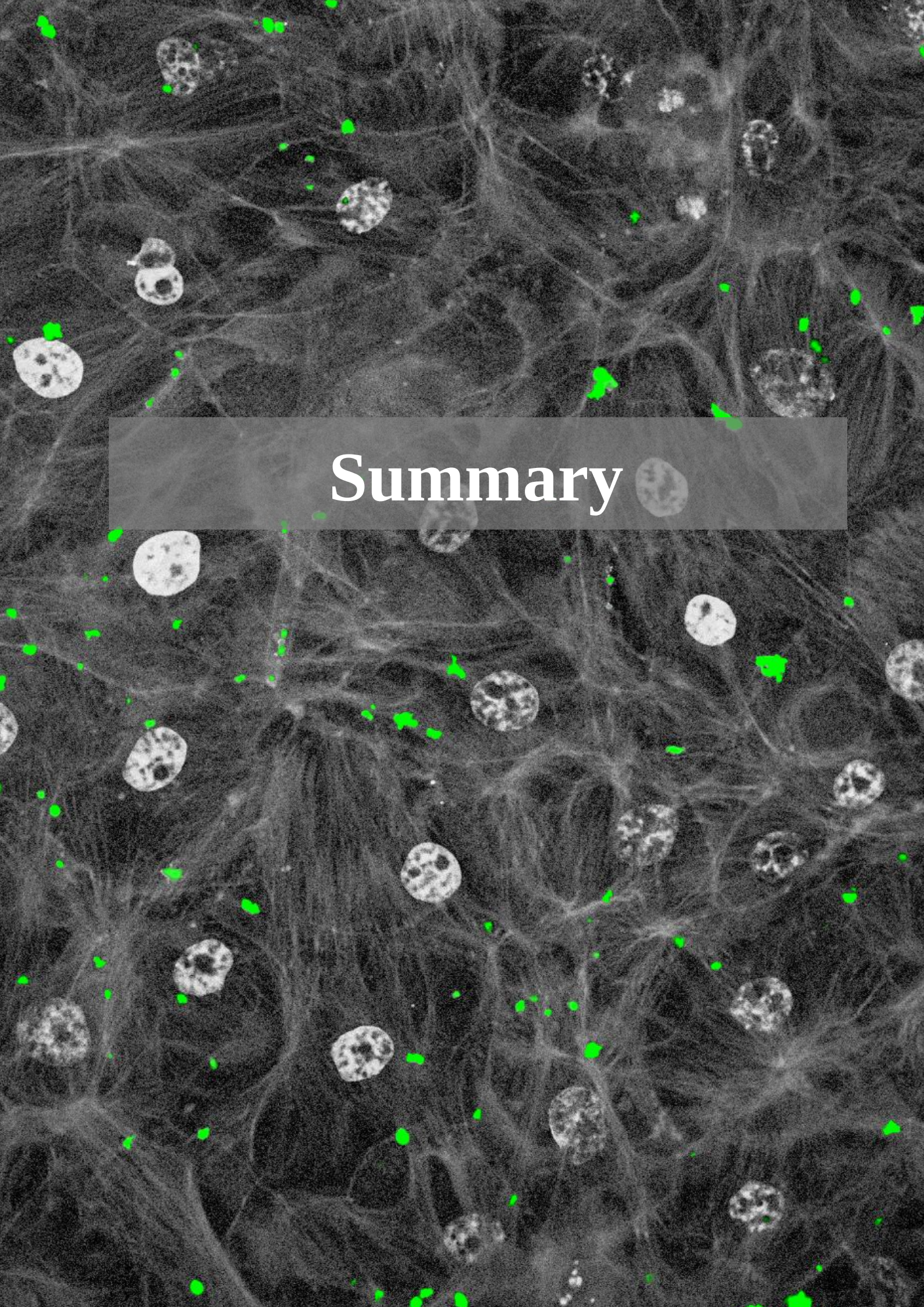
Suppl Figure 1. Expression of CD36 and SCARF1 on porcine neutrophils and macrophages.
The relative expression of CD36 and SCARF1 on porcine neutrophils and macrophages was determined by qPCR in relation to the reference gene GAPDH. The relative C_T values are presented in the graphic. CD36 is highly expressed on porcine macrophages.



Suppl. Figure 2. Uptake of APN-targeted GPs in the lamina propria of porcine intestinal loops.
Pigs were anesthetised under intramuscular anesthesia after an overnight fast as described by Loos et al.[436] Isolated loops of about 15 mm in length were prepared *in situ* from the jejunum without PP.[404, 437] Approximately 500 µl of the GP-suspension was injected into the lumen of the loop. The gut was returned to the abdominal cavity, the abdomen was closed and general anaesthesia was maintained. After 15 minutes, loops were removed and immediately frozen in methocel. Cryosections (18 µm) were cut by a cryotome (Leica CM3050 S), placed on APES-coated glass slides and fixed in aceton for 10 minutes at -20°C. **(A)** Fluorescence image of APN-targeted particles (green) in the jejunal lamina propria is given **(B)** The mean fluorescence intensity of 4 different sections was calculated (mean ± SD, n = 1).***: p<0.001.



Suppl. Figure 3. ROS production by neutrophils stimulated with yeast (mutants).
Neutrophils (2 x 10^5 cells) were stimulated with the living wild type yeast cell (WT1) or the Mnn9 yeast mutant **(A)** or hollow yeast particles **(B)**. ROS production was determined via chemiluminescence. Data are shown as the mean relative light units (RLU) of four pigs.



Summary

Postweaning diarrhoea (PWD) is one of the most frequent causes of heavy economic losses in swine husbandry due to growth retardation, increased drug use and elevated mortality. *Escherichia coli* are most common causative agent. In order to cope with this problem, antibiotics have been used to sustain animal health and performance. However, almost 75% of the piglets in Flanders carry resistant *E. coli*, caused by the excessive use of antibiotics. As these resistant bacteria can also be transmitted to humans via the faeces, a serious risk for human health exists. Thus, an alternative strategy is necessary to control PWD.

Immunomodulators, such as β-glucans, have potential to increase the functionality of the immune system. The immunostimulating mechanism of β-glucans is, however, still a matter of debate. Dectin-1 is considered as the main β-glucan receptor in mice, while studies in man show that complement receptor 3 (CR3) is more important in β-glucan-mediated responses. In pigs, no data are available on the β-glucan receptor usage and the β-glucan signalling pathway in innate immune cells. Elucidating which receptor contributes to the response of innate immune cells towards particulate β-glucans in pigs has been investigated in this thesis.

Besides the use of immunomodulators, active oral vaccination against ETEC infections is another strategy to prevent PWD in piglets. Current oral vaccines, based on attenuated or killed pathogens, have safety risks or elicit an inefficient immune response. Subunit vaccines are a safe alternative, however, are less efficacious and require adjuvants. Different strategies exist to increase the immunogenicity of these vaccines, such as encapsulation in particles. Designing a subunit oral vaccine platform based on particulate β-glucans is another aim of this thesis.

In **the first chapter** a brief introduction is given on the molecular structure of different β-glucans, their receptors and signalling pathways. Furthermore, the current knowledge on the biological effects of orally administered β-glucans is reviewed.

In **chapter 2** a short introduction on the intestinal immune system is given. Furthermore, we summarised current registered human and porcine oral vaccines and gave an overview of different oral vaccine design strategies, focussing on the use of β-glucan microparticles and different targeting strategies.

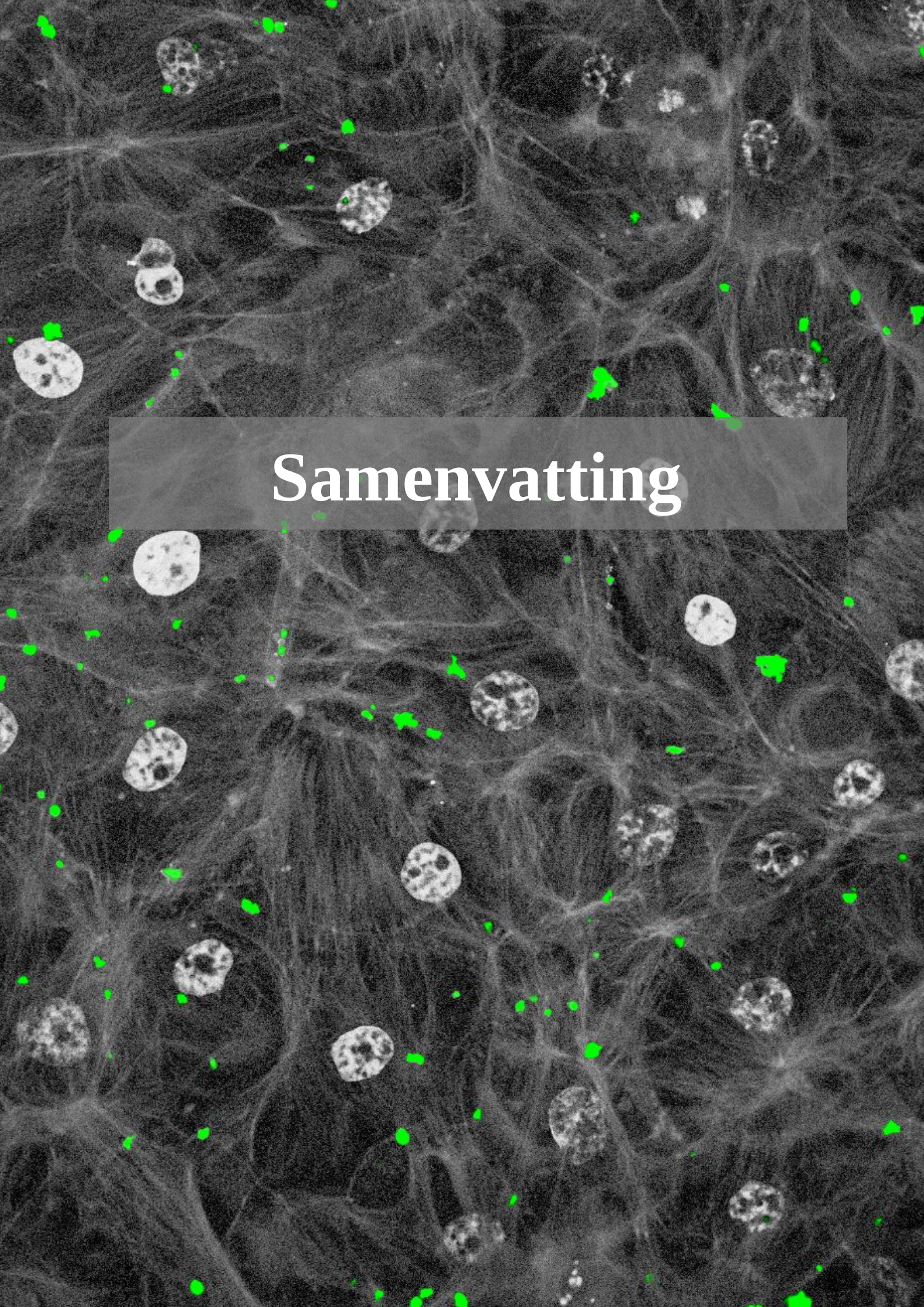
Chapter 3 presents our findings on the use of β-glucan receptors and signalling pathways in porcine innate immune cells. The cell-type specific recognition of particulate β-glucans in porcine neutrophils and macrophages was partially elucidated. Indeed, our results clearly demonstrated an important role of CR3 in β-glucan recognition, as blocking this receptor strongly reduced the phagocytosis of β-glucans and the β-glucan-induced reactive oxygen species (ROS) production by porcine neutrophils. Conversely, dectin-1 does not seem to play a major role in β-glucan recognition in neutrophils. In

porcine macrophages, however, both dectin-1 and CR3 are involved in the β-glucan-mediated responses. In addition, we assume that other receptors, such as scavenger receptors or lactosylceramide, are involved in these responses towards β-glucans by macrophages as well. Interestingly, we have strong indications that CD11R3 is the α subunit of CR3 in pigs, since this receptor is involved in the β-glucan mediated immune responses in porcine neutrophils. In contrast, CD11R1, which cross-reacts with the human CD11b mAbs, is not involved in these responses. Furthermore, we have demonstrated that CR3 signalling through focal adhesion kinase (FAK) was indispensable for β-glucan-mediated ROS production and cytokine production in both neutrophils and macrophages, while the Syk-dependent pathway was only partly involved in these responses. We conclude that CR3 plays a cardinal role in β-glucan signalling in porcine neutrophils, while macrophages use a more diverse receptor array to detect and respond towards β-glucans. Nonetheless, FAK acts as a master switch that regulates β-glucan-mediated responses in neutrophils as well as macrophages. These results in pigs correlate with the receptor usage described in human innate immune cells, while they differ with the rodent system. Therefore, this study supports the use of pigs as a valuable model to evaluate the effects of β-glucans on innate immunity.

Chapter 4 and 5 focus on the design of an oral subunit vaccine platform. β-glucan microparticles (GPs) are promising antigen delivery systems for oral vaccination, because of their dual character as both antigen vehicle and immunostimulants. We first investigated the loading capacity of these particles by incorporating the clinically relevant antigen FedF inside GPs. The incorporation of FedF inside the particles was highly efficient and occurred without antigen degradation. Furthermore, GPs still retain immune-stimulatory properties upon antigen loading, although ROS responses were diminished for reasons yet unknown. In addition, the dual character of these particles was confirmed in a T cell proliferation assay. Indeed, FedF-loaded particles induced a significant higher FedF-specific T cell proliferation than soluble FedF. These results encourage the use of β-glucan microparticles as antigen delivery system for oral vaccination. However, multiple barriers need to be overcome to design oral vaccines, including the poor uptake by the epithelial barrier. Selective targeting to intestinal epithelial cells seems a promising strategy to surmount this obstacle. **Chapter 5** evaluated the capacity of aminopeptidase N (APN)-targeted β-glucan microparticles (GPs) as oral antigen delivery system. APN is expressed on the apical surface of intestinal epithelial cells and is strongly conserved in multiple species. Antibodies against APN were efficiently conjugated to GPs in a correct orientation via the biolinker protein G. The resultant microparticles were analysed for their antigen load, adjuvanticity and interaction with enterocytes and dendritic cells (DCs). Functionalisation of GPs with antibodies neither impedes antigen load nor adjuvanticity. In addition, targeting to APN increased the uptake of microparticles by enterocytes and DCs, leading to an enhanced maturation

of the latter as evidenced by an upregulation of maturation markers and a strong pro-inflammatory cytokine response (TNFα, IL-1β, IL-12p40). Finally, oral administration of APN-targeted antigen-loaded particles to piglets elicited higher serum antigen-specific antibody responses as compared to control particles. Taken together, these data support the use of APN-targeted GPs for oral delivery of antigens.

The final chapter **(chapter 6)** presents the general discussion and future perspectives and applications of the findings.



Samenvatting

Speendiarree is één van de belangrijkste oorzaken van zware economische verliezen voor varkenshouderij als gevolg van groeivertraging, een verhoogd gebruik van geneesmiddelen en sterfte. *Escherichia coli* is de belangrijkste bacterie die deze ziekte veroorzaakt bij biggen. Om dit probleem te bestrijden werden antibiotica gebruikt om de gezondheid en prestatie van het dier te behouden. Overvloedig en langdurig gebruik van antibiotica werken echter het ontstaan van antibiotica resistente bacteriën in de hand. Recent werd aangetoond dat ongeveer 75% van de jonge biggen in Vlaanderen resistente *E. coli* bacteriën dragen.[419] Deze resistente bacteriën kunnen overgedragen worden naar de mens via besmette groenten, wat een risico inhoudt voor de humane gezondheid. Er is dus nood aan alternatieve strategieën om gespeende biggen te beschermen tegen *E. coli* infecties.

Immunomodulatoren, zoals β-glucanen, kunnen dienen als alternatieven voor antibiotica en zijn gericht op het versterken van het immuunsysteem. De immunostimulerende werking van deze β-glucanen is echter nog steeds omstreden. In muizen wordt dectin-1 beschreven als de voornaamste β-glucaan receptor, terwijl bij de mens complement receptor 3 (CR3) belangrijker is in de β-glucaan gemedieerde responsen. Bij varkens is er nog geen data beschikbaar over β-glucaan receptor binding and hun signalisatie wegen. In deze thesis werd er onderzocht welke receptor betrokken is bij de β-glucaan gemedieerde immuunresponsen van aangeboren immuuncellen bij varkens.

Naast het gebruik van immunomodulatoren kan de toediening van een oraal vaccin biggen beschermen tegen speendiarree. De hedendaagse orale vaccins, gebaseerd op verzwakte of dode pathogenen, vertonen echter veiligheidsrisico's of induceren inefficiënte immuunresponsen. Subéénheid vaccines, daarentegen, zijn een veiliger alternatief, maar de werkzaamheid van deze vaccins is echter zwakker en eisen vaak adjuvantia. De immunogeniciteit van deze vaccins kan verhoogd worden door deze in te kapselen in partikels. Het doel van dit deel van de thesis is het ontwikkelen van een subéénheid oraal vaccin platform gebaseerd op partikelvormige β-glucanen.

Hoofdstuk 1 geeft een korte inleiding over de moleculaire structuur van de verschillende β-glucanen, hun receptoren en signalisatie wegen. Daarnaast wordt de huidige kennis over de biologische effecten van oraal toegediende β-glucanen beschreven.

Een korte inleiding over het intestinaal immuunsysteem wordt gegeven in **hoofdstuk 2**. We geven een overzicht over de hedendaagse geregistreerde humane en varkens orale vaccins en de verschillende orale vaccin strategieën, waarbij de nadruk gelegd werd op het gebruik van β-glucaan partikels en verschillende strategieën om vaccins doelgericht af te leveren.

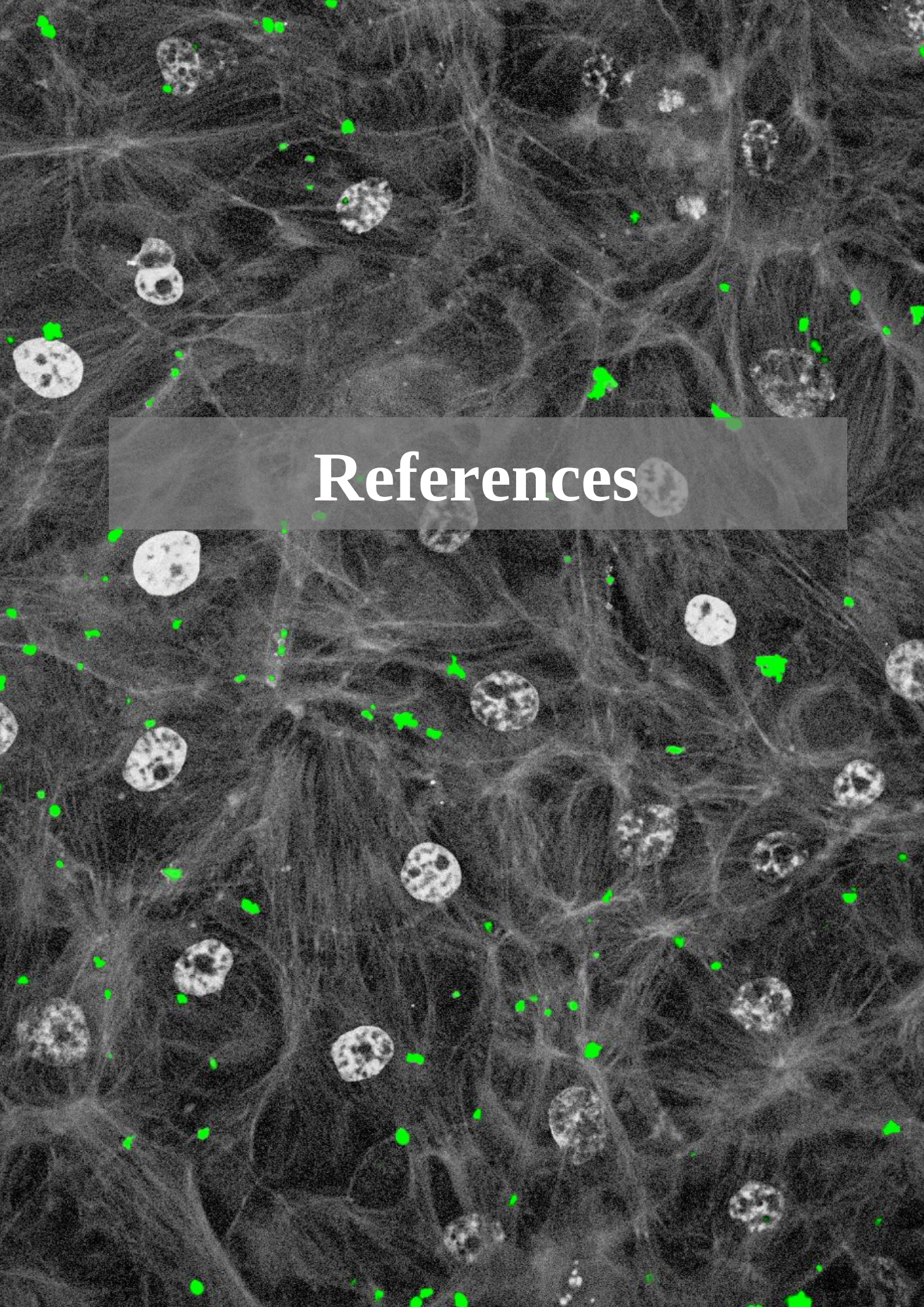
Hoofdstuk 3 geeft het experimenteel werk weer over de belangrijkste β-glucaan receptor en signalisatie wegen in varkens immuuncellen. In dit onderzoek hebben we de celtype afhankelijke

herkenning van β-glucanen in varkens neutrofielen en macrofagen gedeeltelijk ontrafeld. Onze resultaten tonen duidelijk een belangrijke rol in β-glucaan herkenning voor complement receptor 3 (CR3) aan, aangezien het blokkeren van deze receptor de fagocytose van β-glucanen sterk vermindert alsook de β-glucaan geïnduceerde zuurstofradicaal productie door varkens neutrofielen. Dectine-1 daarentegen speelt geen rol in de β-glucaan herkenning in neutrofielen. In varkens macrofagen zijn zowel dectine-1 als CR3 betrokken in de β-glucaan gemedieerde responsen. Bovendien vermoeden we dat andere receptoren, zoals scavenger receptoren of lactosylceramide, ook betrokken zijn in deze reacties tegenover β-glucanen in macrofagen. Bij varkens is het nog steeds omstreden welke receptor de α subunit van CR3 vormt. Aangezien CD11R3 betrokken is in de β-glucaan gemedieerde immuunresponsen in varkens neutrofielen, hebben we sterke vermoedens dat CD11R3 de α subunit van CR3 vormt. CD11R1 is daarentegen niet betrokken in deze responsen. Daarenboven hebben we aangetoond dat CR3-signalisatie via 'focal adhesion kinase' (FAK) onontbeerlijk is voor de β-glucaan gemedieerde zuurstofradicaal productie en cytokine productie in neutrofielen en macrofagen, terwijl de Syk-afhankelijke pathway gedeeltelijk betrokken was in deze responsen. We kunnen dus besluiten dat CR3 een essentiële rol speelt in de β-glucaan signalering in varkens neutrofielen, terwijl macrofagen meerdere receptoren gebruiken om β-glucanen te detecteren. Desalniettemin gedraagt FAK zich als de belangrijkste signaalmolecule om β-glucaan gemedieerde responsen in neutrofielen en macrofagen te reguleren. Deze resultaten met betrekking tot de belangrijkste β-glucaanreceptor bij varkens correleren met wat beschreven is in humane studies, maar verschillen met de resultaten bij knaagdieren. Daarom is het gebruik van varkens een waardevol model om de effecten van β-glucanen op het aangeboren immuunsysteem te evalueren.

Hoofdstuk 4 en 5 leggen de nadruk op het ontwerp van een oraal subéénheid vaccin platform. Het gebruik van β-glucaan micropartikels (GPs) als antigeen afleversysteem is veelbelovend, aangezien deze partikels een duaal karakter bezitten als antigeen vervoersmiddel en immunostimulants. Eerst hebben we de ladingscapaciteit van deze partikels onderzocht door het klinisch relevante antigeen FedF te incorporeren in de partikels. Het laden van FedF in de partikels was zeer efficiënt en gebeurde zonder antigeen degradatie. Daarenboven behouden de gistpartikels hun immunostimulerende eigenschappen na antigeen lading, alhoewel de zuurstofradicaal responsen verminderd waren door nog onbekende redenen. Het duale karakter van deze partikels werd bevestigd in een T cel proliferatie experiment, waarbij we aangetoond hebben dat FedF-geladen partikels een significant hogere FedF-specifieke T cel proliferatie opwekten dan oplosbare FedF. Deze gunstige resultaten moedigen het gebruik van β-glucaan partikels als antigeen afleversysteem voor orale vaccinatie aan. Bij de ontwikkeling van een oraal vaccin moeten er echter enkele barrières overwonnen worden, zoals de zwakke opname van antigenen ter hoogte van de epitheliale barrière.

Het selectief richten van de partikels naar intestinale epitheelcellen is een veelbelovende strategie om dit obstakel te overwinnen. **Hoofdstuk 5** evalueerde de capaciteit van aminopeptidase N (APN)-gerichte β-glucaan micropartikels als oraal antigeen afleversysteem. APN wordt geëxpresseerd op de apicale zijde van intestinale epitheelcellen en is sterk geconserveerd bij verschillende species. Antilichamen tegen APN werden efficiënt en in een correcte oriëntatie geconjugeerd aan GPs via de biolinker proteïne G. De resulterende micropartikels werden geanalyseerd voor hun antigeen lading, adjuvanticiteit en interactie met enterocyten en dendritische cellen (DCs). Functionalisatie van GPs met antilichamen beïnvloedde de antigeen lading of adjuvanticiteit van de partikels niet. Het richten van de partikels naar APN leidde bovendien tot een verhoogde opname van de micropartikels door enteroyten en DCs. Opname van de partikels door DCs resulteerde in een verhoogde opregulatie van de maturatie merkers en een sterke pro-inflammatoire cytokine respons (TNFα, IL-1β, IL-12p40). Ten slotte wekte orale toediening van APN-gerichte antigeen-geladen partikels aan biggen hogere serum antigeen-specifieke antilichaam responsen op in vergelijking met controle partikels. Deze data ondersteunen dus het gebruik van APN-gerichte GPs voor de orale aflevering van antigenen.

Het laatste hoofdstuk **(hoofdstuk 6)** stelt de algemene discussie voor en de potentiële toekomstperspectieven en toepassingen van onze bevindingen.



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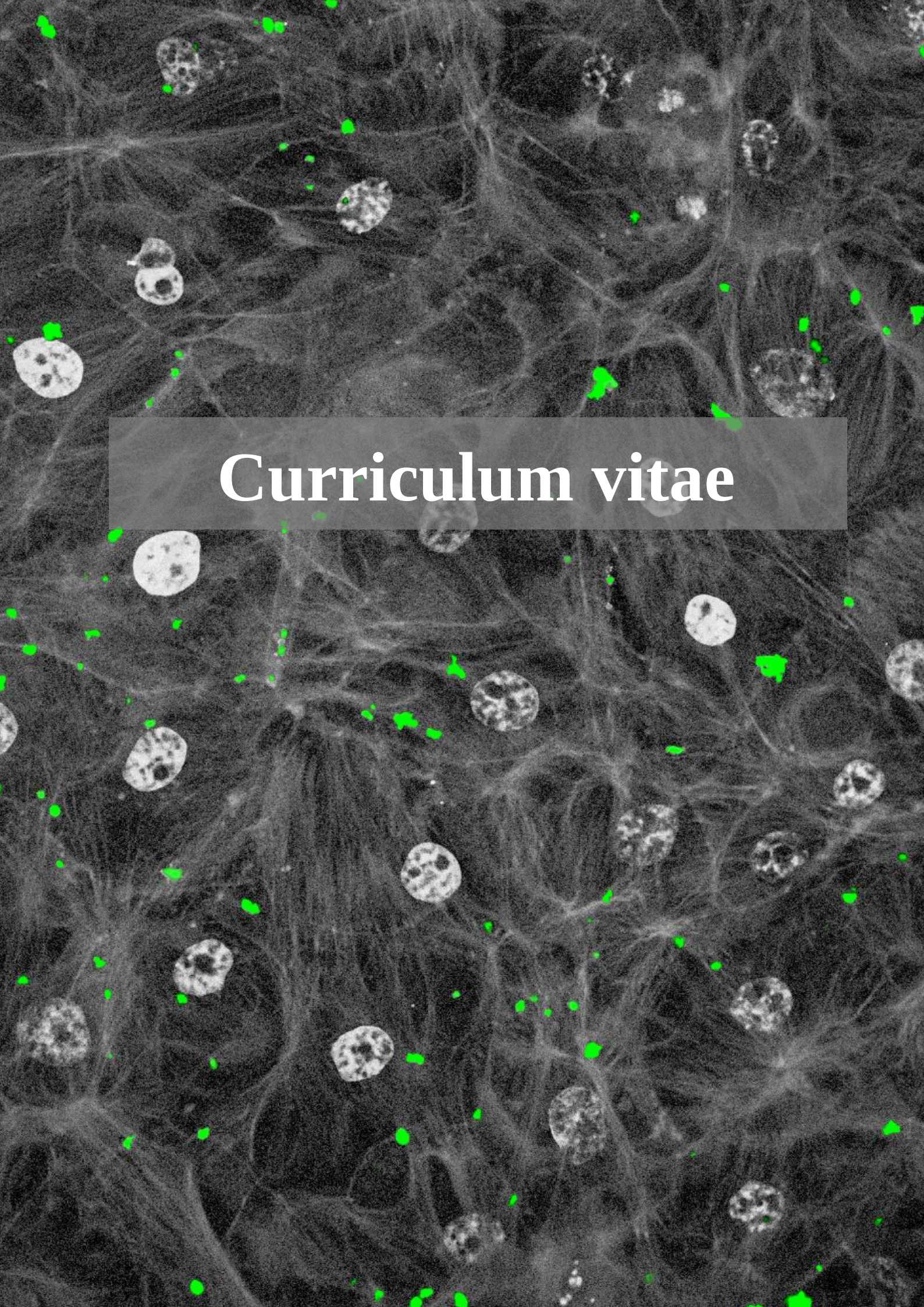
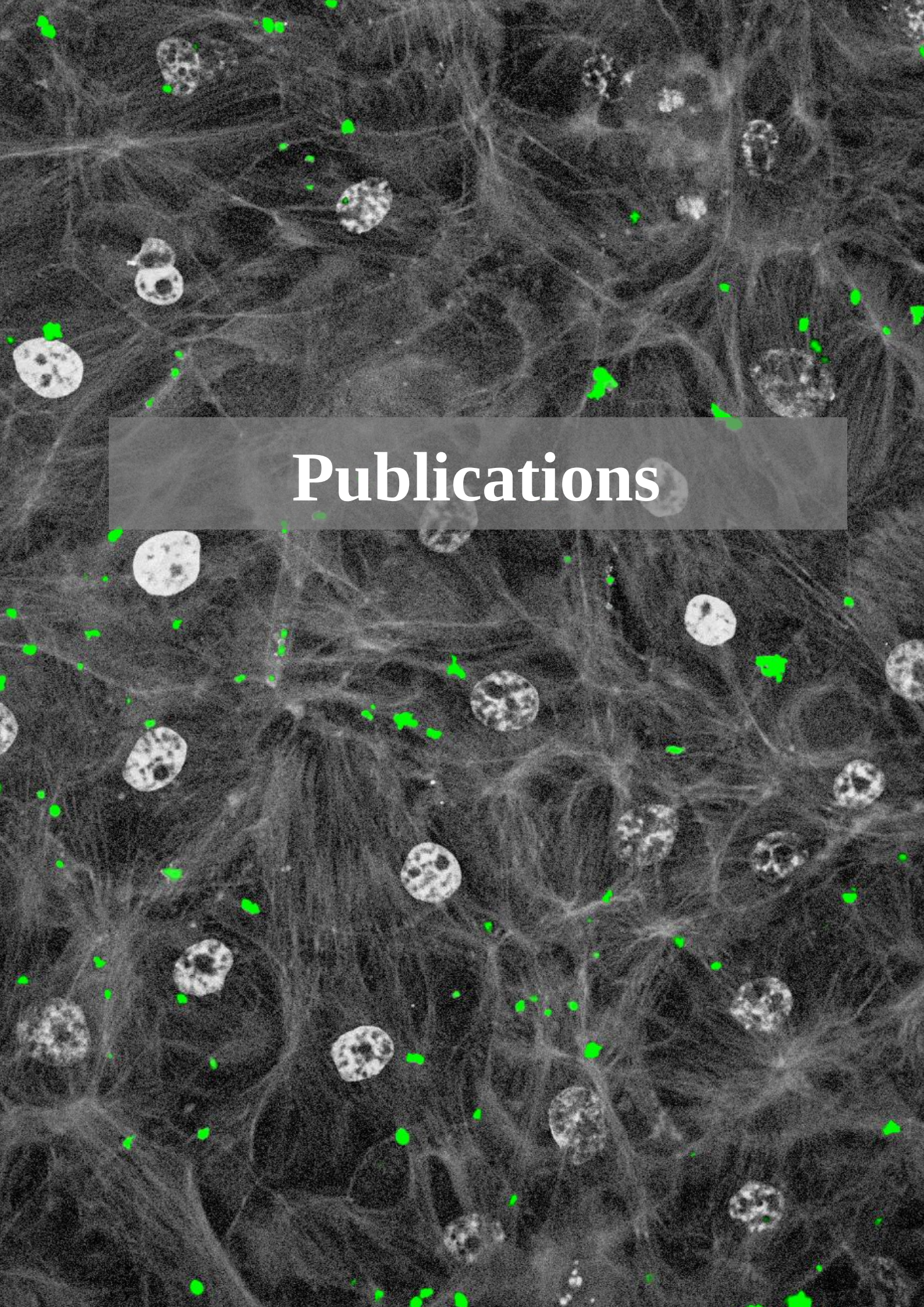


Curriculum vitae

Curriculum vitae

Kim Baert werd geboren op 25 oktober 1987 te Keulen, Duitsland. Zij volgde de secundaire opleiding in de richting Latijn-Wiskunde (8u) aan het Sint-Jans College te Sint-Amansberg. Aan de universiteit van Gent, begon ze in 2005 met de studies Biomedische Wetenschappen en in 2010 studeerde ze af met grote onderscheiding. Geboeid door het wetenschappelijk onderzoek, startte ze in 2010 een doctoraatsstudie aan het Laboratorium voor Immunologie van de Faculteit Diergeneeskunde, Universiteit Gent. Gedurende 5 jaar heeft ze zich toegespitst op de ontwikkeling van een oraal vaccin gebaseerd op β-1,3/1,6-glucanen afkomstig van gistcellen en gericht naar aminopeptidase N (APN). Dit onderzoek werd uitgevoerd onder leiding van Prof. Dr. E. Cox en Dr. B. Devriendt en leidde tot dit proefschrift. Deze studie werd mede gefinancierd door een doctoraatsbeurs van het instituut voor Innovatie door Wetenschap en Techniek in Vlaanderen (IWT-Vlaanderen). Kim Baert is auteur en mede-auteur van meerdere publicaties in internationale tijdschriften en nam actief deel aan meerdere nationale en internationale congressen.



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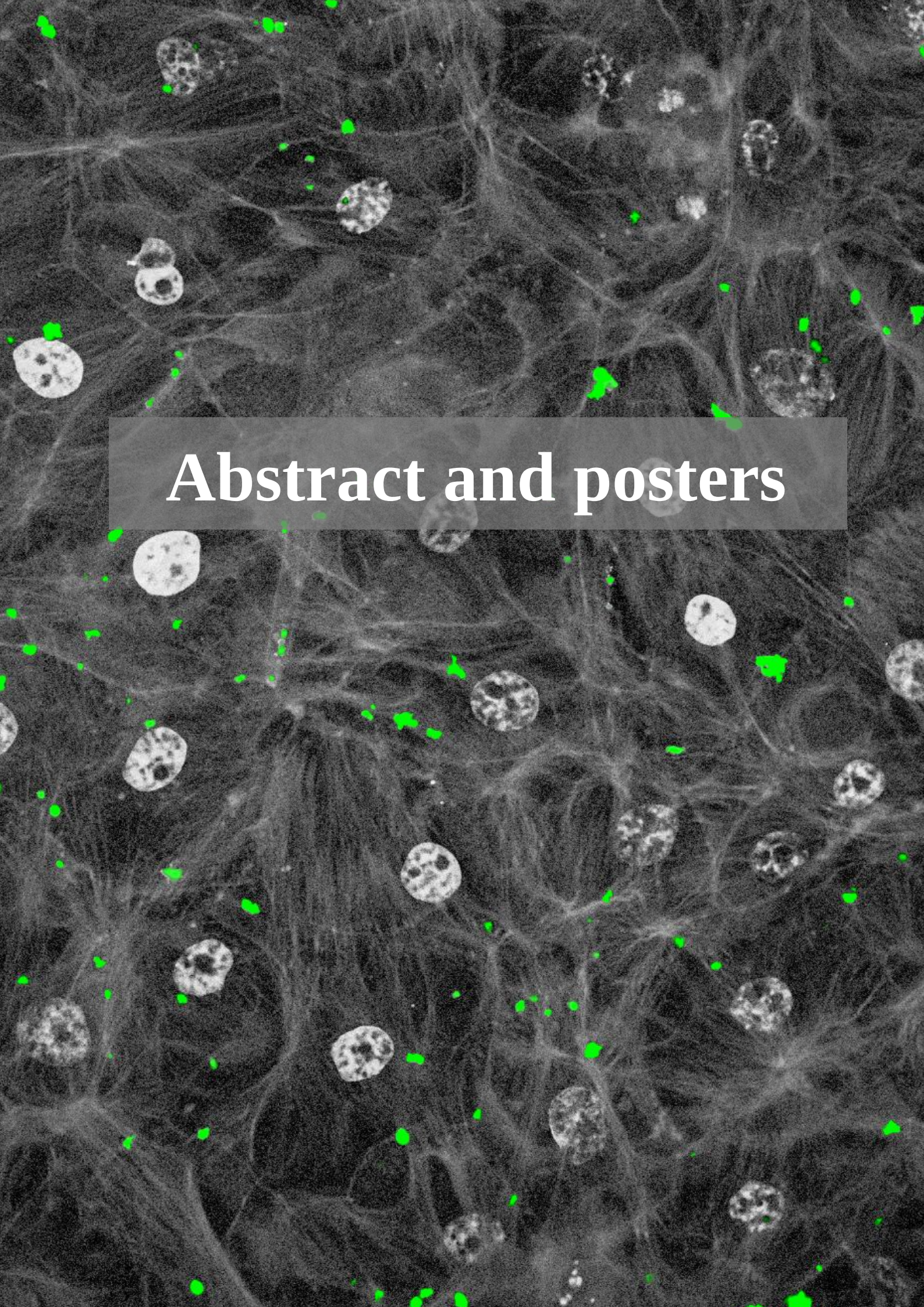

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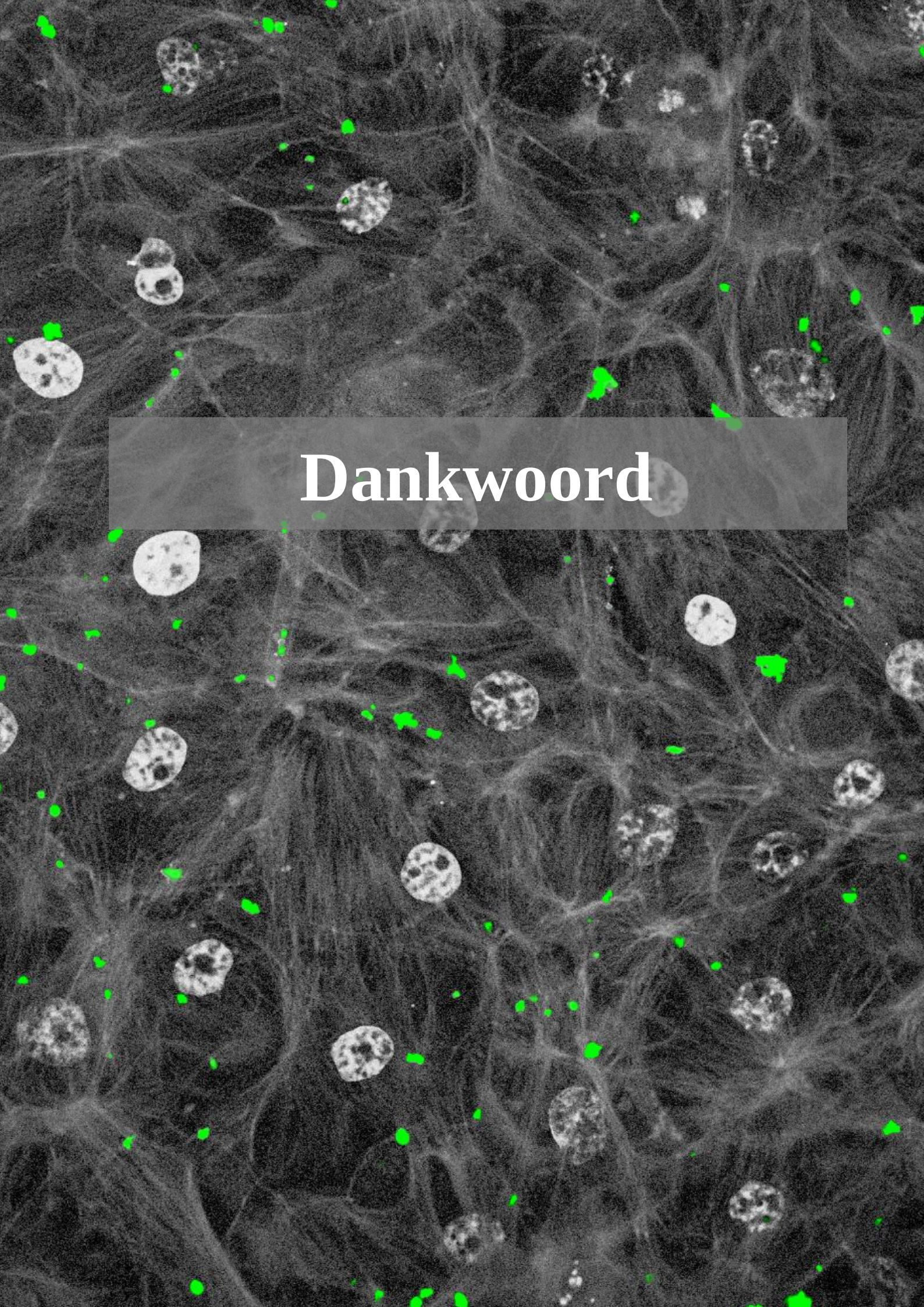

Dankwoord

Dankwoord

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