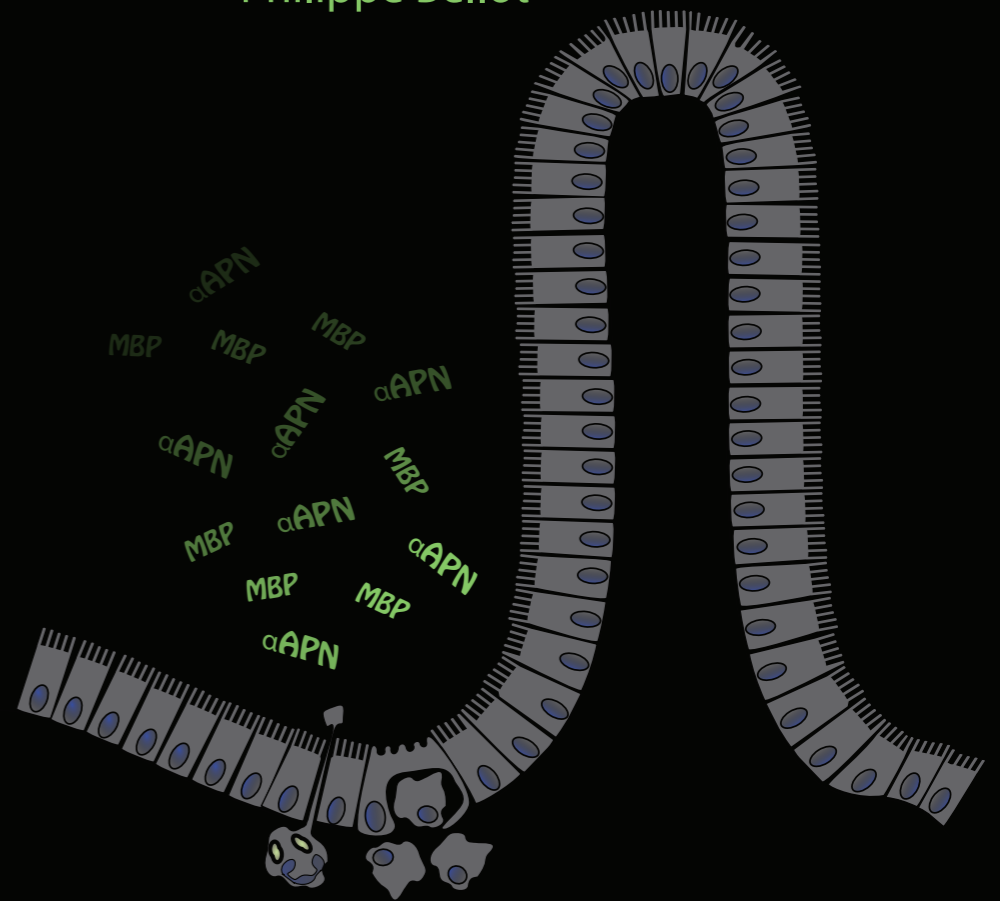New strategies for oral immunization:
targeting soluble antigens or delivery
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# New strategies for oral immunization: targeting soluble antigens or delivery of particles to the intestinal mucosa

**Philippe Bellot**

Promotoren

Prof. Dr. E. Cox

Prof. Dr. B.M. Goddeeris

Dr. V. Melkebeek

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# List of abbreviations

| | |
|---|---|
| ABTS | 2,2'-azinobis(3-ethylbenzthiazolin+e-6-sulfonate) |
| AEC | 3-amino-9-ethylcarbazole |
| APC | antigen presenting cell |
| APN | aminopeptidase N |
| ASC | antibody-secreting cell |
| ATBF1A | AT motif binding factor 1A |
| BCA | bicinchronic acid |
| bestatin | ((2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine |
| BHK-21 | baby hamster kidney cell line |
| BHK-pAPN | pAPN-transfected BHK-21 |
| BSA | bovine serum albumin |
| cpm | counts per minute |
| CT | cholera toxin |
| CTL | cytotoxic T-lymphocyte |
| Da | dalton |
| DC | dendritic cell |
| DMEM | Dulbecco's modified Eagle's medium |
| dppi | days post-primary immunization |
| DTT | dithiotreitol |
| *E. coli* | *Escherichia coli* |
| ELISA | enzym-linked Immunosorbent assay |
| ELISA dilution buffer | PBS + 3% (w/v) bovine serum albumin + 0.2% (v/v) Tween® 20 |
| ETEC | enterotoxigenic *E. coli* |
| F4acR | F4ac-receptor |
| FAE | follicle-associated epithelium |
| FcRn | the neonatal Fc receptor |

| | |
|---|---|
| FCS | fetal calf serum |
| FITC | fluorescein isothiocyanate |
| GALT | gut-associated lymphoid tissue |
| GIS26 | F4ac$^+$ ETEC strain serotype O149:K91, F4ac$^+$, LT$^+$STa$^+$STb$^+$ |
| GIT | gastrointestinal tract |
| HCV | human coronavirus |
| HRP | horse-radish peroxidase |
| Ig | immunoglobulin |
| IL | interleukin |
| IMM01 | F4-specific monoclonal antibody |
| IMM-13 | pAPN-specific monoclonal antibody |
| IPEC-J2 | a non-transformed intestinal epithelial cell line derived from the jejunal epithelium of a neonatal, unsuckled piglet |
| IPEC-J2-pAPN | pAPN-transfected IPEC-J2 cells |
| IPP | ileal Peyer's patch |
| JPP | jejunal Peyer's patch |
| LP | lamina propria |
| LT | heat-labile toxin of *E. coli* |
| mAb | monoclonal antibody |
| MBP | maltose-binding protein |
| MC | monomorphonuclear cell |
| MHC | major histocompatibility complex |
| MFI | mean fluorescence intensity |
| MLN | mesenteric lymph node |
| OD$_{405}$ | optical density measured at a wavelength of 405 |
| pAPN | porcine APN |
| PBMC | peripheral blood monomorphonuclear cell |
| PBS | phosphate buffered saline |
| PBS$^+$ | PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ |

| | |
|---|---|
| PBS-T | PBS plus 0.2% (v/v) Tween® 20 |
| PEDV | porcine endemic diarrhea virus |
| pIgA | polypeptide complex of two IgA monomers |
| pIgR | the polymeric Ig receptor |
| PLGA | poly(DL-lactic-co-glycolic acid) |
| PP | Peyer's patch |
| PRR | pattern-recognition receptor |
| sAPN | soluble form of APN |
| SEM | standard error of mean |
| SGF | simulated gastric fluid |
| SIgA | secretory IgA |
| SI | stimulation index |
| TGEV | transmissible gastroenteritis virus |
| TLR | toll like receptor |
| UEA1 | Ulex europaeus 1 |
| $Zn^{2+}$ | zinc |

# **Introduction**

In the late 18th century, the development of vaccines started with the observations of Edward Jenner, leading to the discovery of the small pox vaccine. He observed that milkmaids were generally immune to smallpox and postulated that the protection arose from prior infection with cowpox, which is less virulent than smallpox (Edward 1909-14). Although today numerous successful vaccines are in use against various pathogens (i.e. Influenza, Rabies, Polio, Tetanus), even with use of modern-day knowledge and techniques, no efficient vaccine exists against a lot of diseases (Plotkin 2009). Actually, only 27 human diseases are recognized by the CDC (Center for Disease Control and Prevention, USA) as preventable by vaccination (CDC 2012). Most infectious diseases are caused by pathogens that colonize and invade the host at mucosal surfaces. For an effective protection of the host pathogen-specific secretory IgA (SIgA) is recommended at the site of infection (Kilian *et al.,* 1988). Nonetheless, the most commercial vaccines are delivered systemically by injection and although they elicit a strong systemic immune response, only a weak pathogen-specific mucosal immunity is acquired (Wang *et al.,* 2004). In contrast, vaccination at mucosal sites can lead to protective mucosal immunity. However, formulation of mucosal vaccines is difficult and the progress in development has been rather slow.

The first chapter, the literature review, will give background information in order to better understand the experiments performed in this thesis. In the first part, the mucosal immune system of the intestinal tract is reviewed. Where necessary, pig specific clarifications are added. Subsequently, different mechanisms are listed of how antigens are taken up in the intestine. Next, a brief overview of conventional vaccines is given, followed by vaccination strategies against enteropathogens. Afterwards several approaches to enhance the efficiency of oral vaccines are clarified. The last two parts give background information on respectively aminopeptidase N (APN) and maltose-binding protein (MBP), two proteins used in the experimental studies.

The aims of this PhD thesis are given in Chapter 2. The experimental part of this thesis is subdivided into two parts. In the first part (Chapter 3 - 5), the role of APN as target to obtain a mucosal immune response was examined. Whereas in the second part, two additional

approaches were used to orally immunize piglets. In Chapter 6, MBP was used as carrier for oral immunization and in Chapter 7, porous pellets loaded with F4ac fimbriae were evaluated.

# Chapter 1

# Review of the literature

## 1.1 The intestinal mucosal immune system

The mucosa-associated lymphoid tissue (MALT) is situated along the surfaces of all mucosal tissues and is sub-divided according to the anatomical region. Its most well-known representatives are the gut-associated lymphoid tissue (GALT), the nasopharynxassociated lymphoid tissue (NALT) and the bronchus-associated lymphoid tissue (BALT). However, the conjunctiva-associated lymphoid tissue (CALT), the lacrimal duct-associated (LDALT), the larynx-associated (LALT) and the salivary gland-associated/duct-associated (SALT/DALT) lymphoid tissue have also been described (Cesta 2006; Liebler-Tenorio and Pabst 2006). At least half of the lymphocytes of the human immune system are located in the MALT (Croitoru *et al.,* 1994). Some reports even state that up to 70% of the human lymphocytes are present in the GALT, underlining the importance of the GALT in the immune system (Brandtzaeg *et al.,* 1989; Corr *et al.,* 2008). Although MALT sites are anatomically separated, they are functionally connected to each other (Mcdermott and Bienenstock 1979). Antigen presentation and B cell activation at one mucosal site can indeed result in IgA secretion at mucosal sites of different organs (Hiroi *et al.,* 1998; Kiyono and Fukuyama 2004). However, this is not common due to regional differences in homing receptors and chemokine profiles between the MALT structures (Brandtzaeg *et al.,* 1999; Brandtzaeg and Johansen 2005). Furthermore, the mucosal immune system can act independent of the systemic immune system (Cesta 2006).

The mucosal immune system can be divided into two general compartments, known as the inductive and the effector sites. The inductive sites, composed of MALT as well as their surrounding regional lymph nodes, contain secondary lymphoid tissues in which IgA class switching and clonal expansion of B cells occurs (Brandtzaeg and Pabst 2004; Mcdermott and Bienenstock 1979; Mcwilliams *et al.,* 1977). After activation and IgA class switch, T and B cells migrate from the inductive sites to the effector sites. These effector sites are present in all mucosal tissues and consist of the lamina propria (LP) and the stroma of both exocrine glands and surface epithelial cells (Brandtzaeg and Pabst 2004).

In accordance with the scope of this thesis, oral vaccination, we will further only focus on the GALT. The intestinal mucosa are constantly in contact with foreign antigens, including food antigens, commensal microflora and pathogens. Consequently, the GALT is able to make a difference between potential harmful and innocent antigens. Antigen uptake, presentation

and immunogenicity of the antigen are critical factors in this distinction and are mainly determined by the properties of the antigen (Garside and Mowat 2001). As illustrated in Figure 1.1, the GALT includes the Peyer's parches (PP) and large numbers of lymphoid cells scattered throughout both the LP and the epithelium of the intestine, the isolated lymphoid follicles (ILF) and intraepithelial lymphocytes (IEL). The occurrence of other GALT-like elements is species-dependent, such as lymphocyte-filled villi (rat and human), cryptopatches (mouse) and lympho-glandular complexes (pig). However, these structures are not involved in B cell induction (Brandtzaeg *et al.,* 2008; Brandtzaeg and Pabst 2004). Hereafter, further description will focus on the GALT of pigs.
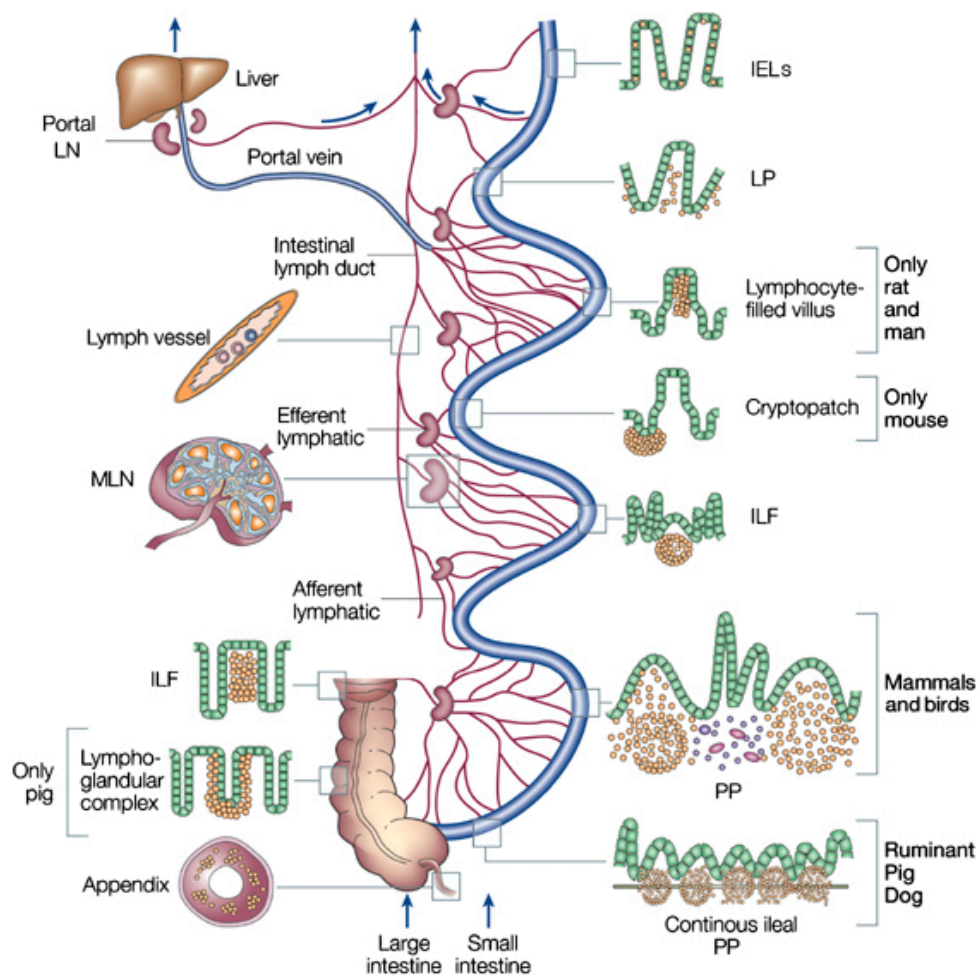


**Figure 1.1** Distribution of lymphoid-cells in various compartments of the intestine. Lymphocytes can leave the gut wall via draining lymphatics afferent to mesenteric lymph nodes (MLNs), or via portal blood reaching the liver where important regulation of immunity takes place. Species differences are indicated where relevant. LN: lymph node; IEL: intraepithelial lymphocytes; LP: lamina propria; ILF: isolated lymphoid follicle; PP: Peyer's patch. Reprinted from Brandtzaeg et al., 2008 with permission from Macmillan Publishers Ltd.

In mature pigs, the intestinal LP is heavily populated with leukocytes. Whereas plasma and B cells mainly are located around the crypts, T cells reside mainly in the villi (Stokes and Bailey 2000). Large numbers of eosinophils and cells of the mast cell/basophil type are also present (Haverson *et al.,* 1994; Pabst and Beil 1989). In addition, a widespread expression of MHC class II is found beneath the epithelial basement membrane in villi and to a lesser extent in crypts (Vegalopez *et al.,* 1993). Pig enterocytes are unable to express MHC class II (Stokes *et al.,* 1996). However, capillary endothelium does express MHC class II, suggesting a role in interactions between antigen-specific T cells and endothelium in regulating peripheral lymphocyte functions (Haverson *et al.,* 2000; Wilson *et al.,* 1996).

The PP have clearly defined T and B cell areas like classical secondary lymphoid organs (Neutra *et al.,* 2001). In pigs, around twenty discrete PPs are distributed throughout the jejunum and their number and position remain constant throughout life (Pabst *et al.,* 1988). Each patch contains multiple B cell follicles separated by interfollicular areas dominated by T cells. In contrast, a single, large PP develops in the ileum of young pigs and is heavily populated with B cells. With age, this patch regresses to a series of isolated patches and its content of B and T cells becomes comparable to that of the discrete patches (Stokes and Bailey 2000). PP are located in the submucosa and are separated from the intestinal lumen by only a single layer of epithelial cells, the follicle-associated epithelium (FAE, Figure 1.2A). The FAE differs intensely from the villus epithelium. Where the villus epithelium is specialized for digestion and absorption of nutrients and therefore is dominated by absorptive enterocytes, mucin-secreting goblet cells and enteroendocrine cells, the FAE contains few or no goblet or enteroendocrine cells (Owen 1999). However, in addition to conventional enterocytes, the FAE contains many lymphoid cells, as well as a unique population of specialized epithelial cells, 'microfold' or M cells (Figure 1.2B), whose function appears to be the uptake and transport of antigen into the lymphoid tissue (Gebert *et al.,* 1996; Gebert *et al.,* 2004; Jang *et al.,* 2004). The antigen transport in M cells is a three stages process. First, the antigens are taken up by endocytosis at the apical membrane, followed by transport of the antigens via an endocytic vesicle to the endosomal compartment and finally exocytosis of the antigens occurs at the basolateral membrane (des Rieux *et al.,* 2005). The M cells are characterized by short irregular microvilli and this, in combination with a thin glycocalyx, promotes the interaction of bacteria, viruses, antigens and particles from the

intestinal lumen with their apical membrane (Bye *et al.,* 1984; Clark *et al.,* 1998; Neutra *et al.,* 1987; Sicinski *et al.,* 1990). Moreover, their basolateral membrane is deeply invaginated, amplifying the cell surface and forming an intraepithelial pocket hosting antigen presenting cells (APC) and lymphocytes (Ermak *et al.,* 1994; Ermak *et al.,* 1990; Farstad *et al.,* 1994). Inflammatory conditions and bacterial stimulation seem not only to increase the amount of M cells but also their transcytotic capacity (Borghesi *et al.,* 1996; Cuvelier *et al.,* 1994; Gebert *et al.,* 2004; Meynell *et al.,* 1999; Velin *et al.,* 2004).
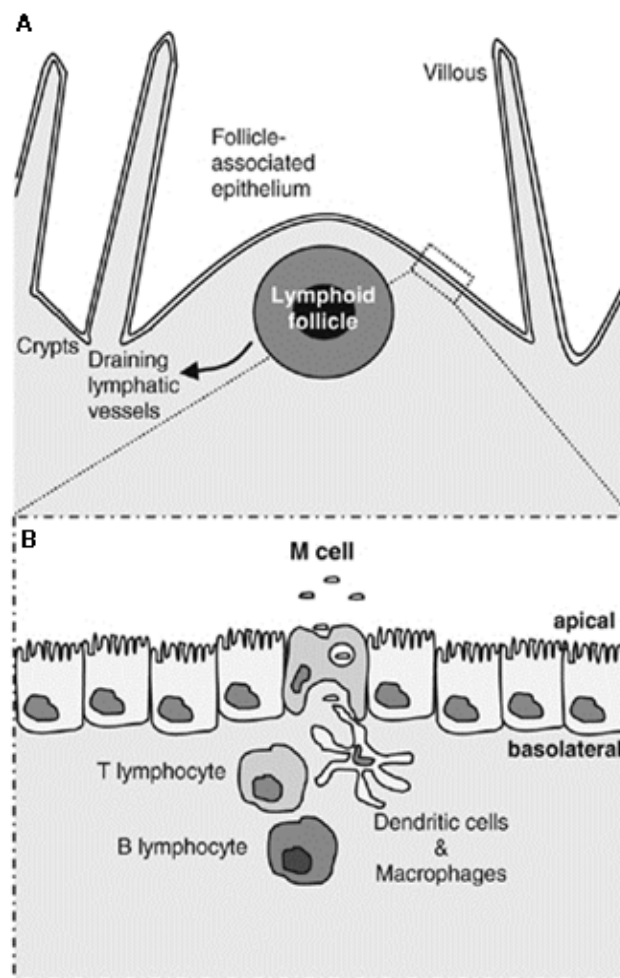


**Figure 1.2** Schematic representation of the follicle-associated epithelium (FAE) in the Peyer's patch (A) and an M cell located within the FAE (B). Reprinted from Corr et al., 2008 with permission from John Wiley and Sons.

The predominant immunoglobulin (Ig) isotype in most mucosal surfaces is secretory IgA (SIgA), a polypeptide complex of two IgA monomers (pIgA) linked by a joining (J) chain (Brandtzaeg *et al.,* 1999; Snoeck *et al.,* 2006a). pIgA is released from plasma B cells, diffuses through the stroma and binds to the polymeric Ig receptor (pIgR) expressed on mucosal

epithelial cells. This complex becomes stabilized by disulfide bonds. Subsequently, the pIgA is transported across mucosal epithelial cells and is secreted into the lumen as SIgA after cleavage from pIgR (Apodaca *et al.,* 1991). SIgA/pIgA protect the mucosal epithelial barrier through a variety of mechanisms: pIgA protects the stromal side of the epithelium by binding to antigens crossing the epithelial barrier (Kaetzel *et al.,* 1991; Robinson *et al.,* 2001). Furthermore, during the pIgR mediated transport, pIgA can bind to newly synthesized viral proteins inside the epithelial cells, preventing virion assembly and neutralizing viral replication (Bomsel *et al.,* 1998; Fujioka *et al.,* 1998; Mazanec *et al.,* 1995). In addition, SIgA can bind to antigens at the luminal side, interfering with the ability of antigens, including viruses, bacteria and bacterial toxins and enzymes, to adhere to and penetrate the mucosa (Brandtzaeg 2003).

The development of memory IgA producing B cells following mucosal immunization has been thoroughly studied in mice (Brandtzaeg 2007; Lycke and Bemark 2010). The constitutive presence of germinal centers in Peyer's patches implies their importance for generating long-living plasma cells and memory B cells (Lycke 2012). For instance, oral immunization with cholera toxin (CT) in mice induced the presence of CT-specific IgA producing plasma cells in the gut LP for up to 6 months after the immunization (Lycke and Holmgren 1987; Lycke and Holmgren 1989). Moreover, memory B cells remained in the GALT and could be triggered by a single oral re-exposure to CT, resulting in a strong IgA plasma cell response in the LP within 3 days. Recently, it is suggested that B cells can migrate from one Peyer's patch to another (Lycke *et al.,* 2012). Therefore the author suggests that after oral priming, activated IgA^{+} B cells can enter existing germinal centers in multiple Peyer's patches, where they can continue proliferating and undergo affinity maturation as illustrated in Figure 1.3 (Lycke 2012). The IgA response can thus be synchronized, leading to the selection of only high-affinity B cell clones, on condition that the antigen is present in the germinal center.
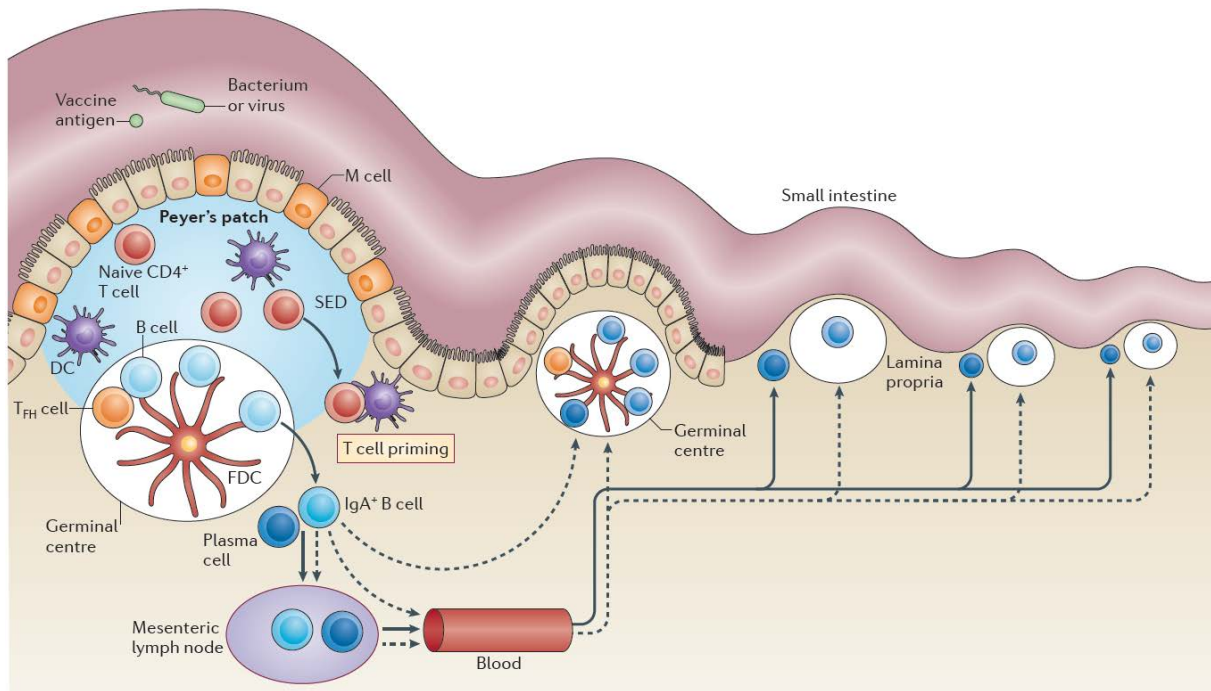
**Figure 1.3** Induction of gut IgA responses. Antigens are taken up by M cells overlaying the Peyer's patches. Subepithelial dome (SED)-resident dendritic cells (DCs) then take up and process these antigens and naive CD4+ T cells are primed in the interfollicular space. T follicular helper (TFH) cells colocalize with B cells in proximity of a follicular dendritic cell (FDC) network, allowing the formation of a germinal center. In the germinal center, the antigen-specific B cells undergo class-switching to IgA and somatic hypermutation to generate higher affinity antibodies. This results in IgA$^+$ long-living plasma and memory B cells. Subsequently, these B cells leave the Peyer's patch through the efferent lymph and migrate first to the mesenteric lymph nodes and then to the blood, from where plasma cells home to bone marrow and to effector sites in the LP of the small and large intestine. Reprinted from Lycke, 2012 with permission from Macmillan Publishers Ltd.
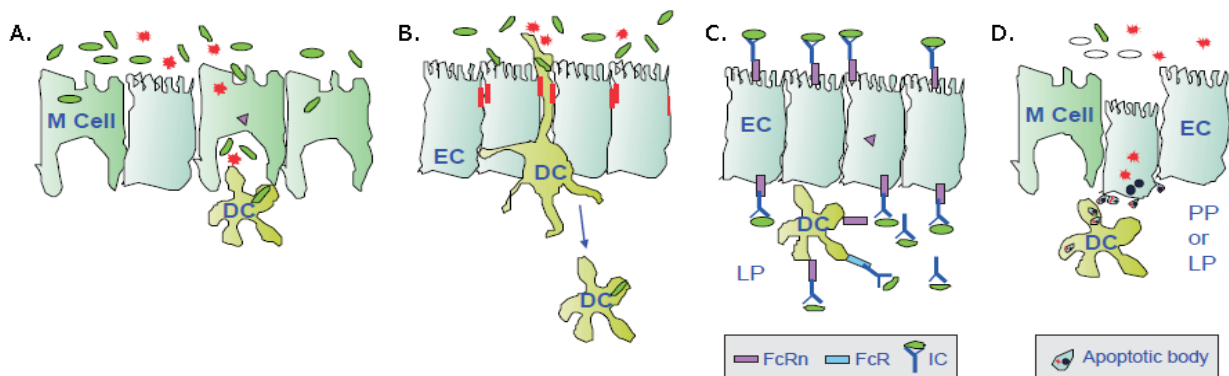


**Figure 1.4** Schematic overview of different antigen uptake mechanisms in the gut. M cells can directly mediate the transport of antigens to the underlying DCs (A), in addition DCs can extend dendrites between intestinal ECs to sample luminal antigens (B). FcRn has the ability to transport IgG to the intestinal lumen, but this receptor can also transport the IgG-antigen complexes formed in the lumen back to the LP (C). Antigens associated with apoptotic epithelial cells can be endocytosed by DCs (D). DCs: Dendritic cells; ECs: Epithelial cells; FcRn: Neonatal Fc receptor; IC: Immune complex; LP: Lamina propria; PP: Peyer's patch. Reprinted from Kelsall and Rescigno, 2004 with permission from Macmillan Publishers Ltd.

## 1.2    Antigen uptake in the gut

Different routes of antigen uptake in the intestines have been described (Devriendt *et al.,* 2011; Devriendt *et al.,* 2012; Kelsall and Rescigno 2004). Besides M cell-mediated transport (Figure 1.4A), antigens/pathogens can, for instance pass the epithelial barrier upon endocytosis by dendrites of dendritic cells (DCs) protruding in the intestinal lumen (Figure 1.4B) (Bimczok *et al.,* 2006; Rescigno *et al.,* 2001). However in healthy pigs, the formation of transepithelial dendrites is rather a rare event, suggesting that these dendrites do not play a significant role in luminal antigen uptake under steady state conditions (Bimczok *et al.,* 2006).

Another mechanism of antigen uptake is by endocytosis of antigen-IgG immunecomplexes after binding to the neonatal Fc receptor (FcRn, Figure 1.4C). FcRn is expressed by adult enterocytes in humans (Israel *et al.,* 1997) and in pigs (Stirling *et al.,* 2005) and is capable of bidirectional transport of IgG across the intestinal barrier. Indeed, not only has FcRn the ability to transport IgG to the intestinal lumen, but this receptor can also transport the IgG-antigen complexes formed in the lumen back to the LP (Yoshida *et al.,* 2004). Several authors suggest an important role of FcRn in the delivery of proteins across the gut in adult species (Baker *et al.,* 2009; Claypool *et al.,* 2004; Devriendt *et al.,* 2012; Dickinson *et al.,* 1999; Hansen *et al.,* 2006; Yoshida *et al.,* 2004). In their study, Stirling and colleagues (2005), demonstrated that after oral administration of bovine IgG to pigs, bovine IgG was transferred into the pigs blood. However, they had to administer 300 ml bovine colostrum for 7 consecutive days and they could not exclude the uptake via non-specific mechanisms. While these studies provide evidence that FcRn can transport IgG in a bidirectional way across the gut, *in vivo* evidence is still lacking.

DCs are also able to endocytose apoptotic bodies from enterocytes dying under steady state conditions or after viral infection (Fleeton *et al.,* 2004; Huang *et al.,* 2000). Moreover, DCs can present peptides derived from the apoptotic bodies on MHC class I and II (Figure 1.4D) (Albert *et al.,* 1998; Huang *et al.,* 2000). Enterocytes are formed in the crypts of intestinal villi, migrate rapidly to the tips (Eastwood 1977) and undergo apoptosis (Iwanaga 1995). The average life span of enterocytes in the distal portion is 10.2 days and in the proximal portion 4.7 days (Fan *et al.,* 2001). However, most of the intestinal epithelial cells are shed into the

intestinal lumen (Huang *et al.,* 2000), suggesting that this route also does not play a significant role in antigen uptake.

While M cells are generally considered to be the preferred route for mucosal vaccine delivery, the potential of enterocytes should not be disregarded. Enterocytes not only outnumber M cells, but they are also able to transcytose macromolecules, such as CT and F4ac fimbriae and transcytose inert particles (Florence 1997; Lencer *et al.,* 1995; Snoeck *et al.,* 2008). The particle absorption of enterocytes is rather limited compared to M cells but the much greater surface area offered by enterocytes combined with specific targeting to enterocytes could make this limitation obsolete.

## 1.3   Vaccination against enteropathogens

Enteropathogens colonize the intestinal tract and cause disease. For instance, enterotoxigenic *Escherichia coli* (ETEC) are an important cause of diarrhea in man and animal. In humans, ETEC infections are the leading cause of travelers' diarrhea and a major cause of diarrhea in developing countries, where it can be life-threatening for children (Clarke 2001; Ratchtrachenchai *et al.,* 2004). In pigs, ETEC infections immediately after birth or after weaning are responsible for significant economic losses in pig farming due to increased mortality, growth retardation and weight loss (Fairbrother *et al.,* 2005). For an effective protection of the host against those enteropathogens, pathogen-specific secretory IgA (SIgA) is required at the site of infection (Kilian *et al.,* 1988). In humans and most animal species such as pigs, vaccination via the oral route is essential to obtain such a protective immunity. Nevertheless, the vast majority of commercial vaccines are delivered systemically by injection and will most likely elicit a strong systemic immunity but only a weak pathogen-specific mucosal immunity (Wang *et al.,* 2004). Systemically administered vaccines primarily lead to the production of circulating IgG, which, in general, is not secreted in sufficient amounts at the intestinal mucosa to induce protection (Neutra and Kozlowski 2006). Furthermore, it does not result in homing of sufficient memory lymphocytes to the mucosa, which is needed for long-term protection. Instead, oral immunization results in the induction of antibody and cell-mediated immune responses at the GALT and often also at the systemic level (Seo *et al.,* 2002; Verdonck *et al.,* 2004a). Further advantages of oral vaccination include ease of administration and non-invasiveness. This improves overall patient/animal compliance and does not necessarily require trained personnel for administration, thereby

reducing costs and facilitating use in mass immunization programs. However, to guarantee effective absorption of orally administered vaccines, several important problems must be surmounted. To start with, the vaccine must endure the hostile gastric and intestinal environments. Subsequently, the vaccine must persist in the intestinal lumen for a sufficient length of time to allow interaction with the intestinal cells, followed by endocytosis. Additionally, the access to the intestinal cell membranes is inhibited by the mucus gel layer, the closely packed microvilli and the cell surface glycocalyx (Clark *et al.,* 2000).

Vaccines can generally be divided into four major groups: the attenuated, the inactivated, the protein based and the genetic vaccines. The first group, the attenuated vaccines are created by adapting living organisms to grow in conditions that attenuate or weaken their virulence (Ulmer *et al.,* 2006). However, attenuated microorganisms are often either too attenuated to induce a long-lasting intestinal immunity, still have residual virulence resulting in clinical signs or are genetically instable (Ehrenfeld *et al.,* 2009). A number of strategies are now available for dealing with those instability issues, like reverse genetics, recombination, deletion mutants, codon deoptimization and control of replication fidelity (reviewed by Plotkin *et al.,* 2009). In addition, vectors can be used for vaccination. Vectors are nonpathogenic strains of various bacteria (*Salmonellae*, *E. coli*, *Lactobacilli*, *Shigellae*, *Listeria*, *Mycobacteria* and *Streptococci*) and viruses (poxviruses, adenoviruses, polioviruses, herpesviruses, influenza and mengo viruses) (Hackett 1990; Mestecky *et al.,* 2008; Panicali and Paoletti 1982), into which genes from pathogens are inserted and from which those genes come to expression. The live attenuated vaccines are effective because they mimic a natural infection and induce cellular, mucosal and humoral immune responses. Their ability to replicate intracellularly results in the production of sustained quantities of antigenic peptides that can be presented by MHC class I molecules for activation of a cytotoxic T-lymphocyte (CTL) response (Burgdorf *et al.,* 2007). However, immune responses against the vectors eventually predominated over time (Tucker *et al.,* 2008; Wells and Mercenier 2008). Indeed, microbial vectors are usually strongly antigenic and induce, especially upon repeated use, dominant immune responses to the vector rather than to the desired antigen. In addition, glycosylated antigens cannot be produced in bacteria (Mestecky *et al.,* 2008). Besides, inactivation during storage due to breaks in the cold chain as well as regain of

virulence in the host are potential risks associated with the use of live vaccines (Plotkin 2009).

An alternative is the use of inactivated vaccines (second approach) or protein based vaccines (third approach). These vaccines can be made by suspensions of killed organisms or by the use of purified or recombinant proteins from the pathogen (Ulmer *et al.,* 2006). Those vaccines can lead to the generation of antibodies, but are overall less effective at inducing protection in comparison with live vaccines, since killed pathogens or proteins cannot enter the intracellular antigen presenting pathways to induce strong CTL responses (Ulmer *et al.,* 2006). In addition, protein based vaccines may be degraded by proteases and have limited bioavailability since they often cannot cross biological membranes (Chadwick *et al.,* 2010).

A fourth vaccination approach is to immunize a host directly with the DNA coding for the antigen of interest (McDonnell and Askari 1996). Host cells take up the foreign DNA, express the foreign gene and translate it to the corresponding protein inside the cell where it can enter the cell's MHC class I pathway. DNA vaccines can induce a CTL response for antigen-specific apoptosis of infected cells and consequently offer the potential for immunotherapy against tumors for example (Polakova *et al.,* 2010). DNA vaccination could lead to vaccines giving lifelong protection, but on the other hand, many problems arise with DNA vaccines. A DNA vaccine needs to overcome difficult cellular and extracellular barriers in order to be successful (Nguyen *et al.,* 2009). Therefore, one of the most commonly cited problems with DNA vaccines, is low levels of gene expression, limiting immune responses (Pack *et al.,* 2005; Ulmer *et al.,* 2006). Numerous clinical trials have been conducted for DNA vaccines yet only one system, Provenge®, has received FDA approval (Kantoff *et al.,* 2010; Liu and Ulmer 2005). In addition, two DNA-based vaccines are licensed and approved for animal use. The West Nile-Innovator® DNA, by Fort Dodge, is licensed by the U.S. Department of Agriculture (USDA) for the protection of horses against the West Nile virus and APEX-IHN®, by Novartis, is approved by the Canadian Food Inspection Agency (CFIA) to prevent infectious haematopoietic necrosis in farm-raised salmon (Chang *et al.,* 2007; Krishnan 2000; Lorenzen and LaPatra 2005; Petersen and Roehrig 2007). Novel strategies are now being designed to increase vaccine immunogenicity by modifying the plasmids, their methods of delivery and/or by using them in combination with other types of adjuvant or vaccine (Klinman *et al.,* 2010).

Commercial oral vaccines against rotavirus, typhoid fever and cholera for example, mainly use live attenuated microorganisms replicating in the gut (Levine 2000; Soares-Weiser *et al.,* 2010). However, as mentioned above, the use of live vaccines has its shortcomings. In particular, the need for a cold chain can be problematic in developing countries. Therefore, non-replicating oral vaccines are an interesting alternative. However, such vaccines need to pass the harsh environment of the gastrointestinal tract (GIT) and cross the intestinal epithelial barrier in sufficient amounts before reaching the GALT where they can induce an effective immune response. Moreover, soluble antigens generally induce oral tolerance instead of a protective immunity. In contrast to whole-organism vaccines, which contain many immunostimulatory components and are naturally invasive, non-replicating vaccines generally do not induce danger signals which initiate innate and adaptive immune responses and insufficiently reach the mucosal immune system. Nevertheless, a limited number of soluble antigens are able to induce immunity after oral delivery, including cholera toxin (CT) (Spangler 1992; Yamamoto *et al.,* 1997), the *E. coli* heat-labile enterotoxin (LT) (Nakagawa *et al.,* 1996; Takahashi *et al.,* 1996) and the F4ac fimbriae of ETEC (Van den Broeck *et al.,* 1999a; Verdonck *et al.,* 2004a).

## 1.4 Enhancing the efficiency of non-replicating oral vaccines

The efficiency of oral vaccines can be improved by enhancing not only the immunogenicity, but also the bioavailability of the vaccines. The intestinal residence time of orally administered vaccines is indeed a major factor limiting antigen absorption (Clark, Hirst et al. 2000). A longer residence time augments the availability and exposure of the antigen to the immune system. However, in relation to postweaning diarrhea caused by ETEC infection in piglets, the oral vaccine needs to be protected from maternal antibodies in the milk. When an oral vaccine is given to piglets during the suckling period, maternal antibodies present in the milk can indeed interfere and neutralize antigens from the vaccine.

The use of the correct vaccine formulation and/or specific mucosal adjuvants is a crucial step in successful oral vaccine design. Mucosal adjuvants are an important tool for achieving an immune response (Cox *et al.,* 2006). The choice of adjuvant can dramatically affect not only the immediate immune response but also the long-term protective effect of a vaccine (Galli *et al.,* 2009; Manicassamy and Pulendran 2009). For example, adjuvants may dramatically

augment the distribution of epitopes recognized by antibodies, leading to the production of higher avidity antibodies with improved neutralizing properties (Dormitzer *et al.,* 2011).

In the next paragraphs, various strategies for increasing both immunogenicity and bioavailability of oral vaccines are described. In addition, the direct targeting of vaccines to the mucosal immune system will be cited as well as general mucosal adjuvants that modulate innate immune responses.

### 1.4.1 Particulated vaccines

Soluble antigens generally induce oral tolerance, a the state of local and systemic immune unresponsiveness induced by oral administration of harmless antigens such as food proteins (Pabst and Mowat 2012). For that reason, particulated delivery systems are preferably used (Ohagan and Davies 1990). A wide variety of such delivery systems have been developed, including polymer based nano- and microparticles, immune-stimulating complexes (ISCOMs), liposomes and yeast shells (Aouadi *et al.,* 2009; Chadwick *et al.,* 2010; Foster and Hirst 2005; Gerdts *et al.,* 2006; Nordly *et al.,* 2009; Rice-Ficht *et al.,* 2010). They are all able to enhance the bioavailability as well as the immunogenicity of the particulated antigens (Rice-Ficht *et al.,* 2010). Microencapsulated antigens are protected from degradation as well as from maternal antibodies in milk, the antigen uptake into the GALT is improved and the slow degradation of the microparticles results in a continuous antigen release, augmenting the availability of antigen to the immune system (Devriendt *et al.,* 2012; OHagan 1996; Rice-Ficht *et al.,* 2010). However, the encapsulation of the antigens is insufficient to induce robust intestinal immune responses. The uptake of the particulated antigens by intestinal epithelia remains poor which results in suboptimal responses (Gerdts *et al.,* 2006). The rapid transit times in the GIT could be a major factor causing this low uptake, encapsulation only partially solves this problem.

An additional way to protect oral vaccines to both the harsh environment of the GIT as well against maternal antibodies in milk, is by the use of an enteric coating. In the pharmaceutical industry, enteric coatings have traditionally been used for the protection of drugs against the environment of the stomach (Marvola *et al.,* 1999). The principle of enteric-coating is based on a highly pH-dependent solubility of their polymers, insoluble in gastric acid but dissolving in intestinal fluid (Wilding *et al.,* 1994). A study in our lab indicated that enteric-

coated pellets of F4ac fimbriae could be used as solid formulation, mixable with creep feed. Since oral vaccination with the pellets was beneficial, a significant reduction in F4$^+$ *E. coli* excretion could be found compared to F4ac fimbriae in solution, upon challenge infecting pigs with virulent F4$^+$ ETEC. However, postweaning diarrhea could not be prevented (Snoeck *et al.,* 2003).

### 1.4.2 Targeting vaccines to the mucosal immune system

The transit time of vaccines can be increased by the use of intestinal bioadhesins. Those bioadhesins are conjugated to mucosally administrated drugs or vaccines and increase their intestinal residence time by attaching to sites within the intestine (Clark *et al.,* 2000). Nowadays, we want to go one step further and selectively target transcytotic receptors present at the apical surface of M cells, enterocytes and DCs (Devriendt *et al.,* 2011; Devriendt *et al.,* 2012). Different strategies to target vaccines to the mucosal immune system are being considered, including the use of both bacterial adhesins and toxins, ligands for the pattern-recognition receptors (PRRs), FcRn ligands, lectins and antibody-mediated targeting. The latter two will be briefly discussed hereafter, while the bacterial toxins and PRR ligands will be discussed in the point about mucosal adjuvants. For additional information the reader is kindly referred to read the reviews of Devriendt *et al.* (2011, 2012).

### 1.4.2.1 Lectins

Lectins are naturally occurring proteins, mainly of plant or bacterial origin, with affinity for sugar residues. They are stable in low pH conditions and able to bind specifically to oligosaccharide moieties on the surface of intestinal cells and hence promote bioadhesion (Gabor *et al.,* 1998). Ulex europaeus agglutinin 1 (UEA1) is the most investigated lectin and in mice, binds to α-L-fucose residues expressed on the apical surface of M cells, but also on goblet and Paneth cells (Gupta *et al.,* 2007). The α-L-fucose receptor is not expressed on human M cells, whereas in pigs its expression is not restricted to M cells (Brayden *et al.,* 2005; Chae 1997; Rajapaksa and Lo 2010).

The use of lectins on its own as targeting ligand remains problematic because of their susceptibility to proteolytic degradation in the GIT and during the preparation of oral drug formulations (Lambkin *et al.,* 2003). Moreover, their toxicity can lead to anti-nutritional properties leading to significant weight loss (Vasconcelos and Oliveira 2004). The production

of recombinant lectins with modified properties could overcome this problem (Eck *et al.,* 1999). Another approach is to select or design peptides or molecules which mimic the function of the lectins. The advantages of these mimics are their small size, increased stability, ease of synthesis and low cost. For example, Lambkin and colleagues (2003) successfully characterized a UEA1 mimic, a tetragalloyl D-lysine amide construct. However, we should also take into account that the glycosylation pattern differs between enterocytes and M cells at different intestinal locations, with age and between species (Clark *et al.,* 2000; Clark *et al.,* 1995; Giannasca *et al.,* 1994; Jepson *et al.,* 1995). The use of sugar binding molecules in vaccine delivery could thus be restricted.

### 1.4.2.2 Antibody-mediated targeting

In mice, it has been shown that both IgA and IgG (Weltzin *et al.,* 1989) selectively adhere to the apical membrane of M cells, regardless of their antigen specificity. In rabbits, this is only demonstrated for IgA (Lelouard *et al.,* 1999; Roy and Varvayanis 1987). In addition, microparticles coated with IgA (Porta *et al.,* 1992; Smith *et al.,* 1995) or IgG (Smith *et al.,* 1995) and liposomes coated with IgA (Zhou *et al.,* 1995) showed an increased uptake by Peyer's patch M cells in mice and rats. However, this increase was lower compared to the increase induced by the plant lectin UEA1 (Foster *et al.,* 1998).

Intestinal targeting can also be achieved by using antibodies directed against apical cell surface antigens of epithelial cells. Pappo *et al.,* (1991) demonstrated the achievability of this approach by showing an increased uptake of polystyrene microparticles coated with a monoclonal antibody (mAb) against an apical surface antigen expressed on both enterocytes and M cells.

### 1.4.3 Mucosal adjuvants

A major category of mucosal adjuvants is without doubt the Toll-Like Receptor (TLR) agonists (Harandi and Medaglini 2010). These adjuvants are based on pathogen-associated molecular patterns (PAMPs) or pattern-recognition receptors (PRRs). In general, they elicit the secretion of proinflammatory cytokines and chemokines leading to the subsequent recruitment of APCs and the priming of effector T and B cell responses (Cox *et al.,* 2006; Pasare and Medzhitov 2004; Sporri and Sousa 2005). For example, the CpG-oligodeoxynucleotide (ODN)-containing formulations, which act through TLR9, have been

used successfully in experimental vaccine models and even in clinical trials (Blaas *et al.,* 2009).

A second category of mucosal adjuvants includes the bacterial enterotoxins CT and LT (LT-I, LT-IIa, LT-IIb and LT-IIc). The toxins function as virulence factors in *V. cholerae* and *E. coli* infections, respectively. They are very similar molecules belonging to a family of structurally related proteins that is divided into two major groups based on genetic, biochemical and immunological characteristics. The type I subfamily consists of CT, LT-I and antigenically related enterotoxins from other enteric bacteria, whereas the type II subfamily consists of LT-IIa, LT-IIb and LT-IIc (Hajishengallis and Connell 2012). The enterotoxins are composed of an A and B subunit of which the B subunit forms a homopentamer structure. The enterotoxins stimulate the production of SIgA against both themselves and co-administered immunogens after stimulation of mucosal inductive sites. The mucosal immune responses arising from CT are probably dependent on DCs (Fahlen-Yrlid *et al.,* 2009).

The B subunits of each enterotoxin are able to bind to specific gangliosides, a structurally complex group of sialic acid decorated glycolipids located on the cell membrane of most nucleated cells (Connell 2007; Sonnino *et al.,* 1986). Unfortunately, these adjuvants can therefore be linked with unwanted side effects. Oral vaccination with CT or LT as adjuvant has already resulted in diarrhea (Levine *et al.,* 1984). In pigs however, CT is a very potent mucosal adjuvant with low toxicity (Cox *et al.,* 2006; Foss and Murtaugh 1999). In previous studies co-administration of 50 to 100 µg CT to orally administered vaccines significantly improved systemic as well as mucosal responses (Foss and Murtaugh 1999; Verdonck *et al.,* 2005).

### 1.4.4 Sophisticated formulations

It is clear that each strategy has its own advantages but unfortunately also its shortcomings. More sophisticated formulations, using both cell-specific targeting approaches and immunomodulation are required. Targeting ligand-coated particles not only to M cells but also to enterocytes, combined with attempts to mimic pathogen entry routes, could lead to increased uptake and successful induction of mucosal immune responses (Corr *et al.,* 2008). Recently, promising results were shown in mice by Zhu and colleagues (2012) with their large intestine-targeted, nanoparticle-releasing oral vaccine. They encapsulated protein

vaccines into biologically compatible poly(DL-lactic-co-glycolic acid) (PLGA). Moreover, they used a combination of several vaccine adjuvants targeting different TLRs: macrophage-activating lipoprotein (MALP-2) for TLR2, polyinosine-polycytidylic acid (poly(I:C)) for TLR3 and CpG-ODN for TLR9. To bypass the denaturing effects of digestive low pH and enzymatic destruction and to selectively deliver the particles to the large intestine, they coated the PLGA nanoparticle surface with a methacrylate-based polymer (Eudragit FS30D). This polymer is pH-sensitive and dissolves in intestinal fluids at pH higher then 7.0, seen only in the terminal ileum. In addition, the coated particles are larger than 10 µm in diameter to avoid premature uptake in the small intestine primarily by Peyer's patches, which are effective at taking up particles up to 1 µm. Despite the promising results, additional studies in other species than mice are necessary to evaluate the efficiency of such sophisticated formulations.

## 1.5   Aminopeptidase N

As previously mentioned, oral immunization of pigs with soluble F4ac fimbriae induces a protective immune response against F4ac[+] ETEC (Van den Broeck *et al.,* 1999a; Verdonck *et al.,* 2004a). We know that orally administered F4ac fimbriae are transcytosed by M cells and follicle-associated enterocytes in the Peyers' patches and by villous enterocytes in the lamina propria (Snoeck *et al.,* 2008). Subsequent uptake and presentation of F4ac by antigen-presenting cells could explain its capacity to induce a mucosal immune response. This implies that targeting selected antigens to the F4ac receptors (F4acRs) involved in transcytosis, may have the potential to elicit efficient mucosal immune responses against these antigens. Recently, our group demonstrated that aminopeptidase N (APN) is involved in the binding and endocytosis of F4ac fimbriae of F4ac-positive ETEC in the small intestine of pigs (Melkebeek *et al.,* 2012; Rasschaert *et al.,* 2011). Comparative proteomic analysis of brush border proteins of F4acR positive and negative pigs which bind F4ac fimbriae, in addition with F4ac fimbriae adherence and internalization experiments on pAPN-transfected cells were used to identify porcine aminopeptidase N (pAPN) as an F4acR. The binding of F4ac fimbriae to pAPN depends on sialic acid containing carbohydrate moieties, and resulted in clathrin-mediated endocytosis of the fimbriae.

APN (E.C number- 3.4.11.2), which is also known as leucine aminopeptidase, membrane alanyl aminopeptidase, amino-oligopeptidase, aminopeptidase M, membrane alanine

aminopeptidase, membrane aminopeptidase I, peptidase E, GP150 or CD13, is a zinc ($Zn^{2+}$)-dependent integral membrane protease belonging to the gluzincins, the M1 family of the metallopeptidase clan (Danziger 2008). The enzyme was first isolated in 1963 from porcine kidneys (Pfleiderer and Celliers 1963). Initially, APN was recognized as a surface glycoprotein expressed on the brush border membranes of kidney proximal tube cells (George and Kenny 1973) and of enterocytes (Louvard *et al.,* 1973; Maroux *et al.,* 1973). Now, APN is known as an ubiquitous enzyme present on a wide variety of organs, tissues and cell types such as hematopoietic, epithelial and endothelial cells, fibroblasts, and on synaptic membranes, astrocytes and pericytes in brain, spinal cord and other nerve tissues (Jardinaud *et al.,* 2004; Luan and Xu 2007). APN is located in the small-intestinal and renal microvillar membrane and also in other plasma membranes involved in the metabolism of regulatory peptides by diverse cell types. On polarized epithelial cells, such as intestinal epithelial cells, APN is located in the apical domain.

### 1.5.1   APN structure

APN exists in two forms, a soluble and a membrane-associated form. The latter is the most common. Membrane-associated APN is a non-covalently linked homodimer with a mass of 150-160 kDa. Each APN monomer consists of 967 amino acids and, as presented in Figure 1.5, contains a short (8-10 amino acids) N-terminal cytoplasmatic domain, a single transmembrane helical spanning domain and a large extracellular domain containing the active site and a stalk region connecting the transmembrane and the extracellular domain (Lendeckel *et al.,* 2000; Olsen *et al.,* 1988; Sjostrom *et al.,* 2000). APN is highly glycosylated, with glycosylation being equivalent to at least 20% of the mass of the protein (Luan and Xu 2007).

The homodimer is non-disulfide-linked and formed intracellularly before the Golgi-associated processing. In fact, APN is cotranslationally N-glycosylated by the addition of high-mannose oligosaccharides in the rough endoplasmatic reticulum (Feracci *et al.,* 1985; Look *et al.,* 1985). The dimerization of APN occurs in this high-mannose glycosylated state. It has been shown that this high-mannose transient form of APN is enzymatically active, indicating that the native folding and the association with $Zn^{2+}$ occur in the very early state of the biosynthesis (Sjostrom *et al.,* 1985). Moreover, Danielsen (1992) demonstrated the importance of correct N-glycosylation for the correct folding of APN. It is suggested that

dimerization may be required for the transport of the intracellular form of APN out of the endoplasmic reticulum (Danielsen 1990a; Danielsen 1990b). APN is also found in the Golgi apparatus. Here O-glycosylation (Noren *et al.,* 1997), the sequential trimming of the N-linked oligosaccharides as well as the addition of terminal hexoses, like sialic acid, occur (Danielsen 1992).

APN isolated from pig intestine contains three polypeptide chains termed A, B and C (Noren and Sjostrom 1980; Sjostrom *et al.,* 1978) or α, β and γ (Svensson 1979) with a molecular weight of respectively 130, 97 and 49 kDa (Vannier *et al.,* 1976). The β and γ chains are derived from the α chain. The γ fragment, which lacks the anchor peptide, is derived from the C-terminal part (Benajiba and Maroux 1981). The three chains for kidney APN have slightly different molecular weights suggesting a different glycosylation pattern (140, 95 and 48 kDa for the α, β and γ chain respectively) (Wacker *et al.,* 1976).
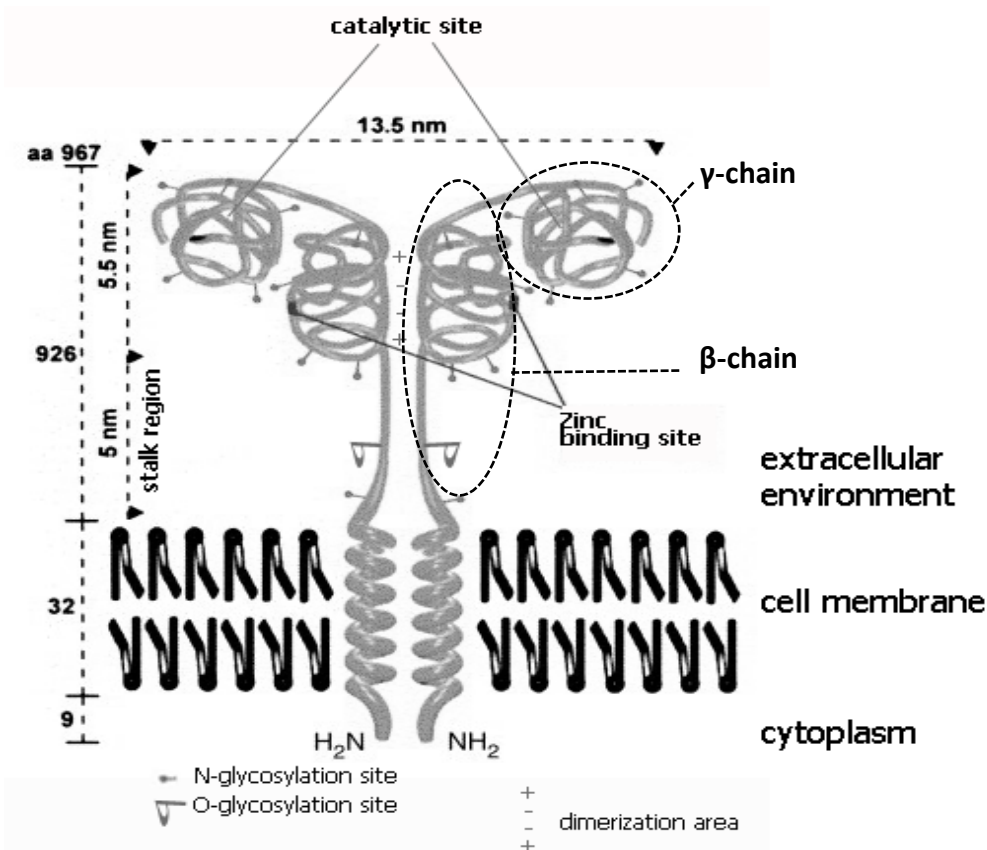


**Figure 1.5** Hypothetic *s*tructure of membrane-associated aminopeptidase N. APN is a non-covalently bonded homodimer and each APN monomer contains a short N-terminal cytoplasmatic domain, a single transmembrane helical spanning domain and a large extracellular domain containing the active site and the stalk region. Adapted from Bauvois and Dauzonne (2006). Information about the dimerization area, catalytic site, O-glycosylation sites and polypeptide chains are from Rieman *et al.,* (1999) and Svensson (1979).

Although APN is a membrane-bound protein, human plasma contains significant amounts of an active soluble form of APN (sAPN) (Favaloro *et al.,* 1993; Jung *et al.,* 1984; Kawai *et al.,* 2003; van Hensbergen *et al.,* 2002). This suggests that certain cells may secrete sAPN, or that APN is released from the plasma membrane by shedding or by specific cleavage. sAPN can block binding of an anti-APN mAb, suggesting sAPN might play a regulatory role in the functions of membrane-bound APN by inhibiting the adhesion of APN ligands via competitive inhibition (Mina-Osorio *et al.,* 2008). However, sAPN is fully enzymatically active and thus its main role *in vivo* might be related to the cleavage of peptides at tumor or inflammation sites. This statement is supported by the fact that an elevated activity of sAPN is found in malignant effusions and in the plasma of cancer patients compared to nonmalignant effusions and plasma of healthy persons (van Hensbergen *et al.,* 2002). The soluble form of APN is also used as a biomarker for liver damage as sAPN is found at high levels in the serum during liver dysfunction (Kawai *et al.,* 2003).

The APN proteins from human, mouse, rat, rabbit, pig, cow, cat, dog and chicken are conserved at the amino acid level, since 70 to 80% amino acid identity is found. The genetic relationship of APN of human, mouse, rat, rabbit, cow, pig and cat is represented as a phylogenetic tree in Figure 1.6. The differences occur primarily in the stalk region immediately downstream from the transmembrane domain.
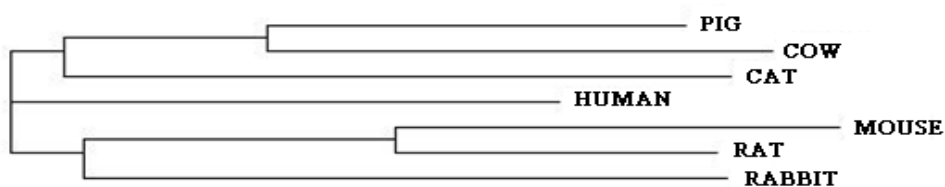


**Figure 1.6** Phylogenetic tree of the APN amino acid sequence of human, mouse, rat, rabbit, cow, pig and cat. The length of each pair of branches represents the distance between sequence pairs.

### 1.5.2   Structure and regulation of the APN gene

The human APN (hAPN) gene has been assigned to the long arm of chromosome 15 (15q25-26), has a coding sequence of 3.5 kb and includes 20 exons (Kruse *et al.,* 1988; Lerche *et al.,* 1996; Look *et al.,* 1986). The porcine APN (pAPN) gene is mapped to the long arm of chromosome 7 (7cen-q21) (Poulsen *et al.,* 1991) and has a similar coding sequence as hAPN. An increased homology is found in 5 exon regions between APN of different species, indicating that those 5 regions represent functional domains. The cytosolic domain, the

transmembrane domain and the stalk are encoded by 1 exon that is not part of the functional domains (Lerche *et al.,* 1996).

Two different promotors, namely the myeloid and the epithelial promotor controlling the expression of both pAPN (Olsen *et al.,* 1991) and hAPN (Shapiro *et al.,* 1991) are identified. The two promotors are arranged in tandem, ±8 kb (human) separated from each other by an enhancer, as shown in Figure 1.7. (Olsen *et al.,* 1997). The epithelial promotor is located closest near the coding sequence and drives the APN transcription in epithelial cell types, such as enterocytes, kidney proximal tubule cells and hepatocytes (Olsen *et al.,* 1991; Shapiro *et al.,* 1991). In contrast, the more upstream myeloid promotor is mainly active in myeloid cell-types, such as monocytes and myeloid leukaemic cells (Shapiro *et al.,* 1991). Both promotors lead to the expression of the same protein. However, the mRNA transcripts differ in the 5' untranslated region as the transcript generated from the myeloid promotor has an additional short non-translated exon. The sequence of the myeloid promotor is CpG rich and does not include a TATA box, which is a DNA sequence found in the promotor region of high number of eukaryotic genes (Shapiro *et al.,* 1991). It is suggested that the specificity of the myeloid promotor in myeloid cells can be explained by the co-expression of Myb and Ets (Norén *et al.,* 1997). Myb and Ets are both transcription factors involved in the regulation of proliferation and differentiation of haematopoietic cells (Shapiro 1995). In addition, Myb and Ets were found in the avian virus E26, which transforms immature haematopoietic cells into myeloid cells (Graf *et al.,* 1992). However, it has been demonstrated that in T cells, the myeloid promotor is induced by mitogenic activation (Lendeckel *et al.,* 1996).
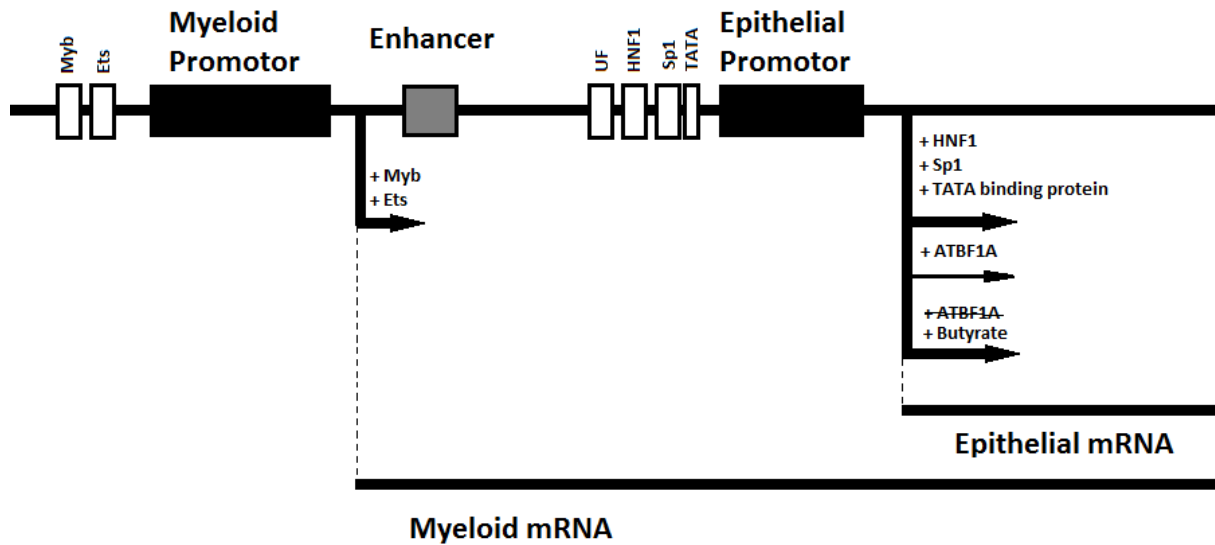
**Figure 1.7** Schematic diagram of the myeloid and epithelial promotors controlling the APN expression. The two promotors (black) are arranged in tandem separated from each other by an enhancer (gray). The binding sites of the transcription factors are drawn in white and named after the binding transcription factor, Myb and Ets for the myeloid promotor and UF, HNF1, Sp1 and TATA for the epithelial promotor. APN is blocked by the AT motif binding factor 1A (ATBF1A), which is expressed in the proliferating stem cells in the intestinal crypts. However, this inhibition can be stopped by butyrate.

The epithelial promotor is regulated by four transcription factors binding sites: the UF, HNF1 and Sp1 sites and the TATA box. Deletion studies revealed that the HNF1 and Sp1 sites in combination with the TATA box are necessary and sufficient for full activity of the epithelial promotor (Olsen *et al.,* 1991). In contrast to the other transcription factors, HNF1 is only found in epithelial cells, implying that the HNF1 element restricts the activity of the epithelial promotor to a limited number of epithelial cells expressing HNF1 proteins (Olsen *et al.,* 1995).

On the other hand it is known that the expression of APN is blocked by the AT motif binding factor 1A (ATBF1A), which is expressed in the proliferating stem cells in the intestinal crypts (Kataoka *et al.,* 2000). However, this inhibition can be stopped by butyrate. As butyrate reduces the ATBF1A expression and consequently induces the expression of APN (Kotunia *et al.,* 2004).

### 1.5.3   Function of APN

APN has many functions as ectoenzyme but its function does not always depend on its enzymatic activity. The biological function of APN depends on its location and is based on the removal of amino acids, with the exception of proline, from the unsubstituted N-

terminus of various peptides (Shipp and Look 1993). APN is most effective on substrates with hydrophobic and basic N-terminal residues and is less effective for acidic residues (Norén *et al.,* 1997). The substrates of APN appear to be small peptides rather than larger proteins, although the enzyme is more effective in removing residues from oligopeptides than dipeptides (Shipp and Look 1993). The different functions of APN are summarized in Figure 1.8. APN facilitates the modulation of bioactive peptide responses (pain management, vasopressin release) and influences immune functions and major biological events (cell proliferation, secretion, invasion and angiogenesis). For example, APN is expressed by immune cells such as B cells, T cells and macrophages where it is involved in the antigen processing by trimming of MHC class I and II associated peptides. In addition, APN is highly expressed on DCs where it participates in DC maturation (Rosenzwajg *et al.,* 2000; van der Velden *et al.,* 2001). APN plays a role in the final digestion of peptides generated from hydrolysis of proteins by gastric and pancreatic proteases in the small intestine (Shipp and Look 1993) and helps to break down neurotransmitter peptides in the brain (Matsas *et al.,* 1985). Its function in proximal tubular epithelial cells and other cell types is less clear. A high level of APN expression is observed in tumor cells such as melanoma, renal, pancreas, colon, prostate, gastric and thyroid cancers (Carl-McGrath *et al.,* 2004; Fujii *et al.,* 1995; Hashida *et al.,* 2002; Ikeda *et al.,* 2003; Ishii *et al.,* 2001; Kehlen *et al.,* 2003; Kitamura *et al.,* 1990; Menrad *et al.,* 1993). It is believed that APN mediates the angiogenesis and metastasis of the tumor. In addition, overexpression of APN also occurs in neutrophils, in several inflammatory diseases like chronic pain, various forms of joint effusions, rheumatoid arthritis, multiplesclerosis, systemic sclerosis, systemic lupus erythematosus, polymyositis/ dermatomyosytis, pulmonary sarcoidosis (Dan *et al.,* 2003; Hafler *et al.,* 1985; Riemann *et al.,* 1993; Riemann *et al.,* 1994; Shimizu *et al.,* 2002; Tani *et al.,* 2000; Ziaber *et al.,* 2000). Consequently, APN is considered to be a useful clinical marker. Besides, the study of Pasqualini *et al.,* (2000) suggested that APN is specifically expressed on the endothelium of tumor vasculature, but not in normal blood vessels. Furthermore, certain APN-specific antibodies bind exclusively to APN expressed in tumors and not to APN expressed in normal tissues, suggesting that a different isoform of APN is expressed in tumors (Curnis *et al.,* 2002).
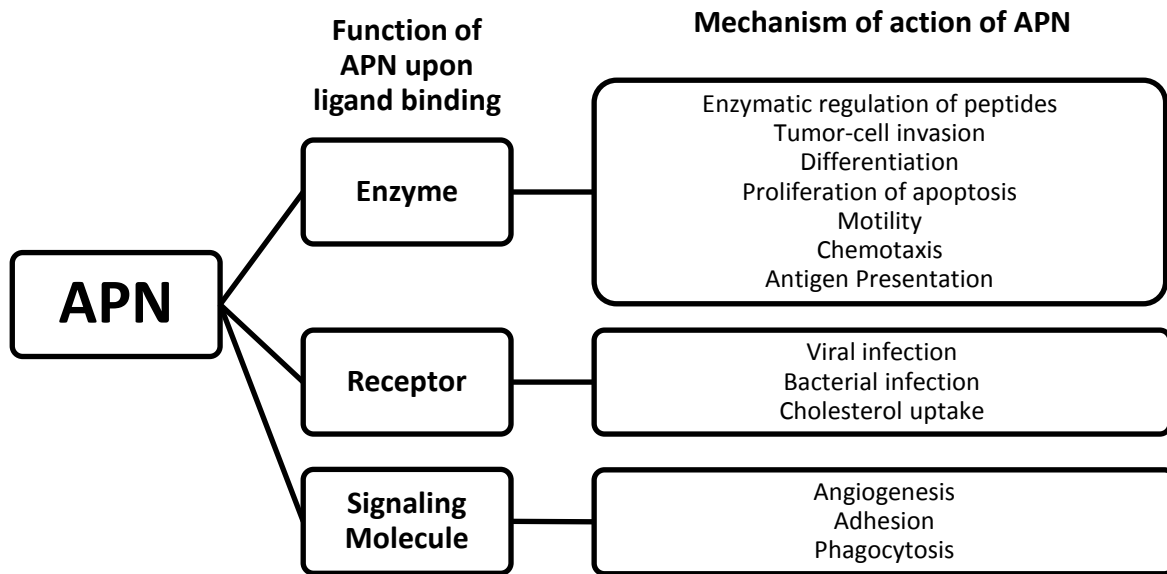
**Figure 1.8** The functions and mechanisms of action of human APN. Adapted from Mina-Osorio (2008).

APN would also be involved in cholesterol uptake. The binding of ezetimibe to APN blocks cholesterol endocytosis and thereby limits intestinal cholesterol absorption (Kramer *et al.,* 2005). In contrast, Garcia-Calvo *et al.,* (2005) stated that not APN but the Niemann-Pick C1-Like 1 (NPC1L1) protein was the direct molecular target of ezetimibe. However, it has been reported that ezetimibe is equally capable of reducing cholesterol uptake in NPC1L1-deficient and wild-type mice (Knöpfel *et al.,* 2007). The enzymatic activity of APN is not affected by the binding of ezetimibe. Instead, it has been proposed that APN participates in the endocytosis of cholesterol by enterocytes (Orso *et al.,* 2006).

In addition, APN also serves as receptor for viruses and bacteria. For instance, APN is considered to be the functional receptor for group I coronaviruses including the human coronavirus (HCV) 229E (Yeager *et al.,* 1992), transmissible gastroenteritis virus (TGEV) (Delmas *et al.,* 1992), porcine endemic diarrhea virus (PEDV) (Li *et al.,* 2009; Nam and Lee 2010; Oh *et al.,* 2003), canine coronavirus (CaCV) (Kolb *et al.,* 1998) and feline infectious peritonitis virus (FIPV) (Tresnan *et al.,* 1996). HCV-229E is causing 15-20 % of the common upper respiratory tract infections in humans (McIntosh 1990), while TGEV is a major source of fatal gastroenteritis in newborn pigs. For those two viruses, the role of APN as receptor was identified by the fact that monoclonal antibodies able to block infection of susceptible

cells specifically recognize extracellular epitopes of the enzyme (Delmas *et al.,* 1994). The specificity of the viruses seems to be species dependent as only the feline APN (fAPN) can act as receptor for the entire group I coronaviruses. By the use of mutational studies of fAPN, the essential determinants were found for the host range of the coronaviruses (Tusell *et al.,* 2007). For example, the presence or absence of a potential N-glycosylation sequence at or near amino acids 288 to 290 is a critical determinant for HCV-229E, whereas a thymine on position 742 is critical for the receptor activity of FIPV, CaCV and TGEV. The activity of APN is not required for the binding of the viruses as no effect in binding is seen after deletions around the catalytic site (Delmas *et al.,* 1994).

Moreover, APN functions as receptor for both the human and murine cytomegalovirus (Kasman 2005; Soderberg *et al.,* 1993) and for the toxin CryIA(c) of *Bacillus thuringiensis* in insects (Knight *et al.,* 1994; Knight *et al.,* 1995).

Recently, our group found another function of APN. As mentioned in the beginning of this part about APN, our group demonstrated that APN is involved in the binding and endocytosis of F4ac fimbriae of F4ac-positive ETEC in the porcine small intestine (Melkebeek *et al.,* 2012). Moreover, we suggest that the interactions between sialic acid residues on pAPN and F4ac fimbriae are essential for binding. In addition, the study shows that the endocytosis of F4ac by pAPN is mediated by clathrin. A clathrin-mediated endocytosis following binding to receptors is also used by several bacterial pathogens to invade host cells, such as *Chlamydia* species, (Hodinka and Wyrick 1986; Soderlund and Kihlstrom 1983; Wyrick *et al.,* 1989), *Campylobacter jejuni* and *Citrobacter freundii* (Oelschlaeger *et al.,* 1993), some strains of enterohemorrhagic *E. coli* (Oelschlaeger *et al.,* 1994) and *Staphylococcus aureus* (Ellington *et al.,* 1999). The F4ac fimbriae are one of the unique molecules that induce an immune response after oral immunization (Van den Broeck *et al.,* 1999a). However, this immune response requires the presence of the F4ac receptor (F4acR) as oral immunization of F4acR-piglets with F4ac fimbriae does not result in the induction of an F4-specific mucosal immune response (Van den Broeck *et al.,* 1999b). pAPN, as F4ac receptor, most likely plays a role in the transport of F4ac fimbriae from the intestinal lumen to the immune cells. However, the exact role of pAPN in the oral immunogenicity needs further clarification.

### 1.5.4  APN inhibitors

APN inhibitors can be useful against virus infection or in therapy of cancer as the inhibition of APN can correct the dysregulated expression of membrane and/or soluble forms of APN (Luan and Xu 2007). The actions of APN inhibitors *in vitro* and *in vivo* are diverse and are summarized in Figure 1.9. In fact, APN inhibitors can directly target cancer cells, or act indirectly against targets by the activation of immune cells (T cells, B cells, neutrophils, natural killer [NK] cells, macrophages) or the alteration of angiogenesis (endothelial cells). In the brain, APN inhibitors exhibit analgesic properties (Bauvois and Dauzonne 2006; Wickstrom *et al.,* 2011). However, the molecular mechanisms underlying these effects are still unclear and most inhibitors lack specificity by inhibiting other metalloproteases (Bauvois and Dauzonne 2006).
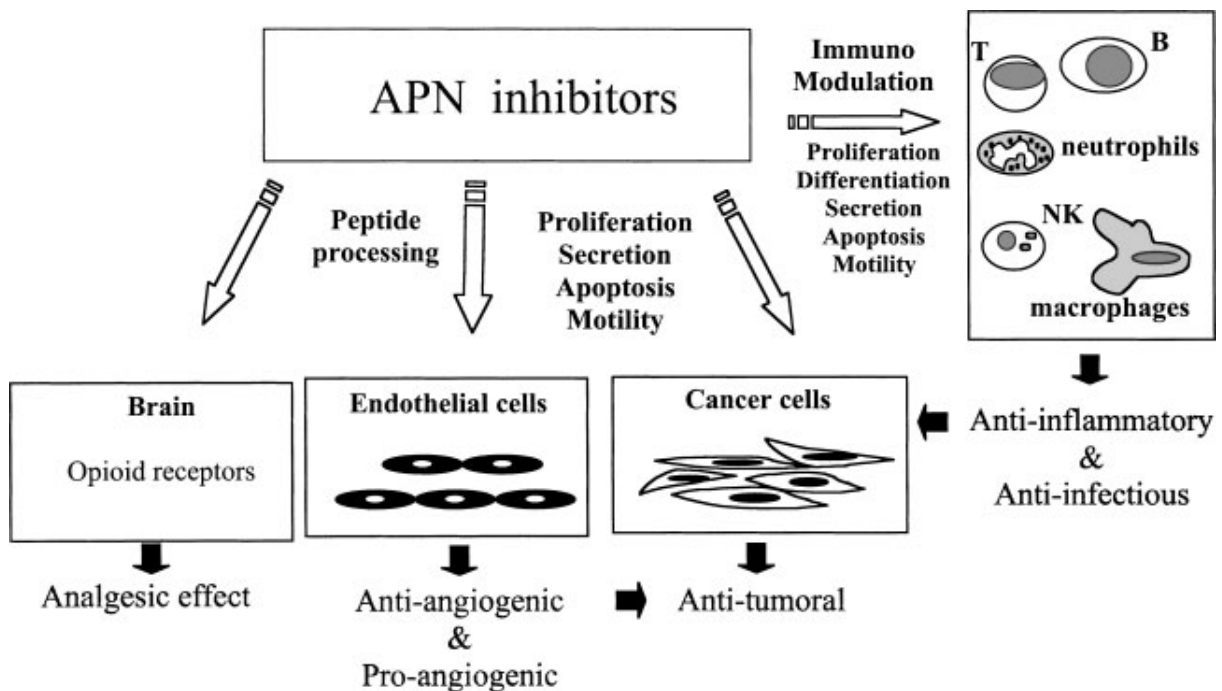


**Figure 1.9** The biological effects of APN inhibitors. Reprinted from Bauvois and Dauzonne, 2006 with permission from John Wiley & Sons.

APN inhibitors can be divided into two groups, the natural and the synthetic inhibitors. The majority of natural APN inhibitors are produced by bacteria belonging to the order of Actinomycetales, especially of the genera Streptomyces (Aoyagi *et al.,* 1978; Aoyagi *et al.,* 1990; Chung *et al.,* 1996; Gordon *et al.,* 1962; Umezawa *et al.,* 1976). Bestatin ((2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine) for example, is produced by *Streptomyces olivoreticuli* and is believed to be promising in APN related therapy (Aozuka *et al.,* 2004;

Chen *et al.,* 2003; Fujisaki *et al.,* 2003; Ichinose *et al.,* 2003; Ueda *et al.,* 1997). In phase III clinical trials in resected stage I squamous cell lung carcinoma, survival was statistically improved for patients treated with bestatin (30 mg/daily during 2 years) as a post-operative adjuvant therapy compared to patients receiving a placebo (Ichinose *et al.,* 2003).

In addition to the natural APN inhibitors, several synthetic small molecules belonging to various chemical families have been reported to inhibit APN activity. The list of APN inhibitors is still growing as new APN inhibitors are still described on a regular basis (Pei *et al.,* 2012; Su *et al.,* 2011; Xu *et al.,* 2012; Yang *et al.,* 2012; Zhang *et al.,* 2011). One of them is NGR, a peptide sequence of asparagine-glycine-arginine recognized by APN expressed in tumor cells (Curnis *et al.,* 2002). In phase II clinical trials, NGR coupled to human tumor necrosis factor (hTNF) showed a stabilization in 50% of patients with malignant pleural mesothelioma that could be maintained for more than 9 months by weekly dosing (Gregorc *et al.,* 2010).

## 1.6  Maltose-binding protein

In a previous study (Tiels *et al.,* 2008), oral administration of an F4-MBPFedF fusion protein adjuvanted with CT to pigs could induce a systemic and local immune response against FedF, the adhesin of F18 fimbriae. Unexpectedly, we also observed a weak priming of a FedF-specific immune response after oral administration of MBPFedF + CT. Using purified F18 fimbriae no such response could be demonstrated suggesting that the maltose-binding protein (MBP) was involved in this response (unpublished results).

MBP is a single-domain protein of 40 kDa that is part of the maltose/maltodextrin system of *E. coli* (Boos and Shuman 1998; Duplay *et al.,* 1984) and is encoded by the *malE* gene of *E. coli* K12. It serves as the initial high affinity receptor for maltose. The actual transport of maltose across the cytoplasmic membrane requires additional proteins forming a membrane-bound hetero-complex, called the maltose/maltodextrin system (Dassa and Hofnung 1985; Froshauer and Beckwith 1984; Gilson *et al.,* 1982; Shuman and Silhavy 1981). MBP is composed of an N- and a C-terminal lobe connected by a hinge region as shown in Figure 1.10 (Spurlino *et al.,* 1991). The maltose binding site is situated in the central cleft between the lobes (Sharff *et al.,* 1992). Upon ligand (maltose) binding, a conformational change occurs from the open conformation into the closed conformation, illustrated in

Figure 1.10 (Bertz and Rief 2009; Sharff *et al.,* 1992). In this transition, the two domains rotate towards each other, resulting in the closure of the central cleft. This conformational change has been used for fluorescence resonance energy transfer (FRET)-, electrochemical-, fluorescent- and enzymatic-based sensing (Medintz and Deschamps 2006).

In molecular biotechnology, MBP is often fused to proteins to improve its yield, to increase the solubility and to facilitate its purification by a one-step amylose affinity chromatography (Butt *et al.,* 1989; Riggs 2000). Moreover, the stability of the fused proteins is enhanced (Butt *et al.,* 1989; Fox *et al.,* 2001; Kapust and Waugh 1999; Schein 1989). The minimal effects of MBP on the bioactivity of the fused proteins contributes to the wide use of MBP as fusion protein for recombinant proteins (Labrie *et al.,* 2002; Rico *et al.,* 2002; Van Neerven *et al.,* 1998).

MBP is utilized as fusion protein in various binding studies (Labrie *et al.,* 2002; Su *et al.,* 1996) and experimental subunit vaccines against pathogenic bacteria (Kang *et al.,* 2005; Lee *et al.,* 1999; Yuzawa *et al.,* 2012) and viruses (Choi *et al.,* 2004; McNeal *et al.,* 2007; Simmons *et al.,* 2001). Interestingly, MBP can enhance the immunogenicity of the recombinant protein and thus lift the effectiveness of the immunization. It is presumed that MBP enhances the interactions of the vaccine with immune cells by increasing the solubility and lengthening the half-life of the fused proteins in immunized animals (Fernandez *et al.,* 2007; Fox *et al.,* 2001).
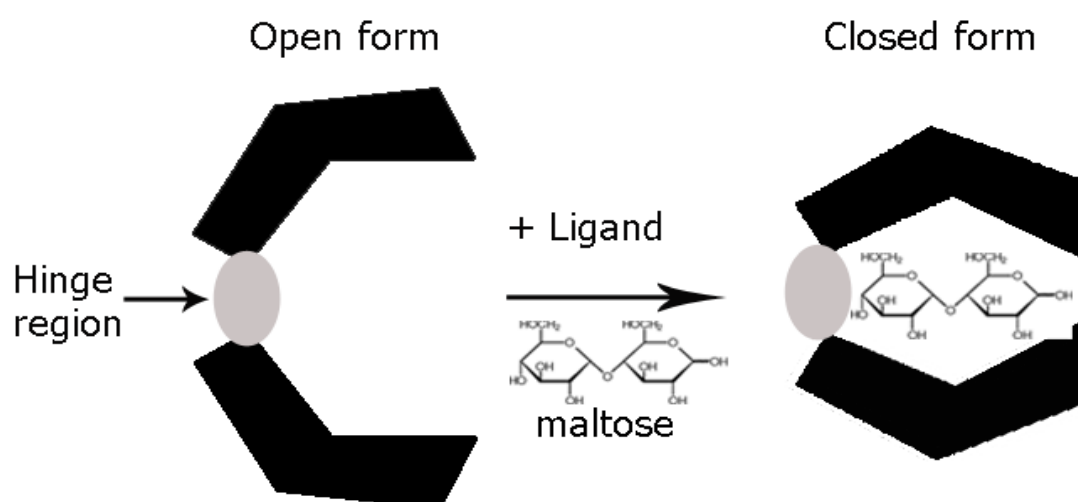


**Figure 1.10** Schematic representation of MBP upon ligand binding. The two domains (lobes) are highlighted in black and are connected by a hinge region (gray). Upon binding of maltose, the lobes rotate and twist laterally +- 8° relative to each other.

However, MBP can also directly influence the immune system as MBP induces the activation of human DCs and the production of proinflammatory cytokines (IL-12p70, tumor necrosis factor alpha, IL-10, IL-6 and IL-1β) (Fernandez *et al.,* 2007). In the study of Fernandez *et al.,* (2007), MBP induced the activation of the nuclear factor-κB (NF-κB) pathway by a process that may occur through TLR4 in DCs, presumably leading to proinflammatory cytokine secretion. Immunization of TLR4 knockout mice with an MBP fusion protein did not induce an immune response in contrast to normal mice immunized with the fusion protein, supporting the idea of TLR4 as MBP receptor (Yuzawa *et al.,* 2012). Furthermore, *in vivo* studies in mice showed that a fusion protein containing MBP could elicit IL-4 and IL-5 producing Th2-type CD4^{+} T cells in a TLR4 dependent manner and thus induce serum IgG, serum IgA and mucosal IgA antibody responses (Du *et al.,* 2011). In addition, a recent study implied that MBP can also bind to human monocytes and modulate their viability via TLR2 (Xiaoxia *et al.,* 2011). Moreover, the same group observed that MBP directly induces the proliferation of murine splenocytes and the secretion of IFN-γ and IL-2 (Xiaoxia *et al.,* 2011). All these findings demonstrate the adjuvant properties of MBP.

# Chapter 2

# Aims of the study

Today, numerous successful vaccines are in use against various pathogens. Most infectious diseases are caused by pathogens colonizing the mucosal tissues. For an effective protection of the host against those pathogens, pathogen-specific secretory IgA (SIgA) is required at the site of infection. In humans and0 most animal species such as pigs, vaccination via the oral route is essential to obtain a protective immunity at the intestinal mucosa. Commercial oral vaccines mainly use live attenuated microorganisms replicating in the gut. Residual inactivation during storage due to breaks in the cold chain as well as regain of virulence in the host are potential risks associated with the use of live vaccines. Therefore, non-replicating oral vaccines are an interesting alternative but soluble antigens generally induce oral tolerance instead of a protective immunity. Nevertheless, a limited number of soluble antigens, including the F4ac fimbriae of ETEC, are able to induce immunity after oral delivery. Recently, we have identified porcine aminopeptidase N (pAPN) as a novel receptor for F4ac fimbriae on small intestinal epithelial cells which is capable of internalizing the F4ac fimbriae. The aim of this thesis was to determine if APN can be used as target for the delivery of other antigens than F4ac to the gut-associated immune system. In addition, another carrier molecule, maltose-binding protein (MBP), was analysed for its potential to target molecules towards the mucosa. Besides delivering soluble antigens in solution, particles can be loaded with antigens to protect them against the harsh environment in the stomach and proximal small intestine. The use of pellets as carrier for oral vaccines was examined.

The following questions were addressed:
1) Can APN be used as target for the delivery of oral vaccines?
   - Can antibodies against pAPN induce an anti-pAPN antibody-specific immune response, similar as for F4ac fimbriae?
   - Which epithelia located in the digestive and respiratory tracts express pAPN?
   - Can the IPEC-J2 cell line transfected with pAPN be used to study the binding, uptake and transport of molecules via this receptor?
2) Is the intriguing difference between F4acR positive and negative pigs caused by a difference in pAPN expression on protein level?
3) Is MBP able to target conjugated antigens towards the GALT?
4) Can the oral administration of porous pellets loaded with F4ac fimbriae improve the mucosal immune response against F4ac?

# Part I

# APN as target to obtain a mucosal immune response

# Chapter 3

# Aminopeptidase N as a target for oral vaccine delivery

## 3.1 Abstract

The F4ac fimbriae of enterotoxigenic *Escherichia coli* (ETEC) are one of the unique molecules that induce an immune response after oral immunization. This immune response requires the presence of the F4ac receptor (F4acR), as oral immunization of F4acR negative (F4acR-) piglets with F4ac fimbriae does not result in the induction of an F4-specific mucosal immune response. Recently, our group identified porcine aminopeptidase N (pAPN) as a novel receptor for F4ac$^+$ ETEC. Aminopeptidase N (APN) is a type II membrane glycoprotein belonging to the family of membrane-bound metalloproteases and is expressed by a variety of cells, including porcine enterocytes. In the present study we determined if antibodies against pAPN, like the F4ac fimbriae are taken up by a receptor-mediated process and are able to induce an anti-pAPN antibody-specific immune response. First the adhesion of the pAPN-specific antibodies to pig enterocytes was demonstrated *in vitro*, by using a pAPN-transfected cell line (BHK-pAPN) as well as *in vivo*, by doing an intestinal loop experiment. In addition, the oral administration of the antibodies against pAPN (anti-pAPN) elicited a strong immune response. Even in the absence of the mucosal adjuvant cholera toxin (CT), strong serum IgA, IgG and IgM responses could be observed after a primary immunization. In addition, a local IgG immune response was observed in pigs orally immunized with anti-APN or anti-APN + CT. Elevated levels of IgA and IgG antibodies were found in the intestinal mucosa of the anti-APN and anti-APN + CT animals nine days after the booster immunization. Furthermore, rabbit IgG-specific IgA ASCs were found in the LP of the anti-APN and anti-APN + CT pigs, indicating the induction of a local IgA immune response against the anti-APN antibodies.

Our results indicate that APN is a promising target for oral delivery of antigens across the small intestinal barrier.

## 3.2   Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are an important cause of diarrhea in man and animal. ETEC infections are the leading cause of travelers' diarrhea and a major cause of diarrhea in developing countries, where it can be life-threatening for children (Clarke 2001; Ratchtrachenchai *et al.,* 2004). Porcine ETEC isolates can express six different types of fimbriae: F1, F4, F5, F6, F18 and F41, of which F4 is most abundant and involved in neonatal and postweaning diarrhea (Moon *et al.,* 1999). Three antigenic variants of F4 have been identified, namely F4ab, F4ac and F4ad, of which F4ac is the most common type. F4$^+$ ETEC adhesion is mediated by specific receptors (F4R) expressed on the brush border of small intestinal enterocytes and is dominantly inherited (Gibbons *et al.,* 1977). Interestingly, oral immunization of pigs with soluble F4ac fimbriae induces a protective immune response against challenge with F4ac$^+$ ETEC (Van den Broeck *et al.,* 1999a). Moreover, oral immunization with F4ac-coupled human serum albumin has demonstrated that F4ac has the potential to serve as a carrier molecule to induce mucosal immune responses against coupled antigens (Verdonck *et al.,* 2005). Orally administered F4ac is transcytosed by M cells and follicle-associated enterocytes in the Peyers' patches and by villous enterocytes in the lamina propria (Snoeck *et al.,* 2008). Subsequent uptake and presentation of F4ac by antigen-presenting cells could explain its capacity to induce a mucosal immune response. This implies that targeting selected antigens to the F4ac receptors (F4acRs) involved in transcytosis, may have the potential to elicit efficient mucosal immune responses against these antigens.

Recently, we have identified porcine aminopeptidase N (pAPN) as a novel receptor for F4ac fimbriae (Melkebeek *et al.,* 2012). Aminopeptidase N (APN) is a type II membrane glycoprotein belonging to the family of membrane-bound metalloproteases and is expressed by a variety of cells, including porcine enterocytes. Its function is based on the removal of the amino acids from the unsubstituted N-terminal of various peptides (Shipp and Look 1993). Moreover, APN also serves as receptor for viruses and bacteria. In humans, APN is a receptor for HCV-229E, which is an important cause of upper respiratory tract infections (Delmas *et al.,* 1992; Yeager *et al.,* 1992). Similarly, transmissible gastroenteritis virus (TGEV), a cause of fatal gastroenteritis in newborn pigs, uses porcine APN as its portal of entry. In both instances, the role of

APN as receptor was found by blocking infection of susceptible cells using monoclonal antibodies, which specifically recognize extracellular epitopes of the enzyme (Delmas *et al.,* 1994).

The aim of present study was to determine if pAPN-specific antibodies, similar to F4ac fimbriae, can be endocytosed by intestinal epithelial cells and can induce an anti-pAPN antibody-specific immune response.

## 3.3 Material and methods

### 3.3.1 Production and evaluation of polyclonal antibodies against pAPN

Polyclonal antibodies against APN were produced by intramuscularly immunizing two rabbits with pig APN (pAPN, Sigma-Aldrich, Bornem, Belgium). For the first immunization (d0), 500 µg pAPN in complete Freund's adjuvant (Sigma-Aldrich) was used, whereas in subsequent immunizations (d21 & d41) incomplete Freund's adjuvant (Sigma-Aldrich) was used. During the experiment, blood was taken from the ear vein using a winged needle. The collected serum was inactivated, treated with kaolin (Sigma-Aldrich) as described by Van den Broeck *et al.,* (1999a) and analyzed by an anti-APN specific ELISA. Briefly, a 96-well microtiter plate (NUNC, Polysorb Immuno Plates; Life Technologies) was coated with pig APN (Sigma-Aldrich) at a concentration of 2 µg/ml in PBS for 2h. In subsequent steps, the remaining binding sites were blocked for 2 hours with PBS supplemented with 3% bovine gelatin (Sigma-Aldrich) and 0.05% (v/v) Tween® 80 (Merck KGaA, Darmstadt, Germany), the treated serum was added in series of twofold dilutions in ELISA dilution buffer (PBS + 3% [w/v] bovine serum albumin [BSA, MP Biomedicals, Belgium] + 0.2% [v/v] Tween® 20 [Merck KGaA, Darmstadt, Germany]), starting from 1/10 for 1h, the wells were treated for 1h with optimal dilutions of anti-rabbit Ig-HRP (Dako, Heverlee, Belgium) and ABTS (Roche, Mannheim, Germany) was added for 1h. Whereafter the $OD_{405}$ was measured. All incubations occurred at 37°C. Between each incubation step, plates were washed three times with PBS-T (PBS plus 0.2% [v/v] Tween® 20). The cut-off values were calculated as the mean $OD_{405}$-value of all sera (dilution 1/10) at day 0, increased with three times the standard deviation. The antibody titer was the inverse of the highest dilution that still had an $OD_{405}$ higher than the calculated cut-off value.

The IgG fraction of the serum was purified by affinity chromatography using a HiTrap protein A column (GE Healthcare, Diegem, Belgium) on an ÂKTA explorer chromatography system

(GE Healthcare). The concentration was determined by bicinchoninic acid (BCA, Pierce®, Thermo Scientific, Rockford, USA) reaction using bovine serum albumin (BSA, MP Biomedicals, Belgium) as standard and calculated with Deltasoft 2.0 software (Bio Metallics, Inc., Princeton, N.J).

Blood of a non-immunized rabbit was also collected and the IgG fraction was purified as described above. This fraction was used as negative control in further experiments.

Experiment and animal management procedures were approved by the animal care and ethics committee of the Faculty of Veterinary Sciences, Ghent University, Belgium (EC2012/030) .

### 3.3.2   Cell line and culture conditions

The BHK-21 and pAPN-transfected BHK-21 (BHK-pAPN) cells (Delmas *et al.,* 1993) were kindly gifted by Dr. Laude of the Unit of Virology and Molecular Immunology of the INRA in France. The BHK-21 cells were cultured in DMEM with 5 % FCS (Greiner Bio-One, Belgium), 1 % L-Glu (Gibco BRL, Life Technologies Inc., Paisley, Scotland), 1 % P/S (Gibco BRL), 1 % sodiumpyruvate (Gibco BRL) and 1 % non-essential amino acids (Gibco BRL). The BHK-pAPN cells were grown in the same medium supplemented with 1.5 mg/ml geneticin G418 (Sigma-Aldrich).

### 3.3.3   Endocytosis assay

The BHK-21 and BHK-pAPN cells were incubated with an optimal dilution of the pAPN-specific polyclonal serum or irrelevant rabbit serum for 1 h at 4°C to allow attachment, but no internalization. Cells were then washed with PBS$^+$ (PBS supplemented with 0.1 mM CaCl$_2$ and 1 mM MgCl$_2$) to remove unbound ligands and shifted to 37°C to start endocytosis. After 0 and 20 minutes incubation, cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with phalloidin-Texas Red (Molecular Probes) and goat anti-rabbit IgG fluorescein isothiocyanate (FITC) (Sigma-Aldrich) in PBS$^+$ for 1 h at 37°C. Confocal images were acquired with a Leica SP5 AOBS confocal microscope (Leica Microsystems, Groot-Bijgaarden, Belgium).

### 3.3.4 *In vivo* endocytosis of polyclonal anti-APN

Two six-week-old pigs (Belgian Landrace) were fasted overnight and sedated by intramuscular injection with a mixture of 0.5 mg/kg midazolam, 0.1 mg/kg morphine and 10 mg/kg ketamine. After 15 min, the pigs were anesthesized by an intravenous injection of 2 mg/kg propofol. The pigs were intubated and anesthesia was maintained by a mixture of isoflurane and oxygen. Following laparatomy, 5-cm-long loops were created in the jejunal lamina propria as described previously (Moon *et al.,* 1966). The loops were separated by an interloop segment of at least 5 cm. Care was taken to minimize surgical trauma and to maintain an adequate blood supply to the ligated segments. Four loops were created in the first pig and two loops in the second pig. In both pigs, polyclonal anti-APN or irrelevant rabbit serum 1 / 20 diluted in 5 ml PBS was injected into the lumen of one loop, whereafter the loops were placed back into the abdominal cavity. After 10 min the additional loops of the first pig were injected with either polyclonal anti-pAPN or irrelevant rabbit serum and 5 min later this pig was euthanized by an overdose of pentobarbital (24 mg/kg). The other pig was kept under anesthesia for 45 min before being euthanized. So, intact mucosa were incubated with the antibodies for 45, 15 or 5 minutes.

### 3.3.5 Immunohistochemistry to localize pAPN-specific antibodies in tissue

Immediately after euthanasia, the loops were excised and flushed with cold PBS, embedded in 2 % (w / v) methocel (Fluka, Bornem, Belgium) in water, snap frozen in liquid nitrogen and stored at – 80°C before use. Approximately 5 μm-thick cryosections were cut and mounted on 3-aminopropyl-triethoxysilane (Sigma-Aldrich)-coated glass slides. After drying for 4 h at 60°C, the sections were fixed in acetone for 10 min at -20°C and stored at -80°C. When needed, the cryosections were air-dried during 1 h, washed for 5 min in PBS and incubated with 10 % goat serum in PBS for 30 min at 37°C in a humidity chamber. Subsequently, the sections were incubated for 1 h at 37°C with goat anti-rabbit IgG-FITC (Sigma-Aldrich) and Phalloidin-Texas Red (Molecular Probes) or with goat anti-rabbit IgG-FITC and anti-cytokeratin peptide-18 (Sigma-Aldrich) in the humidity chamber, followed by a 1h incubation with Texas Red®-X labelled goat anti-mouse IgG (Molecular Probes). Cytokeratin-18 is exclusively present in epithelial cells and stains the enterocytes weakly and goblet cells intensely, whereas phalloidin stains the F-actin filaments present in every cell. The sections

were washed twice for 5 min in PBS before mounting in glycerol containing 0.223 M 1,4-diazobicyclo-(2,2,2)-octane (DABCO, Sigma-Aldrich) to counter photobleaching.

### 3.3.6   Piglets and experimental procedure

Twenty piglets (Belgian Landrace x Piétrain) were weaned at 4 weeks of age, transported to the experimental facilities and subsequently housed in isolation units with food and water *ad libitum*. Experimental procedures and animal management procedures were undertaken in accordance with the requirements of the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2010/117).

The piglets were randomly divided into 4 groups (Table 3.1) and five days after weaning the pigs were orally immunized. The first group with 1 mg purified rabbit anti-pAPN IgG (n=4, anti-APN group) and the second group with 1 mg purified rabbit anti-pAPN IgG and 50µg cholera toxin (CT) (n=6, anti-APN + CT group). As negative control five pigs were given 1 mg purified rabbit IgG (n=4, IgG group) and six pigs 1 mg purified rabbit IgG and 50 µg CT (n=6, IgG + CT group). CT was used as mucosal adjuvant and each immunization was carried out in 5 ml PBS. The pigs were orally immunized at 0, 1 and 2 days post-primary immunization (dppi). These three immunizations are referred as the first immunization. The pigs were orally boosted at 20 dppi. Twenty-four hours before each immunization the acidic gastric pH was increased by orally administrating 20mg rabeprazolum (Pariet, Janssen-Cilag, Berchem, Belgium) as previously described by Verdonck *et al.,* (2004a). Three hours before till two hours after each rabeprazolum treatment and immunization, the pigs were deprived of food and water.

**Table 3.1** Experimental design.

| Group | Total number of pigs | Oral administration |
|---|:---:|---|
| anti-APN | 4 | 1 mg rabbit anti-pAPN IgG |
| anti-APN + CT | 6 | 1 mg rabbit anti-pAPN IgG and 50µg CT |
| IgG | 4 | 1 mg rabbit IgG |
| IgG + CT | 6 | 1 mg rabbit IgG and 50 µg CT |

The local immune response was analyzed in two ways. First, the anti-pAPN antibody specific immune response was analyzed fourteen days following the first immunization and again at seven and nine days after the second immunization for one pig of each group by

enumerating the anti-pAPN antibody-specific IgA and IgG antibody-secreting cells (ASC) in mesenteric lymph nodes (MLN), jejunal Peyer's patches (JPP) and jejunal lamina propria (LP) as previously described (Verdonck *et al.,* 2008). The tissues were sampled following euthanasia of animals by intravenous injection of pentobarbital (24mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) and subsequent exsanguination. Monomorphonuclear cells (MCs) were isolated and suspended at $10^7$ cells/ml in leukocyte medium (RPMI with 10% FCS , 100 IU/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml kanamycin (Gibco BRL), 200 mM L-Glutamine, 100 mM sodium pyruvate and 100 mM non-essential amino acids (Gibco BRL)). Secondly, mid-jejunal tissue extracts were collected nine days after the second immunization for one pig of each group to analyze the local antibody response against rabbit IgG.

During the experiment blood was taken on weekly basis from the jugular vein. The serum was collected, inactivated and treated with kaolin (Sigma-Aldrich) as described by Van den Broeck *et al.,* (1999a).

### 3.3.7   Anti-rabbit specific porcine IgM, IgA and IgG ELISA

For detection of anti-rabbit IgG-specific porcine IgM, IgA and IgG an indirect ELISA was used. The wells of a 96-well microtiter plate (NUNC, Maxisorp Immuno Plates; Thermo Scientific) were coated with purified rabbit IgG at a concentration of 2 µg/ml in PBS. After 2h of incubation at 37°C, the remaining binding sites were blocked overnight at 4°C with PBS supplemented with 0.2% (v/v) Tween® 80 (Merck KGaA, Darmstadt, Germany). Subsequently, the treated pig sera were added in series of twofold dilutions in ELISA dilution buffer, starting from 1/10 and plates were incubated for 1h at 37°C. Thereafter, the wells were treated for 1h at 37°C with optimal dilutions of anti-swine IgG, IgM and IgA monoclonal antibodies (Vanzaane and Hulst 1987). In the next step rabbit anti-mouse Ig-HRP (Dako) was added for 1h at 37°C. Subsequently, ABTS (Roche) was added and the $OD_{405}$ was measured after 15 min of incubation at 37°C. Between each incubation step, plates were washed three times with PBS-T (PBS plus 0.2% [v/v] Tween® 20). The cut-off values and titers were calculated as for the pAPN-specific antibodies.

### 3.3.8    ELISPOT assay for anti-rabbit specific porcine IgA and IgG secreting cells

For enumerating the anti-rabbit specific IgA and IgG ASC, the 96-well microtiter plates were coated and blocked as for the anti-rabbit specific porcine IgA and IgG ELISA. Subsequently, MC suspensions at a concentration of $10^7$ cells/ml in leukocyte medium were added to 10 wells (100 µl/well) and plates were incubated for 18 h at 37°C in a humidified 5% $CO_2$ atmosphere. After removing cells by several washes with PBS-T, plates were treated with anti-swine IgG and IgA and subsequent with anti-mouse Ig-HRP (see ELISA procedure). The substrate solution, consisting of 4 volumes of 3-amino-9-ethylcarbazole (AEC) working solution (0.67 ml AEC stock solution [0.4% in dimethylformamide] in 10 ml Na acetate [0.1 M, pH 5.2] +10 µl 30% $H_2O_2$) and 1 volume of 3% low melting agarose (BIOzym, Landgraaf, The Netherlands), was added. Reactive spots were manually counted after the plates had been incubated overnight in the dark at room temperature. For each MC suspension, spots in 10 wells ($10^6$ MC/well) were counted, so that finally the number of ASC per $10^7$ MC was obtained.

### 3.3.9    Antibody extraction out of tissue samples

Tissue extracts were prepared from an mid-jejunal intestinal segment (2 cm$^2$) without Peyer's patches for one pig of each group nine days after the second immunization. The segments were washed with PBS and cut into pieces of about 3 mm$^3$ which were immediately frozen at -80°C. Antibodies were extracted as described (Bergquist *et al.,* 2000). Briefly, 40 mg tissue was thawed overnight at 4°C in 300 µl PBS with 2% (w/v) saponin, 0.35 mg/ml phenylmethylsulfonyl fluoride (Roche) and 0.1% (w/v) BSA (Sigma-Aldrich). Subsequently, the samples were inactivated at 56°C during 30 min and centrifuged at 13 800g for 5 min after which the supernatant was stored at -80°C until analyzed by ELISA.

### 3.3.10  Statistical analysis

The antibody titers were statistical analyzed with SPSS 19.0 for Windows (IBM, New York, NY) using General Linear Model (repeated measures analysis of variance) with the Bonferoni adjustment for multiple comparisons; $p < 0.05$ was considered as statistically significant.

## 3.4 Results

### 3.4.1 Binding and endocytosis of pAPN-specific antibodies

Like F4ac fimbriae, polyclonal pAPN-specific antibodies bound to and were endocytosed by pAPN-transfected BHK-21 cells, the results are shown in Figure 3.1. These processes were APN-specific as binding to and uptake of anti-pAPN antibodies could not be observed using untransfected BHK-21 cells. As an additional control, irrelevant rabbit serum also did not bind to and was not taken up by pAPN-transfected BHK-21 cells.
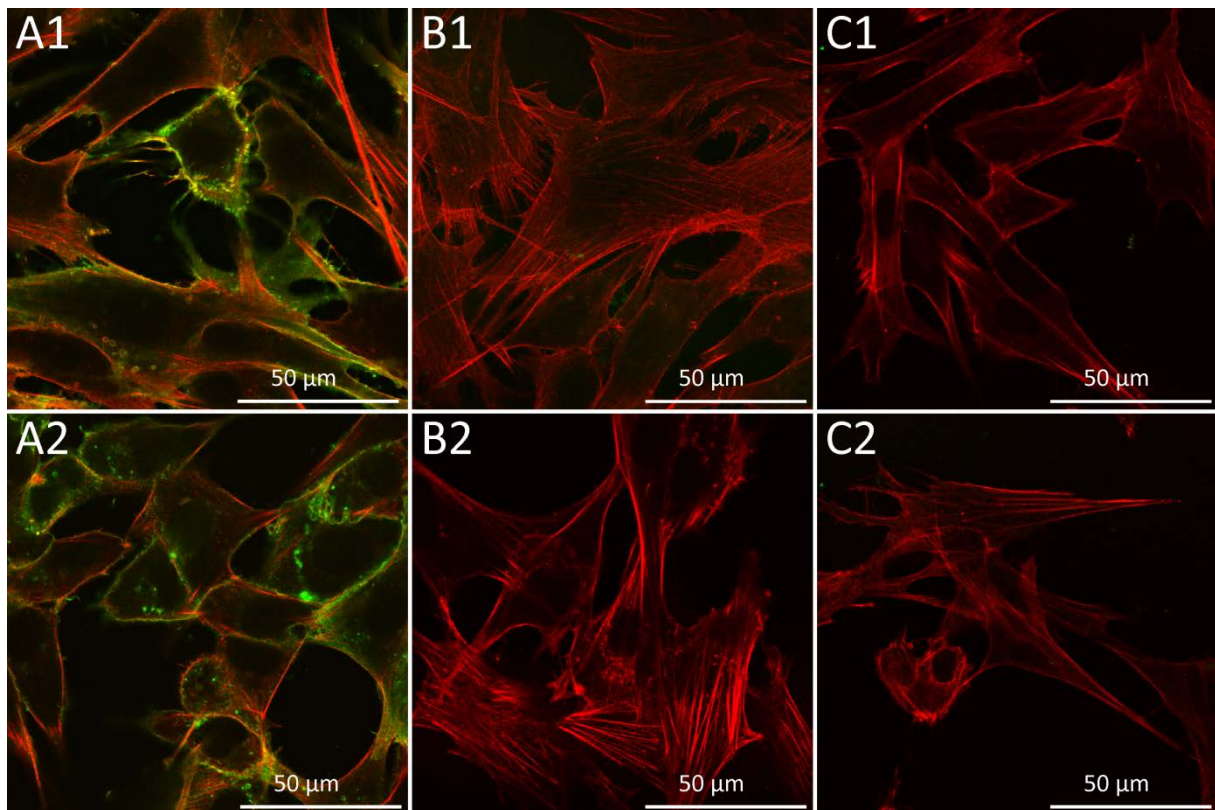


**Figure 3.1** Endocytosis of aminopeptidase N (APN)-specific antibodies *in vitro*. Porcine (p)APN-transfected BHK-21 were incubated with anti-pAPN IgG (**A**) or irrelevant rabbit serum (**B**) and untransfected BHK-21 cells were incubated with anti-pAPN serum (**C**) for 1 h at 4°C to allow binding but no internalization. Then, cells were shifted to 37°C to evaluate endocytosis. After 0 (**A1**, **B1** and **C1**) and 20 min incubation (**A2**, **B2** and **C2**), endocytosis was checked via confocal microscopy. Binding and/or uptake of anti-pAPN antibodies was assessed with anti-rabbit IgG fluorescein isothiocyanate and phalloidin-Texas Red was used to detect the actin cytoskeleton.

Adhesion and endocytosis of polyclonal anti-pAPN antibodies were also evaluated *in vivo* on cryosections of pig jejunal loops incubated for 5, 15, or 45 min with anti-pAPN or with an irrelevant rabbit serum as a control. The results are shown in Figure 3.2. After 5 min, the anti-pAPN antibodies had bound to the brush border membrane of enterocytes and could clearly be observed within the enterocytes after 15 and 45 min of incubation. Conversely, no binding of irrelevant rabbit serum occurred after 5 and 15 min, although binding and some uptake could be detected after 45 min incubation, but to a much lower extent as compared to anti-pAPN serum.
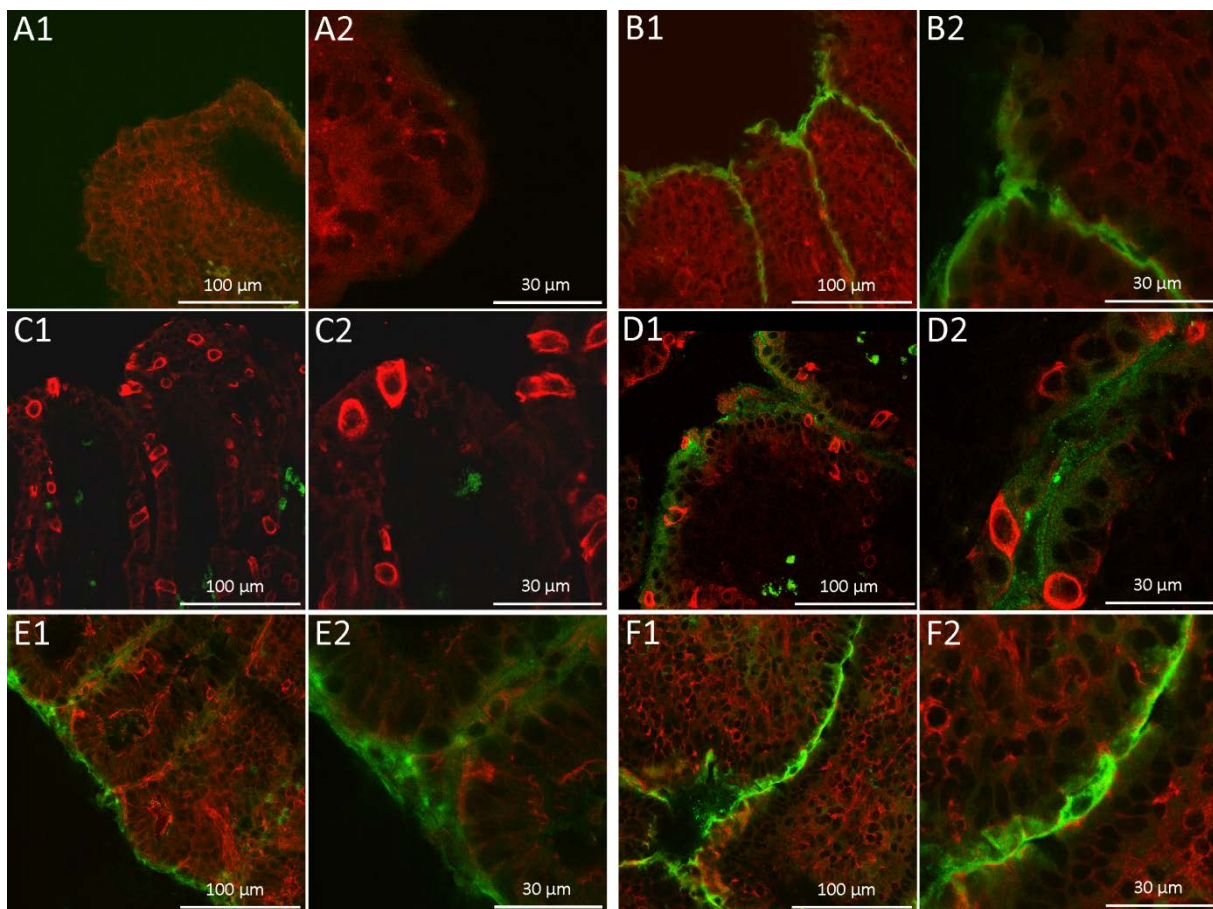


**Figure 3.2** Confocal view of the jejunum of a pig after 5, 15, or 45 min incubation *in vivo* with irrelevant rabbit serum (A1, A2; C1, C2; E1 and E2, respectively) or anti-aminopeptidase N (APN) (B1, B2; D1, D2; F1 and F2, respectively). Binding and/or uptake of anti-pAPN and the irrelevant rabbit serum were assessed with anti-rabbit IgG fluorescein isothiocyanate. In A, B, E and F, the actin cytoskeleton was detected with phalloidin-Texas Red, whereas in C and D, anti-cytokeratin 18 was used to stain the enterocytes.

**3.4.2    Anti-pAPN antibodies induce a systemic immune response after oral immunization**

The serum antibody responses against rabbit IgG are presented in Figure 3.3. The responses have the same pattern for the different isotypes. Although serum IgA, IgG and IgM directed against rabbit IgG only appeared 13 dppi in the IgG group, a serum IgG, IgA and IgM response could be observed as soon as 7 dppi with anti-pAPN IgG. This response reached a maximum 13 dppi (mean IgG, IgA, IgM titers of 320, 113 and 23, respectively, compared to mean IgG, IgA, IgM titers of 44, 11 and 17, respectively, in the IgG group) and was only slightly boosted by a secondary immunization. Furthermore, the serum antibody response in the IgG group remained lower than in the anti-APN group during the course of the experiment. Addition of CT (IgG + CT group) increased the IgA, IgG and IgM response in animals immunized with control IgG to similar titers as observed in the anti-APN group, whereas addition of CT to anti-pAPN IgG further increased IgA and IgG titers at least threefold from day 13 onwards, resulting in significantly higher IgA and IgG titers on days 13, 20 and 27 and significantly higher IgM titers on day 20 in the anti-APN + CT group compared to the IgG group. Significant differences between the other groups were not observed.
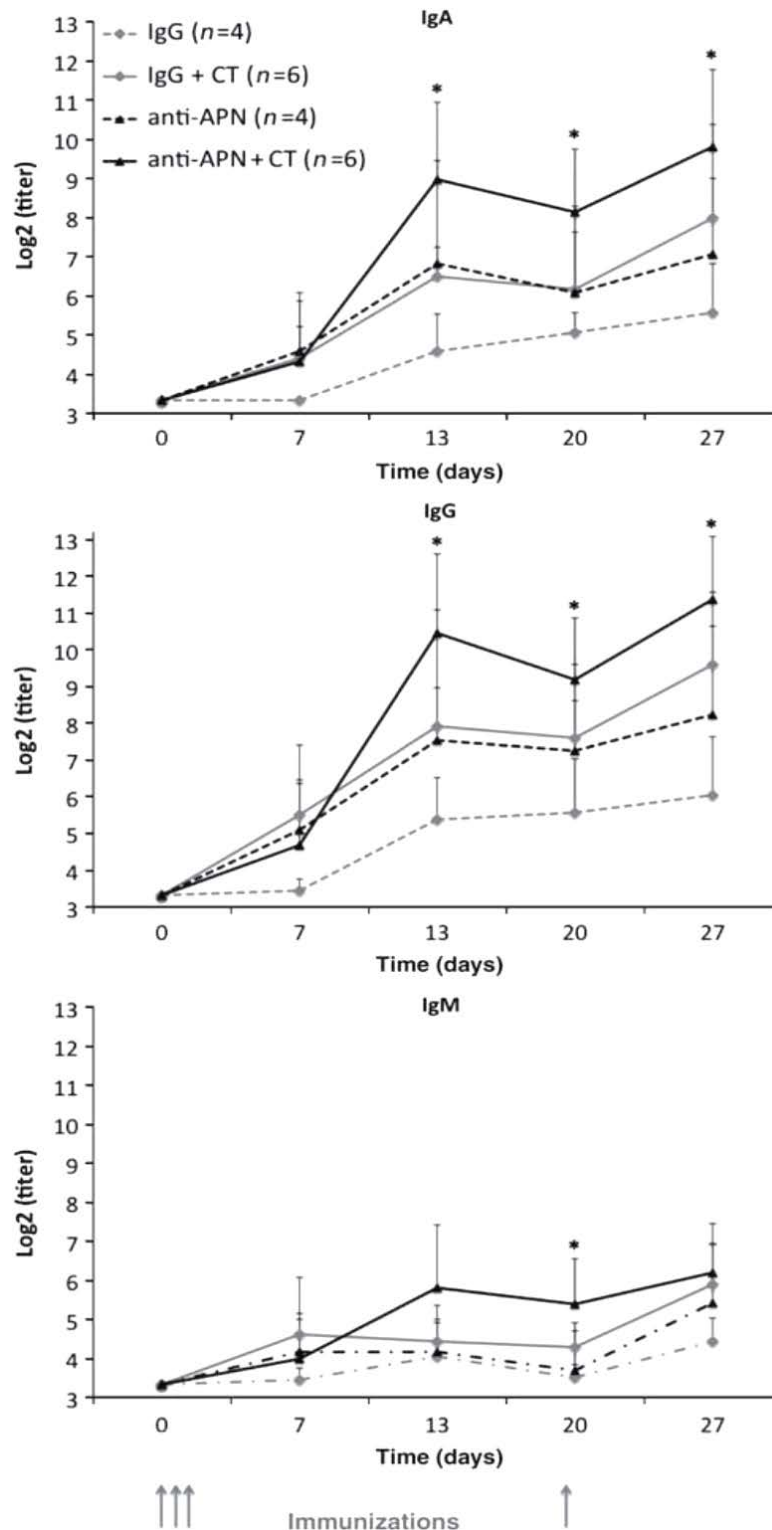
**Figure 3.3** Kinetics of the rabbit IgG-specific serum IgA, IgM and IgG response. Pigs were orally immunized with polyclonal porcine aminopeptidase N (pAPN)-specific rabbit IgG (anti-pAPN; anti-APN group, $n$ = 4), irrelevant rabbit IgG (IgG group, $n$ = 4), anti-pAPN IgG adjuvanted with cholera toxin (CT) (anti-APN + CT group, $n$ = 6), or irrelevant rabbit IgG adjuvanted with CT (IgG + CT group, $n$ = 6) on days 0, 1, 2 and identically boosted on day 20. Serum samples were taken weekly until day 7 after the boost. Results are presented as the mean ± s.d. Significant differences ($p < 0.05$) between the anti-APN + CT group and the IgG group are indicated with an asterisk.

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### 3.4.3 Anti-pAPN induces a local immune response after oral immunization

The local intestinal antibody response against rabbit IgG was analyzed by extracting antibodies out a mid jejunal intestinal segment of one pig of each group nine days after the booster immunization or by counting anti-pAPN antibody-specific ASC numbers in JPP, LP and MLN at 14 days post-primary immunization and 7 and 9 days post booster immunization.

The results for the antibody extraction are presented in Figure 3.4. The animal of the anti-APN + CT group has clearly a higher response for all the isotypes compared to the pigs of the other groups. The anti-pAPN IgG immunized animal has a comparable IgA response as the IgG + CT immunized animal. However, the other isotype responses are higher in the anti-APN animal than in the IgG + CT animal.
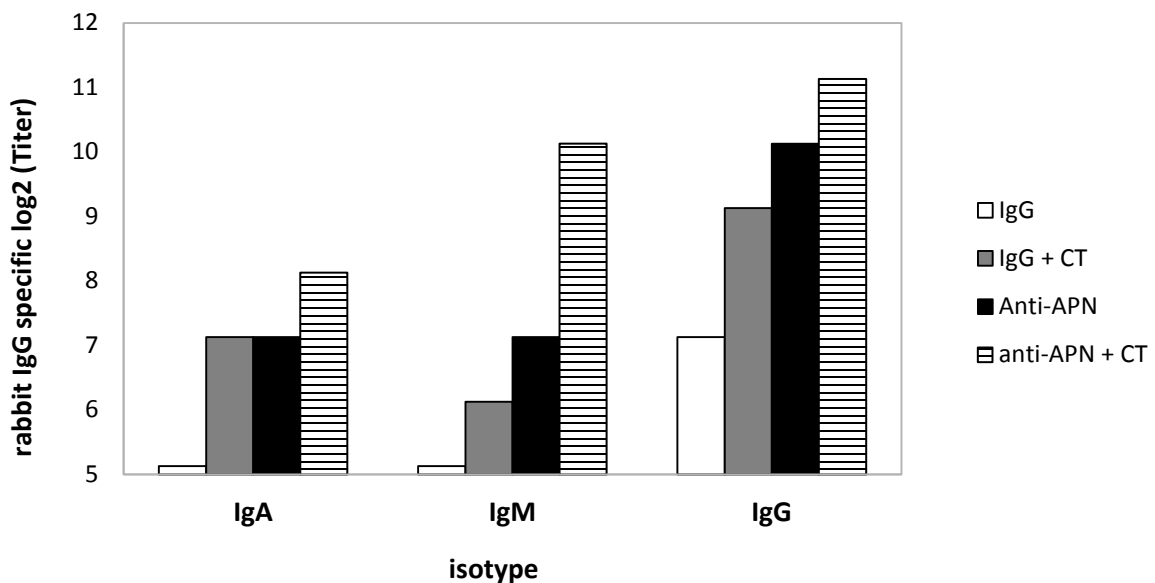


**Figure 3.4** Rabbit IgG-specific IgA, IgM and IgG titers in tissue extracts of the mid-jejunum taken from one pig of each group nine days after the booster immunization. The animals were orally immunized with rabbit IgG (IgG) rabbit IgG and CT (IgG + CT), rabbit pAPN-specific antibodies (anti-APN) and rabbit pAPN-specific antibodies and CT (anti-APN + CT).

The number of rabbit IgG-specific IgA and IgG ASC in JPP, LP and MLN are presented in Figure 3.5 and Figure 3.6. The amount of IgA ASC in JPP and MLN were at background level for the IgG, IgG + CT and anti-APN immunized animals, whereas IgA ASC could be detected in the anti-APN + CT immunized animals in JPP at 7 (14 ASC) and 9 (28 ASC) days after the booster immunization and in MLN at 14 days post-primary immunization (10 ASC), at 7 days after the

boost (15 ASC) and 9 days after the boost (6 ASC). However, in the LP more animals were positive. The anti-APN + CT group has the highest number of ASC at 9 days after the boost (109 ASC), the anti-APN also has a high amount of ASC at 9 days after the boost (70 ASC), whereas the highest number of ASC of the IgG + CT group could be found at 7 days after the boost (53 ASC). These results are comparable with the serum IgA responses.

The amount of IgG ASC was low, the most ASC could be found in the MLN of the anti-APN + CT immunized animal at 7 days after the boost (15 ASC). Nine days after the boost a similar amount of IgG ASC could be detected in the MLN of the anti-APN group at (13 ASC) whereas only low amounts could be detected for the IgG (2 ASC at both 7 and 9 days after the boost) and IgG + CT group (2, 5 ASC at 7 and 9 days after the boost respectively). In the JPP, we could detect ASC at 9 days after the boost in the IgG + CT (3 ASC), anti-APN (5 ASC) and anti-APN + CT (9 ASC) immunized animals. In the LP, ASC could only be found in the anti-APN + CT immunized animal at 9 days after the boost (12 ASC).

**JPP**



**LP**



**MLN**
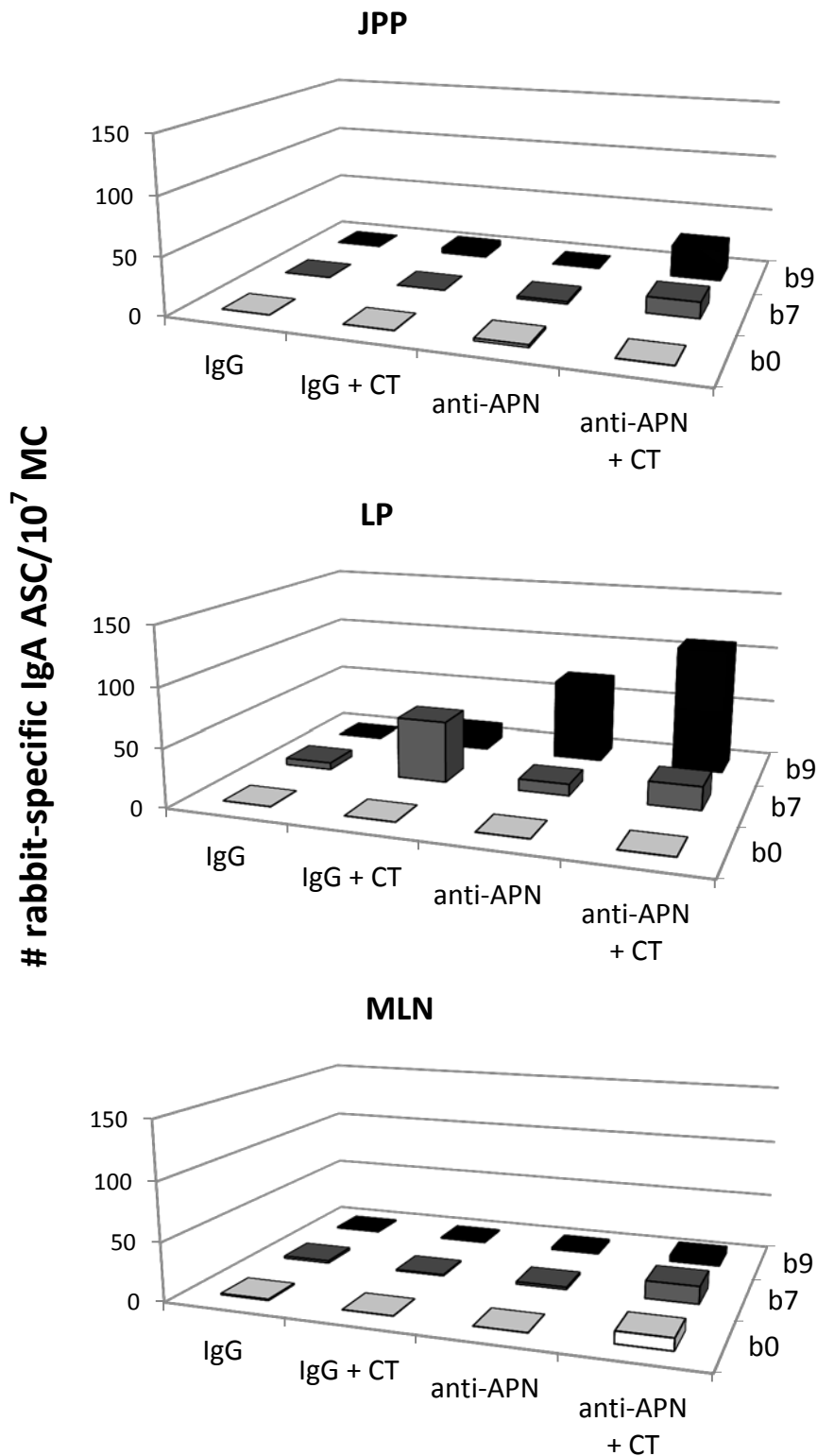


**Figure 3.5** The number of rabbit IgG-specific IgA ASC per $10^7$ MC in jejunal Peyer's patches (JPP), jejunal lamina propria (LP) and mesenteric lymph nodes (MLN) of animals orally immunized with rabbit IgG (IgG), rabbit IgG and CT (IgG + CT), rabbit pAPN-specific antibodies (anti-APN) and rabbit pAPN-specific antibodies and CT (anti-APN + CT) at 14 days post-primary immunization (b0) and 7 (b7) and 9 (b9) days post-booster immunization (n=1 for each group at eachr day).

**Figure 3.6** The number of rabbit IgG-specific IgG ASC per $10^7$ MC in jejunal Peyer's patches (JPP), jejunal lamina propria (LP) and mesenteric lymph nodes (MLN) of animals orally immunized with rabbit IgG (IgG), rabbit IgG and CT (IgG + CT), rabbit pAPN-specific antibodies (anti-APN) and rabbit pAPN-specific antibodies and CT (anti-APN + CT) at 14 days post-primary immunization (b0) and 7 (b7) and 9 (b9) days post-booster immunization (n=1 for each group at each day).

## 3.5 Discussion

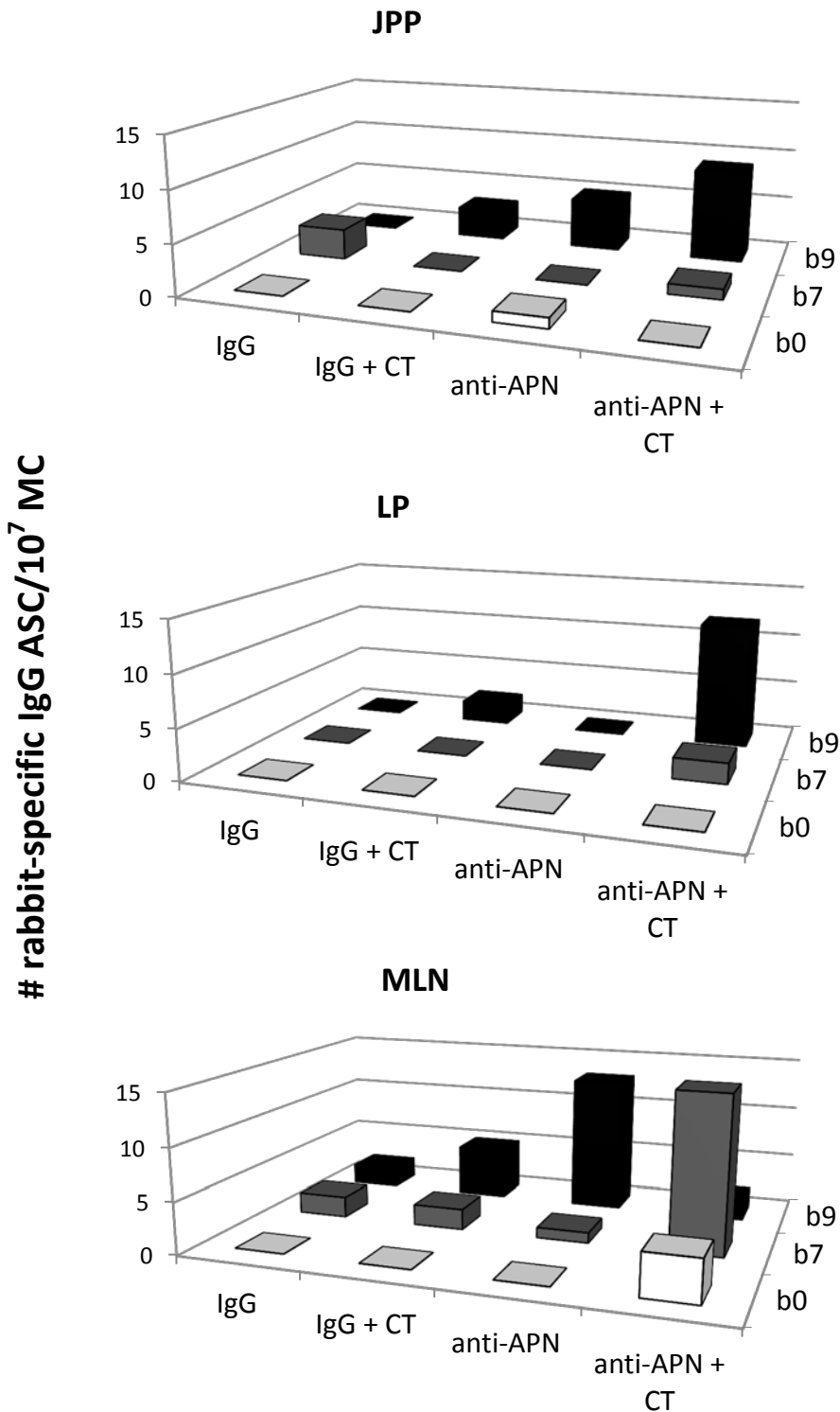Oral administration of large doses of non-replicating antigens can induce a short-lived mucosal IgA response (Van den Broeck *et al.,* 1999a), but mostly results in a systemic state of immunological hyporesponsiveness, called oral tolerance (Garside and Mowat 2001; Stokes and Bailey 2000). On the other hand, receptor-mediated uptake of orally administered antigens may result in the induction of an antigen-specific mucosal immune response (Deaizpurua and Russelljones 1988). A limited number of soluble antigens are able to induce strong mucosal immunity after oral delivery, including CT (Spangler 1992; Yamamoto *et al.,* 1997), the *E. coli* heat-labile enterotoxin (LT) (Nakagawa *et al.,* 1996; Takahashi *et al.,* 1996) and the F4ac fimbriae of ETEC (Van den Broeck *et al.,* 1999a; Verdonck *et al.,* 2004a). However, the F4ac immune response requires the presence of the F4acR as oral immunization of F4acR negative piglets with F4ac fimbriae does not result in the induction of an F4ac-specific mucosal immune response (Van den Broeck *et al.,* 1999b). Recently, pAPN was identified as a receptor for F4ac fimbriae (Melkebeek *et al.,* 2012). Binding to this receptor results in internalization of F4ac in pig enterocytes, leading to F4ac-specific antibody responses. This suggests that targeting APN could be used as a target for the oral delivery of other antigens to pigs. Therefore, we wanted to study if pAPN could also bind and internalize another ligand after oral administration. In the present study, we used rabbit antibodies raised against pAPN (anti-pAPN) and determined that those antibodies are endocytosed and induce a pAPN-specific immune response.

First, we demonstrated that the antibodies against APN adhere to and are taken up by enterocytes, similarly to the F4ac fimbriae. A clear binding of the rabbit pAPN-specific antibodies could be observed *in vitro*, on pAPN-transfected BHK-21 cells. However, BHK-21 cells are used in various studies regarding endocytosis of antigens such as viruses (Dee *et al.,* 1995; Martin-Acebes *et al.,* 2011; Palokangas *et al.,* 1994; Shrivastava *et al.,* 2011), bacteria (Chang *et al.,* 2000) and even immunocomplexes (Engelhardt *et al.,* 1991), suggesting that this cell line easily internalizes antigens upon binding. In addition, being hamster kidney cells, BHK-21 cells are not the best model for endocytosis of porcine enterocytes. Therefore it was needed to determine if endocytosis also occurred *in vivo*, by porcine intestinal enterocytes (jejunum). Binding and uptake could be detected after 15 minutes incubation.

After 45 min incubation irrelevant rabbit serum antibodies were also taken up by enterocytes, but to a much lower extent than the pAPN-specific antibodies.

The oral administration of the antibodies against pAPN elicited a strong serum immune response. Even in the absence of the CT adjuvant, strong serum IgA, IgG and IgM responses could be observed after a primary immunization. Although clearly weaker than observed with APN-specific antibodies, irrelevant rabbit antibodies also interacted with enterocytes and induced a low serum antibody response. This might be explained by the presence of the neonatal IgG receptor, FcRn. This receptor is expressed on porcine and human intestinal epithelium throughout life (Israel *et al.,* 1997; Stirling *et al.,* 2005). Several authors suggest an important role of FcRn in the delivery of proteins across the gut in adult species (Baker *et al.,* 2009; Claypool *et al.,* 2004; Devriendt *et al.,* 2012; Dickinson *et al.,* 1999; Hansen *et al.,* 2006; Yoshida *et al.,* 2004). In their study, Stirling and colleagues (2005), demonstrated that orally administered bovine IgG to pigs was transferred into the pigs blood. However, they had to administer 300 ml bovine colostrum for 7 consecutive days and they could not exclude the uptake via non-specific mechanisms. While these studies provide evidence that FcRn can transport IgG in a bidirectional way across the gut, *in vivo* evidence is still lacking.

But not only a systemic immune response against anti-pAPN IgG was elicited, elevated levels of IgA and IgG antibodies were found in the intestinal mucosa of the anti-APN and anti-APN + CT immunized animals nine days after the booster immunization, indicating a local mucosal response. Furthermore the presence of rabbit IgG-specific IgA antibodies in the jejunum and of rabbit IgG-specific IgA ASCs in the LP of the anti-APN and anti-APN + CT immunized pigs indicate the induction of a local IgA immune response against the pAPN-specific antibodies. Such an IgA response in the gut-associated lymphoid tissue will result in an efficient secretion of dimeric IgA through the polymeric immunoglobulin receptor present on mucosal epithelial cells (Johansen *et al.,* 1999). This secretory IgA is more resistant to degradation in the gut lumen and therefore more efficient than IgG for protecting against enteropathogens (Snoeck *et al.,* 2006a). In contrast to the serum responses, the booster immunization was needed to elicit clear mucosal responses. Indeed, no elevated levels of IgA or IgG ASC could be found 14 days after the primary immunization, whereas an increase could be detected at 7 and particularly at 9 days after the booster. This needs to be taken into account for the further development of vaccines targeting APN. In addition, a local IgG

immune response was observed in the anti-APN and anti-APN + CT pigs in the jejunal intestinal segment. However, the number of IgG ASC in the JPP and LP were low, suggesting that the IgG was not produced locally in the intestine. IgG can be secreted in the intestinal lumen in low amounts via the FcRn receptor as described above. In the lumen IgG can bind to antigens forming antigen-IgG immune complexes. These complexes can bind to the FcRn receptor (Yoshida *et al.,* 2004), can be transported (transcytosis) through the epithelial barrier and finally can be delivered to the dendritic cells of the lamina propria. However, to our knowledge, this pathway is not yet studied *in vivo* in the pig. Besides the FcRn-mediated transport the antigen-IgG immune complexes can pass the epithelial barrier by dendrites of DCs protruding in the intestinal lumen (Bimczok *et al.,* 2006; Rescigno *et al.,* 2001). However, the formation of transepithelial dendrites is rather a rare event in healthy pigs, suggesting that these dendrites do not play a significant role in luminal antigen uptake under steady state conditions (Bimczok *et al.,* 2006).

In the present study, we demonstrated that antibodies against pAPN adhere to and are taken up by pig enterocytes and elicit a strong immune response after oral immunization. Even in the absence of an adjuvant, strong serum IgA, IgG and IgM responses could be observed after primary immunization, indicating that pAPN may represent a promising target for oral delivery of antigens across the epithelial barrier. The use of antibodies as targeting system was already introduced by Pappo *et al.,* (1991). They demonstrated an increased uptake of polystyrene microparticles coated with a monoclonal antibody against an apical surface antigen expressed on both enterocytes and M cells. The fact that APN is present on the intestinal epithelium of many animal species, makes this molecule an interesting candidate for the selective targeting of vaccine antigens towards the mucosal epithelium.

## 3.6 Acknowledgments

# Aminopeptidase N protein expression is not a determining factor for the F4ac receptor status of pigs

Bellot P., Ahasan L.A.S.M., Melkebeek V. and Cox E.

## 4.1 Abstract

Aminopeptidase N (pAPN) was recently identified as a receptor for F4ac fimbriae of F4ac enterotoxigenic *E. coli* (ETEC) involved in the binding and endocytosis of the fimbriae by porcine small intestinal epithelial cells. However, a population of pigs lack the F4ac-receptor(s) (F4acR) and are weak or not affected by an F4ac$^+$ ETEC infection. The first aim of the present study was to investigate if a difference in pAPN expression on protein level could be correlated with presence or absence of the F4acR(s). Since, we have previously shown that pAPN targeting via the oral route elicits a strong immune response. The second aim was to localize pAPN throughout the digestive tract and to determine if pAPN is expressed in the respiratory tracts.

Hetero tissues were sampled throughout the digestive and respiratory tracts of F4acR+ and F4acR- pigs and cryosections were immunofluorescently stained with polyclonal pAPN-specific serum. No difference in pAPN expression could be detected between F4ac receptor positive and negative pigs. Since F4ac fimbriae are lectins binding to carbohydrates, this suggests that variation in glycosylation of pAPN among pigs causes the difference in binding of F4ac fimbriae and thus colonization of F4ac+ ETEC.

The pAPN expression by enterocytes increased gradually from duodenum to ileum. pAPN was not found on the epithelium of the trachea, lung, tongue, oesophagus, stomach, large intestine (caecum and colon) and rectum, suggesting that pAPN targeting can not be used to induce mucosal immunity in the respiratory tract and not in the large intestine.

## 4.2 Introduction

Aminopeptidase N (APN, EC 3.4.11.2) is a conserved type II integral membrane zinc-dependent metalloprotease (Look *et al.,* 1989). This peptidase belongs to the M1 family of ectoenzymes and is also known as leucine aminopeptidase, alanyl aminopeptidase (AlaAP), aminopeptidase M, or CD13. Two forms of APN exist, namely a membrane APN and a soluble form. Membrane-associated APN is a non-covalently linked homodimer with a mass of 150-160 kDa. It is a common enzyme present on a wide variety of organs, tissues and cell types such as hematopoietic, epithelial and endothelial cells, fibroblasts, and on synaptic membranes, astrocytes and pericytes in brain, spinal cord and other nerve tissues (Jardinaud *et al.,* 2004; Luan and Xu 2007).

The function of APN is location dependent and is based on the removal of the amino acids from the unsubstituted N-terminus of various peptides. The substrates of APN appear to be small peptides rather than larger proteins, although the enzyme is more effective in removing residues from oligopeptides than dipeptides (Shipp and Look 1993). In immune cells such as B cells, T cells and macrophages, APN is involved in the antigen processing by trimming of MHC class I and II associated peptides. In DCs it participates in DC maturation (Rosenzwajg *et al.,* 2000; van der Velden *et al.,* 2001). In the small intestine, APN plays a role in the final digestion of peptides generated from hydrolysis of proteins by gastric and pancreatic proteases (Shipp and Look 1993). Its function in proximal tubular kidney epithelial cells and in other cell types is less clear. APN facilitates the modulation of bioactive peptide responses (pain management, vasopressin release) and influences immune functions and major biological events (cell proliferation, secretion, invasion and angiogenesis). Moreover, APN is also used as receptor for different viruses and bacteria. Indeed, APN is a receptor for the human coronavirus (HCV) 229E, an important cause of upper respiratory tract infections (Delmas *et al.,* 1992; Yeager *et al.,* 1992). Similarly in pigs, transmissible gastroenteritis virus (TGEV), a cause of fatal gastroenteritis in newborn pigs, uses porcine APN (pAPN) as its portal of entry. In both instances, the receptor role of APN was suggested by the finding that monoclonal antibodies, able to block infection of susceptible cells, specifically recognized extracellular epitopes of the enzyme (Delmas *et al.,* 1994).

Recently, our group demonstrated that pAPN is involved in the binding and endocytosis of F4ac fimbriae of F4ac-positive enterotoxigenic *E. coli* (ETEC) in the porcine small intestine, resulting in a systemic and intestinal mucosal immune response (Melkebeek *et al.,* 2012;

Rasschaert *et al.,* 2011). Moreover, antibodies against pAPN also adhere to and are taken up by pig enterocytes and elicit a strong immune response after oral immunization. Even in the absence of an adjuvant, strong serum IgA, IgG and IgM responses could be observed after primary immunization, indicating that pAPN is a promising target for oral delivery of antigens across the epithelial barrier. The fact that APN is present on the respiratory and intestinal epithelium of many species makes this molecule a promising candidate for the selective targeting of vaccine antigens towards the mucosal epithelium. However, for this selective targeting, it is important to localize pAPN throughout the digestive and respiratory tracts. This information will be helpful for selecting pathogens for which this targeting could be an interesting vaccination strategy, but it is also important to develop and optimize immunization strategies for these tissues.

Adhesion to specific receptors is a first step in the colonization of the small intestinal villous mucosa by F4ac[+] ETEC. However, not all pigs are susceptible to F4ac[+] ETEC. The adhesion of the bacteria is mediated by specific binding of its F4ac fimbriae to carbohydrates of glycoproteins and/or glycolipids expressed at the brush border of small intestinal enterocytes such as APN and is dominantly inherited (Gibbons *et al.,* 1977). A population of pigs lacks F4ac-receptor(s) (F4acR) and are weak or not affected by an F4ac[+] ETEC infection. The first aim of present study was to investigate if a difference in pAPN expression on protein level could be correlated with the presence or absence of adhesion of F4ac[+] ETEC to small intestinal villi. The second aim was to localize pAPN throughout the digestive and respiratory tracts.

## 4.3 Material and methods

### 4.3.1 Polyclonal antibodies
The polyclonal rabbit antibodies were produced and purified as described in Chapter 3.

### 4.3.2 Cell line and culture conditions
The BHK-21 and pAPN-transfected BHK-21 (BHK-pAPN) cells (Delmas *et al.,* 1993) were kindly gifted by Dr. Laude of the Unit of Virology and Molecular Immunology of the INRA in France. The BHK-21 cells were cultured in DMEM with 5 % FCS (Greiner Bio-One, Belgium), 1 % L-Glu (Gibco BRL, Life Technologies Inc., Paisley, Scotland), 1 % P/S (Gibco BRL), 1 % sodiumpyruvate (Gibco BRL), 1 % non-essential amino acids (Gibco BRL). The BHK-pAPN cells

were grown in the same medium supplemented with 1.5 mg/ml geneticin G418 (Sigma-Aldrich).

### 4.3.3  Specificity of the pAPN-specific antibodies

BHK-21 and BHK-pAPN cells were grown in culture flask (CELLSTAR) until confluence and were collected following dislodging of the cells with sterile PBS supplemented with 1% EDTA (VWR, Leuven, Belgium). Subsequently, the cells were centrifugated (4°C, 400g, 10min) and counted using a Neubauer counting chamber. To examine the binding of pAPN-specific antibodies to the transfected and not transfected cells, 5 x $10^5$ BHK-21 or BHK-pAPN cells were incubated with polyclonal rabbit anti-pAPN or irrelevant rabbit serum (1/100). Subsequently, the cells were incubated with goat anti-rabbit IgG (whole molecule) conjugated with fluorescein isothiocyanate (FITC, Sigma-Aldrich), whereafter the binding could be evaluated using a flowcytometer. Each incubation was performed on ice for 20 minutes and after each incubation step the cells were washed with icecold PBS + 1% P/S. Data of the binding assays were acquired on a FACSCanto flow cytometer (Becton Dickinson, Erembodegem, Belgium) and analyzed with FACSDiva® software.

### 4.3.4  Collecting the tissue

Immediately after euthanasia by intravenous injection of pentobarbital (24mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) and subsequent exsanguination, tissues with the size of ±1 cm² were taken throughout the digestive and respiratory tracts from all seven pigs. The different tissues which were collected are listed in Table 4.1.

**Table 4.1** Tissues collected from the digestive and respiratory tracts of seven pigs. Proximal, mid and distal parts were taken from the tissues marked with an asterix (*).

| **Digestive Tract** |
| --- |
| Tongue |
| Pharynx |
| Oesophagus |
| Stomach: non-glandular, cardia, fundus and pylorus |
| Small intestine: duodenum, jejunum* and ileum* |
| Large intestine: caecum*, colon* and rectum |
| **Respiratory Tract** |
| Larynx |
| Trachea |
| Lung: apical, middle, diaphragmatic and accessory lobes |

After excision, the tissues were washed twice with Krebs (0.12 M NaCl, 0.014 M KCl, 0.001 M KH$_2$PO$_4$, 0.025 M NaHCO$_3$, pH 7.4). Subsequently, they were embedded in 2% (w/v) Methocel® (Fuka, Bornem, Belgium) in distilled water and immediately frozen in liquid nitrogen. From the frozen tissue samples, approximately 10 μm thick cryosections were cut (Jung CM 300®, Leica) and mounted on 3-aminopropyl-triethoxysilane (APES; Sigma-Aldrich) coated glass slides. After drying for 2h at 60°C, the tissue sections were fixed in acetone for 10 min and stored at -20°C till staining.

### 4.3.5 Staining of the tissues with anti-pAPN serum

The cryosections were air-dried during 30 min and subsequently washed in PBS for 5 min after which they were incubated with 10% (v/v) goat serum in PBS for 30 min at 37°C for decreasing the background staining. In the next step, the sections were incubated for 1h at 37°C with either the primary polyclonal anti-rabbit pAPN antibody diluted in PBS or with irrelevant rabbit serum as a negative control. Subsequently the sections were incubated with secondary anti-rabbit IgG-FITC (Sigma-Aldrich) in PBS for 1h at 37°C. The sections were mounted in glycerol containing 0.223 M 1, 4-diazobicyclo-(2, 2 and 2)-octone (DABCO; Sigma-Aldrich) to prevent photo-bleaching. In between each step, the sections were washed twice in PBS for 5 min. The intensity of expression in a tissue was semiquantitatively evaluated by scoring the fluorescence intensity using a negative (-) and strong positive (+++) sample as references. The scores used were negative (-), weak positive (+), moderate positive (++) and strong positive (+++) as shown in Figure 4.2. All the samples of each pig were stained and evaluated on the same day using a fluorescent microscope.

### 4.3.6 *In vitro* villous adhesion assay for F4acR status

The *in vitro* villous adhesion assay  was performed in order to determine the presence or absence of the F4ac receptor on the small intestinal villous enterocytes(Van den Broeck *et al.,* 1999c). Briefly, at the time of slaughter of each pig, a short segment (± 15 cm) was excised from mid jejunum. This segment was washed twice with Krebs-Henseleit buffer (0.12 M NaCl, 0.014 M KCl, 0.001 M KH$_2$PO$_4$ and 0.025 M NaHCO$_3$ adjusted to pH 7.4) and one time with Krebs-Henseleit buffer supplemented with 1% (v/v) formaldehyde (VWR, Leuven, Belgium). Subsequently, the villi were gently scraped from the mucosa with glass slides and stored in the Krebs-Henseleit buffer with formaldehyde at 4°C until used.

For the adhesion test the villi were washed four times with Krebs-Henseleit buffer without formaldehyde. Thereafter the samples were resuspended in PBS solution supplemented with 1 % (w/v) D-mannose (Fluka, Sigma-Aldrich, Bornem, Belgium) to prevent adhesion mediated by type 1 pili. Subsequently, $4x10^8$ F4ac$^+$ ETEC (strain GIS26, serotype O149:K91, F4ac$^+$, LT$^+$STa$^+$STb$^+$) were added to 50 µl villi in a final volume of 1 ml, after which the samples were incubated at room temperature for 30 min while gently rotating. The number of bacteria adhering along 50 µm brush border was counted for 20 randomly selected places using a light microscope (Leica Microsystems, Groot-Bijgaarden, Belgium). Adhesion of <5, 5 to 30, >30 bacteria per 250 µm villous brush border length was considered negative, weak positive and strong positive, respectively (Cox and Houvenaghel 1993).

## 4.4 Results

### 4.4.1 Specificity of pAPN

The polyclonal pAPN-specific antibodies bound to pAPN-transfected BHK-21 cells (BHK-pAPN), The results are shown in Figure 4.1. The binding was APN-specific as binding of pAPN-specific antibodies could not be observed using untransfected BHK-21 cells. As an additional control, irrelevant rabbit serum was tested and neither bound to the pAPN-transfected BHK-21 cells.
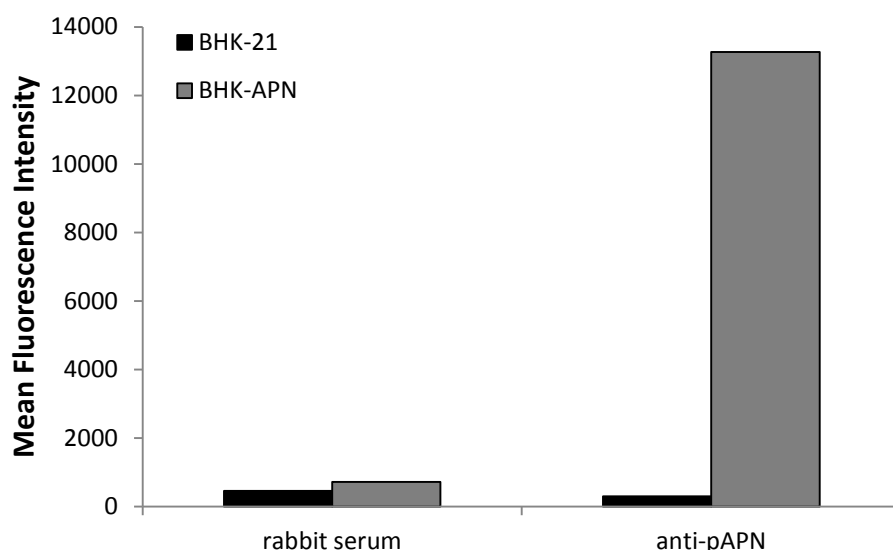


**Figure 4.1** Binding of irrelevant rabbit serum and polyclonal anti-pAPN serum (anti-pAPN) to BHK-21 and pAPN-transfected BHK-21 cells (BHK-pAPN) as determined by flow cytometry.

### 4.4.2   Localization of pAPN

The *in vitro* villous adhesion assay showed that 4 pigs were F4ac-receptor positive (F4acR+) and 3 pigs F4ac-receptor negative (F4acR-). The score for their pAPN protein expression is summarized in Table 4.2. The used scoring system is illustrated in Figure 4.2. No difference in pAPN expression could be detected between the F4acR+ and F4acR- pigs. The irrelevant rabbit serum scored negative for all the samples and pigs.

In the first part of the digestive tract, pAPN is expressed in the lamina propria of the tongue and pharynx (Figure 4.3F). The basement membranes of tongue, oesophagus (Figure 4.3G), and pharynx mucosae stain positive. On the other hand, pAPN is absent in the keratinized squamous epithelium of the tongue, oesophagus and stomach. pAPN is expressed at high levels in the epithelium of the small intestine, only small variations were seen between villous tips, lateral surface of the villi and villous crypts in the different pigs. A gradually increase of pAPN expression was observed from duodenum to ileum as can be seen in Figure 4.3(A–C). pAPN is expressed in lower amounts in the basement membranes and LP of the small intestine compared to the epithelium of the same tissue. No pAPN expression could be detected in the large intestine (caecum and colon, Figure 4.3D) and the rectum (Figure 4.3E).

In the respiratory tract, less pAPN expression was detected. pAPN was absent in the pseudostratified columnar epithelium of trachea and bronchi. Staining occurred at the basement membrane of the larynx. However, in the connective tissue pAPN expression was detected in low amounts in the bronchi, in higher amounts in the perichondrial tissues and the stroma of the laryngeal cartilage.

The secretory epithelium of exocrine glands, mainly tracheal, bronchial, lingual, esophageal, pharyngeal and intestinal glands also express weak to moderate levels of pAPN.
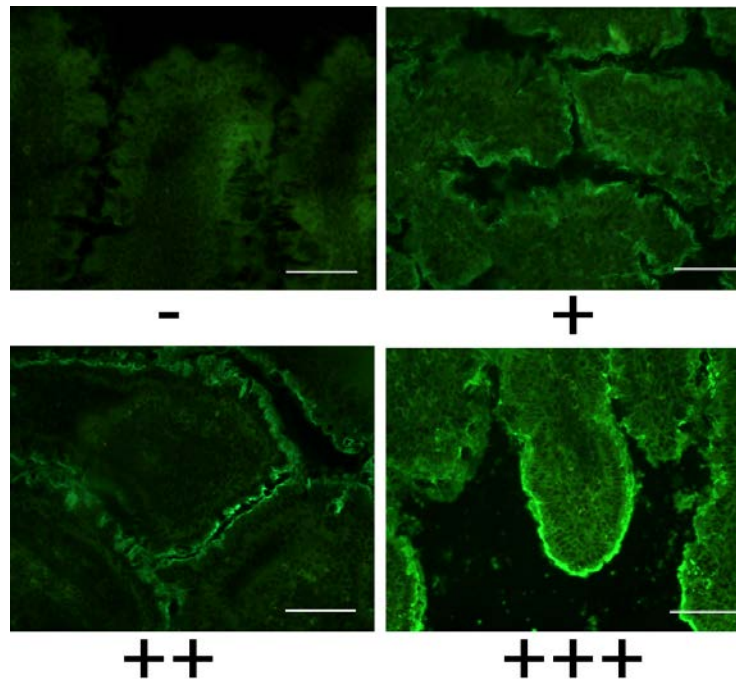
**Figure 4.2** Scoring of the pAPN expression from negative (-), weak (+), positive (++) to strong positive (+++). The white bar represents 50 μm.

**Table 4.2** Expression of pAPN in pig tissues of the digestive and the respiratory tract graded from negative (-), weak (+) to strong positive (+++), as illustrated in Figure 4.2. Expression was determined using immunofluorescence staining. Differences between animals are indicated between brackets. BM: Basement membrane, E: Epithelium (keratinized squamous or pseudostratified columnar), LP: Lamina propria, LG: Lingual gland, OG: Oesophageal gland, TG: Tracheal gland, BG: Bronchial gland, PG: Pharyngeal gland, P: Perichondrium, LS: Laryngeal Stroma, S: Serosa.

| Pig tissue | F4acR+ (n=4) | F4acR- (n=3) |
|---|---|---|
| Tongue | E-<br>BM+<br>LG+(+)<br>LP(-)+ | E-<br>BM+<br>LG+<br>LP(-)+ |
| Pharynx | E-<br>BM+(+)<br>PG +(+)<br>LP+(+) | E-<br>BM+<br>PG+(+)<br>LP+(+) |
| Oesophagus | E-<br>BM+<br>OG+(+)<br>LP- | E-<br>BM+<br>OG+<br>LP- |
| Stomach (all regions) | E-<br>BM-<br>LP- | E-<br>BM-<br>LP- |
| Duodenum | E+<br>BM(-)+<br>LP(-)+ | E+<br>BM(-)+<br>LP(-)+ |
| Jejunum Start | E+(+)<br>BM(-)+<br>LP+ | E+(+)<br>BM(-)+<br>LP+ |
| Jejunum mid | E++<br>BM(-)+<br>LP+ | E++<br>BM(-)+<br>LP+ |
| Jejunum end | E++(+)<br>BM(-)+<br>LP+ | E++(+)<br>BM(-)+<br>LP+ |
| Ileum start | E+++<br>BM(-)+<br>LP+ | E+++<br>BM(-)+<br>LP+ |
| Ileum mid | E+++<br>BM(-)+<br>LP+ | E+++<br>BM(-)+<br>LP+ |
| Ileum end | E+++<br>BM(-)+<br>LP+ | E+++<br>BM(-)+<br>LP+ |

| Pig tissue | F4acR+ (n=4) | F4acR- (n=3) |
|---|---|---|
| Colon start | E-<br>BM-<br>LP- | E-<br>BM-<br>LP- |
| Colon mid | E-<br>BM-<br>LP- | E-<br>BM-<br>LP- |
| Colon end | E-<br>BM-<br>LP- | E-<br>BM-<br>LP- |
| Caecum | E-<br>BM-<br>LP- | E-<br>BM-<br>LP- |
| Rectum | E-<br>BM-<br>LP- | E-<br>BM-<br>LP- |
| Larynx | E-<br>BM+<br>P++<br>LS(-)+ | E-<br>BM+<br>P(-)+<br>LS(-)+ |
| Trachea | E-<br>BM-<br>TG(-)+<br>LP-<br>P- | E-<br>BM-<br>TG(-)+<br>LP-<br>P- |
| Lung | E-<br>BM-<br>BG+(+)<br>LP-(+) | E-<br>BM-<br>BG+(+)<br>LP-(+) |
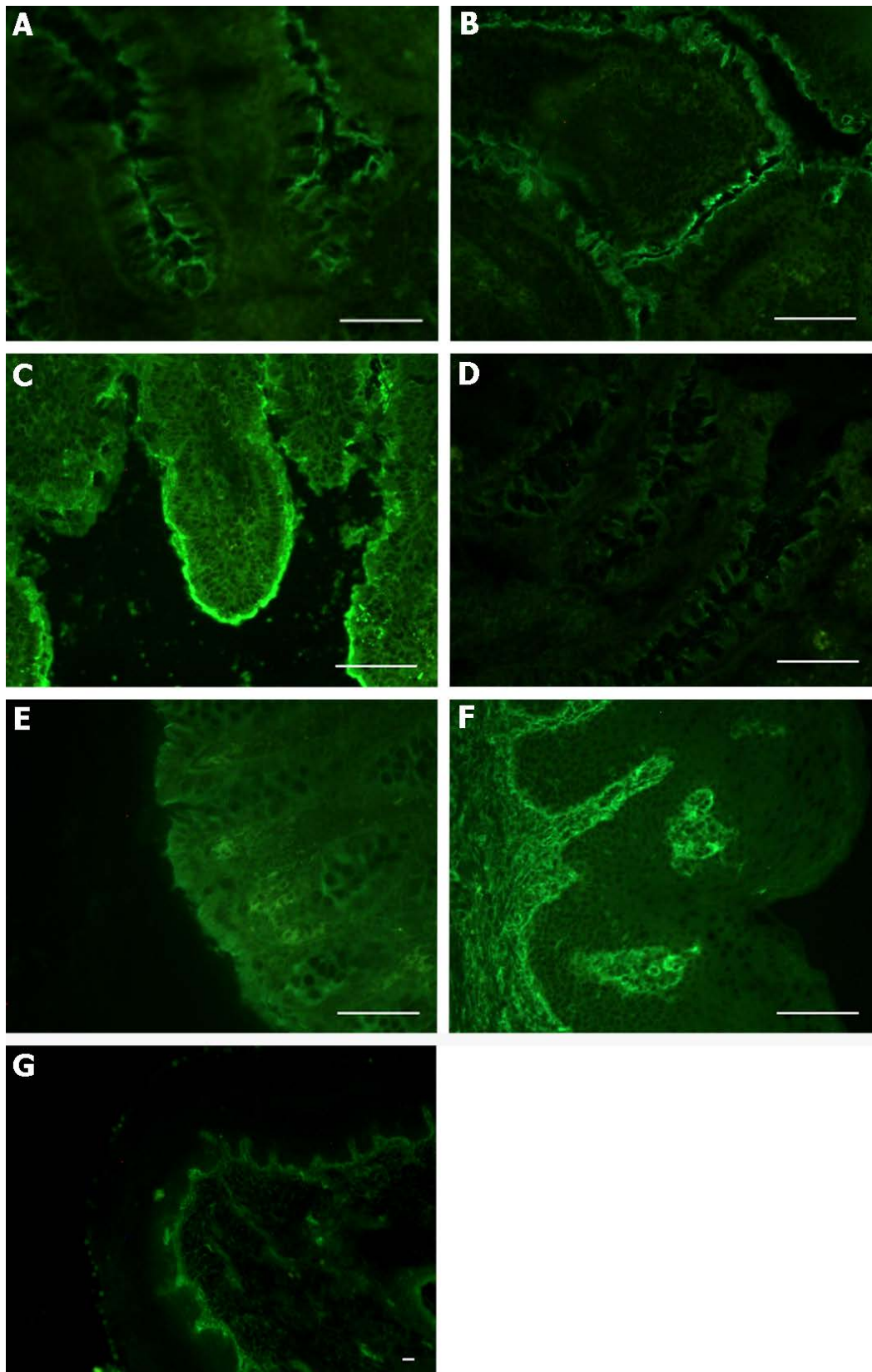
**Figure 4.3** Evaluation of pAPN expression by staining cryosections of pig tissue with rabbit anti-pAPN serum. A gradually increase of pAPN expression was observed in the villi from duodenum to ileum Low pAPN expression is found in the villi of duodenum (A), high expression in the villi of the distal jejunum (B) and even a higher expression in villi of the ileum mid (C). No staining was seen in colon, caecum (D) and rectum (E). The basement membrane, pharyngeal gland and LP of pharynx (F) and the basement membrane of the oesophagus (G) also stained. The white bar represents 50 µm.

## 4.5  Discussion

In the present study, the expression of pAPN in digestive and respiratory tract was assessed by immunofluorescence staining using a pAPN-specific rabbit antiserum. No difference in APN expression could be observed between F4acR positive and F4acR negative pigs, indicating that the difference in F4ac receptor status in both pig populations can not be explained by a difference in the level of APN expression. We know that the interaction between F4ac fimbriae and APN critically depends on the presence of sialic acid containing carbohydrate structures (Melkebeek *et al.,* 2012) and thus it is likely that variations in glycosylation among the pigs causes the difference in F4acR status. Variations in glycosylation could be due to differences in the amino acid sequence of a protein. Therefore, in next experiments the mRNA sequence of pAPN should be compared between F4acR positive and negative pigs. However, also a difference in a glycosyltransferase could result in a different glycosylation pattern between groups. Thirdly, additional molecules could sterically block binding of F4ac fimbriae to its receptor. On the other hand, it can not be excluded that other receptors are also involved in the F4ac binding and then looking at the expression of APN is not enough. Indeed, intestinal mucin-type glycoprotein 1 and 2 have been identified as potential receptors for F4ac (Erickson *et al.,* 1992; Francis *et al.,* 1998), but these have not been reported to initiate endocytosis or a mucosal immune response and are therefore unlikely to be the F4acRs involved in transcytosis and induction of mucosal immunity. A recent study of Nguyen *et al.,* (2012) suggests even the existence of three different receptors. Additional studies are needed to understand why there is no binding of F4ac to pAPN in the F4acR- pigs.

Our results indicate that APN is expressed on enterocytes of the small intestine (duodenum, jejunum, ileum) but not on the epithelium of the trachea, lung, tongue, oesophagus, stomach, large intestine (caecum and colon) and rectum. The high level of APN expression in the small intestine is in accordance with the important role of pAPN in peptide digestion at the small intestine. pAPN is thought to play a role in the final stage of peptide digestion in coordination with gastric and pancreatic proteases (Shipp and Look 1993). Our results indicate that the expression level of pAPN and therefore probably also the enzymatic activity of pAPN gradually increases from duodenum to ileum (Table 4.1). Those results are in accordance with the results of Fan *et al.* (2001) in neonatal pigs. This increase in expression

of pAPN can have different reasons. It could be that it is associated with the longer life span of enterocytes in the distal portion (10.2 days) than in the proximal portion (4.7 days). However, the expression pattern of digestive enzymes in the distal small intestine is found to be similar than in the proximal small intestine (Fan *et al.,* 2001), suggesting that cell life span is not a critical factor. It is known that the expression of APN is blocked by AT motif binding factor 1A (ATBF1A), which is expressed in the proliferating stem cells of intestinal tissue (Kataoka *et al.,* 2000). Butyrate, a product of the bacterial fermentation of undigested carbohydrates in the intestine (Bond and Levitt 1976), reduces ATBF1A expression and consequently induces the expression of APN. As the intestinal flora increases from the proximal to the distal part of the small intestine (Smith 1965), microbial butyrate production could represent one mechanism by which APN expression is influenced in the small intestine. Additionally, in a study of Kotunia *et al.,* (2004), orally ingested Na-butyrate seems to mediate indirectly rather than directly the growth of the distal small intestine and revealed its trophic effect on the jejunal and ileal mucosa but not on the duodenal mucosa. So, it is possible that butyrate levels could explain the lower pAPN expression in duodenum and proximal part of jejunum compared to the distal parts of the intestine (end jejunum and ileum) but no prove of this hypothesis can be given. In next experiments the butyrate level and the expression of ATBF1A should be measured and correlated with pAPN expression to elucidate their role in the different pAPN expression level between the proximal and distal small intestine.

In our study we could only detect a small difference in APN expression between the crypts and villi. However, several studies show that the APN expression is higher at the villi than at the crypts (Fan *et al.,* 2001; Noren *et al.,* 1989; Willing and Kessel 2009). Those previous studies mainly looked at mRNA level of APN or the enzymatic activity of APN to evaluate the APN expression, but it is possible that also a slightly diminished level of APN expression is still enough for a sufficient the binding of our pAPN-specific antibodies. Moreover, preliminary results show that the enzymatic activity of pAPN is not necessary for the binding of our pAPN-specific antibodies as the incubation of pAPN transfected cells with bestatin, a known pAPN inhibitor (Suda *et al.,* 1976), did not influence the endocytosis of the antibodies by pAPN in a negative way (unpublished results).

We could not detect pAPN expression in the colon, , which is in accordance with the absence in the colon of the transmissible gastroenteritis and the porcine epidemic diarrhea virus in pigs (Hooper and Haelterm 1966; Kim *et al.,* 1999). Both viruses use pAPN as receptor for cellular entry. Nevertheless, Quaroni *et al.,* (1992) showed that APN is expressed in low levels in adult human colon by staining the colon with their monoclonal human anti-pAPN. However, other studies failed to detect APN in the human colon (Czernichow *et al.,* 1989; Dixon *et al.,* 1994; Gorvel *et al.,* 1991). The authors stated that the different results could be due to a difference in epitope affinity of the antibodies they used. In our study polyclonal antibodies were used which recognize different epitopes. Still, we could not detect pAPN expression in the colon of healthy animals. Carraway and Hull (1989) pointed out that amounts of glycosyl transferases, their cofactors and substrates differ from tissue to tissue resulting in variation in glycosylation. Such variation in glycosylation can amplify or reduce the binding of the antibodies we used to detect pAPN which are directed against glycosylated pAPN from kidney. A lower affinity together with a lower expression could also explain absence of staining in the large intestine. Therefore, a more in-depth study is necessary to confirm the lack of pAPN expression in the large intestine and the rectum of pigs.

The scattered APN staining found in the lamina propria of different tissues, like tongue, laryngeal stroma, pharynx and small intestine could be due to expression of pAPN by monocytes, basophils, eosinophils and neutrophils (Razak and Newland 1992). We know that APN is also expressed in endothelial and nerve cells, which are both present in the LP (Luan and Xu 2007). The fibrous layer of the perichondrium in the laryngeal cartilage showed pAPN expression. This indicates that pig fibroblasts express pAPN what is in accordance with previous reports of APN expression by human and rabbit fibroblasts (Ghaffari *et al.,* 2010).

pAPN was not found on epithelium of the trachea and lung of the pigs but low expression was detected in the connective tissue of the bronchi. Similar results were previously found in humans although a higher expression was seen in the connective tissue (Van der Velden *et al.,* 1998). It is suggested that the porcine respiratory coronavirus (PRCV) uses APN as receptor in the lungs (Laude *et al.,* 1993), similar to the human coronavirus (HCoV-229E) (Yeager *et al.,* 1992). Therefore, we expected more APN expression in the respiratory tract. However, expression in respiratory tract tissue has not been demonstrated until now.

In conclusion, APN can be used as target for the delivery of molecules to the small intestine but not to the respiratory tract and large intestine. Furthermore, as no differences in expression could be detected between F4acR+ and F4acR- pigs, APN targeting can be used in pigs.

## 4.6   Acknowledgments

# pAPN-transfected IPEC-J2 porcine intestinal cell line: a new tool for studying pAPN-related endocytosis

Bellot P., Melkebeek V. and Cox E.

## 5.1   Abstract

Previous studies demonstrate that pAPN is a promising target for oral delivery of antigens across the epithelial barrier. A good *in vitro* model would allow to study the mechanism of the pAPN-mediated uptake and intracellular transport in more detail and could be a tool in the search for receptor ligands. The IPEC-J2 cell line, a pig intestinal epithelial cell line, expresses pAPN only in low amounts, not enough for pAPN-related binding studies. Therefore, in the present study we describe the transfection of this cell line with pAPN and demonstrate pAPN-related binding and internalization.

The vector containing pAPN, pJAP-1 was cotransfected with the pcDNA3.1(-) vector containing the neomycine marker in IPEC-J2 cells. Flow cytometry was used to identify transfected cells by demonstrating binding of polyclonal rabbit pAPN-specific antibodies. The pAPN-positive cells (IPEC-J2-pAPN) were positively selected using a FACSAria cell sorter and were kept in continuous culture. Endocytosis of anti-pAPN IgG by IPEC-J2 and IPEC-J2-pAPN cells was evaluated following incubation with mono- and polyclonal pAPN-specific antibodies for 45 and 90 minutes. A clear endocytosis of the mono- and polyclonal pAPN-specific antibodies was observed for the IPEC-J2-pAPN cells, whereas no binding was seen for the IPEC-J2 cells. In addition, we observed a colocalization of vesicles containing anti-pAPN IgG and clathrin during anti-pAPN IgG internalization. These results show that the IPEC-J2-pAPN cells are a promising tool for the *in vitro* study of pAPN-mediated binding to and uptake by enterocytes.

## 5.2 Introduction

APN is a promising target for oral delivery of antigens across the epithelial barrier. Not only is porcine APN (pAPN) involved in the binding and endocytosis of F4ac fimbriae of F4ac positive enterotoxigenic *E. coli* (ETEC) in the porcine small intestine (Melkebeek *et al.,* 2012) but, as described in Chapter 3, antibodies against pAPN also adhere to and are taken up by pig enterocytes and elicit a strong immune response after oral immunization. APN is present on the intestinal epithelium of many animal species which makes this molecule an intriguing candidate for the selective targeting of vaccine antigens towards the mucosal epithelium. However, a good *in vitro* model is recommended for further investigation of the pAPN-mediated uptake and the search for receptor ligands. In our laboratory, a baby hamster kidney cell line (BHK-21) transfected with pAPN is available (Delmas *et al.,* 1993). Although the selective binding of F4ac fimbriae to pAPN is demonstrated with this cell line (Melkebeek *et al.,* 2012), a cell line more closely mimicking the *in vivo* situation would allow to study the uptake and transcytosis of F4ac fimbriae. The IPEC-J2 cell line, an intestinal epithelial cell line derived from the jejunal epithelium of a neonatal, unsuckled piglet and maintained as a continuous culture (Berschneider 1989), is a good candidate. Moreover, previous studies have shown that IPEC-J2 cells provide a relevant model for the intestinal epithelial cells and have retained most of their original epithelial nature since they form apical microvilli, express tight junction proteins, produce glycocalyx bound mucin and glycoproteins for bacterial adhesins and are known to express cytokines and chemokines after bacterial stimulation (Burkey *et al.,* 2007; Mariani *et al.,* 2009; Schierack *et al.,* 2006; Skjolaas *et al.,* 2007). However, the IPEC-J2 cells express pAPN only in low amounts (own findings), not enough for pAPN-related binding assays (own findings). Therefore, the aim of this study was to transfect the IPEC-J2 cell line with pAPN to obtain a pig intestinal epithelial cell line expressing sufficient amount of pAPN to study the binding, uptake and transport of molecules via this receptor.

## 5.3 Material and methods

### 5.3.1 Cell line and culture conditions

The IPEC-J2 cells were a kind gift from Berschneider (Berschneider 1989) and were cultured in Dulbecco's Modified Eagle medium/F12 (DMEM/F12 1:1; Gibco BRL, Life Technologies Inc., Paisley, Scotland) supplemented with 5 % fetal calf serum (FCS, Greiner Bio-One,

Belgium), 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium (ITS, Gibco BRL), 100 U/ml penicillin and 100 mg/ml streptomycin (P/S, Gibco BRL) and 5 ng/ml epidermal growth factor (EGF; Invitrogen, Merelbeke, Belgium) at 37°C and 5% $CO_2$ in a humidified atmosphere. The pAPN-transfected IPEC-J2 cells were grown in the same medium supplemented with 1.5 mg/ml geneticin G418 (Sigma-Aldrich, Bornem, Belgium).

### 5.3.2   Transfection of IPEC-J2 cells with pAPN

The vector containing pAPN, pJAP-1 (Delmas *et al.,* 1992; Delmas *et al.,* 1993) was cotransfected in a 10:1 ratio with the pcDNA3.1(-) vector containing the neomycine marker using the Gene Pulser Xcell (Bio-Rad, Hercules, USA). Before transfection, both vectors were linearized with ScaI and BamHI for pJAP-1 and pcDNA3.1(-) respectively, to optimize the insertion of the vectors into the genomic DNA of the IPEC-J2 cells. Cells resistant to the neomycin analogue G418 were selected and assayed for APN expression by evaluating the binding to pAPN-specific antibodies.

### 5.3.3   Mono- and polyclonal antibodies against pAPN

BALB/c mice were injected four times intraperitoneally with 50 µg purified pAPN (Sigma-Aldrich, Bornem, Belgium) in PBS to induce antibodies against pAPN. The first injection occurred in complete Freund's adjuvant (Sigma-Aldrich), followed by two injections (2 & 5 weeks later) in incomplete Freund's adjuvant (Sigma-Aldrich). Finally, the mice were boosted intraperitoneally and intravenously with 50 µg purified pAPN, respectively with or without adjuvant. Four days after the final booster injections a polyethylene glycol mediated fusion was performed between the spleen cells of the mice and SP2/0-Ag-14 myeloma cells according to the procedures described by Harlow and Lane (1988). The hybridomas were cultered in 96-well microtiterplates (CELLSTAR, Greiner Bio-One, Wemmel, Belgium) and cells producing monoclonal antibodies (mAbs) against pAPN were identified using an anti-pAPN antibody-specific ELISA. Briefly, a 96-well microtiter plate (NUNC, Polysorb Immuno Plates; Life Technologies) was coated with pig APN (Sigma-Aldrich) at a concentration of 2 µg/ml in PBS. The remaining binding sites were blocked for 2 hours at 37°C with PBS supplemented with 3% bovine gelatin (Sigma-Aldrich) and 0.05% (v/v) Tween® 80 (Merck KGaA, Darmstadt, Germany). Subsequently, the supernatant was added in series of twofold dilutions in ELISA dilution buffer (PBS + 3% [w/v] bovine serum albumin [BSA, MP Biomedicals, Belgium] + 0.2% [v/v] Tween® 20 [Merck KGaA]), the plates were incubated for

1h at 37°C. Thereafter, the wells were treated for 1h at 37°C with optimal dilutions of anti-mouse Ig-HRP (Dako, Heverlee, Belgium). Subsequently, ABTS (Roche, Mannheim, Germany) was added and the $OD_{405}$ was measured after 1 hour of incubation at 37°C. Between each incubation step, plates were washed three times with PBS supplemented with 0.2% (v/v) Tween® 20. The positive clone was subcloned twice by limiting dilution. The selected mAb (IMM-13) with IgG1 isotype was purified by affinity chromatography using a HiTrap protein G column (GE Healthcare, Diegem, Belgium) on an ÂKTA explorer chromatography system (GE Healthcare). The concentration was determined by bicinchoninic acid (BCA, Pierce®, Thermo Scientific, Rockford, USA) reaction using BSA as standard and calculated with Deltasoft 2.0 software (Bio Metallics, Inc., Princeton, N.J).

The polyclonal rabbit antibodies were produced and purified as described in Chapter 3.

### 5.3.4  Isolation of enterocytes and brush border membrane vesicles (BBMVs)

Enterocytes were isolated as described by Nguyen *et al.,* (2012). Briefly, a jejunal segment of two meter was collected from each pig and rinsed twice with Krebs–Heinseleit buffer (0.12 M NaCl, 0.014 M KCl, 0.001 M $KH_2PO_4$, 0.025 M $NaHCO_3$, pH 7.4) and once with the same buffer supplemented with 1% (v/v) formaldehyde. The segment was then filled with HBSS medium (5.4 mM KCl, 0.44 mM $KH_2PO_4$, 136.88 mM NaCl, 0.58 mM $NaH_2PO_4$, 5.55 mM glucose, pH 7.2) supplemented with 1 mM DTT and 1.5 mM EDTA by clamping the distal and proximal end. Subsequently, the distended segment was immersed in PBS and incubated for 30 min at 37°C. This step was repeated once more and both fluids were pooled. The collected suspension was centrifuged for 10 min at 4°C and 1811g. The pellet was resuspended in HBSS supplemented with 0.1 mM phenylmethanesulfonyl fluoride (PMSF, Fluka, Bornem, Belgium) and washed twice with the same medium.

Starting from the purified enterocyte material, brush border membrane vesicles were prepared (Schmitz *et al.,* 1973). Hereto, we first washed the enterocytes twice with 0.1 mM PMSF in PBS for 10 min at 233g and 4°C. Next, the pellet was resuspended in three times the volume of buffer 1 (50 mM mannitol, 2 mM Tris–HCl, pH 7.1) supplemented with 0.1 mM PMSF and this suspension was homogenized for two minutes using an Ultra Turrax at 24,000 rpm. To precipitate BBMVs, $CaCl_2$ was added immediately until 10 mM to the homogenized suspension. The mixture was incubated on ice for 15 min followed by centrifugation at

3016g and 4°C for 15 min. Subsequently, the pellet was discarded and the supernatant was centrifuged at 27,002g and 4°C for 30 min. The pellet was resuspended in 40 ml buffer 2 (50 mM mannitol, 10 mM HEPES, 50 mM Tris-base, 56 mM HCl, pH 7.5) supplemented with 0.1 mM PMSF and centrifuged again. The same buffer was used to resuspend the pellet. Concentration of BBMVs was estimated based on bicinchonic acid (BCA) reaction using BSA as standard.

### 5.3.5 Testing the monoclonal anti-pAPN by Western blotting

Four µg purified kidney pAPN (Sigma-Aldrich) or 16 µg BBMV of an F4acR+ and F4acR- pig were loaded on 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (GE healthcare). After overnight blocking (5% [w/v] nonfat dry milk in PBS), the membrane was sequentially incubated for 1 hour with the undiluted supernatant of the hybridoma and with rabbit anti-mouse Ig-HRP (Dako). Finally, the membrane was incubated with 3-amino-9-ethylcarbazole and $H_2O_2$ to visualize the bound antibodies. Between each step, blots were washed 3 times for 5 minutes with PBS supplemented with 0.3% (v/v) Tween®20.

### 5.3.6 Binding of pAPN-specific antibodies to IPEC-J2

IPEC-J2 cells were grown in culture flask (CELLSTAR) until 90% confluence and were collected following dislodging of the cells with sterile PBS supplemented with 1% EDTA (VWR, Leuven, Belgium). Subsequently, the cells were centrifugated (4°C, 400g, 10 min) and counted using a Neubauer counting chamber.

To examine the binding of pAPN-specific antibodies to the cells, $5 \times 10^5$ IPEC-J2 or IPEC-J2 cells transfected with pAPN were incubated with both polyclonal rabbit anti-pAPN or irrelevant rabbit serum (1/100) as with 25 µg of purified monoclonal anti-pAPN for 20 minutes on ice. Subsequently, the cells were incubated with goat anti-rabbit IgG (whole molecule) conjugated with fluorescein isothiocyanate (FITC, Sigma-Aldrich) or with sheep anti-mouse IgG (whole molecule)-FITC (Sigma-Aldrich) for 20 minutes on ice, whereafter the binding could be evaluated using a flowcytometer. The cells were washed with icecold PBS + 1% P/S after each incubation step. Data of the binding assays were acquired on a FACSCanto flow cytometer (Becton Dickinson, Erembodegem, Belgium) and analyzed with FACSDiva® software.

### 5.3.7 Sorting APN positive IPEC-J2 cells

pAPN positive IPEC-J2 cells were sorted from the population of transfected cells using a FACSAria cell sorter (Becton Dickinson). The same staining procedure was used as for the binding assay but SYTOX® Red (1/1300, Invitrogen) was added to identify death cells. FACSDiva® software was used for the analysis and for selecting the pAPN-positive cells.

### 5.3.8 Endocytosis assay for pAPN-specific antibodies

The IPEC-J2 and IPEC-J2-pAPN cells were seeded at $5 \times 10^4$ cells/well in a 24-well culture plate (Nunc, Roskilde Denmark) and grown overnight to 50% confluence. The cells were incubated with 100 µg of pAPN-specific mono- or polyclonal antibodies or irrelevant antibodies as negative control in 200 µl DMEM/F12 supplemented with 1% FCS for 1 h at 4°C to allow attachment, but no internalization. Cells were then washed with $PBS^+$ (PBS + 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$), to remove unbound ligands and shifted to 37°C and 5% $CO_2$ to start endocytosis. After 45 or 90 minutes incubation, cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with phalloidin-Texas Red (Invitrogen) and/or goat anti-rabbit IgG (whole molecule)-FITC (Sigma-Aldrich) in $PBS^+$ for 1 h at 37°C. The nuclei were stained with Hoechst (1/100, Invitrogen) for 10 minutes at room temperature. Finally, cells were washed, mounted and analyzed by confocal laser scanning microscopy. Confocal images were acquired with a Leica SP5 AOBS confocal microscope (Leica Microsystems, Groot-Bijgaarden, Belgium).

For the colocalization with clathrin, the IPEC-J2 cells were incubated with 100 µg polyclonal pAPN-specific antibodies at 37°C and 5% $CO_2$ to allow internalization. After 45 minutes, cells were washed with $PBS^+$, fixed and permeabilized as described above. In the next step the cells were stained with 1/30 mouse anti-clathrin heavy chain IgM antibodies (clone CHC 5.9, ICN Biomedicals Inc.,Belgium) in $PBS^+$ and incubated with a 1/100 dilution of rat anti-mouse IgM Texas Red (Molecular Probes, Eugene, OR, USA) for 1 h at 37°C.

## 5.4   Results and discussion

### 5.4.1   Selecting pAPN positive cells

The pJAP-1 vector is a derivative of the pTEJ-4 vector (Delmas *et al.,* 1992). This vector has no selective marker for mammalian cells so we needed to co-transfect pJAP-1 with a vector including a selective marker. The pcDNA3.1(-) vector has a neomycine marker and is a good candidate. After co-transfection and selection of the IPEC-J2 cells with the neomycin analogue G418, seven cell lines grow continuously. The binding of the different cell lines to polyclonal rabbit pAPN-specific antibodies was evaluated by flow cytometry and the results are shown in Figure 5.1. Line 2 and 7 have the highest percentage of pAPN-positive cells, with 61.4% and 63.4% respectively. In all the cell lines, the binding to the irrelevant rabbit antibodies was low, with a maximum of 5.7%. The variation between the cell lines can be explained by the variable efficiency of the co-transfection. The pAPN-positive cells from line 7 were positively selected using a FACSAria cell sorter and kept in continuous culture. Those cells were used in the following experiments and referred to as IPEC-J2-pAPN.
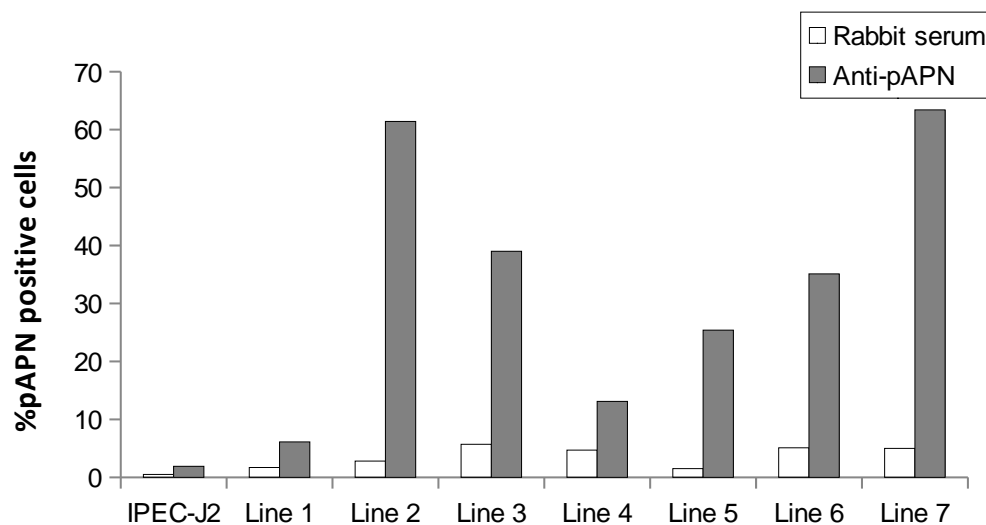


**Figure 5.1** % pAPN positive cells measured with flow cytometry after evaluation of the binding of polyclonal rabbit pAPN-specific antibodies (Anti-pAPN) and irrelevant rabbit serum (Rabbit serum) to IPEC-J2 and different cell lines (1 till 7) of pAPN-transfected IPEC-J2 cells.

### 5.4.2   Evaluation of the pAPN-specific monoclonal antibody

In a next step, we evaluated the binding of our monoclonal anti-pAPN antibody (IMM-13). First, the binding of IMM-13 to kidney pAPN and BBMVs of F4acR+ and F4acR- pigs was assessed by Western blot. The Coomassie staining of the gel is presented in Figure 5.2A. The typical bands for pAPN are shown around 150, 100 and 50 kDa, representing the α, β and γ

chain respectively (Wacker *et al.,* 1976). As shown in Figure 5.2B, a clear binding of IMM-13 can be detected to the α chain of pAPN, additionally a faint binding to the γ chain is shown. On the BBMVs of the F4acR+ and F4acR- pig faint bands around 150 and 50 kDa are also visible. The bands of the BBMVs are weaker due to the lower amount of pAPN in the samples compared to the kidney pAPN. It is assumed that APN represents 5% of the microvillar membrane proteins in enterocytes (Norén *et al.,* 1997). Secondly, the binding capacity of IMM-13 to untransfected and pAPN-transfected IPEC-J2 cells was compared. Flow cytometric analysis demonstrated that IMM-13 antibodies specifically bind to the IPEC-J2-pAPN cells with a mean fluorescence intensity (MFI) of 6570, whereas no binding could be observed to untransfected IPEC-J2 cells (MFI of 300, Figure 5.3). Therefore, IMM-13 can be used to study pAPN-mediated endocytosis or other processes mediated by pAPN.
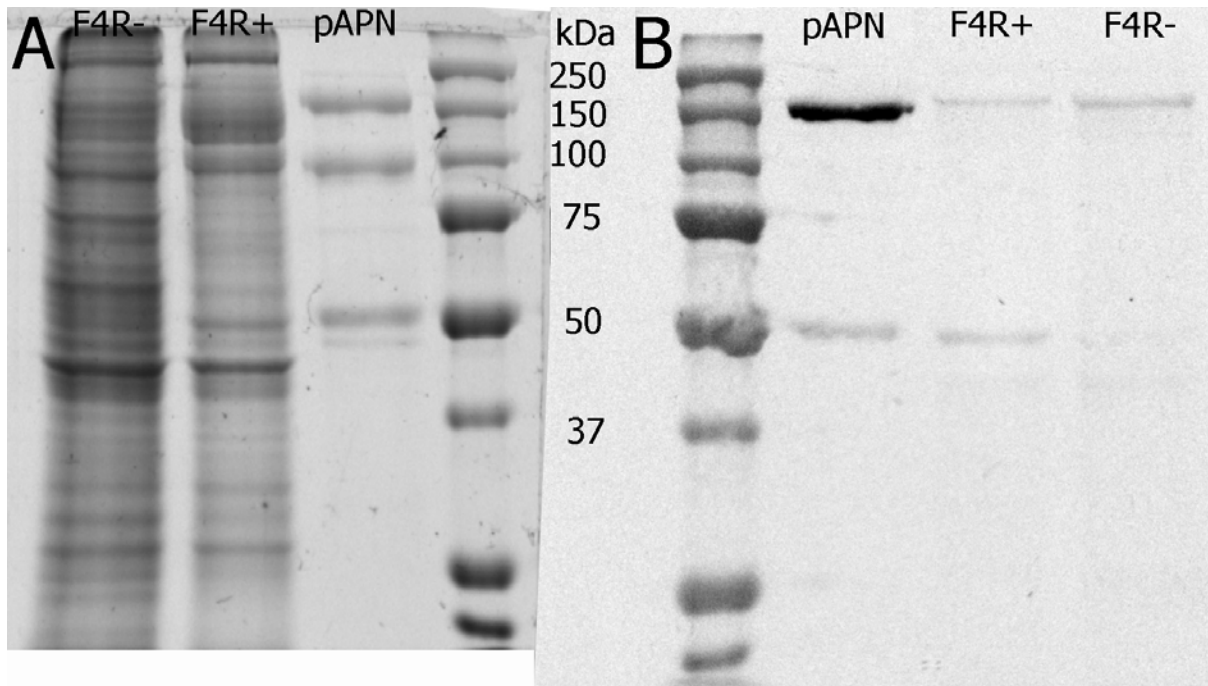


**Figure 5.2** Binding of the anti-pAPN mAb (IMM-13) to kidney pAPN and the brush border membrane vesicles of an F4acR+ (F4R+) and F4acR- (F4R-) pig. The Coomassie staining of the gel is presented in A, whereas the Western blotting of the gel with IMM-13 is presented in B. A clear binding of IMM-13 to the 150 kDa band of pAPN is shown, additionally a faint band at 50 kDa is shown. Also on the BBMVs of the F4acR+ and F4acR- pig faint bands are visible.
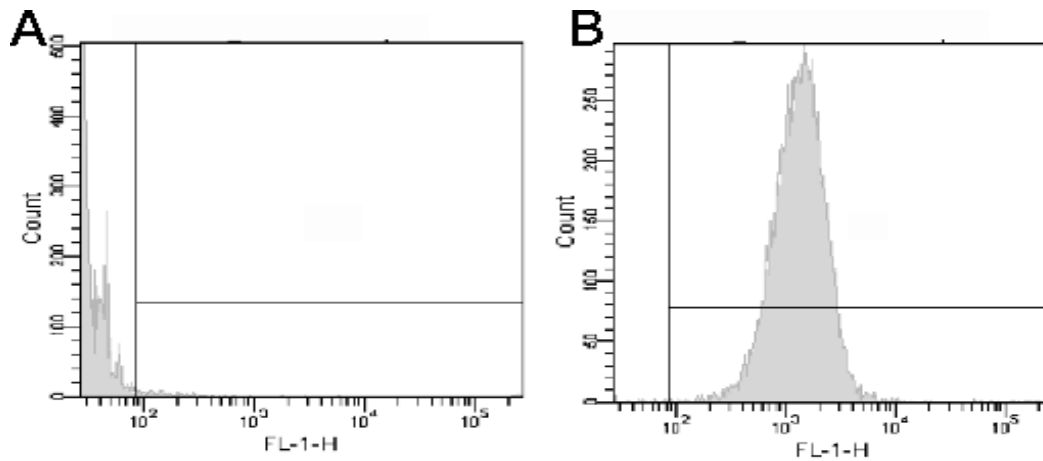
**Figure 5.3** Flowcytometric histogram showing fluorescence intensity of IPEC-J2 (A) and IPEC-J2-pAPN cells (B) after incubation with the monoclonal pAPN-specific antibodies (IMM-13) and subsequent staining with anti-mouse IgG-FITC. A clear binding of IMM-13 to IPEC-J2-pAPN is shown, whereas no binding could be observed to IPEC-J2 cells.

### 5.4.3 Endocytosis of pAPN-specific antibodies by IPEC-J2-pAPN

Baby hamster kidney cells (BHK-21) transfected with pAPN (BHK-pAPN) are able to endocytose F4ac fimbriae in a clathrin-dependent manner (Melkebeek *et al.,* 2012). In the present study, we wanted to investigate if the IPEC-J2-pAPN cells can also endocytose proteins which bind to pAPN, such as pAPN-specific antibodies.

IPEC-J2 and IPEC-J2-pAPN cells were incubated for 45 or 90 min with 100 μg mono- and polyclonal pAPN-specific antibodies. The results after 45 and 90 min incubation were comparable. The results at 90 min are shown in Figure 5.4. A clear endocytosis of the mono- and polyclonal pAPN-specific antibodies was observed in the IPEC-J2-pAPN cells, whereas no binding could be seen to the IPEC-J2 cells. The polyclonal anti-pAPN antibodies colocalized with clathrin after 45 minutes of anti-pAPN internalization (Figure 5.5), similar to the clathrin-mediated endocytosis of F4ac fimbriae by BHK-pAPN (Melkebeek *et al.,* 2012). Several other studies have also linked receptor-mediated endocytosis to clathrin. For example endocytosis of gonococci-expressing galactose-terminating lipooligosaccharide that binds to the human asialoglycoprotein receptor occurs through clathrin-dependent receptor-mediated endocytosis (Porat *et al.,* 1995). Other species, such as different *Chlamydia* sp. (Hodinka and Wyrick 1986; Soderlund and Kihlstrom 1983; Wyrick *et al.,* 1989), *Campylobacter jejuni* and *Citrobacter freundii* (Oelschlaeger *et al.,* 1993), some strains of enterohemorrhagic *E. coli* (Oelschlaeger *et al.,* 1994)*, Staphylococcus aureus* (Ellington *et al.,* 1999), Listeria monocytogenes (Veiga *et al.,* 2007) and even HIV, hepatitis C virus (Blanchard *et al.,* 2006) and ebola virus (Bhattacharyya *et al.,* 2010), also invade host cells by receptor-

mediated endocytosis in a clathrin-dependent manner. Further elucidating of this clathrin-mediated uptake could gain useful insights for the antigen uptake by enterocytes and could lead to new strategies for the production of mucosal vaccines. The clathrin-dependent endocytosis of anti-pAPN IgG can be confirmed by blocking clathrin-coated pit invagination during the early phases of internalization using amantadine-HCl (Phonphok and Rosenthal 1991). However, blocking clathrin could affect other clathrin-dependent pathways and thus indirectly interfere with the receptor-mediated uptake. Indeed, it is known that clathrin plays a role in the bidirectional traffic between the trans-Golgi network and endosomes (Bonifacino and Traub 2003). Furthermore, it is suggested that clathrin has a structural role in the segregation of endosomal membrane domains involved in targeting of growth factor receptors (Goh *et al.,* 2010). Finally, clathrin has been detected at the mitotic spindle where it has been proposed to stabilize microtubules (Royle *et al.,* 2005). Consequently, results obtained after blocking clathrin should be carefully interpreted.
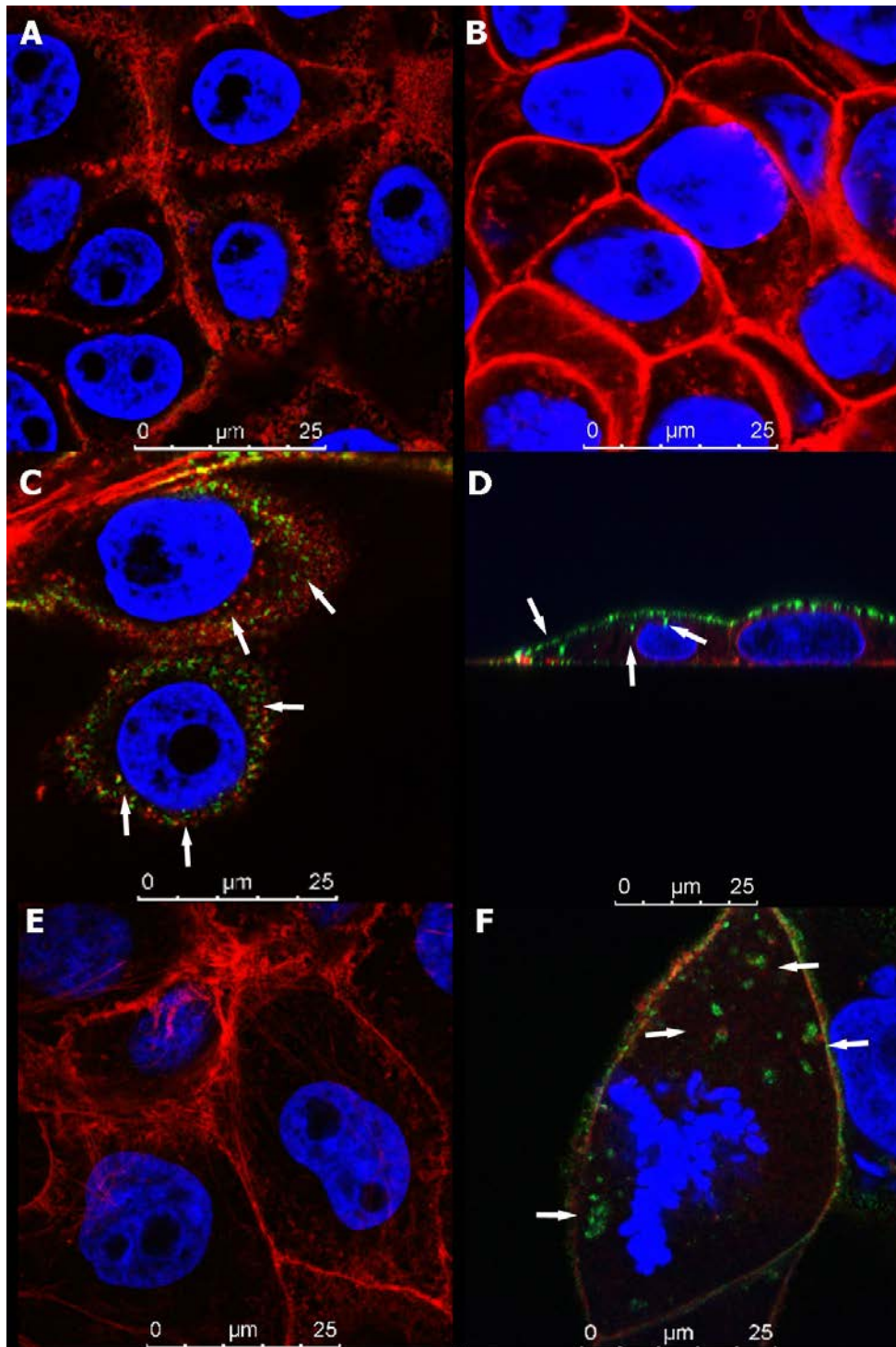
**Figure 5.4** Endocytosis of pAPN-specific antibodies by IPEC-J2-pAPN cells grown on a glass slide and studied by confocal laser scanning microscopy. After 90 min of incubation, cells were fixed and stained with FITC-labeled anti-rabbit IgG or anti-mouse IgG antibodies (green). The actine cytoskelet was counterstained with Phalloidin-Texas Red (red) and nuclei with Hoechst (blue). IPEC-J2 cells were incubated with anti-pAPN rabbit IgG (A) and anti-pAPN mAb (E); IPEC-J2-pAPN cells were incubated with 100 microgram irrelevant rabbit IgG (B), anti-pAPN rabbit IgG (C and D) or anti-pAPN monoclonal antibody (F). Clear endocytosis of the mono- and polyclonal pAPN-specific antibodies was seen in the IPEC-J2-pAPN cells and are indicated with white arrows, whereas no binding could be seen to the IPEC-J2 cells. All the images, except D, represent a single confocal z-section through the middle of the cell, whereas D represent a single confocal y-section. The white bar represents 25 μm.
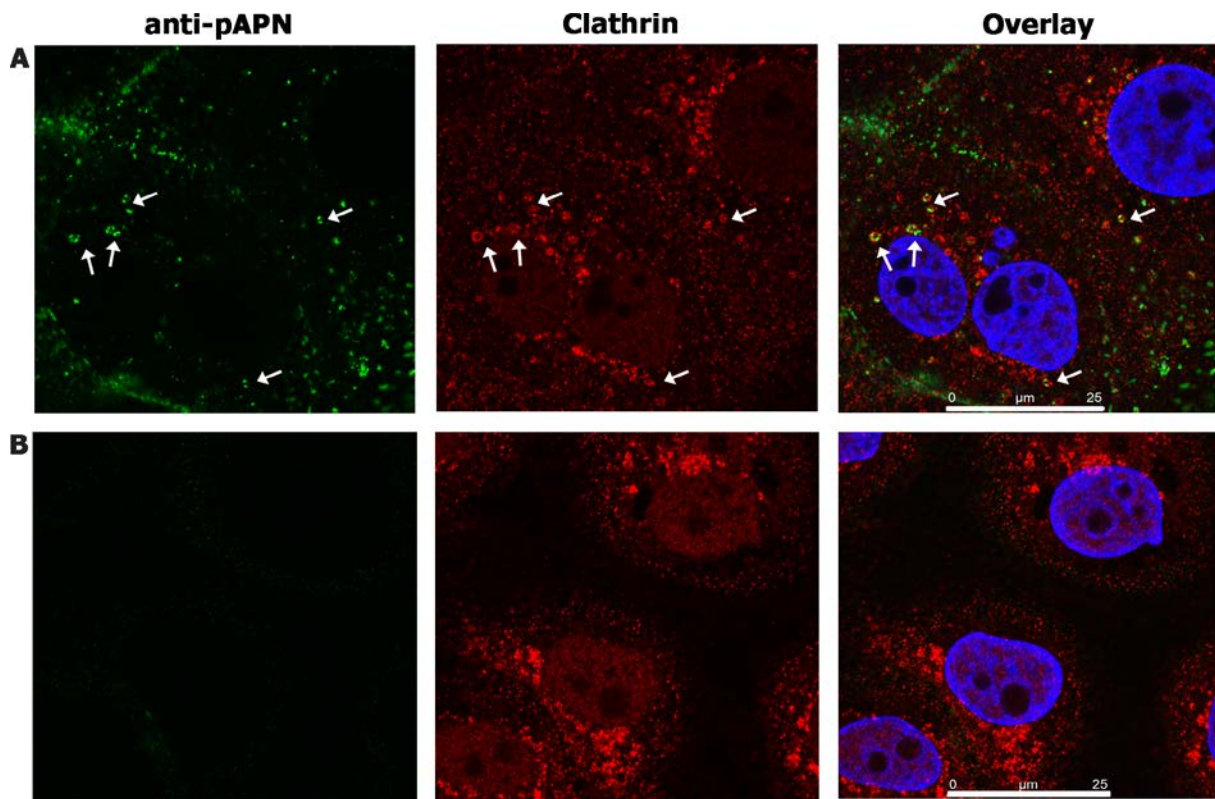
**Figure 5.5** Colocalization of pAPN-specific antibodies (green) and clathrin (red) after endocytosis of the pAPN-specific antibodies by IPEC-J2-APN (A) and IPEC-J2 cells (B), studied with confocal laser scanning microscopy. After 45 min of incubation with anti-pAPN rabbit IgG, cells were fixed and subsequently stained with clathrin antibodies, FITC-labeled anti-rabbit IgG and anti-mouse IgM Texas red. The nuclei were counterstained with Hoechst (blue). Colocalization of the pAPN-specific antibodies and clathrin vesicles was observed in the IPEC-J2-pAPN cells and are indicated with white arrows, whereas no endocytosis could be seen in the IPEC-J2 cells. All the images represent a single confocal z-section through the middle of the cell. The white bar represents 25 μm.

## 5.5 Conclusion

The IPEC-J2-pAPN cells described in this chapter, are a promising tool for the *in vitro* study of pAPN-specific targeting and uptake of antigens such as the APN-specific antibodies. It is important to understand how this endocytosis relates to the IgA response observed following *in vivo* targeting of APN via the oral route (Chapter 3). In addition, a monoclonal antibody against pAPN was produced that will be helpful in the pAPN-related research.

## 5.6 Acknowledgments

# Part II

# Strategies to improve

# oral vaccination

# Maltose-binding protein is a potential carrier for oral immunizations

## 6.1 Abstract

Maltose-binding protein (MBP) is often fused to a relevant protein to improve its yield and facilitate its purification, but MBP can also enhance the immunogenicity of the fused proteins. Recent data suggests that MBP may potentiate antigen-presenting functions in immunized animals by providing intrinsic maturation stimuli to dendritic cells through TLR4. The aim of present study was to examine if an MBP-specific immune response can be elicited by oral administration of MBP. Therefore, in a first experiment the MBP specific immune response was analyzed after oral immunization with MBP or MBP + CT to piglets and both the systemic and mucosal immune responses were examined. Although, no high systemic response was observed in the MBP-group, a local mucosal MBP-specific IgM response was observed in the jejunal Peyer's patches. In the second experiment, MBPFedF was orally administered to piglets. A significant systemic response against MBP and a weak response against FedF were found after oral administration of MBPFedF + CT. The presence of MBP-specific IgA ASC in the lamina propria indicates also that a local intestinal immune response against MBP was induced. Our results suggest that orally administered MBP can pass the intestinal epithelial barrier, implying that MBP could act as a carrier and delivery system for fused proteins to target the vaccine antigens to intestinal immune cells.

## 6.2 Introduction

In humans and most animal species such as pigs, vaccination via the oral route is a prerequisite for the induction of a protective immunity against enteropathogens. Parenterally administered vaccines primarily lead to the production of circulating IgG, which, in general, is not secreted in sufficient amounts at the intestinal mucosa to induce protection. Furthermore, it does not result in homing of sufficient memory lymphocytes to the mucosa needed for long-term protection. Oral immunization is more suited to achieve a protective intestinal mucosal response. The oral route results in the induction of antibody and cell-mediated immune responses at the gut-associated lymphoid tissues (GALT), but often also at the systemic level (Seo *et al.,* 2002; Verdonck *et al.,* 2004a). Live attenuated microorganisms replicating in the gut are used in commercial oral vaccines (Ehrenfeld *et al.,* 2009; Levine 2000; Soares-Weiser *et al.,* 2010). However, these microorganisms are often either too attenuated to induce a long-lasting intestinal immunity or still have residual virulence resulting in clinical signs. Furthermore, inactivation during storage due to breaks in the cold chain as well as regain of virulence in the host are potential risks associated with the use of live vaccines (Plotkin 2009). Therefore, non-replicating oral vaccines would be an interesting alternative. However, such vaccines need to pass the harsh environment of the gastrointestinal tract and cross the intestinal epithelial barrier in sufficient amounts before reaching the GALT where they can induce an effective immune response. Moreover, soluble antigens generally induce oral tolerance instead of a protective immunity. Nevertheless, a limited number of soluble antigens are able to induce immunity after oral delivery, including cholera toxin (CT) (Spangler 1992; Yamamoto *et al.,* 1997), the *Escherichia coli* heat-labile enterotoxin (LT) (Nakagawa *et al.,* 1996; Takahashi *et al.,* 1996) and the F4ac fimbriae of enterotoxigenic *E. coli* (Van den Broeck *et al.,* 1999a; Verdonck *et al.,* 2004a).

Maltose-binding protein (MBP), a 42 kDa protein part of the maltose/maltodextrin system of *E. coli* is often fused to relevant proteins to improve its yield, facilitate its purification by amylose affinity chromatography and enhance its stability and solubility (Butt *et al.,* 1989; Fox *et al.,* 2001; Kapust and Waugh 1999). Because of the minimal effects of MBP on the bioactivity of the fused proteins, MBP has been widely used for the purification of recombinant proteins. Interestingly, MBP can also enhance the immunogenicity of the recombinant protein and thus enhance the effectiveness of a vaccine. It is presumed that

MBP enhances the interactions of the vaccine with immune cells by increasing the solubility and lengthening the half-life of the fused proteins in immunized animals (Fernandez *et al.,* 2007; Fox *et al.,* 2001). However, MBP can also directly influence the immune system. *In vitro* studies have shown that MBP induces the activation of human dendritic cells (DCs) and the production of proinflammatory cytokines. It has been suggested that MBP stimulates DCs through Toll-like receptor 4 (TLR4) (Fernandez *et al.,* 2007). Furthermore, *in vivo* studies in mice showed that a fusion protein containing MBP could elicit IL-4 and IL-5 producing Th2-type $CD4^+$ T cells in a TLR4 dependent manner and thus induce serum IgG, serum IgA and mucosal IgA antibody responses (Du *et al.,* 2011). Another *in vitro* study has shown that MBP directly induces the proliferation of murine splenocytes and the secretion of IFN-γ and IL-2 (Xiaoxia *et al.,* 2011). These findings demonstrate the intrinsic adjuvant-like properties of MBP.

The aim of the present study was to investigate if MBP could induce an immune response via the oral route and if so, to explore its potential to target a conjugated antigen towards the GALT to induce an intestinal immune response against selected enteropathogens.

## 6.3   Material and methods

### 6.3.1   Expression and purification of recombinant MBPFedF and FedF1-280

$FedF_{1-280}$ was expressed and purified as described by Tiels *et al.,* (2007). Recombinant MBPFedF was produced from the construct described by Tiels *et al.,* (2008). Briefly, an ORIGAMI *E. coli* (Novagen, Madison, USA) expression host transformed with an MBPFedF containing *E. coli* expression vector to obtain a His-tagged MBPFedF fusion protein (VIB Protein Service Facility, Belgium), was cultured in Luria Broth media (LB; Life technologies, Paisley, Scotland) supplemented with 100 μg/ml ampicillin (Sigma, Bornem, Belgium) and 50 μg/ml chloramphenicol (Sigma). Subsequently, production of the recombinant protein was induced with 1 mM isopropyl-ß-D-thiogalactoside (IPTG, Sigma) overnight at 18 °C, whereafter the bacteria were harvested and resuspended in 20 mM Tris-HCl pH 7.5. Lysozyme (3.33 μg/ml) was added for 15 min at room temperature. The viscous solution was sonicated on ice six times for 10 s. After centrifugation, the His-tagged MBPFedF was purified from the soluble fraction using Ni-NTA His Bind Resin (Novagen) according to the manufacturer's manual followed by dialysis against PBS.

### 6.3.2 Animals and experimental procedure

Twenty-five piglets (Belgian Landrace x Piétrain) were weaned at the age of 4 weeks. They were transported to the experimental facilities of the faculty and housed in isolation units with water and feed *ad libitum*. Experimental procedures and animal management procedures were undertaken in accordance with the requirements of the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2007/065 and EC2008/034).

### 6.3.2.1 Oral MBP administration

In a first experiment MBP was given orally in the presence and absence of cholera toxin (CT) as adjuvant. Hereto, 8 piglets were randomly divided into 3 groups. Five days after weaning, the piglets were orally immunized either with 1.68 mg purified MBP (New England Biolabs, Ipswich, USA) in 10 ml PBS (MBP group, n = 3), with 1.68 mg purified MBP and 50 µg CT (Sigma) in 10 ml PBS (MBP + CT group, n = 3) or received 10 ml PBS as a negative control (PBS group, n = 2). The first immunization occurred at 0, 1 and 2 days post-primary immunization (dppi). The pigs were orally immunized a second time at 20 dppi and a third time at 52 dppi. Twenty-four hours before each immunization, pigs were orally given 20 mg rabeprazolum (Pariet, Janssen-Cilag, Berchem, Belgium) to increase the pH of the acidic gastric content. The pigs were deprived of feed and water from three hours before till 2 hours after each rabeprazolum treatment and immunizations.

Blood was regularly taken from the jugular vein to determine the MBP-specific serum antibody response. Hereto, the collected serum was inactivated and treated with kaolin (Sigma) as described by Van den Broeck *et al.,* (1999a). The local immune response was analyzed in one pig of each group five days after the third immunization (57 dppi). Therefore, the animals were euthanized by intravenous injection of pentobarbital (24 mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) and subsequent exsanguination. Monomorphonuclear cells (MCs) were isolated from mesenteric lymph nodes (MLN), jejunal Peyer's patches (JPP), jejunal lamina propria (LP), ileal Peyer's patches (IPP) and spleen as previously described (Verdonck *et al.,* 2002; Verdonck *et al.,* 2008). Cells were used to determine the MBP-specific IgA, IgG and IgM ASC by ELIspot assays. To analyze priming of lymphocytes following the oral immunizations, blood was collected on heparin six days after the third immunization from all remaining animals.

**6.3.2.2   Oral administration of an MBPFedF fusion protein**

In a second experiment it was determined if an MBP fusion protein could elicit an immune response against MBP and its cargo. Hereto, 17 piglets were randomly divided into 3 groups. Two weeks after weaning, six piglets were orally immunized with 3 mg MBPFedF supplemented with 50 µg CT in 10 ml PBS (MBPFedF + CT group, n = 6). This amount of MBPFedF was calculated to contain 1.68 mg MBP, the same dose of MBP which was orally administered in the first experiment. As negative controls, piglets were given 10 ml PBS (PBS group, n = 6) or 50 µg CT in 10 ml PBS (PBS + CT group, n = 5). A first immunization occurred during 3 subsequent days (0, 1, 2 dppi). A second and a third immunization occurred at 17 and 41 dppi, respectively. Blood was regularly sampled to determine the serum antibody response and two piglets of each group were euthanized 5 days after the third immunization to analyze the local antibody response in the LP. Hereto, LP MCs were used to enumerate the MBPFedF-specific IgA antibody secreting cells (ASC) by an ELIspot assay. Immunizations, euthanasia and samplings were performed as described for the first experiment.

**6.3.3   MBP and FedF-specific ELISA**

For the detection of MBP-specific antibodies, MBP was coated (2 µg/ml in PBS) directly onto 96-well Maxisorp Immuno Plates (NUNC, Thermo Scientific, Langenselbold, Germany). The plates were blocked with PBS + 0.2% Tween® 80 (Merck KGaA, Darmstadt, Germany). Subsequently, the plates were sequentially incubated with twofold serial dilutions of pig sera, goat anti-pig IgG-HRP (Bethyl, Montgomery, USA) and ABTS (Roche, Mannheim, Germany), whereafter the $OD_{405}$ was measured. All incubations occurred at 37°C for 1 h. Dilutions, except for ABTS, were in ELISA buffer (PBS, pH 7.4 with 0.2% Tween® 20 [Merck kGaA] and 3% bovine serum albumin [BSA, MP Biomedicals, Belgium]). Between each incubation step, plates were washed three times with PBS-T (PBS plus 0.2% [v/v] Tween® 20).

The FedF-specific serum response was obtained using an indirect ELISA. Briefly, an optimal dilution of rabbit polyclonal antibodies against $FedF_{1-280}$ was coated on the maxisorb plates during 2 hours at 37°C. PBS with 1% BSA was used to block the plates overnight at 4°C. Thereafter, 5 µg/ml $FedF_{1-280}$ was added to the plates, followed by the pig serum samples and an optimal dilution of goat anti-pig IgG-HRP (Fitzgerald , Concord, USA). Incubation, washing and detection are similar to the MBP ELISA.

### 6.3.4   Influence of MBP on lymphocyte proliferation

Peripheral blood monomorphonuclear cells (PBMC) were isolated by density gradient centrifugation on Lymphoprep (Nycomed Pharma AS, Belgium) from heparinized blood, sampled 6 days after the third immunization with MBP, CT or PBS (Van der Stede *et al.,* 2003).

The lymphocyte proliferation test was performed by adding MBP (final concentration 15 µg/ml) or medium to the wells of a 96-well flat-bottom microtitre plate (NUNC$^®$) containing 5 x $10^5$ PBMC per well. To evaluate the proliferative capacity of the cells, concanavalin A (Con A, final concentration 10 µg/ml) induced proliferation was used as a control. Each stimulation was performed in triplicate. The cells were incubated at 37°C in a humidified atmosphere with 5% $CO_2$. After 4 to 7 days incubation the cells were pulse-labelled with 1 µCi/ well, [$^3$H]methyl-thymidine (Amersham ICN, Bucks, UK) and 18 h later the cells were harvested (Combi Cell Harvester, Skatron instruments, Norway) onto glass fibre filters (Perkin-Elmer, Life Science, Brussels, Belgium). The radioactivity incorporated into the DNA was measured using a β-scintillation counter (Perkin-Elmer, Life Science, Brussels, Belgium). The results are presented as stimulation indices (SI), calculated as the mean counts per minute (cpm) divided by the background cpm (medium).

### 6.3.5   MBP-, MBPFedF and FedF-specific ELISPOT

The MBP-, MBPFedF or FedF-specific ASC were enumerated in an ELIspot assay. Maxisorb 96-well plates were coated with MBP (2 µg/ml) or with FedF$_{1-280}$ (2 µg/ml) in PBS. Thereafter, the MC suspensions at a concentration of $10^7$ cells/ml in leukocyte medium (RPMI with 10% FCS (Greiner Bio-One, Belgium), 100 IU/ml penicillin (GIBCO$^®$, Life Technologies, Paisley, UK), 100 µg/ml streptomycin (GIBCO$^®$), 100 µg/ml kanamycin (GIBCO$^®$), 200 mM L-glutamine (GIBCO$^®$), 100 mM sodium pyruvate (GIBCO$^®$) and 100 mM non-essential amino acids (GIBCO$^®$)) were added to the plates (100 µl/well). Next, the plates were incubated for 10 h at 37°C in a humidified 5% $CO_2$ atmosphere, whereafter washing with PBS + 0.2% Tween$^®$ 20 was used to remove the cells from the plates. Subsequently, optimally diluted mouse anti-swine IgM/IgA/IgG monoclonal antibodies were added to the wells followed by anti-mouse Ig-HRP. The amount of MBP or FedF-specific IgM/IgA/IgG ASCs per 7 x $10^6$ MC was obtained by counting the spots in 7 wells ($10^6$ MC/well) and subtracting them from the background (spots in uncoated wells).

### 6.3.6    Statistical analysis

The antibody titers, proliferation and ELIspot results were statistical analyzed with SPSS 19.0 for Windows using General Linear Model (Multivariate), adjusting for multiple comparison by Bonferroni. $p < 0.05$ was considered as statistically significant.

## 6.4    Results

### 6.4.1    MBP induces a local and systemic immune response after oral immunization

The total serum antibody response against MBP is presented in Figure 6.1. After the first oral immunization, a clear response against MBP was observed in the MBP + CT group on day 19, whereas in the MBP group the response remained just above background level. The booster immunization at day 20 seems to enhance the response in the MBP + CT group, whereas no effect was seen in the MBP group. At day 40 in the MBP group and day 49 in the MBP + CT group the antibody titer showed a decrease. No significant differences could be detected between the groups as a result of the low number of animals in each group in this experiment.
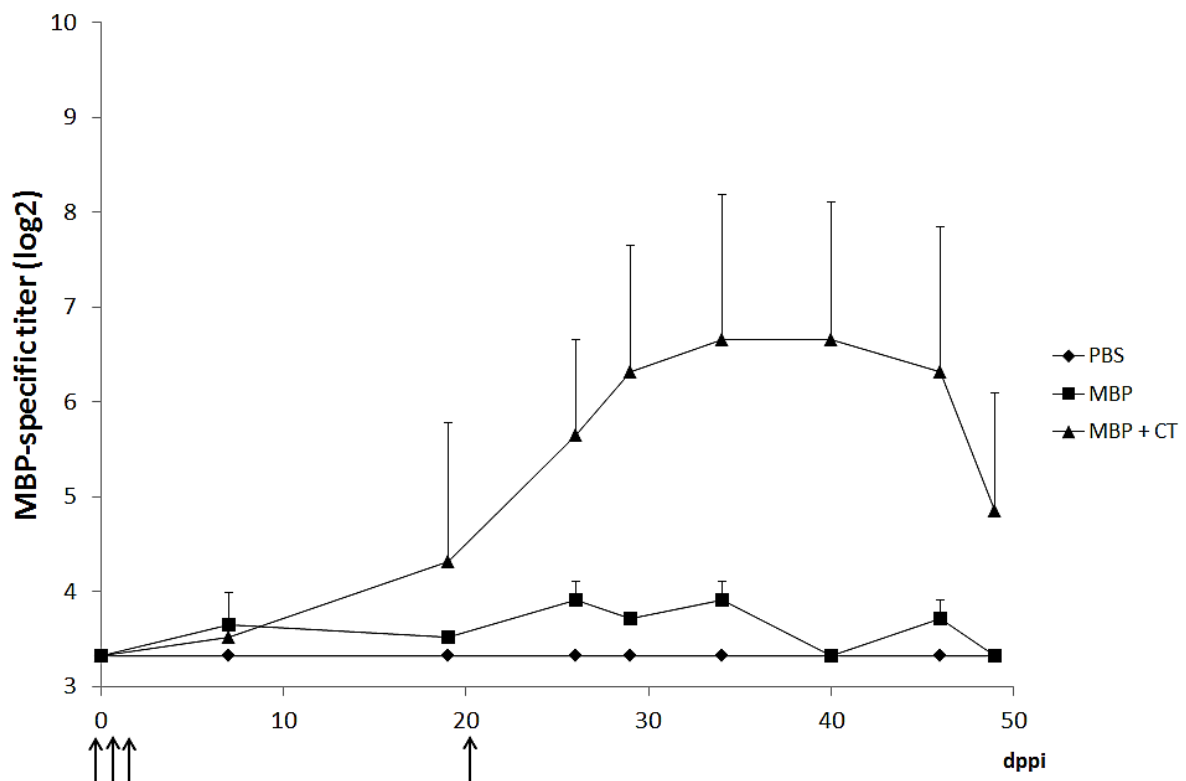


**Figure 6.1** Mean total MBP-specific serum antibody response (± standard error of the mean (SEM)) of pigs immunized with PBS (n = 2), MBP (n = 3), MBP + CT (n = 3). Black arrow: immunization day.

To analyze priming of lymphocytes by the oral MBP administration, MBP-induced lymphocyte proliferation of PBMCs was determined six days after the third immunization after 5-8 days of *in vitro* incubation with MBP and stimulation indices (SI) were calculated for each group (Figure 6.2). Only after 8 days of incubation, the MBP-specific SI was clearly higher for the MBP-group than for the PBS and the PBS + CT-group. However, this difference was not significant due to the limited number of animals. The ConA-induced (10 µg/ml) proliferation of the lymphocytes is shown in Table 6.1.
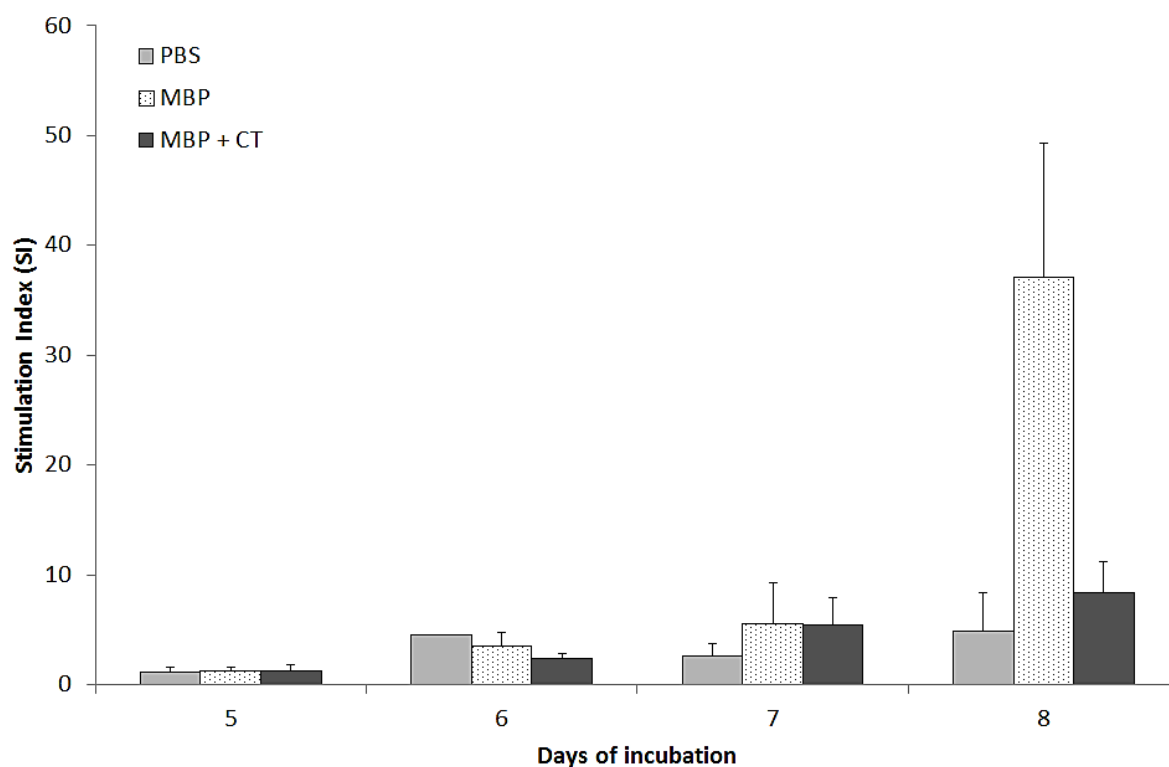


**Figure 6.2** Influence of MBP on PBMC proliferation indicated as stimulation index (±SEM) for the different groups (PBS, n =2; MBP, n = 3; MBP + CT, n = 3) measured after 5 until 8 days incubation.

**Table 6.1** Influence of ConA on PBMC proliferation indicated as stimulation index (±SEM) for the different groups (PBS, n =2; MBP, n = 3; MBP + CT, n = 3) measured after 5 until 8 days of incubation.

| | ConA stimulation index ± SEM | | | |
| --- | --- | --- | --- | --- |
| | **5 days** | **6 days** | **7 days** | **8 days** |
| **PBS** | 135.43 ± 34.80 | 80.56 ± 8.65 | 28.53 ± 16.74 | 139.92 ± 77.65 |
| **MBP** | 317.04 ± 163 | 214.07 ± 78.32 | 382.25 ± 135.96 | 279.21 ± 76.03 |
| **MBP + CT** | 214.16 ± 103.18 | 113.90 ± 32.15 | 169.29 ± 134.08 | 118.71 ± 45.83 |

Five days after the third immunization, one pig of each group was euthanized in order to localize the MBP-specific IgA, IgM and IgG ASC in spleen, MLN, JPP, IPP and LP. No MBP-specific ASCs were found in the spleen and LP. The results for MLN, JPP and IPP are presented in Figure 6.3. In the MBP as well as the MBP + CT immunized animal, IgM ASC were found in the JPP, IPP and the MLN, with the highest number in the JPP of the MBP immunized animal, whereas IgG ASCs were found in the MLN and the JPP (10 per $10^7$ MCs) but not in the IPP. The numbers were cleary higher in the MBP + CT pig than in the MBP pig. IgA ASCs could only be found in low numbers in the JPP of the MBP + CT pig.
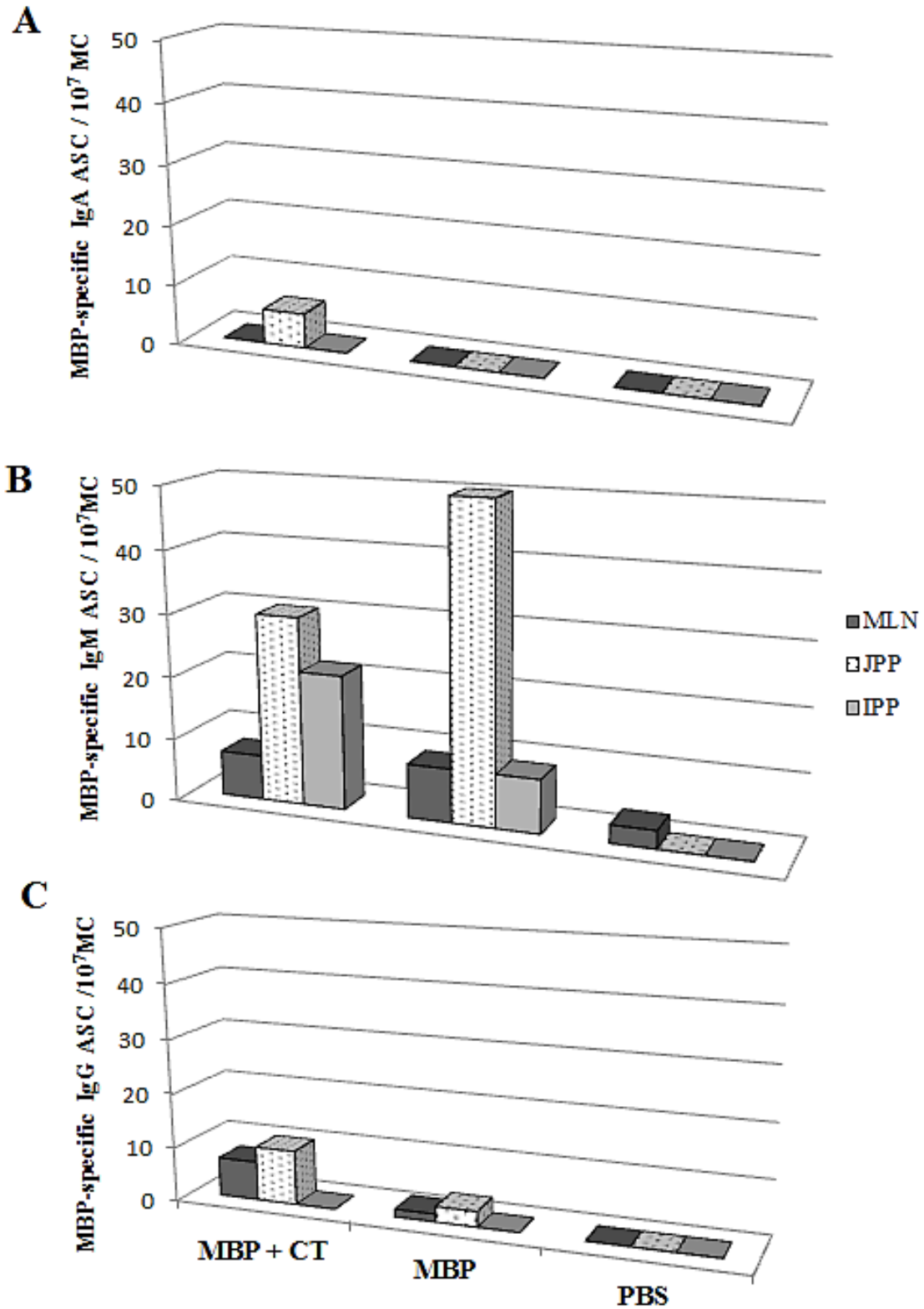
**Figure 6.3** MBP-specific IgA (A), IgM (B) and IgG (C) ASCs per $10^7$ MCs in the mesenteric lymph nodes draining the middle jejunum (MLN) and in jejunal (JPP) and ileal Peyer's patches (IPP) for one piglet of each group (MBP + CT, MBP and PBS) 5 days after the third immunization.

**6.4.2   MBP functions as carrier system to fused antigens**

The oral administration of CT-adjuvanted MBP was thus capable to induce a systemic and local MBP-specific immune response. This incited us to evaluate the potential of MBP as a carrier system for conjugated vaccine antigens. As shown in Figure 6.4, the fusion protein MBPFedF induced a systemic and local antibody response against MBP in the presence of CT whereas only a weak priming of a FedF-specific immune response was induced. The MBP-specific serum antibody response in the MBPFedF + CT group was similar to the MBP-specific response in the MBP + CT group in the first experiment. Here, the titer was significantly higher in the MBPFedF group than in the PBS + CT group ($p < 0.05$) from the second immunization onwards (21 dppi). The third immunization did not boost the antibody response against MBP or FedF.

Five days after the third immunization, two pigs of the PBS + CT and the MBPFedF + CT group were euthanized to analyze the local MBP- and FedF-specific IgA response in the lamina propria. Oral administration of CT-adjuvanted MBP-FedF (MBPFedF + CT group) clearly induced an MBP-specific IgA response in both pigs (15 and 28 ASC per 5 x $10^6$), while only one pig showed an elevated but weaker FedF-specific IgA response (6 ASCs per 5 x $10^6$).
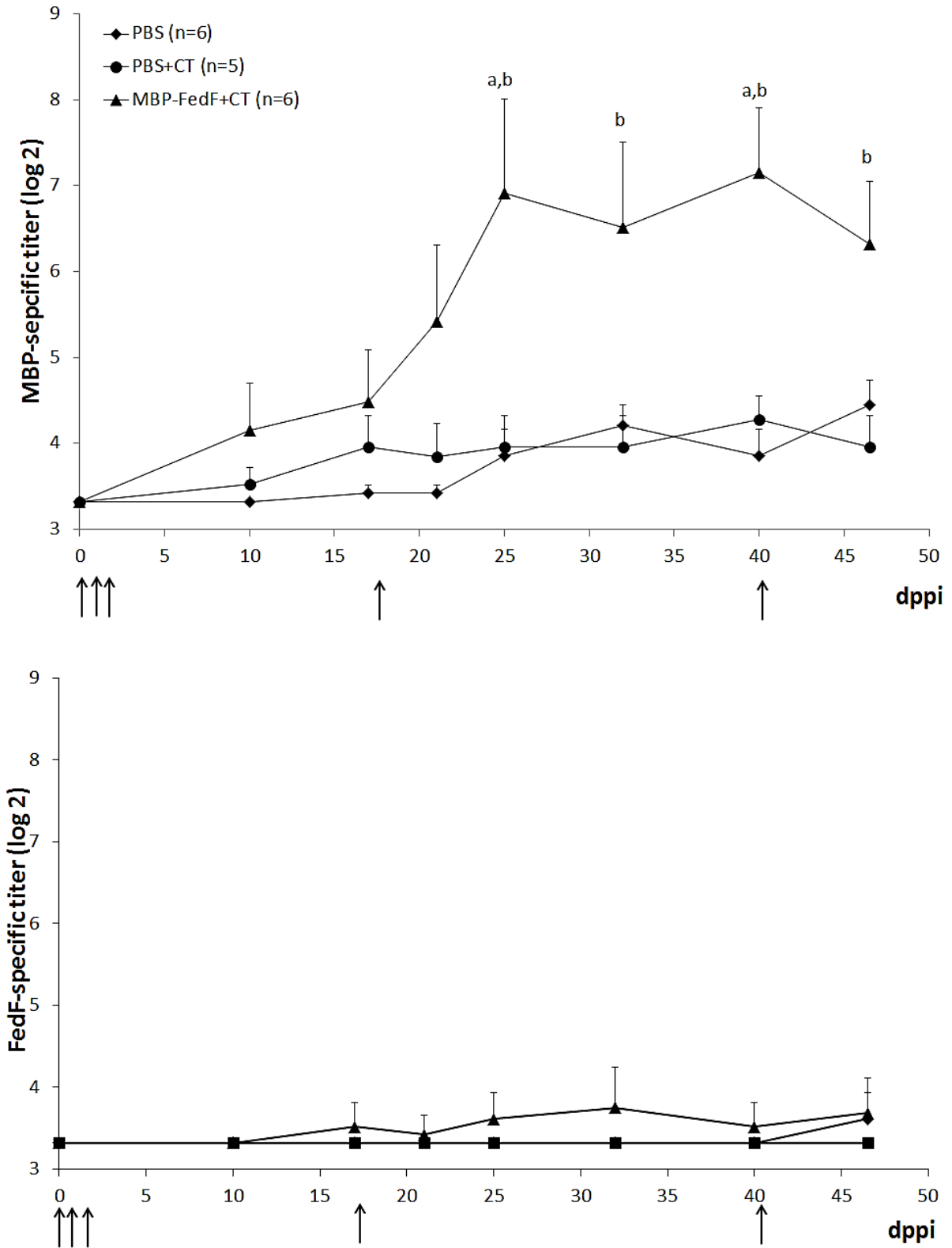
**Figure 6.4** Mean total MBP- and FedF-specific serum antibody response (± SEM) of pigs immunized with PBS (n = 6), PBS + CT (n = 5 ), MBPFedF + CT (n = 6). Black arrow: immunization day. Significant differences ($p < 0.05$) between groups are represented by letters: (a) significant difference between PBS and MBPFedF + CT and (b) between PBS + CT and MBPFedF+ CT.

## 6.5 Discussion

A variety of studies (Du *et al.,* 2011; Fernandez *et al.,* 2007; Kushwaha *et al.,* 2001; La *et al.,* 2004; Xiaoxia *et al.,* 2011) demonstrated the potent immunomodulatory properties of MBP when used as a fusion protein. However, the capacity of MBP to enhance immune responses to fused antigens after oral administration has not been assayed before. In the present study, we evaluated the ability of MBP as a carrier for oral immunizations in the pig. MBP has been widely used for the purification of recombinant proteins because of its minimal effects on the bioactivity of fused proteins. There is evidence that MBP not only enhances the interaction of a vaccine with the immune system in a passive way by increasing the solubility of the immunogen and lengthening its half-life, but also in an active way by stimulating dendritic cells through TLR4 (Fernandez *et al.,* 2007).

In a previous study (Tiels *et al.,* 2008), we demonstrated that F4-MBPFedF adjuvanted with CT could induce a systemic and local immune response against FedF, the adhesin of F18 fimbriae, after oral administration to pigs. Unexpectedly, we also observed a weak priming of a FedF-specific immune response after oral administration of MBPFedF + CT. Using purified F18 fimbriae no such response could be demonstrated suggesting that MBP was involved in this response (unpublished results).

In the first part of the present study, the MBP-specific serum antibody response and the local IgA response against MBP were determined after an oral immunization of pigs with MBP with or without CT. The aim was to investigate the potential of MBP to target the intestinal mucosal immune system. From the second immunization onwards, a significant serum antibody response could be observed in the MBP + CT group. However, lymphocyte proliferation was mainly observed in the MBP group. Why proliferation was almost absent in the MBP + CT group was not clear. A reason could be a different kinetic of the response or the high antibody titer in the latter group at the moment of the third immunization neutralizing the antigen so that no real boost of T-lymphocytes could occur. Nevertheless, the presence of MBP-specific IgA ASCs in the jejunal Peyer's patches in the MBP + CT pig indicates that at least in that pig a local intestinal immune response against MBP was induced. In the MBP pig the intestinal antibody response was IgG directed. IgG can be secreted in the intestinal lumen via the FcRn receptor, but possibly only in low amounts, insufficient to neutralize enteropathogens. In contrast, an IgA response in the gut-associated

lymphoid tissue will result in a much more efficient secretion of dimeric IgA via the polymeric immunoglobulin receptor present at the intestine. This secretory IgA is more resistant to degradation in the gut lumen and therefore more efficient than IgG for protecting against enteropathogens such as F18[+] *E. coli* (Snoeck *et al.,* 2006a). Results clearly demonstrated that both a systemic and a mucosal immune response against MBP can be obtained after oral immunization in combination with the mucosal adjuvant CT. CT is a very potent mucosal adjuvant with low toxicity in pigs (Cox *et al.,* 2006; Foss and Murtaugh 1999). In previous studies co-administration of 50-100 µg CT to orally administered vaccines significantly improved systemic as well as mucosal responses (Foss and Murtaugh 1999; Verdonck *et al.,* 2005).

To induce an intestinal and systemic response, MBP has to pass the intestinal mucosal barrier. Different routes of antigen uptake in the intestine have been described (Devriendt *et al.,* 2012). Fernandez *et al. (*2007) provided evidence that MBP could induce maturation of human dendritic cells (DCs) through interaction with TLR4. Wassef *et al.,* (2004) showed that TLR4 is also expressed by pig enterocytes and binding to TLR4 can result in endocytosis. However, TLR4 mRNA expression could not be demonstrated in the pig small intestine (Thomas *et al.,* 2006). Moreover no direct binding of MBP to porcine enterocytes could be observed *in vitro* (Smeds *et al.,* 2003; Tiels *et al.,* 2008). A recent study implied that MBP can bind on human monocytes and modulate their viability via TLR2 (Xiaoxia *et al.,* 2011). TLR2 is also expressed by porcine M cells as well as enterocytes (Tohno *et al.,* 2005). So, M cells might be involved in the transcytosis of MBP through the epithelial barrier in a TLR2 dependent manner. Besides M cell-mediated transport, antigens/pathogens can pass the epithelial barrier upon endocytosis by dendrites of DCs protruding in the intestinal lumen (Bimczok *et al.,* 2006; Rescigno *et al.,* 2001). Obviously, further investigation is needed to elucidate how MBP can cross the epithelial barrier and induce an intestinal immune response.

Since a strong systemic antibody response as well as a local IgA response occurred against MBP when piglets were orally given MBP with CT, a second experiment was performed to determine if a similar immune response could be obtained against MBP and FedF, with the MBPFedF fusion protein. The amount of fusion protein was chosen so that a similar amount of MBP was given as in the first immunization experiment. Like in the first experiment, a high

MBP-specific serum antibody response was observed from the second immunization onwards.

According to Du *et al.,* (2011) unlike adjuvants, MBP should be fused to the target antigen to activate the immune response, since no detectable antibody titers were induced after co-administration of MBP with their antigen after nasal immunization in mice. This suggests that MBP acts more as a carrier to transport the fused protein through the intestinal barrier and to deliver it to immune cells than as an adjuvant, like CT, that activates/modulates the mucosal immune system to enhance the immunogenicity of the antigen. In the present study, one of the two piglets orally immunized with MBPFedF showed a local IgA response against FedF in the lamina propria, suggesting the induction of an intestinal mucosal immune response against FedF.

Our results suggest that MBP can pass the epithelial barrier after oral administration to pigs, implying that MBP can act as a carrier for targeting heterologous antigens to the gut-associated immune system. Our results also show that this immune response can be boosted after a second immunization. However, the observation that a third immunization was ineffective in further amplifying the systemic antibody response could mean that the antigen was either neutralized in the gut lumen or in the circulation by the induced antibodies, which then could interfere with the use of MBP as carrier for other antigens. Hence, further investigations are needed to study the application of MBP as carrier for oral vaccines.

Our group previously demonstrated that it is possible to induce immunity against enteropathogens by targeting antigen towards enterocytes (Van den Broeck *et al.,* 1999b). More recently, we demonstrated that a protective immune response against F18[+] verotoxigenic *E. coli* was induced after oral administration of a F4-MBPFedF fusion protein to 4-week-old pigs (Tiels *et al.,* 2008). Oral immunization of pigs with MBPFedF in combination with the mucosal adjuvant CT did not lead to a serum immune response against FedF neither to a significant protection against an F18 + *E. coli* infection, even though there was a tendency towards lower excretion near the end of the infection. Nevertheless, the present study demonstrates an MBPFedF-specific intestinal immune response, as evidenced by MBPFedF and FedF-specific IgA antibody secreting cells in the jejunal lamina propria 5 days after a third oral immunization.

## 6.6   Acknowledgments

# Oral immunization with F4ac fimbriae loaded porous pellet induces strong IgA responses

Bellot P.*, Cosijns A.*, Melkebeek M., Vervaet C., Remon J.P. and Cox E.

* These two authors contributed equally to this work.

## 7.1 Abstract

Enterotoxigenic *Escherichia coli* (ETEC) are implicated as an important causal agent in diarrhea and mortality in domestic animals and humans. *E. coli* infections in pigs, immediately after birth or post weaning are responsible for significant economic losses in pig farming. In the present study, the use of porous pellets consisting of Avicel PH 101 was evaluated for the oral vaccination of suckling piglets with F4ac fimbriae. As the pellets consist of an interconnecting pore network, the F4ac fimbriae can penetrate inside the pellet, likely resulting in protection against acids, bile and enzymes present in the stomach and duodenum. Microscopic evaluation of sliced pellets confirmed that F4ac was homogeneously distributed throughout the pellet. F4ac retained most of its activity after freeze-drying and the release of F4ac in simulated gastric fluid was slow, indicating that F4ac is available for interaction with the mucosa at the jejunal Peyer's patches. For the *in vivo* study, piglets were orally immunized with F4ac fimbriae in PBS (F4 solution group), F4ac fimbriae in porous pellets (F4 pellet group) or only PBS (PBS group). Even though higher mucosal and IgA serum titer responses occurred in the F4 pellet group, a better protection against a challenge infection could not be demonstrated in comparison with the F4 solution group. The faecal $F4^+$ *E. coli* excretion was lower in the pellet group than in the soluble group until 6 days after the challenge. The duration of the excretion was the same in both groups and was 1 day shorter than in the PBS group. Nevertheless, in the piglets immunized with F4ac fimbriae in solution faecal excretion of $F4^+$ *E. coli* was more severe than in previous experiments. The cause of this difference is unclear. Therefore no final conclusions could be drawn on the usefulness of the pellets for immunizing piglets.

## 7.2 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) infections are implicated as an important causal agent in diarrhea and mortality in domestic animals and humans. In pigs, *E. coli* infections, immediately after birth or post-weaning, are responsible for significant economic losses in pig farming (Fairbrother *et al.,* 2005). Neonatal infections can be prevented by passive colostral and lactogenic immunity (Deprez *et al.,* 1986; Rutter and Jones 1973), but an active intestinal immunity is needed for protection of newly weaned piglets since they are deprived of passive lactogenic immunity. An active intestinal immunity can occur following oral infection but is not obtained by parenteral immunization, which tends to stimulate the systemic rather than the mucosal immune system (Moon and Bunn 1993). There is clearly a need for competent oral vaccines to induce mucosal protection. Therefore, vaccination of piglets against post-weaning infections is still an important challenge.

Some of the ETEC strains bear F4ac fimbriae, a surface antigen which enables the bacteria to adhere to F4ac-specific receptors (F4acR) present on the brush borders of the villous enterocytes and subsequently enables them to colonize the small intestine. The presence or absence of F4acR is genetically determined and piglets without the F4ac receptor are resistant to F4ac$^+$ *E. coli* infections, indicating that F4ac-mediated adhesion is a prerequisite for infection (Gibbons *et al.,* 1977; Rutter *et al.,* 1975). Antibodies against these fimbriae prevent ETEC adhesion in a direct or indirect manner (van Zijderveld *et al.,* 1998). Consequently these fimbriae are candidate antigens to induce a protective mucosal immune response. It has been demonstrated that newly weaned F4acR-positive piglets can be orally immunized with purified F4ac fimbriae in solution (Van den Broeck *et al.,* 1999a). However, to prevent post-weaning diarrhea, an active mucosal immunity is needed at the moment of weaning. Therefore, the piglets have to be immunized during the suckling period. This could occur using a vaccine formulation that can be mixed with creep feed to facilitate administration. Previously, enteric coated pellets have been used for oral administration of vaccines, as they protect F4ac fimbriae against the detrimental effects in stomach and duodenum (Snoeck *et al.,* 2003). However, incompatibility between the protein and the enteric coating polymer was noticed (Huyghebaert *et al.,* 2005).

In the present study the use of porous pellets was evaluated for oral vaccination of suckling piglets with F4ac fimbriae (Cosijns *et al.,* 2009). As the pellets consisted of an interconnected

pore network, the F4ac fimbriae can penetrate in the porous matrix of the pellets which might offer protection against acids, bile, enzymes, antibodies and other glycoproteins during gastrointestinal transit. Consequently, the release of the protein at the target site (major inductive sites; jejunal Peyer's patches) in an immunogenic conformation can be obtained (Snoeck *et al.,* 2006b). Since binding of F4ac to the F4ac receptor on the villous enterocytes is a prerequisite for the induction of a protective intestinal immune response, it is important that the conformation of the fimbriae is maintained within the pellets.

## 7.3    Material and methods

### 7.3.1   Purification of F4ac fimbriae

To prepare the F4ac stock solution, F4ac fimbriae of F4ac$^+$ ETEC bacteria were isolated as described by Van den Broeck *et al.,* (1999b). The protein concentration of the isolated solution was determined using the bicinchoninic acid protein assay kit (Sigma-Aldrich, Bornem, Belgium). The purity was assessed by electrophoresis on 12% SDS polyacrylamide slab gels.

### 7.3.2   Production and loading of the porous pellets

The pellets were manufactured by An Cosijns at the laboratory of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Ghent University (Cosijns *et al.,* 2009). NaCl was grinded in a ball mill (Pulverisette 6, Fritsch, Idar-Oberstein, Germany) during 10 min and the sieve fraction <125 μm was collected. Avicel PH 101 (FMC Biopolymer, Cork, Ireland) and NaCl were dry mixed (30/70, w/w) in a planetary mixer (Kenwood Major Classic). Next, 42.5 % (w/w) water was added to the mixture and the wet mass was granulated for 10 min. Extrusion was performed using a single screw extruder (Dome extruder lab model DG-L1, Fuji Paudal, Tokyo, Japan) at 50 rpm, equipped with a 1 mm perforated screen. The extrudates were spheronized on a spheronizer (Calvea model 15) with a cross-hatched friction plate, operating at 1000 rpm with a residence time of 5 min. The pellets were oven-dried at 40°C, followed by sieving whereby the 710-1400 μm pellet fraction was collected. The NaCl fraction was removed from the pellets by aqueous extraction: 30 g pellets were brought onto a 500 ml bottle top filter (0.22 μm) (Corning, New York, USA), the filter was placed on a 2 L flask and connected to a vacuum pump. 2 L water was brought onto the filter in steps of 250 ml to extract the NaCl fraction. Next, the pellets were oven-dried at 40°C.

In order to load the pellets with F4ac fimbriae, 15 ml vials were filled with 500 mg pellets (glass type 1, Gaash Packaging, Mollem, Belgium) and spiked with 500 µl F4ac solution (3.35 mg/ml). As the solution was completely absorbed by the porous pellets, each vial contained 1.68 mg F4ac. The pellets were dried by freeze-drying (Amsco-Finn Aqua GT4, Amsco, Germany). The samples were frozen to -45°C within 175 min. Primary drying was performed during 13 h at -15°C and at a pressure varying between 0.8 and 1 mbar, followed by secondary drying for 7 h at elevated temperature (10°C) without changing the pressure. After freeze-drying the vials were closed and kept by -20°C until analysis or administration.

### 7.3.3 Evaluation of the pellets

#### 7.3.3.1 F4ac biological activity

In order to investigate if F4ac fimbriae retained their activity during the manufacturing process, 500 mg freeze-dried pellets (n=3) were soaked in 100 ml phosphate buffered saline (PBS). After crushing the pellets, using a magnetic stirrer and centrifugation at 2500g, the supernatant was collected and stored at -20°C until analysis. The amount of biologically active F4ac was determined via ELISA, as described by Verdonck *et al.,* (2004b). Briefly, the wells of a 96-well microtiter plate (NUNC® Brand Products, Polysorp Immuno Plates, Life Technologies, Merelbeke, Belgium) were coated with F4ac-specific swine polyclonal antiserum in PBS. This polyclonal antiserum was able to bind native as well as denatured F4ac fimbrial FaeG. After 2 h incubation at 37°C, the remaining binding sites were blocked overnight with PBS + 0.2% Tween® 80 (Merck kGaA, Darmstadt, Germany). Subsequently, F4ac stock solution (1 µg F4ac in PBS) and the pellet supernatant, diluted so that the F4ac concentration would be equal to this of the F4ac stock solution if all F4ac would release from the pellets, were added in series of two-fold dilutions (1-0.001 µg/ml) in ELISA buffer (PBS, pH 7.4 with 0.2% Tween® 20 [Merck kGaA] and 3% bovine serum albumin [BSA, MP Biomedicals, Belgium]). Thereafter, an optimal dilution of the F4-specific monoclonal antibody (mAb) IMM01 was added to the wells for 1 h at 37°C. Subsequently, rabbit anti-mouse Ig horseradish peroxidase (HRP) conjugated serum (Dako, Glostrup, Denmark) diluted in ELISA buffer and supplemented with 2% (v/v) pig serum was brought onto the plates for 1 h at 37°C. An ABTS (2,2'-azinobis 3-ethylbenzthiazoline-6-sulfonic acid, Roche, Mannheim, Germany) solution containing $H_2O_2$ was added, whereafter the $OD_{405}$ was measured.

### 7.3.3.2 Distribution of F4ac in the porous pellet

Pellets were fixed in formol. A cassette containing the pellets was placed overnight in a Tissue-Tek-VIP machine and consecutively soaked in formol, ethanol and methanol in order to dehydrate the pellets. Next, the pellets were rinsed in xyleen, followed by embedding in paraffin (Medite TBS 88 paraffin embedding system, Klinipath). Slices (4 μm) were made and dried during 10 min at 60°C. To visualize the F4ac fimbriae, the slices were immunostained by incubating them with anti-F4 rabbit serum followed by adding anti-rabbit IgG (whole molecule)-FITC (Sigma-Aldrich, Schneldorf, Germany). In order to ensure that no background fluorescence was detected, slices incubated in PBS followed by adding anti-rabbit IgG-FITC, were used as control. The slices were examined using a fluorescence microscope.

### 7.3.4  *In vitro* F4ac release from the porous pellets

### 7.3.4.1  *In vitro* dissolution assay

To simulate feed preparation 500 mg pellets (containing 1.68 mg F4) were immersed for 5 min in a pH 7.4 PBS solution. The amount of F4ac released after 5 min was determined via ELISA. Secondly, after removing the pellets from PBS, a dissolution test was performed using the reciprocating cylinder method (USP apparatus 3) (Bio-Dis, Vankel, NJ, USA) at a dip rate of 21 dpm with two consecutive media (250 ml): during 2 h in simulated gastric fluid (SGF, pH 3.0) (USP 27) and during 5 h in PBS pH 7.4 to simulate the gastric and intestinal passage, respectively (Snoeck *et al.,* 2004). Samples were taken after 1 and 2 h in SGF and 1, 3 and 5 h dissolution in PBS. As positive control, the same test was performed without pellets, but by adding 1 ml F4ac solution (1.68 mg/ml) to the dissolution media. The amount of F4ac in the samples was determined using ELISA.

### 7.3.4.2  *In vitro* assay for competitive inhibition of villous adhesion

An *in vitro* villous adhesion/inhibition test was carried out to determine if the released F4ac had the capacity to bind to the F4ac receptor as described by Cox and Houvenaghel (1993). Intestinal villi of an F4acR+ pig were incubated with supernatants obtained after dissolution of the F4 pellets in PBS, F4 solution (+ control) or PBS (- control) for 1h at room temperature while gently rotating. The PBS solution was supplemented with 1 % (w/v) D-mannose (Fluka, Sigma-Aldrich, Bornem, Belgium) to prevent adhesion by type 1 pili. Afterwards, $4 \times 10^8$ F4ac$^+$ ETEC (strain GIS26, serotype O149:K91, F4ac$^+$, LT$^+$STa$^+$STb$^+$) were added followed by

incubation at room temperature for 30 min while gently rotating. Villi were examined by phase contrast microscopy at 600x magnification and the adhesion of the bacteria was evaluated quantitatively by counting the number of bacteria adhering along a 50 μm villous brush border at 20 different places, after which the mean bacterial adhesion per 250 μm villous length was calculated. Adhesion of more than 30 bacteria per 250 μm villous length was noted as strong adhesion, less than 30 bacteria per 250 μm villous length was classified as weak adhesion.

### 7.3.5   *In vivo* F4ac release from the porous pellets: an oral immunization experiment

For the *in vivo* study, 14 F4acR+ pigs were selected using a PCR-RFLP assay based on the *mucin 4* gene (Jørgensen *et al.,* 2004; Rasschaert *et al.,* 2007) what was confirmed after slaughter by a villous adhesion test. At the age of 9 weeks (63 days), 4 animals received an oral dose of 1 mg F4ac fimbriae in 5 ml PBS (F4 solution group) on three successive days (orally administered using a syringe). Six animals received a similar immunization with F4ac fimbriae (1.68 mg) formulated in porous pellets (F4 pellet group). The amount of F4ac administered to pigs using the porous pellets was higher because not all the F4ac is released from the porous pellets, the 1.68mg is calculated for an estimate release of 60% based on the *in vitro* release of F4ac from the pellets. The pellets were orally administered in 5 ml PBS. The direct oral administration of a suspension of the pellets allowed to give the same dose of F4ac as in the solution group. This would be impossible if the pellets were mixed with the feed. Another four animals were not immunized, but received 5 ml PBS orally (PBS group) as negative control. Both vaccinated groups received a booster vaccination at day 15. Finally, at day 30, all animals were infected with a virulent F4ac[+] ETEC strain (GIS26) as described by Cox *et al.,* (1991). Briefly, piglets were treated with florfenicol in PBS (20mg/kg/day) (Nuflor, Schering-Phough, Brussels, Belgium) at day 3 and 2 pre-infection. They were fasted overnight and were deprived of water 3 h pre-infection. Subsequently, they were orally infected with $10^{10}$ GIS26 in 10 ml PBS, 15-30 min after neutralizing the acidic gastric pH with 60 ml of $NaHCO_3$ (1.4% (w/v) in distilled water). Faecal samples were collected daily from the day of challenge till 8 days after.

Blood was sampled from the jugular vein on a weekly basis, starting from the first immunization till 3 weeks after challenge, for determining the total antibody titer in serum.

Serum was collected and inactivated for 30 min at 56°C and subsequently treated with kaolin (Sigma-Aldrich, Bornem, Belgium) to decrease the background reading in ELISA.

The local immune response was analysed 61 days post-infection (at the age of 154 days) for one randomly chosen pig of each group. Hetero, the anti-F4-specific IgA, IgG and IgM antibody-secreting cells (ASC) were enumerated in mesenteric lymph nodes (MLN), spleen, jejunal (JPP) and ileal Peyer's patches (IPP) and jejunal lamina propria (LP) as previously described (Verdonck *et al.,* 2008). The tissues were sampled following euthanasia by intravenous injection of pentobarbital (24mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) and subsequent exsanguination. Monomorphonuclear cells (MCs) were isolated and suspended at $10^7$ cells/ml in leukocyte medium (RPMI with 10% FCS (Greiner Bio-One, Belgium), 100 IU/ml penicillin (GIBCO®, Life Technologies, Paisley, UK), 100 µg/ml streptomycin (GIBCO®), 100 µg/ml kanamycin (GIBCO®), 200 mM L-Glutamine (GIBCO®), 100 mM sodium pyruvate (GIBCO®) and 100 mM non-essential amino acids (GIBCO®)).

### 7.3.5.1   ELISA for the F4-specific serum antibody response

The wells of a 96-well microtiter plate were coated with 5 µg/ml purified F4ac fimbriae in PBS. After 2 h incubation at 37°C, the remaining binding sites were blocked overnight at 4°C with PBS supplemented with 0.2% (v/v) Tween® 80. In subsequent steps, treated sera were added for 1 h in series of twofold dilutions starting from 1/10, the wells were incubated with optimal dilutions of anti-swine IgG, IgM and IgA monoclonal antibodies (Vanzaane and Hulst 1987), rabbit anti-mouse Ig-HRP (Dako) was added and thereafter ABTS. Whereafter the $OD_{405}$ was measured. All incubations occurred at 37°C for 1 h. Dilutions, except for ABTS, were in ELISA buffer. Between each incubation step, plates were washed three times with PBS-T (PBS plus 0.2% [v/v] Tween® 20). The cut-off values were calculated as the mean $OD_{405}$-value of all sera (dilution 1/10) at day 0, increased with three times the standard deviation. The antibody titer was the inverse of the highest dilution that still had an $OD_{405}$ higher than the calculated cut-off value.

### 7.3.5.2   Faecal excretion of F4+ ETEC

The faecal F4$^+$ *E. coli* excretion was determined as described by Van den Broeck *et al.,* (1999b). Briefly, faecal samples were inoculated onto blood agar plates (Becton Dickinson, Erembodegem, Belgium) supplemented with 5% sheep blood (Biotrading, Mijdrecht, the

Netherlands) at 37°C for 24 h. Hemolytic colonies were examined for the production of F4ac fimbriae by agglutination with the IMM01 mAb. When excretion of $F4^+$ ETEC was demonstrated, bacteria were quantified by inoculating 0.5 ml of a 1% faecal suspension onto blood agar plates. After incubation (24 h, 37°C), the colonies were blotted onto polyvinylidene fluoride membranes (Gelman Sciences, Leuven, Belgium) during 2 h at room temperature. Subsequently, the remaining binding sites were blocked overnight with blocking solution (5% [w/v] nonfat dry milk in PBS). After the membranes were rinsed in PBS, IMM01 was added at a concentration of 0.4 μg/ml of blocking solution and the membranes were incubated for 1 h at room temperature. Subsequently, the membranes were washed three times in PBS whereafter HRP-conjugated rabbit anti-mouse Ig (Dako), diluted 1/1000 in blocking solution, was added for 1 h of incubation at room temperature. After three washes in PBS, the substrate solution (0.67 ml of 3-amino-9-ethylcarbazole [AEC] stock solution [0.4% (w/v) in dimethylformamide] in 10 ml of sodium acetate [0.1M, pH 5.2] plus 10 μl of 30% $H_2O_2$) was added for 5 min, the reaction was stopped by rinsing the membranes with $H_2O$. Developed brown-red dots were counted and the average was calculated.

### 7.3.5.3 Elispot assay for F4-specific IgA , IgM and IgG secreting cells

For enumerating the F4-specific IgA, IgM and IgG ASC, the 96-well microtiter plates were coated and blocked as described for the F4-specific serum antibody ELISA. Subsequently, MC suspensions at a concentration of $10^7$ cells/ml leukocyte medium were added to 10 wells (100 μl/well) and plates were incubated for 3 h at 37°C in a humidified 5% $CO_2$ atmosphere. After removing the cells by three washes with PBS-T, plates were treated with optimal dilutions of anti-swine IgG, IgM and IgA and subsequent with anti-mouse Ig-HRP (see ELISA procedure). The substrate solution, consisting of 4 volumes of AEC working solution in 10 ml sodium acetate (0.1 M, pH 5.2) + 10 μl 30% $H_2O_2$) and 1 volume of 3% low melting agarose (BIOzym, Landgraaf, The Netherlands), was added.

Red spots were counted after the plates had been incubated overnight in a dark at room temperature. For each MC suspension, spots in 10 wells ($10^6$ MC/well) were counted, so that finally the number of antigen-secreting cells (ASC) per $10^7$ MC could be determined.

### 7.3.6 Statistical analysis

The antibody titers and ETEC shedding results were statistical analyzed with SPSS 20.0 for Windows (IBM, New York, USA) using General Linear Model (repeated measures analysis of variance) with the Bonferoni adjustment for multiple comparisons. $p < 0.05$ was considered as statistically significant.

## 7.4 Results

### 7.4.1 F4ac biological activity

The F4ac fimbriae were incorporated in the pellets after extrusion/sferonization. The only process that could influence the stability of the antigen during manufacturing of the formulation was freeze-drying. The ELISA results of the supernatant of crushed freeze-dried pellets and of an F4ac solution (standard, 100% activity) were compared: 79.8 ± 3.8 % of the F4ac activity was recovered in the porous pellets. For both the standard solution and the pellets, similar sigmoid $OD_{405}$ curves with a steep linear phase between 1 and 64 ng/ml were found (Figure 7.1, only linear part of the curve is shown).
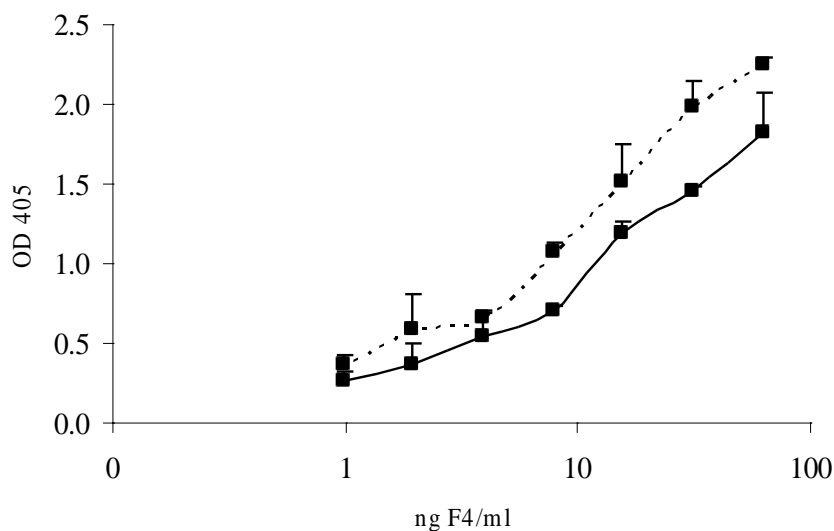


**Figure 7.1** Influence of manufacturing process on stability of the F4ac fimbriae (n=3): standard solution (----) and supernatant of crushed pellets (____).

### 7.4.2 Distribution of F4ac in the porous pellets

To visualize the distribution of F4ac in the porous pellets, slices were made from pellets which were incubated with an F4-specific antibody followed by adding a FITC-labeled anti-rabbit IgG. Figure 7.2 shows that F4ac was not only present on the surface of the pellets but

was found in the entire pellet. After spiking the pellets with the F4ac solution, the pellets absorbed the solution and due to the presence of an interconnected pore network, the solution passes through the entire pellet whereby F4ac fimbriae becomes distributed throughout the entire pellet.
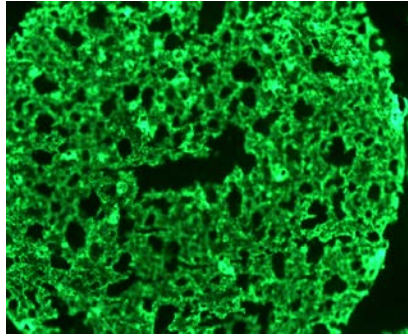


**Figure 7.2** Image obtained by fluorescence microscopy of a cross section of a porous pellet loaded with F4ac fimbriae after incubation with anti-F4 rabbit antiserum followed by the addition of anti-rabbit IgG-FITC.

### 7.4.3   *In vitro* study: release of F4ac fimbriae from the porous pellets

During the *in vivo* experiment, the pellets will be suspended in PBS before administration to the piglets to ensure that the animals in the pellet group receive the same dose of F4ac as in the solution group. To determine if there will be loss of F4ac from the pellets during this procedure pellets were immersed in PBS during 5 min. Only a small amount (10.8 ± 2.5%) of F4ac was released, indicating that F4ac loss from the pellets will be limited.

To investigate the release of F4ac in the stomach and the gut, a reciprocating dissolution test was performed on the pellets after sequentially immersing them for 2 h in SGF and 5 h in PBS. The diffusion-controlled release during 2 h in SGF was slightly slower (13.9 ± 4.5 %) whereas during 5 h in PBS 68 ± 2.7 % of the F4ac fraction was released. In addition, a villous adhesion/inhibition assay was done on the supernatant of the porous pellets after 5 h dissolution in PBS to determine if the released F4ac were still able to adhere to F4ac receptors. The mean number of bacterial adhesion per 250 µm villous length was 8.3 (weak adherence) and 86 (strong adherence) for the F4ac (positive control) and PBS solution (negative control), respectively. After dissolution of pellets in PBS the mean number adhering bacteria was 8.5 (weak adherence), indicating that the F4ac receptors on the brush borders were occupied by F4ac fimbriae present in the sample. This indicated that F4ac

fimbriae released from the pellets in PBS solution retained their binding capacity, even after residence in the acid medium.

### 7.4.4    *In vivo* study: oral immunization of piglets with F4ac loaded porous pellets

#### 7.4.4.1   Systemic F4ac-specific antibody response

The solution as well as the pellet group were orally vaccinated at day 1, 2 and 3 (age of 63, 64 and 65 days) and again at day 15 (age of 78 days). The control group was not immunized. At day 30 (age of 93 days) all animals were inoculated with the virulent F4ac$^+$ ETEC strain. The F4-specific serum antibody response was analyzed till day 45 (age of 108 days) and is presented in Figure 7.3. The serum IgG response was the most pronounced. Following primary vaccination, an increase in F4-specific IgG was detected in the solution as well as in the pellet group, but not in the control group. The solution group reached a maximum on day 15 (titer of 6.97 ± 1.03). The second vaccination resulted in a further increase in the antibody titer for the pellet group, reaching a maximum on day 20 (titer of 6.59 ± 0.73). No raise in IgG was observed in the solution group. The titers of both immunized groups stabilized around 6.5 and were significantly different from the control group from 15 till 29 days post-primary immunization (dppi) as shown in Table 7.1. Following challenge infection, all groups displayed a further increase in IgG with a titer around 9 (9.16 ± 0.37; 9.57 ± 0.54; 8.99 ± 0.80 for the pellet, solution and control group respectively) sixteen days post-challenge.

For serum IgA, an increase in F4-specific IgA was detected in the solution as well as in the pellet group after primary vaccination, but not in the control group. Similar to the IgG response, the solution group reached a maximum at 15 dppi (4.57 ± 0.22), whereas the response of the pellet group further increased after the second vaccination to a maximum at 29 dppi (6.41 ± 0.72). The response of the pellet group was higher than the response of the solution group, being significantly higher than the IgA in the control group from 15 till 29 dppi (Table 7.1). After the challenge infection, the titer increased to the same level for all 3 groups (7.82 ± 0.39; 7.82 ± 0.43; 7.16 ± 0.67 for the pellet, solution and control group respectively).The serum IgM responses remained low after the immunizations in all the groups. After challenge infection, there was a similar increase in all 3 groups (5.1 ± 0.3; 5 ± 0.3; 5.2 ± 0.4 for the pellet, solution and control group, respectively).
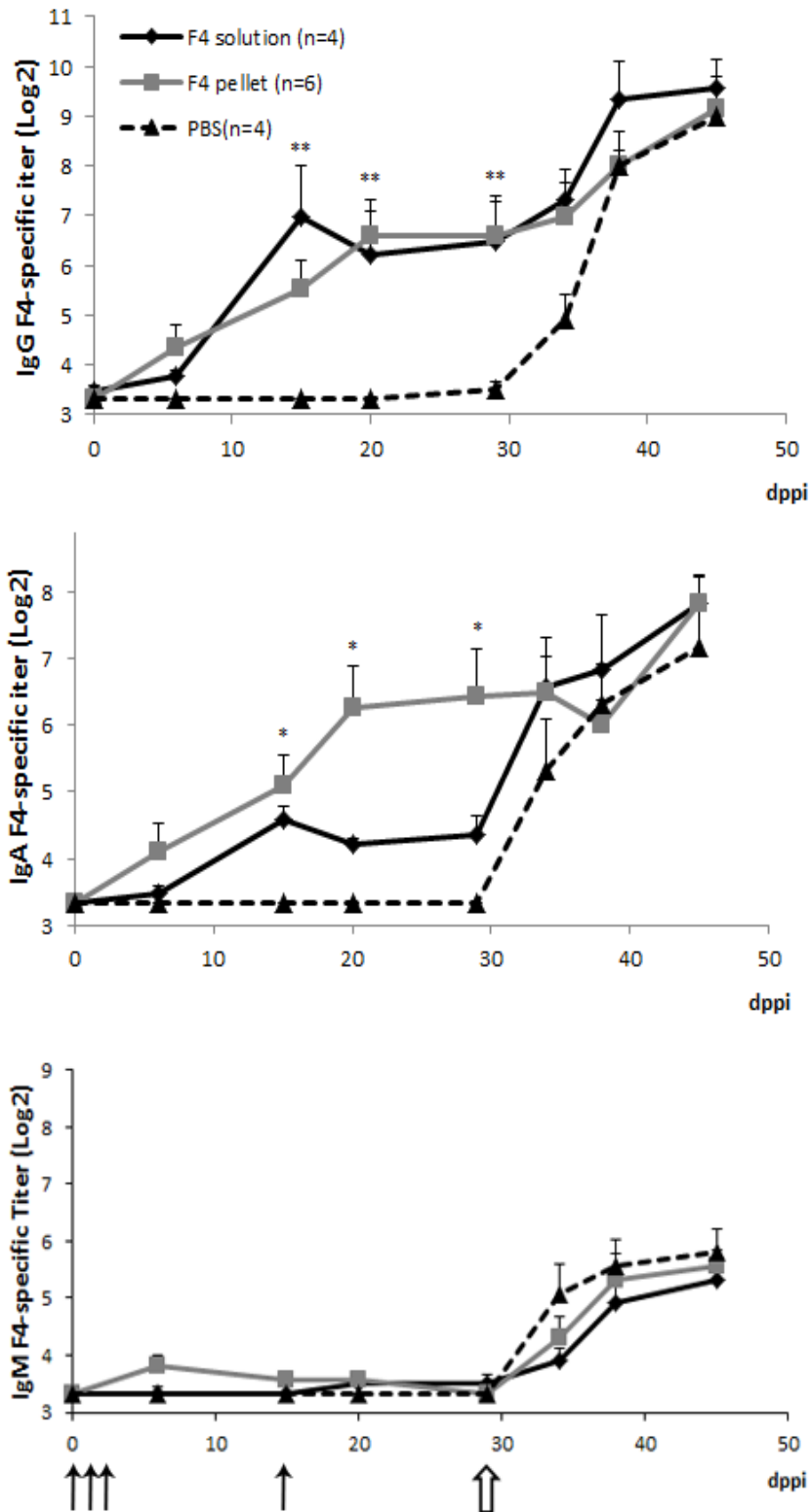
**Figure 7.3** Kinetics of the F4ac-specific IgG, IgA and IgM antibody titer (± standard error of the mean (SEM)) in the control group (n=4), the pellet group (n=6) and the solution group (n=4). Black arrows indicate the vaccinations on day 1, 2, 3 and the booster vaccination on day 15, the white arrow the challenge with virulent F4[+] ETEC on day 30. Significant differences (p < 0.05) between the pellet and the control group are indicated with an asterix (*), a double asterix (**) indicates significant differences between on one hand the pellet and the control group and on the other hand the solution and the control group.

**Table 7.1** Statistical differences of the IgA and IgG titers between the different immunized groups (PBS, F4 solution and F4 pellet) after 15, 20 and 29 days post-primary immunization (dppi) calculated using the General Linear Model (repeated measures analysis of variance) with the Bonferoni adjustment for multiple comparisons. p < 0.05 was considered as statistically significant and marked with an asterix (*).

| Dppi | Groups compared | p-value | |
|------|-----------------|---------|---|
| | | IgA | IgG |
| 15 | PBS <-> F4 solution | 0.329 | 0.007* |
| | PBS <-> F4 pellet | 0.032* | 0.043* |
| | F4 pellet <-> F4 solution | 1.000 | 0.467 |
| 20 | PBS <-> F4 solution | 1.000 | 0.038* |
| | PBS <-> F4 pellet | 0.002* | 0.009* |
| | F4 pellet <-> F4 solution | 0.039* | 1.000 |
| 29 | PBS <-> F4 solution | 1.000 | 0.044* |
| | PBS <-> F4 pellet | 0.009* | 0.018* |
| | F4 pellet <-> F4 solution | 0.134 | 1.000 |

### 7.4.4.2 Faecal shedding of F4ac+ *E. coli*

Examination of the faecal samples revealed that all groups excreted hemolytic F4+ ETEC after the challenge infection (Figure 7.4). No statistically significant differences could be demonstrated between the groups. The duration of the faecal F4+ *E. coli* excretion was the same in both immunized groups and was 1 day shorter than for the PBS group.
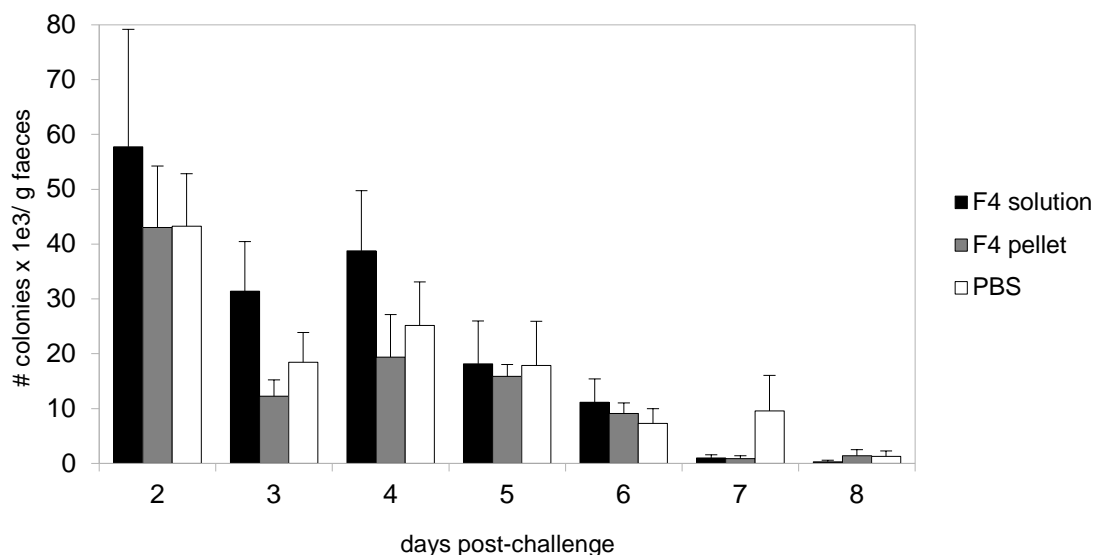


**Figure 7.4** Faecal excretion of hemolytic F4+ ETEC/g faeces (± SEM) after oral challenge with F4+ ETEC of the pellet group (n=6), the soluble group (n=4) and the control group (n=4).

### 7.4.4.3 Mucosal immune response

By analyzing the local F4-specific immune response 60 days after the F4$^+$ ETEC challenge, we checked whether the oral administration of F4ac in the pellets primed the intestinal immune system. This was done by counting the F4-specific IgA, IgM and IgG ASC in spleen, MLN, JPP and IPP after euthanasia of one pig of each group.

The results for the different groups are presented in Figure 7.5. The piglet of the pellet group had high numbers of F4-specific IgA and IgM ASC in the ileal and jejunal Peyer's patches and low numbers in the mesenteric lymph nodes and spleen, whereas the piglet receiving an F4ac solution showed only a high response in jejunal Peyer's patches. The IgG response was similar to the IgA response, high amounts of ASC in IPP, JPP and low amounts in MLN and spleen for the F4ac pellet piglet and only a high amount of ASC in JPP for the piglet of the F4ac solution group. The control piglet (PBS group) only showed a weak response at the inductive sites, with mainly IgM in the mesenteric lymph nodes and spleen. Only very few IgA ASC could be observed in the control piglet and some IgG response could be detected in IPP and MLN.
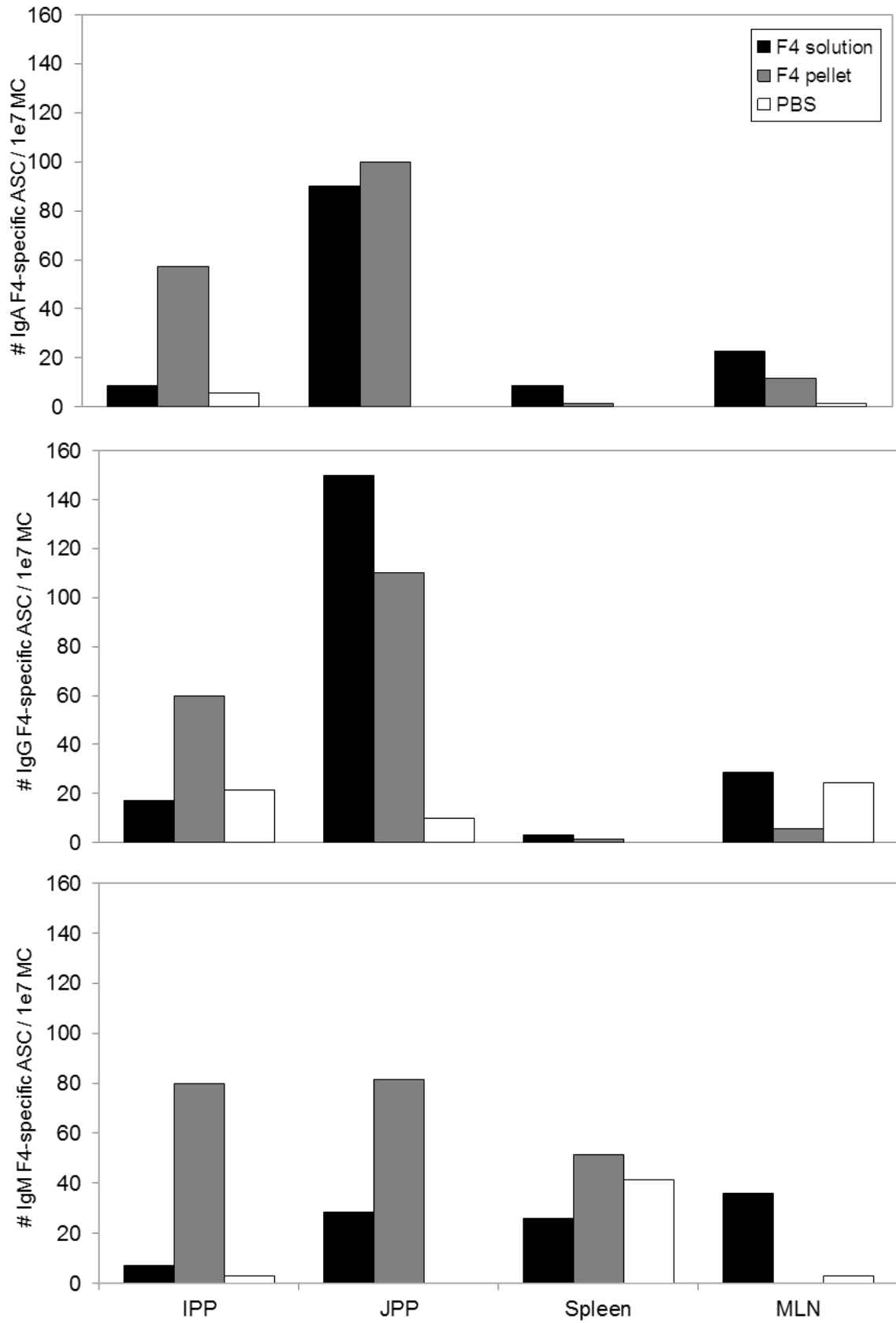
**Figure 7.5** F4-specific IgA, IgM and IgG ASC per $10^7$ MCs in the ileal Peyer's patches (IPP), the jejunal Peyer's patches (JPP), the spleen and the mesenteric lymph nodes (MLN) for one piglet of each group (F4 solution, F4 pellet and PBS).

## 7.5 Discussion

F4ac fimbriae represent one of the rare molecules that induce a strong mucosal immune response following oral administration. Enterotoxigenic *Escherichia coli* (ETEC) producing F4ac fimbriae are an important cause of diarrhoea and mortality in neonatal and recently weaned piglets (Alexander 1994; Hampson 1994). To prevent this diarrhoea, an active mucosal immunity is needed at the moment of weaning. A practical vaccine formulation would be a formulation that could be mixed with creep feed to facilitate administration, that could protect the antigens against the detrimental effects in stomach and duodenum and that would deliver the antigens at a high concentration to the right place. In the present study, we used a porous pellet (particles with size between 500 and 1500 μm) consisting of Avicel PH 101 that was loaded with F4ac fimbriae. Pellets disperse freely in the gastro-intestinal tract, what contributes to maximum drug absorption, reduced peak plasma fluctuations and less side effects (Ghebre-Sellasie 1989; Krämer and H. 1994). As the pellets consisted of an interconnected pore network, the F4ac fimbriae can penetrate in the porous matrix of the pellets which can offer protection to acids, bile, enzymes, antibodies and other neutralizing molecules during gastrointestinal transit, thus ensuring release of the protein at the target site. Furthermore, pellets also allow the formulator to modify the drug release by coating the pellets. A mixture of pellets with different release characteristics can be used to obtain the desired release profile (Ghebre-Sellasie 1989; Krämer and H. 1994).

The biological activity of F4ac was not influenced by the loading process since F4ac released from the pellet, was still able to bind in an ELISA assay and to pig villi in an adhesion/inhibition assay. A more shallow curve for the pellets in the ELISA assay would indicate a lower affinity of the monoclonal antibody for F4ac fimbriae, most likely due to a conformational change in the epitope recognized by mAb, resulting in a lower activity (Verdonck *et al.,* 2004b). As this was not observed, the decrease in F4ac activity in the pellet formulation is mainly contributed to an incomplete release of F4ac from the pellets. Indeed, we noticed that not all the F4ac was released, 79.8 ± 3.8 % of the F4ac activity was recovered after the loading process. The release of F4ac in the stomach and the gut was simulated by performing a reciprocating dissolution test on the pellets after immersing them for 5 min in PBS. The release during 2 h in SGF was slightly slower (13.9 ± 4.5 %) whereas 68 ± 2.7 % of the F4ac fraction was released during 5 h incubation in PBS. This indicated that

most of F4ac, absorbed in the internal pellet structure will be protected during gastric passage as most of the F4ac dose is only released in the intestine. Hence, a larger fraction of F4ac fimbriae will be available for interaction with the mucosa at the jejunal Peyer's patches, which are the major inductive site of the F4-specific immune response (Snoeck *et al.,* 2006b). As previous studies have shown that F4ac fimbriae retain their antigenicity during at least 4 h at pH 3.0 (Snoeck *et al.,* 2004), the limited amount of F4ac detected in SGF is only attributed to the slow release of F4ac from the pellets and not to inactivation of F4ac in SGF. Furthermore, pellets have a limited residence time in the stomach as their gastric emptying is similar to that of fluids. The slow release of F4ac from the porous pellets can be attributed to the size of F4ac fimbriae: 0.1-1 µm length and 2.1 nm diameter (Mooi and Degraaf 1985; Stirm *et al.,* 1967), whereas the size of conventional drugs is in the Angstrom range (0.1 nm).

The systemic IgG immune response after oral vaccination of piglets with F4ac-containing porous pellets was not significantly higher compared to the response after oral vaccination with an F4ac solution. We could detect significant differences in serum IgG responses between both the pellet group and the control group and between the solution group and the control from 15 days after the primary vaccination until the day of the challenge (29 dppi). However, in contrast to the F4 solution group, the response of the pellet group was boosted after the second vaccination to reach similar titer as the solution group. The kinetics of the serum IgA responses were similar to those of the IgG responses. The titer of the solution group quickly raised to its maximum, whereas the titer of the pellet group gradually increased to reach a plateau 5 days after the booster vaccination at a higher titer than the solution group. Significant differences in serum IgA responses could be found between the pellet group and the control group from 15 till 29 dppi. The high IgA responses suggests a better protection following immunization with the pellet. Indeed, an IgA response in the gut-associated lymphoid tissue will result in a much more efficient secretion of dimeric IgA via the polymeric immunoglobulin receptor present at the intestine. This secretory IgA is more resistant to degradation in the gut lumen and therefore is expected to be more efficient than IgG for protecting against enteropathogens such as F4$^+$ *E. coli* (Snoeck *et al.,* 2006a). However, no difference in protection against F4$^+$ *E. coli* could be detected. Variation in shedding was observed among the different piglets. Especially in the solution group during the first four days, we could identify high and low shedders resulting in fluctuating means

and high error bars. The duration of the faecal F4[+] *E. coli* excretion was the same for the pellet and the soluble group and seemed one day less compared to the control group. In contrast, the piglet of the pellet group had high numbers of F4-specific IgM and IgA ASC in the ileal and jejunal Peyer's patches and low numbers in the mesenteric lymph nodes, whereas the piglet receiving an F4ac solution showed only a high response in jejunal Peyer's patches suggesting a less pronounced priming compared to the F4ac pellets. On the other hand, it is possible that a full uptake of the F4ac in solution already takes place in duodenum and proximal jejunum, whereas the pellet ensures a more distributed delivery of F4ac throughout the intestine. However, a previous study shows an equal distribution of the local F4ac-specific response throughout the small intestine following oral immunization with F4ac in solution (Snoeck *et al.,* 2006b). The control piglet only showed a weak response in the inductive sites, with mainly IgM in the mesenteric lymph nodes and very few IgA. In contrast, both vaccinated groups had an IgA response, consistent with a primary response following infection in the control pig and a secondary response in both immunized piglets.

Although a higher mucosal and serum IgA titer occurred using porous pellets for oral immunization, a better protection against infection could not be demonstrated compared to piglets immunized with soluble F4. However, the low protection following the immunization with F4ac in PBS as compared to previous studies needs further clarification (Van den Broeck *et al.,* 1999b; Verdonck *et al.,* 2004a). Therefore, the use of this pellet as F4ac carrier for oral immunization against ETEC needs further study. Furthermore, the protection value of the pellet against the low gastric pH could be questioned as the pellets might absorb gastric fluid when passing through the stomach, filling the pores with this acidic medium and creating an acidic microenvironment inside the pellet. So additional studies with acid-sensitive antigens are needed to determine if the pellet is able to protect and deliver such antigens more efficient to the intestinal mucosa. Nevertheless, the pellet could protect the F4ac fimbriae or other antigens against neutralization by milk during the suckling period. Indeed, milk can inhibit the adhesion of F4ac fimbriae to the villous brush border, even in the absence of F4-specific antibodies. This is probably caused by receptor analogues present at fat globule membranes or in milkserum (Atroshi *et al.,* 1983). Additional studies are needed to assay this hypothesis.

However, delivery to the mucosa will not be enough to induce a mucosal immune response. Although, the pellet is capable to increase the intestinal residence time, more sophisticated formulations, using both cell-specific targeting approaches and immunomodulation might be required to induce a mucosal immune response against most antigens after oral immunization with antigens.

## 7.6 Acknowledgments

**Chapter 8**

**General discussion and future perspectives**

M cells are generally considered as the gateways for mucosal vaccine delivery. Nevertheless, the potential of enterocytes should not be disregarded. Enterocytes not only outnumber M cells, but they are also able to transcytose macromolecules, such as CT, F4ac fimbriae, and very small inert particles (Florence 1997; Lencer *et al.,* 1995; Snoeck *et al.,* 2008). The ability of enterocytes to take up particles is rather limited compared to M cells. However, this limitation could become negligible due to the bigger surface area offered by enterocytes combined with specific targeting to enterocytes. In this thesis, we introduced APN as a promising candidate for the selective targeting of vaccine antigens towards the mucosal epithelium. We showed that polyclonal rabbit antibodies against pAPN adhere to and are taken up by pig enterocytes in a similar way as the F4ac fimbriae of F4ac$^+$ ETEC. Even in the absence of the mucosal adjuvant CT, strong serum IgA, IgG, and IgM responses could be observed after a first oral immunization of pigs with polyclonal rabbit antibodies against pAPN. In addition to a serum antibody response, a local mucosal pAPN-specific IgA response was induced. Since pAPN seems to be expressed by small intestinal enterocytes and not by epithelial cells of the large intestine or the respiratory tract. We conclude that APN can be used as target, rather for the delivery of molecules to the small intestine than to the respiratory tract or large intestine. No differences in pAPN protein expression could be detected between F4acR+ and F4acR- pigs, indicating that the APN targeting can be used in the whole pig population. Besides APN-specific antibodies, results in my thesis suggest that MBP is an alternative carrier for delivering antigens towards the gut-associated lymphoid tissue. Indeed results suggest that MBP can pass the epithelial barrier after oral administration to pigs.

Commercial oral vaccines that are available today mainly use live attenuated microorganisms replicating in the gut (Levine 2000; Soares-Weiser *et al.,* 2010). The use of live vaccines involves potential risks (Ehrenfeld *et al.,* 2009) and therefore, non-replicating oral vaccines are an interesting alternative. Nevertheless, the main issue for these vaccines is the induction of oral tolerance instead of a protective immunity. To increase the success rate, APN binding molecules and MBP could be part of a sophisticated formulation, combining both cell-specific targeting, immunomodulation and protection against the harsh environment of the gastrointestinal tract. A schematic representation of such an oral vaccine is given in Figure 8. pAPN binding molecules (i.e. the anti-pAPN mAb) as well as MBP can be

used to target vaccines towards the mucosal intestinal epithelium. Those targeting molecules can be coated together with other immunomodulating molecules such as lipopolysaccharide (LPS) and CpG-oligodeoxynucleotides [ODN] (Cox *et al.,* 2006; Zhu *et al.,* 2012) on the surface of biologically compatible nanoparticles, which contain the vaccine antigens. To bypass the denaturing effects of the low gastric pH and the enzymatic destruction in the gastrointestinal tract and to selectively deliver the particles to the small intestine, the nanoparticles should be coated with a pH-sensitive polymer which dissolves in intestinal fluids but not in gastric content.
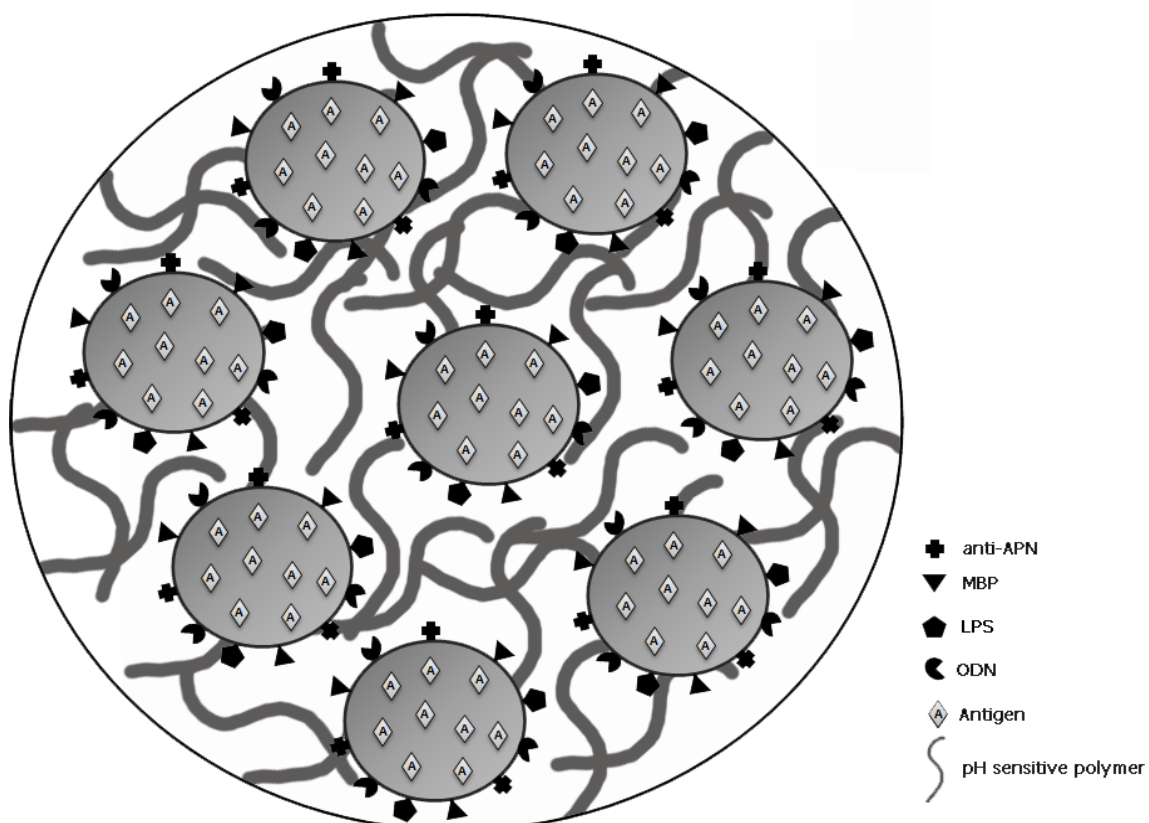


**Figure 8** Schematic presentation of a sophisticated oral vaccine formulation combining both cell-specific targeting (anti-pAPN monoclonal antibodies [anti-pAPN] and maltose-binding protein [MBP]) and immunomodulation (lipopolysaccharide [LPS] and CpG-oligodeoxynucleotides [ODN]). Nanoparticles, containing the vaccine antigens (A) are on their surface coated with mAb anti-pAPN, MBP, LPS and ODN. These particles are enveloped with a pH-sensitive polymer dissolving in intestinal fluids to bypass the denaturing effects of low gastric pH and enzymatic destruction and to selectively deliver the particles to the small intestine.

For piglets the pH in the small intestine varies between 5.1 and 6.5 in suckling piglets, between 4.2 and 6.1, at the moment of weaning, between 3.2 and 5.8, one week post-weaning, and between 2.4 and 6.0, two weeks post-weaning. From 1/16 of the length of the

small intestine onwards, pH values lower than 4.6 were never found (Snoeck *et al.,* 2004). So the enteric coating should dissolve between pH 4.0 and 6.4, this can be achieved by a methacrylate-based polymer (e.g. chitosan-Eudragit L100-55 polymer) (Jelvehgari *et al.,* 2010; Wang and Zhang 2012). To be easily mixable with the feed, the coated particles can be manufactured in different sizes, depending on the feed used. In addition, the formulation should be stable at room temperature what can be achieved by freeze-drying. In Chapter 7 we demonstrated that F4ac retained most of its activity after freeze-drying, obviously this should be tested for the antigens incorporated in the oral vaccine. F4ac fimbriae and FedF molecules could both be incorporated in the vaccine to protect piglets against postweaning diarrhea caused by $F4^+$ and $F18^+$ ETEC. The priming of the immune response has to occur in suckling piglets because $F4^+$ *E. coli* infections appear early after weaning. However, the suckled milk contains maternal antibodies and receptor-analogues, preventing the contact of the vaccine with the mucosal immune system. The pH-sensitive polymer from our proposed vaccine formulation could protect against the milk. Consequently, the vaccine should be administered as a liquid to the piglets and the size of the particles needs to be small to assure an easy to administer suspension. After weaning, the vaccine particles could be mixed with the feed or in the drinking water. However, previous studies with oral vaccines showed that individual drench application offers a better preventive protection than vaccination via drinking water (McOrist and Smits 2007; Pejsak *et al.,* 2009). Elaborate studies for determining the best administration of the vaccine will be required.

The polyclonal pAPN-specific antibodies used in this thesis, are an interesting research tool, but are not optimal for targeting antigens due to batch differences of different polyclonal antibody stocks. Further development of monoclonal antibodies targeting different pAPN-specific epitopes is needed. In addition, the use of the monoclonal antibodies could give interesting information on the conformation and expression of pAPN in different tissues. Our immunohistochemistry results indicate a gradual increase of pAPN expression along the small intestine of pigs. On the other hand, pAPN was not found in the epithelium of the trachea, lung, tongue, oesophagus, stomach, large intestine (caecum and colon) and rectum. Either absence of expression or weak expression with an insufficient sensitivity of the used polyclonal antibodies to demonstrate this weak expression could explain our finding. The use of monoclonal antibodies, in combination with quantitative pAPN mRNA expression will be

necessary to elucidate this. In new experiments, the butyrate level and the expression of ATBF1A should also be measured and correlated with pAPN expression to get indications of their role in the pAPN expression level in different parts of the intestinal tract.

The produced IPEC-J2-pAPN cell line can be used as a tool for further pAPN-related research. It can for instance, help in a screening for molecules interacting with pAPN. These molecules could perhaps be used as carriers for the delivery of antigens across the epithelium. Another application could be to block pAPN, making it unavailable for pathogens which use pAPN as a receptor, like F4ac$^+$ ETEC, transmissible gastroenteritis virus (TGEV) (Delmas *et al.,* 1992) and the porcine endemic diarrhea virus (PEDV) (Li *et al.,* 2009; Nam and Lee 2010; Oh *et al.,* 2003). The binding of F4ac fimbriae to pAPN depends on sialic acid containing carbohydrate moieties since this binding is reduced after treatment of brush border membranes with neuraminidase from *Arthrobacter urefaciens* removing 2-3,6,8,9-linked sialic acids. Therefore, it would be interesting to identify the sugar structure on pAPN involved in the binding of the F4ac fimbriae and investigate if a difference exists between F4acR positive and negative pigs. These variations in glycosylation could be due to differences in the amino acid sequence of pAPN but a difference in a glycosyltransferase could also result in a different glycosylation pattern. Identification of the sugar structures could also lead to the production of F4ac binding inhibitors that can be supplemented to the feed and could prevent F4ac$^+$ ETEC infections. For characterization of the sugar structures, several high resolution techniques are available such as high performance liquid chromatography (Guile *et al.,* 1996), capillary electrophoresis (Suzuki and Honda 1998) and mass spectrometry (Rudd and Dwek 1997). However, this will not be an easy task because of the high and often complex branching degree of the carbohydrates.

Additional investigation of the uptake of molecules by pAPN will be necessary to resolve the mechanisms of the pAPN-mediated transcytosis. To further confirm the clathrin-dependent endocytosis of pAPN-specific antibodies, clathrin can be blocked during the early phases of internalization by inhibition of clathrin-coated pit invagination at the plasma membrane using amantadine-HCl (Phonphok and Rosenthal 1991). However, blocking clathrin could affect other clathrin-dependent pathways and thus indirectly interfere with the receptor-mediated uptake as clathrin plays a role in the bidirectional traffic between the trans-Golgi network and endosomes (Bonifacino and Traub 2003).

Since APN is present on the intestinal epithelium of many animal species (Norén *et al.,* 1997) and our polyclonal pAPN-specific antibodies successfully bind to the enterocytes of humans and sheep but not to mice (unpublished results), it would be interesting to evaluate APN as target in these species. Seventy to 80% amino acid identity is found between the APN proteins from human, mouse, rat, rabbit, pig, cow, cat, dog and chicken. In addition, a homology is found between the bfinding to APN of different group I coronaviruses including the human coronavirus (HCV) 229E (Yeager *et al.,* 1992), transmissible gastroenteritis virus (TGEV) (Delmas *et al.,* 1992), porcine endemic diarrhea virus (PEDV) (Li *et al.,* 2009; Nam and Lee 2010; Oh *et al.,* 2003), canine coronavirus (CaCV) (Kolb *et al.,* 1998) and feline infectious peritonitis virus (FIPV) (Tresnan *et al.,* 1996). Therefore, it is plausible that also in other species than pig, APN is able to internalize targeted molecules. Then APN could be used as an universal target for the delivery of antigens to mucosal tissues.

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# Summary

Even with use of modern-day knowledge and techniques, still no efficient vaccines exist against a lot of diseases. Most infectious diseases are caused by pathogens that colonize and/or invade the host at mucosal surfaces. For an effective protection of the host, pathogen-specific secretory IgA (SIgA) is required at the site of infection. This mucosal immunity prevents the pathogens to colonize and/or invade the mucosa. Nonetheless, most commercial vaccines are delivered systemically by injection and although they elicit a strong systemic immune response, only a weak pathogen-specific mucosal immunity sometimes appears. In contrast, vaccination at mucosal sites can lead to protective mucosal immunity. However, formulation of mucosal vaccines is difficult and the progress in development has been rather slow. In this thesis, we evaluated new strategies for the oral immunization of pigs by directly targeting soluble antigens to the intestinal mucosa.

The F4ac fimbriae of enterotoxigenic *Escherichia coli* (ETEC) are one of the unique molecules that induce an immune response after oral immunization. This immune response requires the presence of the F4ac receptor (F4acR), as oral immunization of F4acR negative piglets with F4ac fimbriae does not result in the induction of an F4ac-specific mucosal immune response. Recently, our group identified porcine aminopeptidase N (pAPN) as a novel receptor for F4ac$^+$ ETEC. In this thesis, we evaluate the use of APN as target for the delivery of oral vaccines. Moreover, two additional approaches were used to orally immunize piglets, i.e. maltose-binding protein (MBP) as carrier for antigens and porous pellets containing F4ac fimbriae.

**Chapter 1** reviews the current knowledge of the intestinal mucosal immune system, antigen uptake in the gut, oral vaccination and ways to enhance the immune response. In addition, background information is provided about APN and MBP.

**Chapters 3 to 7** describe the experimental work, which is subdivided into two parts. The first part deals with APN as target to obtain a mucosal immune response, whereas the second part evaluates the use of MBP as antigen carrier and targeting molecule and the use of porous pellets for oral immunization of pigs.

*Summary*

Following main questions were addressed:

1) Can APN be used as target for the delivery of oral vaccines?
2) Is the difference between F4acR positive and negative pigs caused by a difference in APN expression on protein level?
3) Is MBP able to target conjugated antigens towards the GALT?
4) Can oral vaccination with porous pellets loaded with F4ac fimbriae improve the immune response against F4ac?

In **Chapter 3** we determined if antibodies against pAPN are taken up by a receptor-mediated process and induce an anti-pAPN antibody-specific immune response when given orally, similar to the response against F4ac fimbriae. First the adhesion of the pAPN-specific antibodies to pig enterocytes was demonstrated *in vitro* by using a pAPN-transfected cell line (BHK-pAPN) as well as *in vivo* using intestinal loops. In the next step, pigs were orally immunized with purified antibodies against pAPN (anti-pAPN) or with purified rabbit IgG as negative control. The oral administration of anti-pAPN antibodies elicited a strong immune response in the pigs, even in the absence of the mucosal adjuvant cholera toxin (CT). Strong serum IgA, IgG, and IgM responses could already be observed after a primary immunization. Elevated levels of IgA, and IgG were found in the intestinal mucosa of the anti-pAPN and anti-pAPN + CT immunized animals nine days after the booster immunization. Furthermore, rabbit IgG-specific IgA ASCs were found in the LP in the anti-pAPN and anti-pAPN + CT immunized pigs, indicating the induction of a local IgA response against the pAPN-specific antibodies.

These results and the fact that APN is present on the respiratory and intestinal epithelium of many species makes this molecule a promising candidate for the selective targeting of antigens towards the mucosal epithelium. However, in pigs it has not yet been demonstrated which epithelia of the digestive tract and respiratory tract express APN. This is necessary to know which mucosa can be targeted. In **Chapter 4,** the expression of pAPN was evaluated for F4acR positive and negative pigs. A difference in pAPN expression on protein level between F4acR positive and negative pigs would hamper the use of pAPN targeting in pigs. No difference in pAPN protein expression could be detected between the receptor positive and negative pigs. Either differences in APN glycosylation, in a molecule sterically hindering

adhesion of F4ac fimbriae to the intestinal mucosa and/or other F4ac receptors involved in the initial binding could explain F4acR positive and negative pigs. We found a gradually increasing pAPN protein expression from duodenum to ileum. pAPN expression was not seen on the epithelium of the trachea, lung, tongue, oesophagus, stomach, large intestine (caecum and colon) and rectum. As a consequence, APN can be used as target for the delivery of molecules to the small intestine but not to the respiratory tract and large intestine.

A good *in vitro* model is recommended for studying the pAPN-mediated uptake and transcytosis of antigens as well as for screening potential receptor ligands. The IPEC-J2 cell line, a pig intestinal epithelial cell line, is a good candidate as it is a relevant model for intestinal epithelial cells. But the IPEC-J2 cells express pAPN only in low amounts, not enough for pAPN-related binding studies. Therefore, in **Chapter 5** we described the transfection of the IPEC-J2 cell line with pAPN. Flow cytometry was used to identify transfected cells which express pAPN by evaluating the binding of polyclonal rabbit pAPN-specific antibodies to the cells. Subsequently, the successfully transfected cells (IPEC-J2-pAPN) were positively selected using a FACSAria cell sorter and kept in continuous culture. In addition, a monoclonal antibody against pAPN was produced. Endocytosis of pAPN-specific antibodies by the IPEC-J2-pAPN cells could be demonstrated, whereas no binding was observed using the IPEC-J2 cells. Vesicles containing anti-pAPN antibodies and clathrin colocalized during the anti-pAPN antibody internalization as seen for F4ac fimbriae and clathrin. This indicates that the IPEC-J2-pAPN cell line is a promising tool for the *in vitro* study of pAPN-mediated binding and uptake.

In the second part of the experimental chapters, two additional approaches were used to orally vaccinate piglets. Recent data suggest that the maltose-binding protein (MBP) may potentiate antigen-presenting functions in immunized animals by providing intrinsic maturation stimuli to dendritic cells via TLR4. The aim of **Chapter 6** was to examine if an MBP-specific immune response can be elicited by oral administration of MBP. In a first experiment MBP or MBP + CT was orally administered to piglets and both the systemic and mucosal immune responses were examined. From the second immunization onwards, a significant serum antibody response could be observed in the MBP + CT group. Although no high systemic response was observed in the MBP-group, a local mucosal MBP-specific IgM

response was observed in the jejunal Peyer's patches. In a second experiment, MBPFedF was orally administered. A significant systemic response against MBP and a weaker response against FedF were found after oral administration of MBPFedF + CT. The presence of MBP-specific IgA ASC in the lamina propria indicated that a local intestinal immune response against MBP was induced. Our results suggest that MBP passes the epithelial barrier after oral administration to pigs, implying that MBP could act as a carrier and delivery system for targeting fused proteins to the intestinal immune system.

In **Chapter 7**, porous pellets consisting of Avicel PH 101 were evaluated for oral vaccination of suckling piglets with F4ac fimbriae. As the pellets consist of an interconnecting pore network, the F4ac fimbriae can penetrate inside the pellet what can offer a protection in the stomach and duodenum against acids, bile, enzymes antibodies and other glycoproteins binding to the fimbriae present. Fourteen F4acR+ pigs were selected, randomly divided into 3 groups and were orally immunized with either F4ac fimbriae in PBS, F4ac fimbriae in porous pellets or only PBS. Two weeks after the final vaccination, all animals were infected with a virulent F4ac$^+$ ETEC strain. Although there were higher mucosal and serum IgA responses using porous pellets than soluble F4ac, a better protection against an induced infection could not be demonstrated. The F4ac$^+$ ETEC excretion was lower in the pellet group compared to the soluble group until 6 days after the challenge but not in comparison with the PBS group. The duration of the faecal F4ac$^+$ ETEC excretion was the same for both immunized groups and seemed 1 day shorter than for the PBS group. F4ac fimbriae are quite resistant to an acidic pH. Additional studies with more acid-sensitive antigens are needed to determine if the pellet is able to protect and deliver such antigens more efficient to the intestinal mucosa.

The final chapter (**Chapter 8**), presents the general conclusions and future perspectives. In this thesis, we introduced APN as promising candidate for targeting of vaccine antigens towards the mucosal epithelium and towards antigen presenting cells. We conclude that APN can be used as target for the delivery of molecules to the small intestine, but not to the large intestine or the respiratory tract. No differences in pAPN expression could be detected between F4acR+ and F4acR- pigs, so APN targeting is applicable in both groups of pigs. The IPEC-J2-pAPN cell line as well as the pAPN monoclonal antibody can be used as tools for pAPN-related research. In addition, our results suggest that MBP can also pass the epithelial

barrier after oral administration to pigs, implying that it is a potential carrier for targeting heterologous antigens towards the intestinal mucosal immune system.

# Samenvatting

Ondanks de huidige kennis en knowhow bestaan er tegen vele ziekten nog steeds geen doeltreffende vaccins. Besmettelijke ziekten worden zeer dikwijls veroorzaakt door pathogenen die de mucosale oppervlakten koloniseren en/of binnendringen. Voor een doeltreffende bescherming van de gastheer tegen deze infectie is op de plaats van infectie meestal pathogeen-specifiek gesecreteerd IgA (SIgA) nodig. Deze mucosale immuniteit belet de pathogenen de mucosa te koloniseren en/of te invaderen. Bijna alle commerciële vaccins worden systemisch toegediend via injecties en induceren bijgevolg een sterke systemische maar slechts een zwakke mucosale immuniteit. Vaccinatie ter hoogte van de mucosa kan daarentegen wel leiden tot een beschermende mucosale immuniteit. De formulering van mucosale vaccins is echter moeilijk omdat voldoende antigenen de mucosale epitheellaag moet passeren zonder ziekte of tolerantie te veroorzaken. In dit proefschrift evalueerden we nieuwe strategieën voor de orale immunisatie van biggen door oplosbare antigenen te richten naar het intestinaal weefsel.

De F4ac fimbriae van enterotoxigene *Escherichia coli* (ETEC) behoren tot een beperkte groep van unieke moleculen die in staat zijn om een immuunrespons te induceren na orale immunisatie. Deze immuunrespons vereist de aanwezigheid van de F4ac receptor (F4acR), aangezien de orale immunisatie van F4acR negatieve biggen met F4ac fimbriae niet leidt tot een F4ac-specifieke mucosale immuunrespons. Recent identificeerde onze onderzoeksgroep het varkens aminopeptidase N (pAPN) als nieuwe receptor voor F4ac$^+$ ETEC. In dit proefschrift evalueerden we het gebruik van pAPN als doelwit voor de aflevering van orale vaccins. Bovendien werden twee bijkomende benaderingen gebruikt om biggen oraal te vaccineren, namelijk het gebruik van het maltose-bindend eiwit (MBP) als antigeendrager en poreuze pellets geladen met F4ac.

**Hoofdstuk 1** beschrijft de huidige kennis over het intestinaal mucosaal immuunsysteem, antigeenopname ter hoogte van de darmen, orale vaccinatie en manieren om de immuunrespons te verhogen. Bovendien wordt achtergrond informatie verstrekt over APN en MBP.

**Hoofdstukken 3 tot 7** omvatten de experimenten uitgevoerd in dit proefschrift. Het experimenteel werk is onderverdeeld in 2 delen. Het eerste deel handelt over APN als doelwit voor inductie van een mucosale immuunrespons en in het in het tweede deel worden zowel MBP als poreuze pellets gebruikt voor de orale vaccinatie van biggen.

De volgende algemene vragen werden beantwoord:

1) Kan APN gebruikt worden als doelwit voor de aflevering van orale vaccins?
2) Wordt het intrigerend verschil tussen F4acR+ en F4acR- varkens veroorzaakt door een verschil in APN eiwitexpressie?
3) Kan MBP gebruikt worden om antigenen af te leveren ter hoogte van het intestinaal mucosaal immuunsysteem?
4) Kan de orale toediening van poreuze pellets geladen met F4ac de immuunrespons verbeteren?

In **Hoofdstuk 3** onderzochten we of antilichamen tegen pAPN na orale toediening, op een gelijkaardige receptor-gemedieerde manier als F4ac fimbriae worden opgenomen door het intestinaal epitheel en een immuunrespons induceren. In eerste instantie werd de adhesie van de pAPN-specifieke antilichamen aan varkensenterocyten aangetoond, eerst *in vitro* met behulp van de BHK-pAPN cellijn, en vervolgens *in vivo*, via darmlussen. In een volgende stap werden biggen oraal geïmmuniseerd met opgezuiverde pAPN-specifieke antilichamen (anti-pAPN) of met opgezuiverd konijn IgG als negatieve controle. De orale toediening van anti-pAPN induceerde een sterke immuunrespons bij biggen en dat zelfs in de afwezigheid van het mucosaal adjuvans cholera toxine (CT). Sterke serum IgA, IgG en IgM responsen traden reeds op na een primaire immunisatie. Tevens werden negen dagen na de booster immunisatie, verhoogde hoeveelheden konijn IgG-specifiek IgA en IgG gevonden ter hoogte van de intestinale mucosa van de dieren geïmmuniseerd met anti-pAPN en anti-pAPN + CT. Konijn IgG-specifieke IgA secreterende cellen werden aangetroffen in de lamina propria van de dieren geïmmuniseerd met anti-pAPN en anti-pAPN + CT.

Deze resultaten samen met het feit dat APN aanwezig is in zowel het ademhalingsstelsel als het intestinaal epitheel van verschillende diersoorten, maken dat APN een veelbelovende kandidaat is voor de selectieve aflevering van antigenen ter hoogte van het mucosaal epitheel. Bij biggen is echter nog niet aangetoond welke epithelia van het spijsverterings- en

het ademhalingsstelsel APN tot expressie brengen. Deze informatie is noodzakelijk om deze mucosa te identificeren waar APN als doelwit zou kunnen gebruikt worden. In **Hoofdstuk 4** bestudeerden we de expressie van APN bij F4acR+ en F4acR- biggen. Verschillen in APN eiwitexpressie tussen F4acR+ en F4acR- biggen zouden het universeel gebruik van pAPN als doelwit voor immunisatie van biggen hypothekeren. Er kon echter geen verschil in pAPN eiwitexpressie aangetoond worden tussen F4acR+ en F4acR- biggen. Verschillen in glycosylaties van APN, in aan- of afwezigheid van een molecule die sterisch de adhesie van F4ac fimbriae aan de intestinale mucosa kan hinderen en/of de aanwezigheid van andere F4ac receptoren kunnen een verklaring geven voor het verschil tussen F4acR+ en F4acR- biggen. Een geleidelijke verhoging van pAPN expressie werd waargenomen van duodenum naar ileum. pAPN expressie was echter afwezig op het epitheel van de luchtpijp, longen, tong, slokdarm, maag, dikke darm (caecum en colon) en rectum. Als gevolg kan APN als doelwit gebruikt worden in de dunne darm maar niet in het ademhalingsstelsel en de dikke darm.

Een goed *in vitro* model is aangewezen voor de studie van zowel pAPN-gemedieerde opname en transcytose, als voor de zoektocht naar moleculen die binden aan deze receptor. De IPEC-J2 cellijn, een cellijn afkomstig van varkens dunne darmepitheel, is een goede kandidaat aangezien het een relevant model is voor darmepitheelcellen. Maar de cellen brengen van nature te weinig pAPN tot expressie voor bindingsstudies. Daarom werd in **Hoofdstuk 5** de transfectie van de IPEC-J2 cellen met pAPN beschreven. Met behulp van flow cytometerie werd de binding van polyklonaal konijn anti-pAPN serum aan de getransfecteerde cellen nagegaan. De succesvol getransfecteerde IPEC-J2 cellen (IPEC-J2-pAPN) werden vervolgens positief geselecteerd door gebruik te maken van een FACSAria celsorteerder en werden in continue cultuur gehouden. Bovendien werd een monoklonaal antilichaam tegen pAPN geproduceerd. Opname van pAPN-specifieke antilichamen door IPEC-J2-pAPN werd aangetoond, terwijl geen binding werd waargenomen bij gebruik van de IPEC-J2 cellen. Tijdens opname werd tevens een co-lokalisatie waargenomen van pAPN-specifieke antilichamen en clathrine bevattende vesikels, zoals waargenomen bij F4ac fimbriae. Dit impliceert dat de IPEC-J2-pAPN cellijn een veelbelovende middel is voor de *in vitro* studie van pAPN-gemedieerde binding en opname.

In het tweede luik van het experimenteel deel, werden twee bijkomende benaderingen aangewend voor de orale vaccinatie van biggen. Recente onderzoeken suggereren dat het maltose-bindend eiwit (MBP) de antigeenpresenterende functies in geïmmuniseerde dieren kan versterken door maturatie van dendritische cellen te induceren via TLR4. Het doel van het onderzoek in **Hoofdstuk 6** was om na te gaan of een MBP-specifieke immuunrespons opgewekt kan worden door orale toediening van MBP. In een eerste experiment werd de MBP-specifieke immuunrespons geanalyseerd na orale immunisatie van biggen met MBP of MBP + CT. Zowel de systemische als de mucosale antilichaamrespons werd onderzocht. In de MBP + CT groep werd een significante systemische respons gezien vanaf de tweede immunisatie. In de MBP groep was dit niet het geval, maar werd er wel een lokale MBP-specifieke IgM respons waargenomen ter hoogte van de jejunale Peyerse platen. In een tweede experiment werd MBPFedF oraal toegediend om na te gaan of MBP als drager van een ander antigeen kan functioneren voor inductie van een mucosale immuniteit tegen dat antigeen. FedF is het adhesine van de F18 fimbriae van porciene ETEC en verotoxigene *E. coli*. Een significante respons werd gevonden tegen MBP en een lage respons tegen FedF na de orale vaccinatie met MBPFedF + CT. De aanwezigheid van MBP-specifieke IgA secreterende cellen in de lamina propria duidde op de inductie van een lokale intestinale immuunrespons tegenover MBP. Onze resultaten suggereren dat MBP na orale vaccinatie doorheen de epitheelbarrière kan gaan en impliceren tevens dat MBP als carrier kan fungeren voor de aflevering van antigenen ter hoogte van het intestinaal immuunsysteem.

In **Hoofdstuk 7** werd een poreuze pellet bestaande uit Avicel PH 101 geëvalueerd voor orale immunisatie met F4ac fimbriae. De F4ac fimbriae kunnen binnendringen in de pellets aangezien de pellets uit een coherent poriënnetwerk bestaan. Dit kan bescherming bieden tegen zuren, gal, enzymen, antilichamen en andere glycoproteïnen die binden aan de fimbriae in maag en/of duodenum. Veertien biggen werden willekeurig verdeeld over 3 groepen en werden oraal geïmmuniseerd met F4ac fimbriae in PBS, F4ac fimbriae in pellets of enkel PBS. Twee weken na de laatste vaccinatie werden alle biggen geïnfecteerd met een virulente F4ac$^+$ ETEC stam. Hoewel het gebruik van de pellets een hogere mucosale en serum IgA titer veroorzaakte in vergelijking met de F4ac fimbriae in PBS, kon geen betere bescherming aangetoond worden. De F4ac$^+$ ETEC uitscheiding lag tot 6 dagen na de inoculatie lager in de pellet groep in vergelijking met de F4ac in PBS groep maar niet in

vergelijking met de PBS groep. De duur van de F4ac$^+$ ETEC uitscheiding was echter gelijk in beide geïmmuniseerde groepen en één dag korter dan voor de PBS groep. F4ac fimbriae zijn redelijk bestendig tegen zure pH. Bijkomende studies met zuur- gevoelige antigenen zijn noodzakelijk om na te gaan of de pellet in staat is om de antigenen te beschermen in maag en duodenum en af te leveren ter hoogte van de jejunale/ileale mucosa.

In het laatste hoofdstuk (**Hoofdstuk 8**) worden de algemene besluiten en toekomst- perspectieven overlopen. In dit proefschrift werd APN geïntroduceerd als veelbelovende kandidaat voor de aflevering van vaccin antigenen ter hoogte van het intestinaal mucosaal epitheel en aan antigeen-presenterende cellen. We besluiten dat APN als doelwit kan fungeren voor de aflevering van moleculen ter hoogte van de dunne darm, maar niet ter hoogte van de dikke darm of het ademhalingsstelsel. Geen verschil in APN expressie werd waargenomen tussen F4acR+ en F4acR- biggen waardoor de APN-gemedieerde aflevering van antigenen bruikbaar is in beide groepen. Zowel de IPEC-J2-pAPN cellijn als het monoklonaal tegen pAPN zijn nuttige tools voor verder onderzoek rond pAPN targeting. Bovendien suggereren onze resultaten dat ook MBP de epitheelbarrière kan passeren na orale immunisatie, wat impliceert dat het een potentiële carrier is voor aflevering van eiwitten aan het intestinaal mucosaal immuunsysteem.

# Curriculum vitae

Philippe Bellot werd geboren op 9 februari 1983 te Brussel. In 2001 beëindigde hij zijn secundaire opleiding, richting Wetenschappen-Wiskunde, aan het Sint-Pieterscollege te Jette. In hetzelfde jaar begon hij aan de studie Bio-ingenieur aan de Vrije Universiteit Brussel, waar hij in 2004 het diploma Kandidaat Bio-ingenieur behaalde met onderscheiding. In 2004 verhuisde Philippe naar de Universiteit van Gent om in 2007 het diploma van Bio-ingenieur in de cel- en genbiotechnologie met onderscheiding te behalen. Vervolgens trad hij in dienst aan het Laboratorium voor Immunologie waar hij in 2008 een doctoraatsbeurs ontving van het agentschap voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen (IWT-Vlaanderen). Het onderzoek spitste zich toe op nieuwe strategieën voor de orale immunisatie van biggen en werd uitgevoerd onder leiding van Prof. Dr. E. Cox. Philippe Bellot is auteur en medeauteur van wetenschappelijke publicaties in internationale tijdschriften.

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**Bellot P.**, Ahasan L.A.S.M., Melkebeek V. and Cox E. Aminopeptidase N protein expression is not a determining factor for the F4ac receptor status of pigs, in preparation.

Melkebeek V.*, Rasschaert K.*, **Bellot P.***, Tilleman K., Favoreel H., Deforce D., De Geest B. G., Goddeeris B. M. and Cox E. (2012). Targeting aminopeptidase N, a newly identified receptor for F4ac fimbriae, enhances the intestinal mucosal immune response. Mucosal Immunology, 5(6): 635-645. * These authors contributed equally to this work.

**Bellot P.***, Tiels P.*, Melkebeek V., Devriendt B., Goddeeris B. M. and Cox E. (2012). "Maltose-binding protein is a potential carrier for oral immunizations." Vet Immunol Immunopathol. doi: 10.1016/j.vetimm.2012.09.013. * These two authors contributed equally to this work.

# Abstracts en posters

**Bellot, P.**, Tiels, P., Melkebeek, V., and Cox, E. (2011). Maltose-binding protein is a potential carrier for oral immunizations. The 15th International congress of Mucosal Immunology, abstract and poster, 5-9 July, Paris, France.

Cox, E., **Bellot, P.**, and Melkebeek, V. (2011). Aminopeptidase N is a target for a very rapid IgA response upon a primary oral immunization. The 15th International congress of Mucosal Immunology, abstract and poster, 5-9 July, Paris, France.

**Bellot, P.**, Melkebeek, V., and Cox, E. (2011). Maltose-binding protein (MBP) is a potential carrier for oral immunizations. ECMIS: Escherichia coli and the Mucosal Immune System: interaction, modulation and vaccination, abstract and poster, 2-5 July, Ghent, Belgium.

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**Bellot, P.**, Cosijns, A., Stuyven, E., Vervaet, C., Remon, J. P., and Cox, E. (2008). Porous pellets as carriers of F4 fimbriae used for oral vaccination of suckling piglets against enterotoxigenic *Escherchia coli* infections. The first International conference Modern Mucosal Vaccines, Adjuvants and Microbicides, abstract and poster, 22-24 Oct., Porto, Portugal.

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