Expert Opinion

Designing oral vaccines targeting intestinal dendritic cells

Bert Devriendt, Bruno G De Geest† & Eric Cox
†*Ghent University, Laboratory of Pharmaceutical Technology, Belgium*

Introduction: Most pathogens colonize and invade the host at mucosal surfaces, such as the lung and the intestine. To combat intestinal pathogens the induction of local adaptive immune responses is required, which is mainly achieved through oral vaccination. However, most vaccines are ineffective when given orally owing to the hostile environment in the gastrointestinal tract. The encapsulation of antigens in biodegradable microparticulate delivery systems enhances their immunogenicity; however, the uptake of these delivery systems by intestinal immune cells is rather poor. Surface decoration of the particulates with targeting ligands could increase the uptake and mediate the selective targeting of the vaccine to intestinal antigen-presenting cells, including dendritic cells.

Areas covered: In this review, current knowledge on dendritic cell subsets is discussed, along with progress in the development of selective antigen targeting to these cells, in addition to focusing on data obtained in mice and, where possible, the pig, as a non-rodent animal model for humans. Moreover, the potential use and benefits of Fcγ receptor-mediated targeting of antigen delivery systems are highlighted.

Expert opinion: In conclusion, dendritic cell targeting ligands grafted on antigen carrier systems should preferably bind to a conserved endocytotic receptor, facilitating the design of a multispecies vaccine platform, which could elicit robust protective immune responses against enteric pathogens.

Keywords: antigens, dendritic cells, ligands, particles, vaccines

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1. Introduction

Vaccine development is a rapidly expanding research area, as vaccination is regarded as the most effective way to control infectious diseases. Indeed, with the exception of water sanitation, only vaccination has spared millions of individuals, mainly children, from the morbidity and mortality associated with infectious diseases [1]. However, most infectious diseases are caused by pathogens that colonize and invade the host at mucosal surfaces and require the induction of pathogen-specific secretory IgA (SIgA) at the site of infection for an effective protection of the host. In spite of this, the vast majority of commercial vaccines are directed against systemic pathogens, and parenteral immunization with these vaccines generally does not induce a pathogen-specific mucosal immunity owing to the systemic homing specificity of effector lymphocytes activated in the peripheral lymph nodes. Therefore, vaccines should be delivered to the intestinal mucosa by means of the oral route to induce a protective immunity against intestinal pathogens, especially for non-invasive pathogens, such as enterotoxigenic *Escherichia coli* (ETEC), in which vaccine-induced protection is mediated almost exclusively by locally produced SIgA antibodies [2,3].

The oral route for vaccine delivery, however, is the most challenging and difficult to achieve, and progress in oral vaccine development has been rather slow. Indeed, inactivated vaccines are sometimes ineffective and although vaccines containing live

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microorganisms (attenuated or vectors) are more effective, they carry the risk of reversion to virulence and the induction of disease in immunocompromised individuals. Therefore, new vaccines consisting of protein antigens are now being developed. However, the effectiveness of these vaccines is hampered owing to the physiological and immunological barriers posed by the gastrointestinal tract. For example, proteins have to survive the low gastric pH and degradation by proteolytic enzymes present in the gastrointestinal tract. A promising strategy to overcome these obstacles is the entrapment of vaccine antigens in biodegradable particulate delivery systems, which can protect them against the gastrointestinal degradation and interference by the lactogenic immunity, such as neutralizing antibodies and milk factors. Other problems associated with the oral route of vaccine delivery are the poor transport of antigens across the intestinal epithelium to reach the underlying gut-associated lymphoid tissue (GALT) and the induction of oral tolerance instead of protective immunity by the GALT. The intestinal mucosa continuously encounters a myriad of foreign antigens, including innocuous food antigens, commensal microflora and a variety of microbial, viral or fungal pathogens, and has the complex task of responding to the vast numbers of signals generated by these antigens. As a consequence, the intestinal immune system has to fulfil two opposing functions in normal physiological conditions. On the one hand, potent immune responses are required against pathogens to prevent infection and on the other hand the intestinal mucosa has to be immunologically hyporesponsive to innocent food antigens and commensals. Accordingly, the default response to harmless gut antigens is a local and systemic immunological tolerance, known as oral tolerance [4,5]. Indeed, to be effective as a vaccine, antigens have to activate the innate immune system and subsequently evoke intestinal adaptive immune responses. However, oral administration of antigens may induce immunosuppressive mechanisms, resulting in a state of immunological hyporesponsiveness towards the administered antigen [2,4]. The current data suggest that specific intestinal dendritic cell subpopulations, which are abundant in the GALT, may be specialized in the induction of this non-inflammatory environment [6].

A single layer of columnar intestinal epithelial cells provides a physical barrier separating the luminal environment from the underlying mucosal immune cells. Although the intestinal epithelium seems impervious to luminal antigens, several antigen-sampling mechanisms exist, which allow the intestinal immune system to monitor constantly the luminal environment [7,8]. Antigen-sampling routes could be exploited to enhance uptake and transport of particulate antigen formulations by the intestinal epithelium, resulting in improved access of the immune cells present in the GALT to the vaccine antigens. Understanding these mechanisms is important in oral vaccination, as one could exploit these mechanisms for enhanced antigen uptake. Targeting of antigen-loaded delivery systems to epithelial receptors could enhance the uptake and transepithelial transport of antigens and could potentially overcome the induction of oral tolerance because receptor-mediated endocytosis mostly induces antigen-specific mucosal immune responses. In addition, the incorporation of mucosal adjuvants in particulate delivery systems could further enhance the activation of the intestinal immune system. In the following sections, the different antigen-sampling mechanisms in the gut are reviewed, and the distribution and function of small intestinal dendritic cells (DCs) under steady-state conditions are reviewed; possible strategies to target these intestinal DCs are discussed, with an emphasis on data obtained in murine and porcine animal models.

2. Antigen sampling in the gut

In the steady-state, with an intact intestinal barrier, four mechanisms for antigen uptake have been described (**Figure 1**). Perhaps the best known is antigen uptake by M cells present in the follicle-associated epithelium (FAE), overlying the B-cell follicles of the Peyer's patch (PP) [9]. M cells have been identified in varying proportions in the FAE of a wide variety of species, such as laboratory, domestic and farm animals and man. These highly specialized M cells have some unique structural and functional features that facilitate the endocytosis and transport of macromolecules and bacteria. In contrast to enterocytes, M cells lack an extensive mucus layer, display a poorly organized brush border with irregular shaped microvilli, and have a thin glycocalyx and a reduced enzymatic activity [10-12]. In addition, the basolateral membrane is invaginated to form an intraepithelial pocket, in which lymphocytes and antigen-presenting cells (APCs) are present, which allows a rapid transfer of antigens to immune cells (**Figure 1A**) [13]. Furthermore, Jang *et al.* [14] observed that M cells are scattered in the absorptive epithelium, where they could transport antigens to the lamina propria (LP). Nonetheless, the number of M cells is limited and thus it seems likely that further mechanisms of antigen uptake are important. Intriguingly, their numbers can be rapidly increased in inflammatory conditions and on bacterial stimulation, along with an enhanced transcytotic capacity, although the cellular and molecular mechanisms for this phenomenon remain elusive [15-18].

A second mechanism of sampling was initially described by Rescigno *et al.* [19]. In the lamina propria, dendritic cells can sample both commensal and pathogenic bacteria from the gut lumen by extending dendrites through the intestinal epithelium without disturbing the integrity of the barrier through the expression of tight junction proteins, such as occludin, claudin and zonula occludens (**Figure 1B**) [19]. These processes appear to form 'balloon bodies' on reaching the gut lumen, as visualized by two-photon microscopy [20]. The formation of these sampling dendrites appears to involve the epithelial secretion of CX_3CL1 (fractalkine), as in $CX_3CR1^{-/-}$ mice DCs failed to form dendrites. These mice also showed impaired bacterial uptake and an enhanced susceptibility to

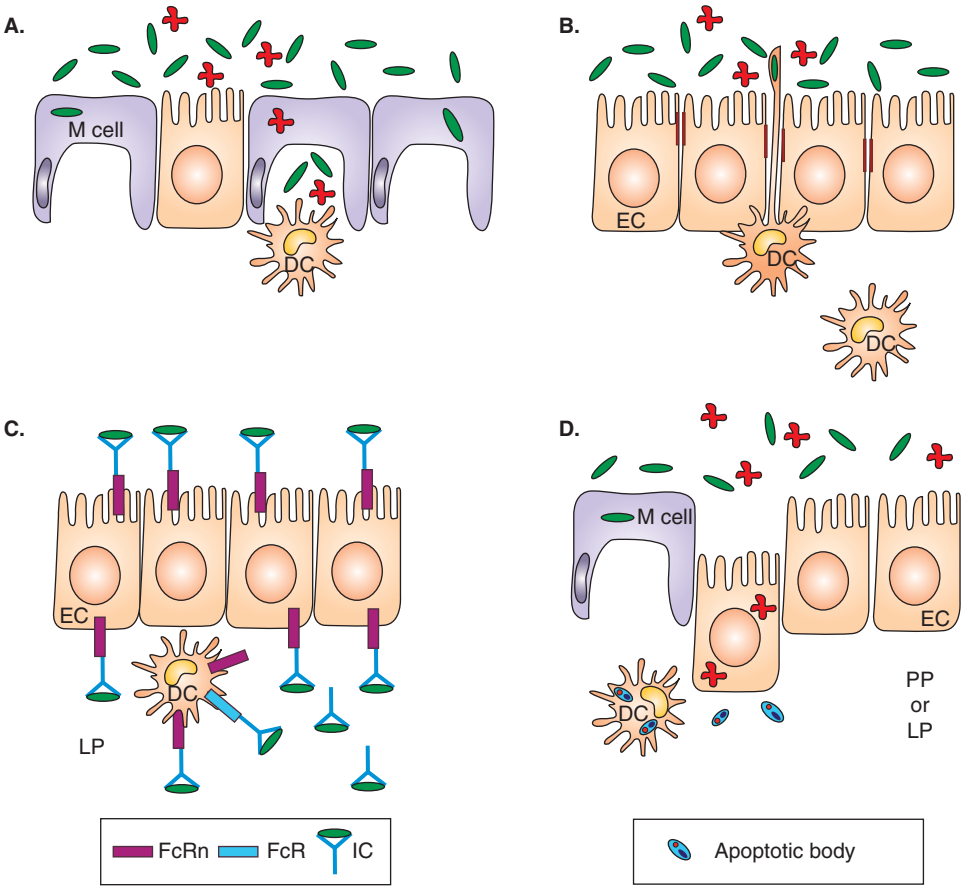

Figure 1. A. M cells can transport antigens directly to the underlying DCs, whereas **B.** DCs can extend dendrites between intestinal ECs to sample luminal antigens. **C.** The FcRn mediates bidirectional transport of IgG, resulting in transport into the lumen and trafficking back to the LP of Ag–Ab complexes. **D.** Antigens associated with apoptotic epithelial cells can be endocytosed by DCs in the steady-state or after viral infection.

DCs: Dendritic cells; ECs: Epithelial cells; FcRn: Neonatal Fc receptor; IC: Immune complex; LP: Lamina propria; PP: Peyer's patch.

Salmonella infection [21]. This model was, however, opposed by Chieppa *et al.* [20], who demonstrated that CX_3CR1^- cells could also form sampling dendrites. Nevertheless, it seems that epithelial Toll-like receptor (TLR) signaling is the driving force behind the formation of these dendrites because in $MyD88^{-/-}$ mice as well as in mice subjected to oral antibiotic treatment the induction of these sampling dendrites is diminished [20,21]. In pigs, Bimczok and co-workers [22] also identified DCs protruding their dendrites across the small intestinal epithelium, although they suggested this was a rare event under steady-state conditions.

A third mechanism involves the neonatal Fc receptor (FcRn), which is expressed by adult enterocytes of the human and porcine intestine [23,24]. This receptor transports IgG across the intestinal epithelial barrier and, after binding its cognate antigen in the lumen, recycles these antigens as immune complexes back to the LP, where they are delivered to DCs (**Figure 1C**) [25]. Finally, DCs are able to endocytose apoptotic bodies from epithelial cells both in the steady-state as well as following viral infection and, on processing, present these to T cells in the mesenteric lymph nodes (**Figure 1D**) [26,27].

Although these mechanisms seem important for the surveillance of the intestinal lumen and M cells are generally considered as the gateways for antigen entry to the GALT [28], the importance of enterocytes should not be overlooked because these cells vastly outnumber M cells and possess a transcytotic capability for macromolecules, such as cholera toxin (CT) and F4 fimbriae, and inert particles [29-31]. Moreover, enterocytes are able to phagocytose and transcytose bacteria across the epithelial barrier in a TLR4-mediated fashion [32]. Thus, targeting to enterocytes may provide an easier and more effective approach for intestinal vaccine delivery, although the potential for inducing adaptive immunity following endocytosis by enterocytes is unclear.

3. Dendritic cells in the gut

Dendritic cells represent the most potent antigen-presenting cells, which play a crucial role in orchestrating both innate

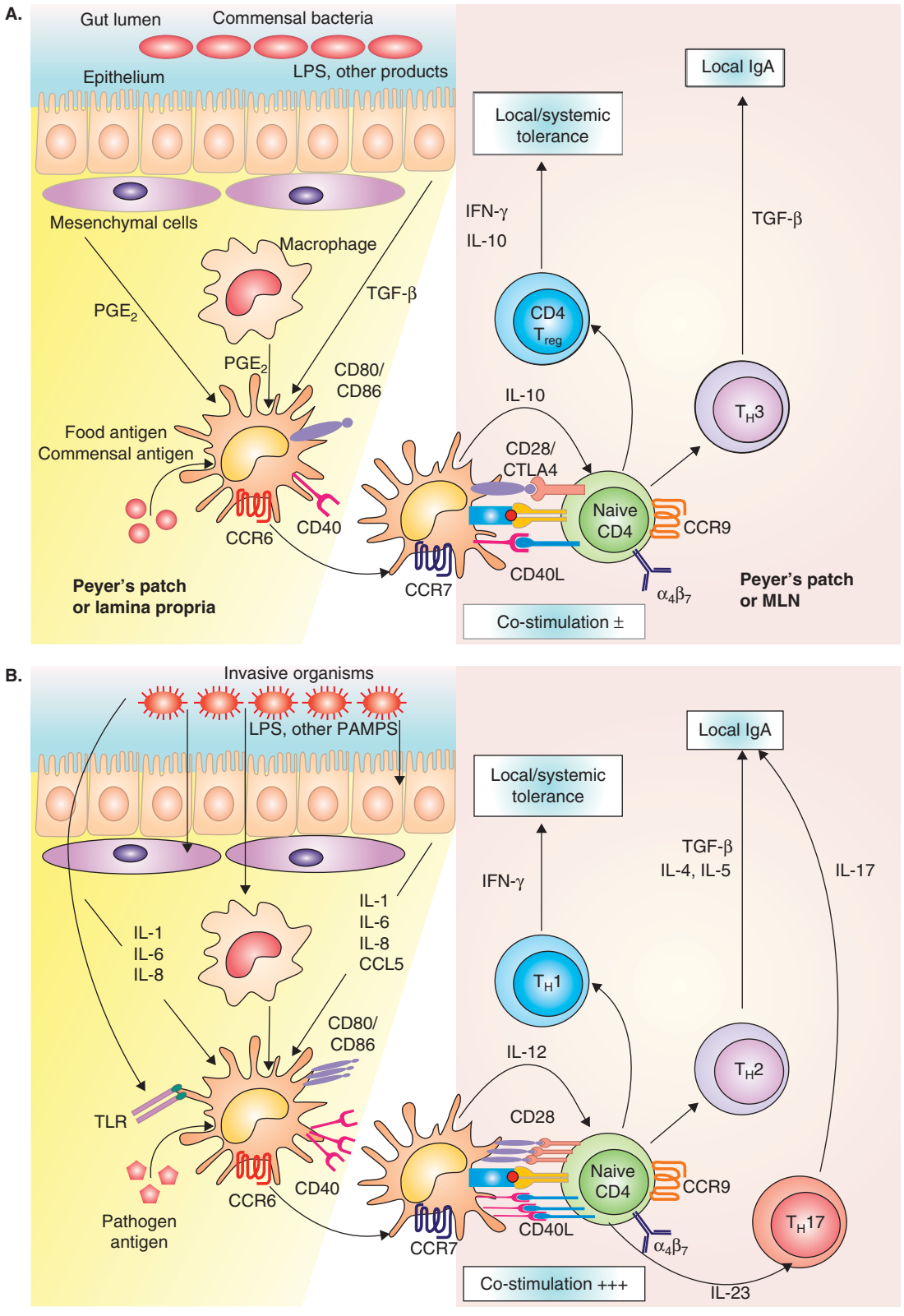

Figure 2. Model of intestinal DC maturation and T-cell polarization during the steady-state and inflammatory conditions (continued).

and adaptive immune responses and are therefore of pivotal importance for the induction of immune responses to control and eliminate pathogen infections [33]. Under steady-state conditions, circulating DC precursors continuously enter the mucosal LP, PP and mesenteric lymph node (MLN), where they differentiate into immature DCs. Although the nature of these DC precursors remains obscure, DC migration into these different intestinal regions is dictated by the constitutive expression of chemokines, such as CCL9, CCL19, CCL20 and CCL21, by the local microenvironment [34]. At these mucosal tissues, immature DCs are located at the main sites of pathogen entry, where they act as the sentinels of the immune system by continuously sampling antigens from their environment by the aforementioned pathways [35]. On antigen encounter and processing, these DCs undergo a complex maturation process, which involves not only the translocation of MHC class II (MHCII) from an intracellular pool to the cell surface and the upregulated expression of several costimulatory molecules, such as CD40, CD80 and CD86, but also a reduced Ag internalization capacity [34,36]. Concomitant with this maturation, Ag-loaded DCs upregulate the expression of CCR7, which is required for the migration of DCs to the MLN [37,38]. In the T-cell areas of the local lymph nodes, mature DCs have the unique feature of activating naive T cells through the secretion of T-cell polarizing cytokines and chemokines, which subsequently can influence the outcome of the ensuing immune responses (**Figure 2**) [2,33]. In the absence of inflammation, the endocytosis of harmless antigens by DCs results in their partial maturation. These semi-mature DCs induce T cells to differentiate into either tolerizing T cells (T_H3) or regulatory T cells (T_{reg}) owing to the secretion of IL-10. It seems that the phenotype of these semi-mature DCs is in part influenced by local factors such as prostaglandin E_2 (PGE_2) and TGF-β produced by intestinal epithelial cells. By contrast, in the face of infection, TLR recognition of pathogens by intestinal epithelial cells and DCs results in complete maturation of the latter, and activation of pathogen-specific effector T cells (T_H1, T_H2 and/or T_H17) will occur, leading to clearance of the pathogen. However, DC function is not restricted solely to providing antigenic and co-stimulatory signals to lymphocytes; they also can educate these lymphocytes to come back to the mucosal LP by upregulating chemokine and homing molecules [39-42]. Although several DC subsets have been identified that display unique functions, which are in part dictated by the local microenvironment, DCs are nevertheless characterized by a high functional plasticity [43,44]. The intestinal immune system contains an extensive network of DC subsets, which induce either protective immunity to pathogens or tolerance to innocuous antigens, including food antigens and commensal bacteria. Knowledge on the distribution of the distinct DC subtypes in the intestine and their functions is fundamental to targeting Ag to the proper DC subpopulation.

3.1 Distribution of dendritic cell subsets in the intestine

Dendritic cells are found throughout the intestine, including the LP of the small and large intestine, PP and MLN, and in these locations several DC subsets, which display unique functions, have been described in mice (**Table 1**) [43-47]. Despite the description of these different subsets, it seems that small intestinal LP DCs can be divided in two major subsets, which can be distinguished based on the expression of CD103 (the α_E chain of the $\alpha_E\beta_7$ integrin), the receptor for the epithelial cell adhesion molecule E-cadherin, and CX_3CR1 [44,48,49]. $CD103^+$ DCs are also present in the MLN and these presumably arise from migratory $CD103^+$ LP DCs, as their numbers were reduced in CCR7-deficient mice [50].

Although in mice much work has been done to characterize intestinal DC subpopulations and elucidate their immune functions, the distribution of DC subpopulations in the porcine intestine has been explored only recently [51]. Since the initial characterization of immature $MHCII^+$ DCs within the porcine jejunal LP and PP regions [52,53], which induced proliferation in mixed leukocyte reactions, recent studies have expanded our knowledge of porcine DC subsets within the various intestinal anatomical locations [22,54,55]. In fact, four intestinal $MHCII^+$ DC subsets were detected based on the expression of signal immunoregulatory protein α (SIRPα) and CD11R1, which is the porcine homologue of human CD11b [54,56]. LP DCs were mainly $CD11R1^+/SIRP\alpha^+$ and were rarely observed within the epithelium. By contrast, PP DCs were predominantly $CD11R1^-/SIRP\alpha^+$ in the

Figure 2. Model of intestinal DC maturation and T-cell polarization during the steady-state and inflammatory conditions (continued). A. Food proteins and commensal bacteria are endocytosed by dendritic cells, which in the absence of inflammation and under the influence of PGE_2, TGF-β and IL-10 results in the partial maturation of PP or LP DCs. These semi-mature DCs then present antigen to naive $CD4^+$ T cells in the MLN or PP, which will differentiate into either regulatory T cells (T_{reg}) and/or tolerogenic T_H3 cells. The immunological consequences are local IgA production, systemic tolerance and intestinal immune homeostasis. **B.** When pathogens are encountered, local inflammation is induced through the recognition of pathogens by TLRs expressed by DCs, macrophages and intestinal epithelial cells. This results in the complete maturation of DCs with a concomitant secretion of IL-12. Following migration to the MLN, these mature DCs prime T_H1, T_H2 and/or T_H17 cells, leading to clearance of the pathogen.

DCs: Dendritic cells; LP: Lamina propria; MLN: Mesenteric lymph node; PP: Peyer's patches; PAMP: Pathogen-associated molecular pattern; PGE_2: Prostaglandin E_2; TLR: Toll-like receptor.

Table 1. Dendritic cell distribution and function in the murine intestine.

Dendritic cell subset	Localization	Function
PP		
CX_3CR1^+	SED	Unknown
$CCR6^+$	SED	T_H1-polarizing ability
$CD11c^+CD11b^+CD8\alpha^-$	SED	T_H2-polarizing ability; IgA class switching
$CD11c^+CD11b^-CD8\alpha^+$	IFR	T_H1-polarizing ability
$CD11b^-CD8\alpha^-B220^-$	SED	T_H1-polarizing ability
Small intestinal LP		
$CD11c^+CD11b^+CD103^+$		T_{reg}-polarizing ability
$CD11c^+CX_3CR1^+CD70^+CD11b^+CD103^-$		T_H17-polarizing ability; transepithelial dendrites
$CD11c^+CD11b^+TLR5^+$		T_H17-polarizing ability; IgA class switching
$CD11c^+iNOS^+TNF\alpha^+$		IgA class switching
MLN		
$CD11c^+CD103^+$		T_{reg}-polarizing ability; gut-homing T-cell imprinting
$CD11c^+CD103^-$		T_H stimulatory

Details in the table were derived from [44,164].

IFR: Interfollicular region; iNOS: Inducible nitric oxide synthase; LP: Lamina propria; MLN: Mesenteric lymph node; PP: Peyer's patches; SED: Subepithelial dome; T_H: T helper cell; T_{reg}: Regulatory T cell.

subepithelial dome (SED), where they are situated directly adjacent to M cells and $CD11R1^-/SIRP\alpha^-$ in the interfollicular region (IFR), whereas MLN DCs consist of a large $CD11R1^+/SIRP\alpha^-$ and a minor $CD11R1^+SIPR\alpha^+$ population [54,57]. Following pseudo-afferent cannulation of the intestinal lymph duct only the $CD11R1^+/SIRP\alpha^+$ and $CD11R1^+/SIRP\alpha^-$ DC subsets were detected, indicating that DCs mainly migrate from the LP to the MLN. Although these cells expressed high levels of MHCII and CD80/86, they were unable to induce peripheral blood mononuclear cells (PBMC) proliferation, indicating that these semi-mature steady-state migrating DCs could be involved in the induction of oral tolerance to food antigens or commensal bacteria [54].

3.2 Unique functions of intestinal dendritic cells

Intestinal DCs have been proposed to be involved in the induction of protective immunity to pathogens, tolerance to commensal bacteria as well as tolerance to food antigens and self-antigens [34], and accumulating evidence supports the concept that intestinal DC subsets possess unique functions that are optimally suited for these different tasks (**Table 1**). However, it should be noted that intestinal dendritic cells can display a significant phenotypical and functional plasticity, depending on the nature of the encountered antigen or pathogen and the local microenvironment.

$CD11c^+CD103^+CX3CR1^-$ LP DCs are the main migratory DC subset to the MLN and are involved in the regulation of immune responses. Both $CD103^+$ LP and MLN DCs drive the *de novo* conversion of naive T cells into $Foxp3^+$ regulatory T cells in a TGF-β- and retinoic acid (RA)-dependent manner [41,58,59]. $CD103^+$ cells are also found in human MLN, probably reflecting migratory DCs from the LP, and have recently been shown to induce T_{reg} differentiation [60,61]. Retinoic acid can be derived from dietary vitamin A through conversion by retinal dehydrogenases, which are expressed in both DCs and enterocytes [62,63]. Recently, $CD103^+$ MLN DCs were shown to lose their tolerogenic capacity, indicating that this subset can adapt to environmental cues [64].

As noted above, $CD103^-$ LP DCs are involved in antigen sampling through the formation of transepithelial dendrites, which depends on epithelial TLR engagement and requires the expression of CX_3CR1, particularly in the terminal ileum, where the ligand for this receptor is abundantly expressed [20,21,65]. Both invasive and non-invasive bacteria are sampled by LP DCs through this process, suggesting a possible role for these cells in presenting both commensal and pathogenic bacterial antigens to naive T cells in the MLN [19-21,65]. Although *in vitro* CX_3CR1^+ DCs preferentially support the differentiation of T_H1 and T_H17 cells, recent *in vivo* data indicate that small intestinal CX_3CR1^+ LP APCs are a non-migratory cell population, which are phenotypically indistinguishable from macrophages. It was hypothesized that these CX_3CR1^+ LP APCs could transfer antigen to DCs or modulate the microenvironment to control antigen uptake or DC migration [65-67].

In mice, T_H17 cells are present in the LP and have been implicated in the generation of protective immunity to pathogenic *Citrobacter*, *Klebsiella* and *Streptococcus pneumoniae* infection [68-71]. The differentiation of these T_H17 cells also depends on the presence of other DC subsets, such as $CD70^+CX_3CR1^+$ and $CD11c^+CD11b^+TLR5^+$ [72,73]. Intriguingly, $CD11c^+$ LP DCs either express low TLR levels in comparison with DCs from other intestinal locations or are refractory to stimulation with most TLR ligands, implying that these $TLR5^+$ LP DCs may still be responsive to flagellated pathogens and are important for the induction of intestinal adaptive immunity [73,74]. In pig, $CD11R1^+$ LP DCs are functionally mature in response to TLR5 stimulation,

implying that a similar mechanism could be at play in the porcine small intestinal LP [75].

Besides T-cell activation, intestinal DCs also induce B-cell activation. In the gut, commensals are prevented from reaching the lymphoid tissues by the induction of commensal-specific SIgA, which mediates immune exclusion. Peyer's patch DCs mediate B-cell IgA class switching through the secretion of IL-10, TGF-β, IL-6 and RA, which are all required in IgA class switching [42,76,77]. In the PP, T-cell-dependent IgA class switching is regulated by TNF-α/inducible nitric oxide synthase (iNOS)-producing $CD11b^+$ DCs (TipDC), whereas in the LP a DC subset expressing these two factors also seems to favor IgA class switching [78]. These intestinal DCs express iNOS on TLR activation by microbial ligands. This iNOS expression allows intestinal DCs to produce nitric oxide, which not only enhances the secretion of the IgA switch factors, a proliferation-inducing ligand (APRIL) and B-cell-activation factor of the TNF family (BAFF), by DCs, but also the secretion of TGF-β by T cells. In addition, nitric oxide upregulates the expression of the TGF-β receptor on B cells [78,79]. In addition, the $TLR5^+$ LP DC subset is also involved in RA-dependent IgA class switching on flagellin stimulation [73]. Moreover, PP DCs can induce B cells to undergo IgA switching independently from T cells, a process that is mediated by the production of IL-5, IL-6 and RA [42].

Intestinal DCs have an important role in dictating the homing potential of recently activated lymphocytes. For example, PP, MLN as well as small intestinal LP DCs promote the expression of the gut-homing receptors $\alpha_4\beta_7$ integrin and CCR9 on T and B cells [39,40,50]. In the MLN, the ability to imprint the expression of these gut-homing receptors on $CD4^+$ and $CD8^+$ T cells resides within the $CD103^+$ DC subset and depends on the ability of these DCs to convert vitamin A to RA [41,50]. CCR9 binds to CCL25 produced by small intestinal epithelial cells (IECs) [80], whereas $\alpha_4\beta_7$ binds to MAdCAM-1 expressed on the intestinal vascular endothelium of the LP and PP [81]. Both MAdCAM-1 and CCL25 are constitutively expressed, implicating constitutive migration of T and B cells to the small intestinal LP. Thus, it appears that intestinal DCs utilize the vitamin A metabolic pathway to educate T and B cells for gut-homing migration. Although imprinting of intestinal lymphocyte homing by intestinal DC has not been reported in pigs, tissue-specific homing does occur and both the small intestinal-specific expression of CCL25 and CCR9 as well as the strong expression of $\alpha_4\beta_7$ on intestinal T and IgA^+ B cells have been confirmed [82,83].

3.3 Influence of the local microenvironment on dendritic cell function

Recent evidence indicates that conditioning of DCs in their local microenvironment has an important role in shaping DC function and the ensuing immune response. An extensive crosstalk between intestinal IECs, innate immune cells and DCs is likely to be an integral part of this regulation. Indeed, IECs, once considered as merely providing a physical barrier to the external environment, are pivotal in the regulation of adaptive immunity and intestinal homeostasis. Human IECs secrete immunoregulatory mediators, including TGF-β, RA and thymic stromal lymphopoietin (TSLP), which drive the development of non-inflammatory or tolerogenic DCs, necessary for maintaining immune homeostasis [84-86]. TLSP is constitutively expressed by IECs, inhibits IL-12p70 production by DCs on bacterial stimulation and drives the development of T_H2-polarizing DCs [85]. Studies by Zaph *et al.* [87] have confirmed the significance of IEC-derived TSLP in regulating intestinal immune responses *in vivo*. In addition, exogenous RA imprints a mucosal phenotype on porcine and human monocyte-derived dendritic cells (MoDC) [60,88]. The latter expressed CD103 and inhibited the development of both T_H1 and T_H17 cell differentiation [60]. Similarly, RA-treated porcine MoDC induced the expression of gut-homing molecules on T cells, secreted TGF-β and promoted IgA responses [88]. Furthermore, RA induced the expression of retinal dehydrogenases, and inhibition of this enzyme in RA-treated DCs abrogated their ability to elicit mucosal homing receptors on T cells [51]. Thus, it seems that in the steady-state these IEC-derived factors act synergistically to imprint a tolerogenic intestinal phenotype on DCs. These IEC-educated DCs are irreversibly blocked in their tolerogenic phenotype [85], implying that the induction of protective immune responses against pathogens would require IECs to recruit unconditioned DCs either from circulating monocytes or intestinal DCs located beyond the local influence sphere of the intestinal epithelial cells, to generate the so-called inflammatory-type DCs [89].

IECs are the first cells to encounter intestinal pathogens and they can act as immunological sensors by recognizing pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRR), including TLR and NOD-like receptors (NLR) [90,91]. On PRR ligation, epithelial cells secrete pro-inflammatory cytokines and chemokines, alerting underlying mucosal cells, such as DCs, macrophages and neutrophils. This results in their recruitment and activation, culminating in the induction of innate defences and adaptive immune responses, ultimately leading to the eradication of the infectious agent. PAMPs are, however, shared by both pathogens and commensals, but IECs can respond differently to commensals and pathogenic bacterial species in terms of their cytokine secretion profile. For example, on TLR5 ligation by flagellin from pathogenic but not from commensals, IECs secrete the chemokine CCL20, which in turn recruits pro-inflammatory $CCR6^+$ DCs to the intestinal epithelium [92]. Similarly, porcine IECs respond to flagellin by secreting pro-inflammatory cytokines and chemokines [93]. Interestingly, incorporation of chemokines such as CCL20 in biodegradable microspheres resulted in DC migration through a collagen matrix *in vitro*, implying that this strategy could be used to attract inflammatory-type DCs to the vaccination site *in vivo* [94].

Beyond the conditioning of DCs, epithelial cells may directly interact with intestinal lymphocytes to modulate the immune response. For example, IECs secrete APRIL and BAFF, which supports T-cell-independent IgA class switching in the LP. In addition, TSLP favors the release of APRIL and BAFF by IEC-conditioned DCs and may exert direct effects on B cells [95-97]. BAFF is also expressed by porcine dendritic cells and presumably provides a survival signal for activated memory B cells in a T-cell-independent fashion [98].

Epithelials are not solely responsible for the modulation of DC function; macrophages also appear to fine-tune the inflammatory potential of DCs [99]. Furthermore, other innate immune cells, such as eosinophils, neutrophils, mast cells and NK cells, could be activated by IEC-secreted pro-inflammatory cytokines or directly on pathogen detection (the PRR repertoire expressed by these cells is largely unknown) [100]. Following their activation, these cells could in turn secrete pro-inflammatory cytokines and chemokines, thereby sustaining the local inflammatory environment. This could result in an enhanced recruitment and activation of DCs, amplifying their activation and modulating their functions.

4. Antigen targeting to dendritic cells

In spite of the tolerogenic intestinal environment under steady-state conditions, in the face of infection, DCs are still the central and key players for the induction of efficacious adaptive immunity [101]. Thus, the efficacy of vaccination will depend on the efficient interaction of the vaccine with DC subsets. This interaction can be enhanced by targeting vaccines to DC surface molecules. Ideally, this DC receptor should be as DC-specific as possible to reduce unwanted side effects resulting from binding to other cells. Unfortunately, there are very few, if any, DC-specific markers. Nevertheless, these cells possess a broad spectrum of cell surface receptors involved in endocytosis and the induction of immune responses, such as C-type lectins, scavenger receptors, TLRs and FcR (FcγR, FcαR and FcεR, which bind IgG, IgA and IgE, respectively) (**Figure 3**) [102].

These receptors offer the potential to target vaccines to DCs. However, depending on the specific receptor being targeted, the outcome of the ensuing immune response can differ. Moreover, the expression of these receptors can vary between DC subsets and their location. In general, ligation of PRR elicits DC activation with enhanced Ag internalization, the induction of pro-inflammatory cytokine secretion and the upregulation of co-stimulatory molecules. Indeed, TLR ligands deliver a strong activation signal to DCs, which forms the basis for their potent adjuvant properties. By contrast, the C-type lectin receptor DEC-205 and the mannose receptor are more involved in enhanced endocytosis, although DC activation has been achieved with DEC-205 targeting [103].

4.1 Antibody-mediated targeting

A simple and straightforward approach for DC targeting is the application of antibodies specific for DC receptors. Several studies of antibody-mediated targeting of antigen to DC receptors have been performed in murine models for vaccine delivery [104,105]. One of the most studied receptors for DC targeting encompasses DEC-205. Antibody-mediated targeting of antigen to DEC-205 enhanced antigen presentation [103], and in the presence of a strong DC maturation stimulus (such as anti-CD40 or TLR ligands), this strategy permits the induction of effective cellular and humoral immune responses to antigens conjugated to the anti-DEC-205 mAb [106-109]. However, in the absence of a DC maturation stimulus, DEC-205 targeting generated T_{regs}, resulting in the induction of tolerance to conjugated antigens [106,110]. This approach has been used recently to prevent the onset and progression of autoimmune diseases, by targeting autoantigens to DEC-205 in experimental mouse models of type I diabetes [111].

Similar to DEC-205, antigen targeting to other DC surface molecules such as Dectin-1, the mannose receptor and Clec9A requires the co-administration of adjuvants to evoke effective cellular immunity [112-114]. In contrast to Dectin-1 and the mannose receptor, targeting to Clec9A elicits strong antibody responses in the absence of adjuvants [115]. Clec9A is a C-type lectin-like molecule with a higher specificity for murine and human DCs than most markers and functions as a recognition receptor for dead cells [105,115]. Hence, it seems that targeting to Clec9A directs antigens to cell compartments involved in the efficient processing of exogenous antigens.

Another example of antibody-mediated DC targeting is found with the C-type lectin DC-SIGN (DC-specific intercellular adhesion molecule-3 grabbing non-integrin), which has been used to target vaccines by means of specific antibodies in both murine and non-human primate models [116,117]. Moreover, retroviral vectors gain improved immunogenicity if the viral envelope is engineered to target DC-SIGN on DCs [118].

Siglecs (sialic acid-binding Ig superfamily lectin) represent a family of immune cell receptors that bind specific sialic acid-containing carbohydrate structures on different pathogens, thereby increasing their endocytosis or phagocytosis. Many members have been identified in mouse, human and swine (Siglec-1, -2, -5, -7, -10, -15 and -H) [119]. Antibody-mediated antigen targeting to Siglec-H on murine DC and Siglec-5 on human monocytes resulted in enhanced endocytosis [120,121]. However, most members of the Siglec family are inhibitory receptors either carrying cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIM) (Siglec-7 and -10) or recruiting adaptor proteins, which deliver inhibitory signals to the cell (Siglec-H and -15), making their application in vaccine delivery questionable. In contrast to these inhibitory Siglecs, Siglec-1, also known as CD169 or sialoadhesin, lacks the ITIM motif and seems to be involved

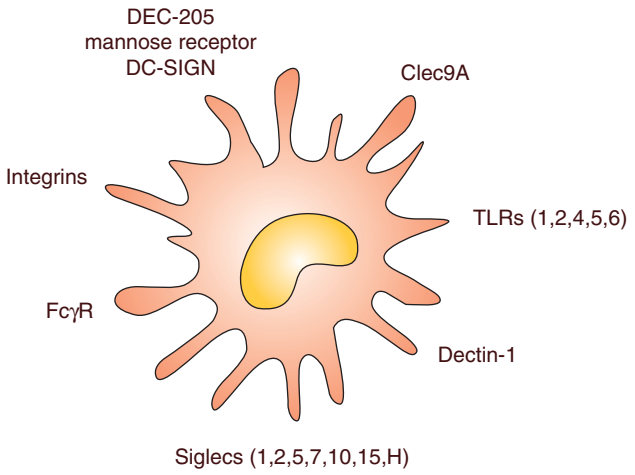

Figure 3. Examples of known receptors on DCs, which may have potential for targeting vaccines, based on experiments performed with vaccine targeting to DCs or from analyses of the ligands with which the receptors interact.
DCs: Dendritic cells; TLR: Toll-like receptor.

in modulating immune responses, at least in mice [119,122]. Sialoadhesin is the receptor for porcine reproductive and respiratory syndrome virus (PRRSV), mediating binding to and internalization of PRRSV into porcine macrophages [123]. This receptor is also expressed on porcine DCs and has recently been demonstrated to enhance antigen-specific T-cell proliferation *in vitro* on targeting with anti-CD169 mAb [124], indicating that Siglec-1 targeting may prove to be a useful vaccine targeting strategy in pigs. However, a main risk associated with this strategy is the possibility that the host evokes immune responses to the heterologous antibody used for targeting, compromising the overall application of such an approach.

4.2 Ligand-mediated dendritic cell receptor targeting

As an alternative to applying mAb to target antigen to DC receptors, one could use the ligands of these receptors. For most of the above-mentioned receptors the specific carbohydrate structures have been identified, which could be useful as targeting moieties. In this regard, mannan has been found to interfere with bacterial binding by DC-SIGN [125]. Moreover, mannan enhanced antigen endocytosis by DCs, resulting in DC activation and the induction of immune responses in a mouse model [126]. Mannan and other mannosylated structures bind to DC-SIGN as well as to the mannose receptor CD206, and mannosylation of biodegradable particulate vaccine carriers either with synthetic structures or with vaccine antigens with inherent mannose-containing carbohydrates has been used to target immune cells [127,128]. Indeed, mannosylated liposomes effectively targeted C-type lectin receptors on both human and murine DCs *ex vivo*, resulting in their maturation and the enhancement of Ag-specific T-cell proliferation in comparison with Ag alone or with non-targeted liposomes [129,130]. Furthermore, intraperitoneal administration of *Leishmania* antigen entrapped in mannosylated liposomes protected mice from subsequent lethal challenge [131]. Recently, mannosylation of PEGylated PLGA nanoparticles elicited higher antigen-specific IgG serum responses in mice on intraduodenal administration in comparison with non-grafted nanoparticles [128]. The enhanced transepithelial transport of the mannosylated nanoparticles across Caco-2 monolayers, the strong uptake by rat PP and the higher endocytosis by macrophages and DCs *in vitro* could account for this effect [128,130,132].

CyaA, the adenylate cyclase toxin derived from *Bordetella pertussis*, binds to $\alpha_M\beta_2$ integrin (CD11b/CD18) on APCs and a recombinant detoxified toxin has been used to target conjugated viral antigens to DCs to elicit protective antiviral immunity [133,134].

Recently, a specific DC-binding peptide, derived from a bacteriophage library, was identified. This short peptide binds to *in vitro*-generated DCs from several species, including human, rhesus monkey, chimpanzee and mice. On oral delivery of recombinant *Lactobacillus* species, which secrete fusion proteins consisting of a *Bacillus anthracis* antigen and this specific DC-targeting peptide, robust immune responses were induced, protecting mice against a lethal challenge with *B. anthracis*. The receptor for this DC-targeting peptide is unknown, although it seems to be conserved and involved in the endocytotic pathway [135,136]. Alternatively, these peptides could be grafted on microparticles, allowing specific targeting of these carrier systems to DCs in several species.

Although targeting of these receptors mediates enhanced endocytosis of antigen by DCs and subsequently can improve immune responses, most of these studies have been performed *in vitro* or in murine models [137]. Hence, vaccination approaches targeting specific murine DC subsets may not be applicable in other species. Moreover, whether these targeting strategies can be applied for oral administration is unknown at present. Further studies are required to analyze the behavior of these delivery systems in the gastrointestinal tract and how they interact with intestinal DCs. Indeed, the mechanisms of cellular uptake, antigen retention and processing are not well characterized for particulate vaccine delivery systems, especially for surface-modified particles that target PRR on DCs. This is important as, depending on the targeted PRR, the intracellular fate of the particle could differ. Furthermore, DC activation should be monitored when targeting to PRR, as DC activation and maturation are required for the efficient induction of adaptive immunity. Therefore, a danger signal, such as TLR ligands, could be added to the vaccine formulation to ensure proper DC maturation.

One should also be aware that the intestinal epithelium has to be crossed for efficient immunization on oral administration. As FcRn targeting seems a promising strategy to allow transcytosis of delivery systems across the epithelium,

this would favor the endocytosis of Ag or delivery systems by FcγR-expressing antigen-presenting cells. In addition, intestinal lamina propria DCs are known to extend dendrites across the intact epithelium. Although it can be assumed that specific receptors are expressed on these protruding dendrites, the identity and functionality of these receptors remain unknown. In pigs, a $MHCII^+/CD11R1^+/FcγRIII^+$ LP DC subpopulation extends dendrites into the gut lumen, although this was a rare event in steady-state conditions [22]. Nevertheless, DCs could sample orally administered Fc-conjugated delivery systems directly from the gut lumen, enabling DC-specific vaccine delivery.

4.3 Fcγ receptors as potential targeting molecules

FcγR are a family of membrane glycoproteins belonging to the Ig superfamily that bind the Fc fragment of IgG molecules [138]. These receptors can be classified based on their molecular mass, IgG binding affinity, IgG subclass specificity and cellular distribution. Three major subclasses of FcγR have been identified in mice, human and livestock species: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (**Figure 4**) [139,140]. In addition, a fourth subclass, FcγRIV, has been described in mice, although this receptor appears to be expressed exclusively in mice [141]. FcγRI is a high-affinity receptor, which binds both monomeric and multimeric IgG. This receptor is constitutively expressed on antigen-presenting cells such as macrophages and dendritic cells. On the contrary, FcγRII and FcγRIII are low-affinity receptors, which bind only multimeric IgG and are constitutively expressed on a more diverse cell population [140,142]. On crosslinking by immune complexes, these FcγR activate a signaling pathway which can be stimulatory or inhibitory in nature, depending on the signal transduction motif in the cytoplasmic domain of the FcγR. Stimulatory signals are generated by an immunoreceptor tyrosine-based activation motif (ITAM), whereas the signal motif responsible for generating the inhibitory signals is called the immunoreceptor tyrosine-based inhibition motif [139]. These activating (FcγRI, FcγRIIa and FcγRIII) and inhibitory FcγR (FcγRIIb) mediate opposing functions, underlining the role of these receptors in regulating adaptive immune responses [143].

Numerous studies have demonstrated that Ag targeting to FcγR *in vitro* and *in vivo* can enhance both humoral and cellular immune responses [144-147]. This has been achieved with Ab–Ag immune complexes (IC), FcγR-specific-Ag chemical conjugates and anti-FcγR-Ag fusion proteins [144,146-148]. However, the mechanisms by which FcγR targeting enhances antigen presentation and antibody-mediated immunity *in vivo* are still largely unresolved. Previous *in vitro* experiments with human, murine and porcine DCs have demonstrated that Ag incorporated in IC are internalized more efficiently than their soluble counterparts [138,149-152]. This enhanced endocytosis of IC by DCs can be influenced by the IgG subclass, the valency of the ligand and the number of FcγR expressed on the DC surface [153]. Furthermore, FcγR engagement by IC enhances the maturation of DCs, resulting in a strongly enhanced efficiency of Ag presentation to $CD4^+$ and $CD8^+$ T cells owing to the increased expression of MHC and T-cell co-stimulatory molecules on DCs [138,144,150,151]. The ITAM signaling cascade is required for this IC-mediated DC maturation and antigen presentation [152,153]. Moreover, targeting to FcγR promotes not only efficient MHCII, but also MHCI-restricted Ag presentation [154]. This process, by which extracellular Ag is presented through MHCI, is called cross-presentation, and occurs at a 1000-fold lower Ag concentration for FcγR-mediated versus fluid-phase uptake of Ag by murine DCs. As a result of the enhanced Ag internalization, the induction of DC maturation and the enhanced Ag presentation to T cells, antigen targeting to FcγR dramatically reduces the dose of Ag required for the induction of immunity compared with non-targeted antigen [138].

Besides the enhanced antigen internalization on FcγR engagement, it is possible that targeting also improves antigen processing and presentation as well as intracellular trafficking. Certain properties of IC, such as IgG subclass and valency, could affect the ability of the IC to interact with particular FcγR, leading to alteration in these mechanisms. Indeed, elegant studies by Qiao *et al.* [155] demonstrated that only multimeric IC elicited strong, dose-dependent stimulation of Ag-specific T cells. Intriguingly, interplay between FcRn and FcγR has been demonstrated in antigen processing on FcγR engagement. The T-cell proliferative capacity of DCs on IC stimulation was severely reduced when using DCs from $FcRn^{-/-}$ mice or when stimulating wild-type DCs with IC in which the Fc region was engineered to carry mutations that abrogated FcRn binding, implicating FcRn in the trafficking of IC towards MHCII loading. Indeed, FcRn co-trafficks with the MHCII pathway-associated invariant chain molecule [156]. Moreover, wild-type IC trafficked to lysosomal compartments, whereas IC with mutant IgG, unable to bind to FcRn, was not observed in these compartments. Together, these experiments demonstrated that multimeric IC is directed towards a degradative pathway, leading to efficient antigen processing and presentation by DCs. This is in contrast to the recycling pathway for monomeric IgG in epithelial and endothelial cells, but in line with the observation that multimeric IC has a shorter half-life compared with monomeric IC or uncomplexed IgG *in vivo* [155,157]. These studies point out that FcγR targeting in immunizations strategies should use multimeric IC or particulates on which the density of the Fc regions is high enough to ensure sufficient FcγR aggregation.

Although *in vivo* studies have demonstrated that when targeting antigen to FcγR adjuvants are not required and both humoral and cellular immunity can be enhanced, offering protection against both intracellular and extracellular pathogens [146,147,158], one important caveat associated with FcγR targeting is the possibility that IC can interact with the inhibitory receptor, FcγRIIb. This receptor is expressed on

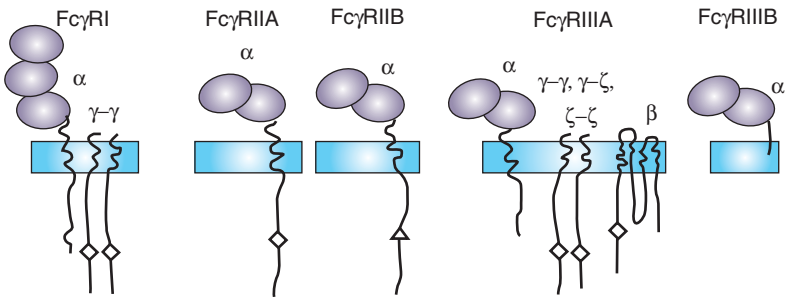

Figure 4. Schematic representation of Fcγ receptors. The filled ovals represent the extracellular Ig-like domains. The associated γ- or ζ-chains for FcγRI, FcγRIII and the β-chain for FcγRIII are indicated by symbolic characters. The activating ITAM and inhibitory ITIM motifs in the cytoplasmic tail are depicted as diamonds and triangles, respectively.
Adapted from [140].
ITAM: Immunoreceptor tyrosine-based activation motif; ITIM: Immunoreceptor tyrosine-based inhibition motif.

various immune cells, including DCs, and is the only FcγR expressed on B cells [159]. Engagement of FcγRIIb results in the activation of an inhibitory signaling cascade through its ITIM motif and coligation of FcγRIIb and activating FcγR results in a dominant inhibitory signal in cells that express both types of FcγR. In human MoDC, a blockade of this inhibitory receptor is required to induce full DC maturation on FcγR ligation [160]. Moreover, FcγRIIb engagement on B cells inhibits their activation and subsequent Ab production [159]. Indeed, in FcγRII$^{-/-}$ mice increased antigen-specific antibody titers have been observed on vaccination compared with their wild-type littermates [161,162]. However, in spite of the interaction of IC with FcγRIIb on B cells and DCs, which potentially attenuates both Ag-specific Ab responses and DC-mediated T-cell activation, several studies have demonstrated that FcγR targeting *in vivo* does result in enhanced humoral and cellular immunity, indicating that FcγRIIb does not prevent, but rather dampens IC-mediated immunity [146,147,158].

Moreover, the IgG subclass may influence the degree of FcγRIIb involvement. Indeed, surface plasmon resonance experiments have pointed out that the activity of inhibitory and activating FcγR is strongly influenced by the IgG subclass, primarily owing to the different binding affinities of IgG subclasses to particular FcγR [141,142,159]. Hence, selecting the appropriate IgG subclass to generate IC could on the one hand minimize engagement of the inhibitory FcγRIIb, while on the other hand augment the interaction with activating FcγR. Whereas in most laboratory species the IgG subclasses have been identified, in pig the genes encoding IgG molecules have been described only recently. Eleven genomic gene sequences representing six putative IgG subclasses have been identified, and comparative gene analyses predicted that most subclasses should be able to bind to FcγR. However, owing to the inability to purify physicochemically porcine IgG subclass proteins, recombinant expression and purification of these subclasses will be necessary to elucidate the binding affinities to particular porcine FcγR [163].

5. Expert opinion

Vaccination programs have had a major impact on global health, with the eradication of infectious diseases such as smallpox. Despite this success, there is still a need for new prophylactic and therapeutic vaccines as most pathogens invade the host at mucosal surfaces and the vast majority of the current commercial vaccines are mostly effective at preventing systemic infections. Mucosal pathogens require the induction of pathogen-specific secretory IgA at the site of infection for effective protection of the host. Indeed, protection against the intestinal ETEC pathogens is mediated almost exclusively by SIgA. However, oral administration of antigens remains a major challenge for vaccinologists owing to the hostile environment in the gastrointestinal tract. The entrapment of antigens in biodegradable microparticulate delivery systems has the potential to accelerate the development of oral vaccines as this will render antigens more immunogenic, mimicking polymeric antigens and pathogen dimensions. However, uptake of these particles by intestinal epithelial or immune cells remains poor. Several strategies are now being investigated to enhance the uptake by M cells and enterocytes.

Besides this epithelial targeting, a variety of DC-specific receptors are now under investigation for the selective targeting of antigens and delivery systems to these professional antigen-presenting cells. However, under steady-state conditions, it seems that most of the intestinal DCs are imprinted with a tolerogenic function. The incorporation of either adjuvants or chemokines could guarantee the attraction of DCs with a more immunogenic character. Antigen targeting to this immunogenic or inflammatory DC subset will require knowledge of the membrane protein expression pattern to select an appropriate receptor target. This receptor would preferably be conserved among several species, which would enable a multispecies vaccine platform to be generated.

The use of IgG molecules or Fc fragments in vaccine design could offer several advantages. Fc-coated microparticles could

allow efficient transcytosis of the decorated microparticles through the intestinal epithelium via FcRn and, subsequently, FcγR-expressing intestinal antigen-presenting cells, such as dendritic cells, can be targeted with a concomitant induction of maturation and ensuing adaptive immune responses. Furthermore, this strategy offers the potential to eliminate or reduce the need for adjuvants and increase dose sparing, and provides a mechanism for simultaneously enhancing humoral and cellular immunity against a variety of intestinal pathogens. Despite the general lack of knowledge regarding the function of FcγR in mucosal immunity, which limits the ability to use a knowledge-based approach in vaccine design, another caveat associated with this strategy is the ability of antibodies or Fc domains to interact with the inhibitory FcγRIIb. However, this can be circumvented by careful selection of the IgG subclass or by the insertion of mutations into the IgG Fc domain to limit the binding to the inhibitory FcγRIIb receptor. The data discussed in this review were obtained from monogastric animal models and indicate that several targeting strategies are being developed for use in humans. However, to be successful as oral vaccine in other species, such as ruminants or poultry, these strategies probably need to be tailored, or a completely new strategy needs to be developed. Nevertheless, surface conjugation of Fc fragments to antigen-loaded microparticles could pave the way for the development of a versatile multispecies oral antigen delivery system.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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Affiliation

Bert Devriendt[1], Bruno G De Geest[†2] & Eric Cox[1]
[†]Author for correspondence
[1]Ghent University,
Faculty of Veterinary Sciences,
Laboratory of Immunology,
Salisburylaan 133,
9820, Merelbeke, Belgium
[2]Ghent University,
Laboratory of Pharmaceutical Technology,
Harelbekestraat 72,
9000, Ghent, Belgium
E-mail: br.degeest@ugent.be