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Targeting antigens to sialoadhesin-expressing macrophages as a vaccination strategy

Karen Ooms

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Promoters: Prof. dr. ir. P.L. Delputte & Prof. dr. H.J. Nauwynck

Laboratory of Virology

Department of Virology, Immunology and Parasitology

Faculty of Veterinary Medicine

Ghent University

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List of abbreviations

APC(s)	antigen-presenting cell(s)
AUC	area under the curve
C	constant
CDR(s)	complementarity-determining region(s)
CHO	Chinese hamster ovary
CHO-pSn	Chinese hamster ovary cells expressing porcine sialoadhesin
CSF-1	Colony-stimulating factor 1
DCs	dendritic cells
DEAE	Diethylaminoethyl
EAE	experimental allergic encephalitis
Fab	fragment antigen binding
FcRn	neonatal Fc receptor
GBS	Guillain-Barré syndrome
GnRH	gonadotropin releasing hormone
GP4	glycoprotein 4
GS	glycine-serine
HEL	hen egg lysozyme
HIV-1	human immunodeficiency virus 1
HSA	human serum albumin
i.m.	intramuscular
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNKT cell(s)	invariant natural killer T cell(s)
IPMA	immunoperoxidase monolayer assay
ISCOM(s)	immune-stimulating complex(es)
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KLH	keyhole limpet hemocyanin
LN	lymph node
LT	Lymphotoxin
LV	Lelystad virus
mAb(s)	monoclonal antibody – antibodies
MDSC(s)	myeloid-derived suppressor cell(s)

MGL-1	macrophage galactose-type C-type lectin 1
MMM	marginal metallophilic macrophages
MR	mannose receptor
MZM	marginal zone macrophages
NK	natural killer
NPs	nanoparticles
OVA	ovalbumin
p41D3	plasmid 41D3
PAM	porcine alveolar macrophages
PRRSV	porcine reproductive and respiratory syndrome virus
PSGL-1	P-selectin Glycoprotein Ligand-1
pSn	porcine sialoadhesin
rec13D12	recombinant antibody 13D12
rec41D3	recombinant antibody 41D3
scFv	single-chain fragment variable
SCS	subcapsular sinus
SER	sheep erythrocyte receptor
Siglec(s)	sialic acid-binding immunoglobulin-like lectin(s)
Sn	sialoadhesin
Sn^+	sialoadhesin-expressing
TAM(s)	tumor-associated macrophage(s)
Teffs	effector T cells
TGE	transient gene expression
TLR(s)	toll-like receptor(s)
TNF-α	tumour necrosis factor-α
Tregs	regulatory T cells
V	variable
VLP(s)	virus-like particle(s)
VN	virus-neutralizing
VSV	vesicular stomatitis virus

Chapter 1

Introduction

1.1 TARGETING ANTIGENS TO ANTIGEN-PRESENTING CELLS AS A VACCINATION STRATEGY

1.1.1 INTRODUCTION

Vaccination is one of the most successful public health initiatives ever achieved [1]. While sanitation has markedly decreased deaths from infectious disease, vaccination has done so as well. The global eradication of the life-threatening disease smallpox, announced by the World Health Organization in 1979, is being considered “the greatest triumph of vaccination” [2]. In addition, the global eradication of rinderpest, declared eradicated by the World Organization for Animal Health in 2011, can be considered the greatest triumph of veterinary vaccination [3, 4]. The term vaccination stems from the Latin word for cow “vacca” [5]. Edward Jenner introduced this term based on the observation that a previous infection with cowpox, which elicits a mild illness in humans, could prevent disease upon exposure to smallpox [5]. This first milestone in the era of vaccine development was followed by Louis Pasteur’s discovery of attenuation. During his summer holiday in 1881, a culture of bacteria provoking chicken cholera was left on the bench. At his return in autumn, he used this culture to inoculate chickens and noticed no disease could be monitored. To his surprise however, a second inoculation with a fresh, pathogenic culture did not provoke disease either, hence leading to the concept of attenuation of a pathogen to induce immunity to a subsequent challenge with the original pathogen [6]. Ever since, multiple strategies for attenuation or complete inactivation of pathogens have been applied for the development of successful vaccines rendering several diseases vaccine-preventable (e.g. poliomyelitis, mumps, rubella,…) [6]. Nevertheless, many infectious diseases still exist for which development of an effective vaccine remains elusive based on these classical approaches (e.g. HIV, malaria, tuberculosis,…). In addition, continuously increasing safety demands for novel vaccine formulations encourage the search for more rational vaccine designs. As a result, a wide range of vaccines currently under development are based on isolated peptides, proteins or polysaccharides of pathogens, or naked DNA encoding a protective antigen. Whilst these can be safer and more immunologically defined compared to existing classical vaccines, they are often poor immunogens, and therefore require additional immunostimulation to induce protective immune responses [7]. The use of adjuvants however needs to be tightly controlled as there is a risk for serious adverse reactions, such as a systemic inflammatory response or the induction of autoimmunity [8]. As a consequence, finding safe, yet efficient ways to boost immunity has become an increasingly important area of research in current-day vaccinology.

The ultimate goal of a vaccine is to induce an antigen-specific memory immune response. To facilitate this, an interaction between antigen-loaded antigen-presenting cells (APCs) and

naïve lymphocytes needs to take place in secondary lymphoid tissues [2]. Therefore, one way to improve the efficiency of a vaccine is to improve the efficiency of the uptake of antigen by APCs [9]. As such, targeting antigen to uptake receptors expressed on the surface of APCs is increasingly studied. As the dogma establishes that dendritic cells (DCs) are the most efficient APCs stimulating naïve lymphocytes, most targeting studies have been focused on DCs and their different subtypes. However, recently also sialoadhesin-expressing (Sn^+) macrophages are gaining increased attention as targets for vaccination strategies as their location in spleen and lymph nodes appears to be ideal for antigen capture and presentation [10]. These macrophages and more precisely the immune stimulatory effect of directly targeting antigens towards them, is the major subject of this dissertation.

In this first part of the thesis, an overview will be given of the lessons learned from APC targeting studies for vaccination purposes up till now. Furthermore, a thorough introduction will be given on Sn (CD169, Siglec-1) and Sn^+ macrophages to demonstrate the targeting potential of this cell type. Finally, targeting molecules to mediate antigen targeting will be summarized and compared.

1.1.2 STATE-OF-THE-ART

Three types of professional APCs exist: macrophages, B cells and DCs [2]. As DCs are generally considered to be the most potent APCs, most targeting studies performed aimed at targeting DCs. However, many receptors evaluated as candidates for targeting antigen to DCs are not DC-specific and can target macrophages and/or B cells as well. Below, each cell subset will be briefly introduced and an overview will be given of studies performed and lessons learned.

Targeting dendritic cells

A major contributor to the field of DC targeting has been Ralph Steinman, who has been fascinated by this cell type ever since he discovered it up until he passed away in 2011, just two and one-half days before receiving the Nobel Prize for Physiology or Medicine. An inspiring review of his own hand written just before he died describes some crucial steps from the discovery of DCs up till where we are now and challenges that remain for the future [11]. For DC targeting, his main focus has been on the C-type lectin endocytic receptor DEC-205. As the natural ligands for DEC-205 were still unknown, it was decided early on to use antibodies as surrogate ligands [11]. The heavy chain of the anti-DEC-205 monoclonal antibody (mAb) was engineered to express different antigens. In this way, the consequences of targeting antigens to DCs could be studied *in vivo* without having to isolate the DCs or generate them from precursors. The first antigen that was engineered into an anti-DEC-205

mAb was a peptide from hen egg lysozyme (HEL) that dominates the $CD4^+$ T cell response of H-2k mice [12]. The injected antibody targeted to the DCs in the T cell area, but instead of an enhanced immune response to HEL, mice became specifically tolerant to HEL [12]. This led to the speculation that DCs perform tolerogenic functions under steady-state conditions and that inflammatory stimuli or adjuvants are required to enhance the induction of antigen-specific immune responses. When such adjuvants were administered, DCs matured, expressed costimulatory molecules and the antigen dose required for an effective immune response could be greatly reduced by targeting the antigen specifically to DCs via DEC-205 [13, 14]. Later on, Dudziak *et al.* elegantly showed that differential DC subsets *in vivo* process antigen in different ways, by targeting ovalbumin (OVA) either to $CD8\alpha^+$ DCs by a DEC-205-specific antibody or $CD8\alpha^-$ DCs by a DCIR2-specific antibody [15]. $CD8\alpha^+$ DCs preferentially induced $CD8^+$ T cell immunity, while $CD8\alpha^-$ DCs preferentially induced $CD4^+$ T cell immunity. At present, it is clear that DCs are not a homogenous population of cells, but represent a complex group of subsets that differ in ontogeny and specialized functions (Fig. 1) [16].

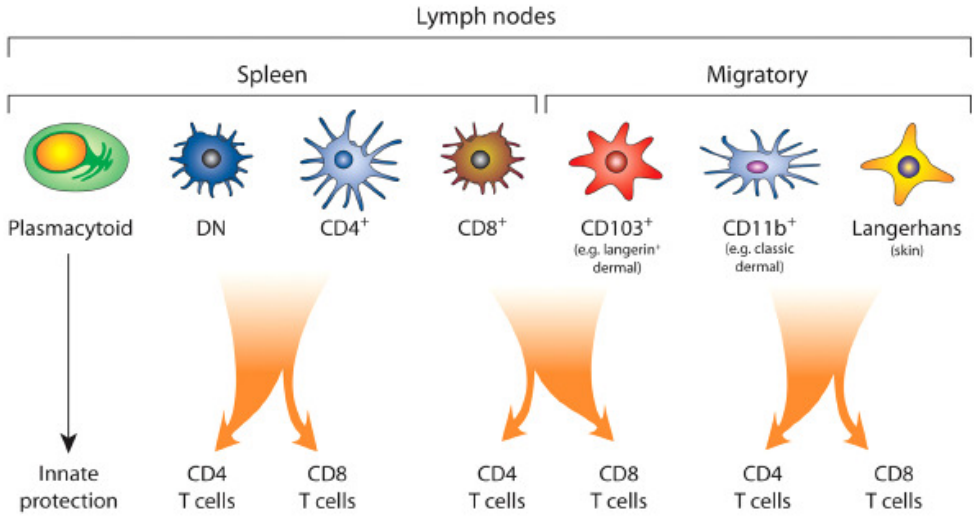

Figure 1. The complex group of DC subsets in mice
Plasmacytoid DCs provide an innate barrier against viral pathogens by the efficient production of type I interferon. Conventional DCs, which include both the lymphoid tissue-resident DCs and migratory DCs, drive the adaptive immune response. In the mouse spleen, the lymphoid tissue-resident DCs are divided into those that express CD8α ($CD8^+$), CD4 ($CD4^+$), or those that express neither CD4 or CD8α, the double negative (DN) DC subset. The lymph nodes also contain migratory DCs, which can be further segregated into at least three subsets: the $CD103^+$ DCs, $CD11b^+$ (dermal) DCs, and Langerhans cells. There is functional specialization between the DC subsets, where the $CD103^+$ DCs and $CD8^+$ DCs are most proficient at cross-presentation and activation of $CD8^+$ T cells. By contrast, splenic DN and $CD4^+$ DCs and $CD11b^+$ DCs and Langerhans cells are more efficient at driving $CD4^+$ T cell responses. Nevertheless, under certain conditions $CD4^+$ DCs, DN DCs and Langerhans cells have been shown to cross-present antigen [17, 18]. Figure adapted from Caminschi *et al.* [16].

The understanding that different DC subsets differ in their functional specialization has led to a search for subset-specific uptake receptors to enable fine-tuning of the immune response

generated [8]. This is of particular interest for the development of vaccines against intracellular pathogens for which it is believed that a strong cellular immune response is necessary to eliminate the cells that are infected with the causative agent, as most current preventative vaccines are designed to initiate protective humoral immune responses [19]. Prominent examples of such diseases are HIV-induced acquired immune deficiency syndrome, virus-induced hepatitis C or *Mycobacterium*-induced tuberculosis [20]. Also for therapeutic vaccines to treat cancer, the induction of a strong cellular antitumor immune response is required [16].

For a long time it has been difficult to relate mouse DC subsets to human DC subsets due to difficulties of isolating large quantities of DCs from human tissue [21]. At present however, human BDCA3$^+$ DCs are the proposed equivalent of the murine CD8α$^+$ DCs, human dermal CD1a$^+$ DCs of murine dermal CD103$^+$ DCs, human BDCA1$^+$ DCs of murine CD8α$^-$ DCs, while Langerhans cells and plasmacytoid DCs are equally present in both species [8]. Although DC subsets in human and mouse can be equivalent in function, they do not necessarily express identical uptake receptors, which can hamper translation from animal models towards clinical outcomes. An overview of uptake receptors identified on human and mouse DC subsets is given in Table 1.

Table 1: Uptake receptors identified on human and mouse DC subsets

DC subset	Uptake receptor
BDCA3$^{+\,H}$ CD8α$^{+\,M}$	Clec9A, Langerin^M, DEC-205, Clec12A, DCAR1^M
Langerhans	Langerin, DEC-205, Dectin-1, Dectin-2, DCIR^H
Dermal CD1a$^{+\,H}$ Dermal CD103$^{+\,M}$	Langerin, DEC-205, MGL^H, DCIR^H
BDCA1$^{+\,H}$ CD8α$^{-\,M}$	DCIR2^M, Clec12A, DCIR^H, Dectin-1^H, DEC-205^H, mMGL1^M, mMGL2^M
Monocyte-derived DC$^{H\bullet}$	DC-SIGN, DEC-205, MR, DCIR, Clec12A
Plasmacytoid DC	Siglec-H, BST-2, BDCA-2^H, Clec9A^M, Clec12A, Dectin-2, DEC-205^H, DCIR^H, Dectin-1^M, mMGL1^M

M, mouse; H, human.
$^\bullet$ *In vivo* inflammatory-type DCs
Table adapted from Kreutz *et al.* [8].

As seen in Table 1, most uptake receptors are not specific for one DC subset. In addition, many of these receptors are also expressed on other cell types apart from DCs (Table 2). Therefore, it has not been possible up till now to deliver an antigen specifically to one DC subset [16]. Ubiquitous expression of a receptor is of importance, as it can reduce its targeting efficiency, which can result in less efficient stimulation of the envisaged immune response. Apart from not specifically targeting the DC subset that would most efficiently guide the immune response in the desired direction, expression by other cell types not involved in antigen presentation can clear the antigen from the system, thus reducing

vaccine efficiency. An overview of the expression of 'DC-specific' uptake receptors used for targeting applications is given in Table 2.

Table 2: Expression pattern in mice and men of DC uptake receptors

Receptor	Synonyms	Expression in men	Expression in mice	Reference
DEC205	CD205; LY75; CLEC13B	BDCA1$^+$ DCs; monocytes; B cells; low levels on NK cells; pDCs and T cells	Cortical thymic epithelium; CD11c$^+$CD8$^+$ thymic medullary/splenic/lymph node DCs, dermal/interstitial DCs and Langerhans cells	[22-25]
Mannose Receptor	CD206; MRC1; CLEC13D	Tissue macrophages and a subpopulation of DCs; lymphatic and hepatic epithelium; kidney mesangial cells	Tissue macrophages and a subpopulation of DCs; lymphatic and hepatic epithelium; kidney mesangial cells	[25-29]
DC-SIGN	CD209; CLEC4L	DCs; specific macrophage subsets	-	[25, 30, 31]
hMGL	CD301; CLEC10A; CLECSF14	Immature DCs; macrophages	-	[25, 32]
mMGL1	CLEC10A; CD301a	-	Macrophages; interstitial DCs; pDCs	[25, 32-34]
mMGL2	CD301b	-	Macrophages; DCs in dermis, small intestines and lymph nodes	[25, 32-34]
Langerin	CD207; CLEC4K	Langerhans cells	Langerhans cells, CD103$^+$ DCs and CD8α$^+$ DCs	[25, 35-38]
hDCIR	LLIR; CLEC4A; CLECSF6	Blood myeloid DCs; dermal DCs; pDCs; monocytes; macrophages; B cells; neutrophils; granulocytes	-	[39, 40]
mDCIR2	CLEC4A2	-	CD8α$^-$ splenic DCs	[15]
CLEC9A	DNGR-1	BDCA3$^+$ DCs	CD8α$^+$ DCs; pDCs; pulmonary CD103$^+$ DCs	[25, 41-44]
Dectin-1	CLEC7A; CLECSF12; β-glucan receptor	DCs; monocytes; macrophages; neutrophils; B cells; eosinophils; T cell subsets	Monocytes; macrophages; DCs; neutrophils; T cell subsets	[45, 46]
Dectin-2	CLECSF10; CLEC6A; CLEC4N	DCs; monocytes	DCs; neutrophils; macrophages	[47, 48]
CLEC12A	CLL1; KLR1; MICL; DCAL2	Monocytes; granulocytes; DCs; NK cells*	DCs; macrophages; B cells	[49, 50]
DCAR1	CLEC4B2	-	CD8α$^+$ DCs; CD11b$^+$ myeloid cells in bone marrow and spleen	[47, 51]
Siglec-H	-	-	pDCs; low levels on macrophages	[52]
BST-2	CD317; PDCA-1; tetherin; HM1.24	Monocytes; conventional DCs	pDCs; low levels on conventional DCs, macrophages, B cells and T cells	[53]
BDCA-2	CD303; CLEC4C; CLECSF7; HECL; DLEC; CLECSF1; CLECSF11	pDCs	-	[47, 54, 55]
CD11c	CR4; Itgax	DCs; monocytes; macrophages; polymorphonuclear leukocytes	DCs; monocytes; macrophages; polymorphonuclear leukocytes	[9, 56, 57]

* Conflicting data on expression on NK cells [50]
h, human; m, mouse; pDCs, plasmacytoid dendritic cells

When aiming at specific immune responses, not only the choice of DC subset is taken into consideration, but also the type of adjuvant used in the vaccine formulation. Indeed, different types of adjuvants have been shown to skew the type of immune response generated (e.g. Th2 versus Th17) [16, 58]. From the DC targeting studies performed to date, it appears to be a general rule that in order to induce substantial cell-mediated immunity, the addition of adjuvants to mature the targeted DCs is required and can affect the type of immune response generated [16]. The induction of humoral immunity on the other hand can sometimes be obtained without the use of adjuvants [59]. A prominent example hereof is the targeted delivery of antigens to Clec9A [41, 60]. One proposed explanation for this phenomenon is that the restricted expression pattern of Clec9A prevents the clearance of antibody-antigen complexes by irrelevant cells, which then leads to a sustained antigen presentation that promotes the generation of follicular helper T cells driving B cell germinal centre development [59]. Also, the observation that Clec9A targeting can induce robust humoral immune responses, even without the addition of adjuvant, shows that the nature of the DC-surface molecule targeted can sometimes override DC subset specific antigen presentation biases, as Clec9A is present on $CD8\alpha^+$ DCs, which are supposed to primarily induce antigen cross-presentation and thus predominantly cell-mediated immunity [16].

Although the general rule for current DC-targeting studies appears to be to target particular DC subsets as specific as possible, also ‘more general’ APC receptors were evaluated for their potential as targeting receptor. These include MHC molecules [61-65], Toll-like receptors (TLRs) [66, 67], Fc receptors [68-70] and costimulatory molecules [71-73]. In the case of TLRs and costimulatory molecules, the targeting moieties can perform a dual role: targeting to APCs on the one hand and inducing an immune stimulatory (adjuvant) function on the other hand by influencing the maturation state of the DC. TLR and costimulatory molecule ligands are also often applied in studies merely for their adjuvant function and thus separated from the antigen and its targeting moiety (Table 3). To date, it is still a matter of debate whether it is best to physically link the antigen with the adjuvant to ensure co-delivery to the same cell or whether the adjuvant should be supplied by mere co-administration [8]. A recent study employing nanoparticles to deliver antigen and adjuvant showed better humoral immune responses upon injection of both agents in separate particles than when combined in a single particle [74]. Nevertheless, many studies aiming to induce cellular responses show that physically linking the antigen and adjuvant improves T cell responses [8]. One proposed explanation for this phenomenon is that antigens are better processed and presented due to co-localization of both antigen and stimulus in the same phagosome [75]. At the same time, this approach ensures activation of the cells that have seen the antigen, which is important for efficient T cell priming by DCs [2].

Another point of attention is the endocytic capacity of the targeted receptor. A recent study by Chatterjee *et al.* targeted identical antigens to either CD40, the mannose receptor (MR) or DEC-205 by the use of respective mAbs [76]. They evaluated the amount of antigen presentation via MHCI or cross-presentation and found an inverse relationship between internalization or antigen degradation and cross-presentation. Antigens destined for more degradative late endosomes were poorly cross-presented relative to the same antigens targeted to early endosomes, an effect that was independent of the amount of antigen internalized. In addition, also the targeting mAb itself can affect the immunological outcome. In the absence of adjuvant one anti-Clec9A mAb induced follicular helper T cells [60], whereas another induced regulatory T cells (Tregs) [77]. This observation could be due to the different genetic background of the mouse species used (BALB/c versus C57/BL6 respectively). Lahoud *et al.* suggest that a rat IgG1 mAb lacks appropriate epitopes for a direct response in C57/BL6 mice, while it contains the appropriate epitopes for a direct response in BALB/c mice, which was supported by unpublished experiments of their research group [60]. Furthermore, they suggest differences in antibody affinity, signal transduction upon receptor engagement or serum persistence of the antibody can contribute to the differences in immune outcome described.

Taken together, it is clear that multiple factors can have an impact on the immune outcome of a DC-targeted vaccine formulation. Whether antigen targeting to DC results in the induction of a strong (humoral and/or cellular) immune response depends on an interplay between the DC subset targeted, the nature of the targeting receptor and its expression pattern, the targeting moiety, the antigen dose and co-administered adjuvants. In addition, also the route of immunization can have an impact on the efficiency of antigen encounter by the DCs and the subsequent immune response [78]. How these theoretical concerns turn out *in vivo* often have to be determined empirically [79]. In addition, although promising results in mouse models have been obtained, the translation of animal-derived experimental data into regimes applicable to human vaccination remains challenging [79]. An overview of DC targeting approaches studied for vaccine design is given in Table 3.

Table 3: Overview of APC vaccine targeting approaches showing representative *in vivo* targeting studies grouped by cell type, species and targeting receptor.

Species	Receptor	Delivery system	Antigen targeted	Adjuvant[1]	Type of response[2]	Route[3]	REF
APC targeting							
Mouse	TLR2	Pam2cys	OVA, *L. monocyto-genes* antigen, Inluenza antigen	Pam2cys*	Ab, CTL, IFNγ, antitumor activity	s.c., i.v., i.n.	[66]
	MHCII	M1 modified super-antigen	OVA	α-GalCer	CD4, CD8, CTL, IFNγ, antitumor activity	i.v.	[65]
	CD40	CD40 Ligand-bearing adenovirus	Melanoma peptides	CD40 Ligand*	IFNγ, antitumor activity	i.d.	[73]
	FcγRI	scFv	Pneumo-coccal surface protein A	-	Ab, IFNγ, protection from lethal challenge	i.n.	[70]•
DC targeting							
Mouse	DEC-205	Ab	OVA, HIV-1 gag p24, HEL peptide, CSP, HER2/neu antigen	αCD40 Ab, MALP-2, PAM_3Cys, polyI:C, polyICLC, LPS, R848, CpG	CD4, CD8, CTL, IFNγ, Ab, antitumor activity	s.c., i.v., i.p.	[12-15, 80, 81]
			OVA	CpG*	CTL, IFNγ, antitumor activity	s.c./i.d.	[82]
		Ab-conjugated MP	OVA	Particle composition*	CTL, IFNγ	s.c.	[83]
		Ab-conjugated PLGA NP	OVA	PolyI:C*, R848*	CD8, CTL, IFNγ	i.v.	[84]
		scFv-conjugated liposome	OVA	IFN-γ* or LPS*	CTL, antitumor activity	i.v.	[85]
	CD11c	scFv-conjugated liposome	OVA	IFN-γ* or LPS*	CTL, antitumor activity	i.v.	[85]
	DCIR2	Ab	OVA	αCD40 Ab	CD4	i.p., i.v.	[15]
	MR	Ab	OVA	CpG	Ab, CD4, CD8, CTL, antitumor activity	s.c., i.p.	[86]•
		Mannose-conjugated liposome	ErbB2 peptide epitopes	PAM_3CAG*, PAM_2CAG*, PAM_2CGD*	IFNγ, antitumor activity	s.c.	[87]
	Langerin	Ab	HIV gag p24	PolyI:C, PolyICLC, αCD40 Ab	IFNγ, CD8	i.p.	[88]
	DC-SIGN	Ab	KLH	-	T cell proliferation, antitumor activity	s.c.	[89]••
	DC-SIGN	scFv	Tetanus toxoid peptides	-	T cell proliferation	s.c.	[89]••

Species	Receptor	Delivery system	Antigen targeted	Adjuvant[1]	Type of response[2]	Route[3]	REF
	Dectin-1	Ab	OVA	Polyl:C	CD4	s.c., i.v.	[78]
	CD103	Ab	OVA	LPS, αCD40 Ab, Polyl:C	CD4, CD8, IFNγ, CTL, Ab	i.p.	[90]
			OVA	-	Tolerance	i.t.	[90]
	Clec9A	Ab	OVA, HIV gag p24, melanocyte differentiation antigen peptides	Polyl:C, PolylCLC, αCD40 Ab, Curdlan	CD4, IFNγ, Ab, CD8, antitumor activity	i.v., s.c./i.d., i.p.	[77, 88, 91]
	Clec12A	Ab	OVA	LPS, CpG, Polyl:C, αCD40 Ab	Ab, CD4, CD8, minor CTL	i.v.	[50]
	MGL	Tn antigen	Tn antigen	CpG, alum, αCD40 Ab	Ab	i.d., s.c.	[92]
	BST-2	Ab	OVA, HEL peptide	CpG, Polyl:C	CD4, CD8, Ab, IFNγ, CTL, antitumor activity	i.p.	[93]
	DCAR1	Ab	OVA	αCD40 Ab	Ab, CD4, CD8	i.v.	[51]
	LOX-1	Ab	OVA	IFA	Antitumor activity	s.c.	[94]
	CD36	scFv	OVA	αCD40 Ab	CD4, CD8, Ab, IFNγ, CTL, antitumor activity	s.c./i.d.	[95]
Human	MR	Ab	hCG-β	R848, PolyICLC, GM-CSF	Ab, IFNγ, minor beneficial clinical outcome	i.d., i.v.	[96]
B cell targeting							
Mouse	B cell receptor	D domain of protein A	OVA, Influenza antigen	QuilA and A1 subunit of cholera toxin-containing ISCOM*	T cell proliferation, IFNγ, Ab	i.n.	[97]
	CD19	Ab	OVA, MUC1 peptide	CpG	CD4, CD8, IFNγ, Ab, antitumor activity	i.v.	[98]
		Diabody	Idiotype	-	Ab, IFNγ, antitumor activity	i.d.	[99]
Macrophage targeting							
Mouse	Sn	Ab	OVA	αCD40 Ab	CD8, IFNγ, CTL, antitumor activity	i.v.	[100]
		Ab	OVA	-	CD8	s.c./i.d.	[101]
		Glycan-coated liposome	α-GalCer	-	iNKT activation	i.v.	[102]
Pig	Sn	Ab	HSA	-	Ab	i.m., i.v.	[103]
		Ab	Ab	-	Ab, T cell proliferation	s.c.	[104]
	CD163	Ab	Ab	-	Ab, T cell proliferation	s.c.	[104]

Ab, antibody; -, none; scFv, single chain fragment variable; REF, reference; CSP, circumsporozoite protein *Plasmodium yoelii*; MP, microparticle; NP, nanoparticle; PLGA, poly(lactic-co-glycolic acid); ISCOM, immune stimulating complex
[1] Adjuvants followed by * were physically linked to the antigen
[2] CD4, CD4 T cell proliferation; CD8, CD8 T cell proliferation; CTL, cytotoxic T cell mediated killing; IFNγ, T cell IFNγ production; Ab, antibody induction
[3] i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous; i.d., intradermal; i.t., intratracheal
• human receptor transgenic mice
•• human immune cell engrafted mice

Targeting B cells

B cells are conventionally considered to be antibody-secreting cells and are, just like macrophages, much less appreciated for their APC capacity than DCs. However, B cells can serve as professional APCs to prime both $CD4^+$ and $CD8^+$ T cells and were also shown to be able to induce T cell tolerance [105-109]. Also, noncognate B cells can carry antigen in the follicles towards follicular DCs, which can present the antigen for days to weeks to allow encounter of the antigen by cognate B cells [110]. In the lymph nodes, these noncognate B cells can either directly retrieve antigen that arrives through specialised channels (conduits) or through gaps in de subcapsular sinus (SCS; low molecular weight antigens) or they can retrieve antigen from SCS macrophages displaying the antigen on their surface [111, 112]. Recently, Ng *et al.* reasoned that a low molecular weight molecule targeting B cells that carries an antigen should be able to reach the follicle B cells by passing through the special conduits and generate potent immune responses [99]. Indeed, using such low molecular weight molecule (diabody) targeting CD19 on B cells, an antibody response was evoked against a tumour antigen which was absent when the tumour antigen was coupled to a non-targeting molecule and partial protection was observed in a subsequent tumour protection model. Nevertheless, in a study by Ding *et al.*, similar results could be obtained using a conventional B cell targeting αCD19 antibody coupled to a tumour antigen [98]. Lastly, Helgeby *et al.* made use of the affinity of protein A for immunoglobulins to target their antigen towards the B cell receptor [97]. Via intranasal immunization they were able to prime T cells and induce antigen-specific antibody responses at both mucosal and systemic sites. These few studies performed are also listed in Table 3.

Targeting macrophages

Elie Metchnikoff, who received the Nobel Prize in Medicine in 1908, was the first person to use the term “macrophage” to describe large mononuclear phagocytic cells he observed in tissues [113, 114]. The term is derived from the Greek words “macros”, meaning large, and “phagein”, to eat, and refers to the observation that these cells are able to take up and digest harmful bodies such as pathogens, much alike the process of digestion in micro-organisms. Since their discovery, macrophages have been found in every organ and in different tissues of the human body where they show functional specialization which varies with the organ or

tissues [115, 116]. These functions include immune surveillance, repair and remodelling, as shown in Figure 2.

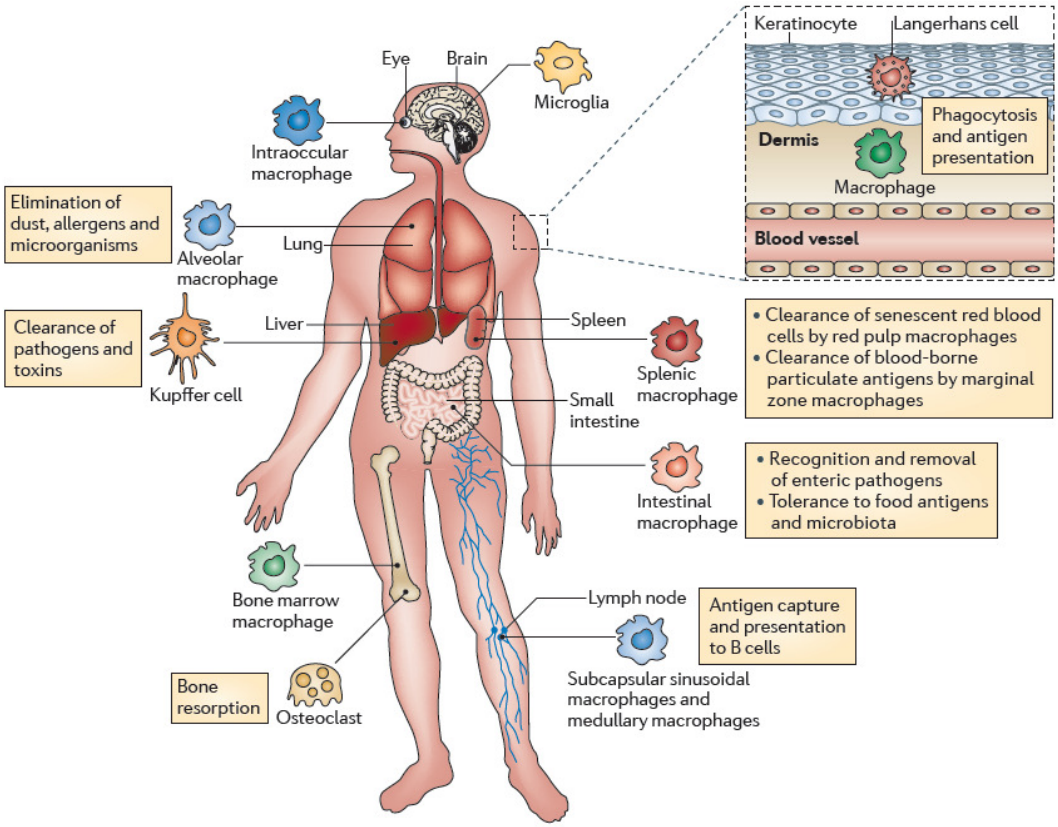

Figure 2. Tissue macrophages show functional specialization based on their anatomical location
Specialized tissue-resident macrophages include osteoclasts (bone), alveolar macrophages (lung), histiocytes (interstitial connective tissue) and Kupffer cells (liver). The gut is populated with multiple types of macrophages and DCs, which work together to maintain tolerance to the gut flora and food. Secondary lymphoid organs also have distinct populations of macrophages that perform unique functions, including subcapsular sinus macrophages of lymph nodes (LNs), which clear viruses from the lymph and initiate antiviral humoral immune responses. Distinct macrophage subpopulations also patrol so-called immune-privileged sites – such as the brain (microglia), eye and testes – where they are assumed to have central functions in tissue remodelling and homeostasis. These tissue-specific macrophage subpopulations ingest foreign materials and recruit additional monocytes from the circulation to differentiate into macrophages during an infection or following injury. Figure adapted from Murray and Wynn [116].

Besides performing functions during steady-state conditions, macrophages can also become activated and adopt context-dependent phenotypes [115, 116]. These 'inflammatory macrophages' can either be locally activated resident tissue macrophages or monocytes recruited from the blood to differentiate into mature macrophages in the tissues. As such, several macrophage subsets with distinct functions have been described. Classically activated macrophages (M1 macrophages) mediate defence of the host from a variety of bacteria, protozoa and viruses, and have roles in antitumor immunity [116, 117]. Alternatively activated macrophages (M2 macrophages) have anti-inflammatory function and regulate wound healing. 'Regulatory' macrophages can secrete large amounts of interleukin-10 (IL-10) in response to FcγR ligation [118]. Tumor-associated macrophages (TAMs) suppress

antitumor immunity, and myeloid-derived suppressor cells (MDSCs) are linked to TAMs and may be their precursors [119]. Thus, when stimulated, macrophages adopt context-dependent phenotypes that either promote or inhibit host antimicrobial defence, antitumor immunity and inflammatory responses. It is generally believed that macrophages represent a spectrum of activated phenotypes rather than discrete stable subpopulations [116, 119]. Indeed, numerous studies have documented flexibility in their functional programming, with macrophages switching from one functional phenotype to another in response to the variable signals of the local micro-environment [120-122].

Macrophages are sometimes described as a classical double-edged sword [116, 117]. Their actions on the one hand help to integrate many physiological functions, yet on the other hand also result in their contribution to many pathological processes. Macrophages have been implicated in the onset and/or progression of various pathogenic conditions, either through normal pro-inflammatory or pro-angiogenic activities or through defects in their activity [123]. Under- or overactivity of macrophage clearance, immune effector functions and responses to metabolic abnormalities contribute to common disorders such as autoimmunity, rheumatoid arthritis, chronic granulomatous disease, atherosclerosis, diabetes, systemic lupus erythrematosis, Wiskott-Aldrich syndrome, Alzheimer's disease and the growth and spread of malignant tumours [114, 116, 117, 124]. In addition, certain pathogens can parasitize these cells and may replicate within them, using these macrophages as a safe haven and a reservoir [125-127]. As such, macrophages have attracted attention for their potential as therapeutic target, to either eliminate, activate of immunomodulate them. Examples of macrophage surface receptors under study for targeted therapy are the folate receptor [128], CD163 [129] and also Sn [130].

As macrophages were not as much appreciated for their antigen-presenting cell capacity, few studies have been performed to date to specifically target antigens towards them as a vaccination strategy. One study investigates Ag targeting to CD163 in pigs [104] and as the MR was first described to be a macrophage-restricted receptor [131], some early studies might fit better in the Chapter targeting macrophages than DCs. Other receptor targeting strategies that will reach macrophages as well include the more general APC receptors under study for vaccination strategies (Table 3), which obviously also target macrophages. In addition, it should be mentioned that many receptors studied to specifically target DCs are also expressed on macrophages. This observation is perhaps not so surprising, as macrophages and myeloid DCs both arise from common myeloid precursors and are thus likely to express common cell surface markers [116]. The most prominent example might well be CD11c, a marker widely used to specifically isolate DCs, which appears to be expressed by almost all macrophages as well. At present, it has even been argued that it might be

impossible to distinguish macrophages and DCs from each other and that it might be better to speak of a mononuclear phagocyte system that comprises both cell types and their different subsets that complement each other in function [132]. Indeed, recent evidence indicates that certain cells defined as macrophages can migrate, some macrophages are only weakly phagocytic and TIP-DCs (tumour-necrosis factor/inducible nitric oxide synthase-producing DCs) might rather be inflammatory macrophages than DCs [116, 133]. Be this as it may, it is nonetheless clear that many markers studied to specifically target antigens towards DCs are also expressed by cell subsets defined as macrophages and consequently evoked immune responses are often hard to establish as purely DC (and DC-subset)-mediated.

Only recently, Sn^+ macrophages have received attention for vaccination strategies as well. In spleen and lymph nodes, these macrophages are situated at the border of circulating fluids and thus appear to be ideally positioned for antigen capture and processing [134]. Studies targeting Sn have been performed and they will be discussed in more detail in the next Chapter on Sn and Sn^+ macrophages.

1.1.3 SIALOADHESIN AND SIALOADHESIN-EXPRESSING MACROPHAGES

Sialoadhesion: a member of the sialic acid-binding immunoglobulin-type lectin (Siglec) family

Sialoadhesin (Sn; also referred to as CD169 or Siglec-1) belongs to the family of sialic acid-binding immunoglobulin-like lectins or Siglecs [135, 136]. This family name reflects their binding specificity for carbohydrates containing sialic acids and the presence of immunoglobulin (Ig)-like domains in their protein structure. Siglecs therefore represent a subgroup of Ig-type or I-type lectins belonging to the Ig superfamily, one of the largest protein families in the vertebrate proteome.

Siglecs are type 1 transmembrane proteins comprising a sialic acid-binding N-terminal variable (V) Ig-like domain followed by a variable number of constant (C) Ig-like domains, a transmembrane region and a cytosolic tail (Fig. 3) [137]. A conserved arginine residue in the V domain is essential for the sialic acid binding capacity of all Siglecs. Based on sequence similarities and evolutionary conservation, two primary subsets of Siglecs have been identified: the first includes Sn, Siglec-2 (CD22), Siglec-4 (myelin-associated glycoprotein or MAG) and Siglec-15, which are quite distantly related (~25–30% sequence identity) but have orthologues in all mammalian species studied and are therefore referred to as well-conserved [52, 137]. The second, rapidly evolving, subset is designated the CD33-related Siglecs, which share ~50-99% sequence identity, yet very poor species homology, thereby impeding the assignment of orthologues [138]. As a consequence, a different numbering

system is used for the human and mouse CD33-related Siglecs. An overview of the structure and nomenclature of the Siglec family is given in Figure 3.

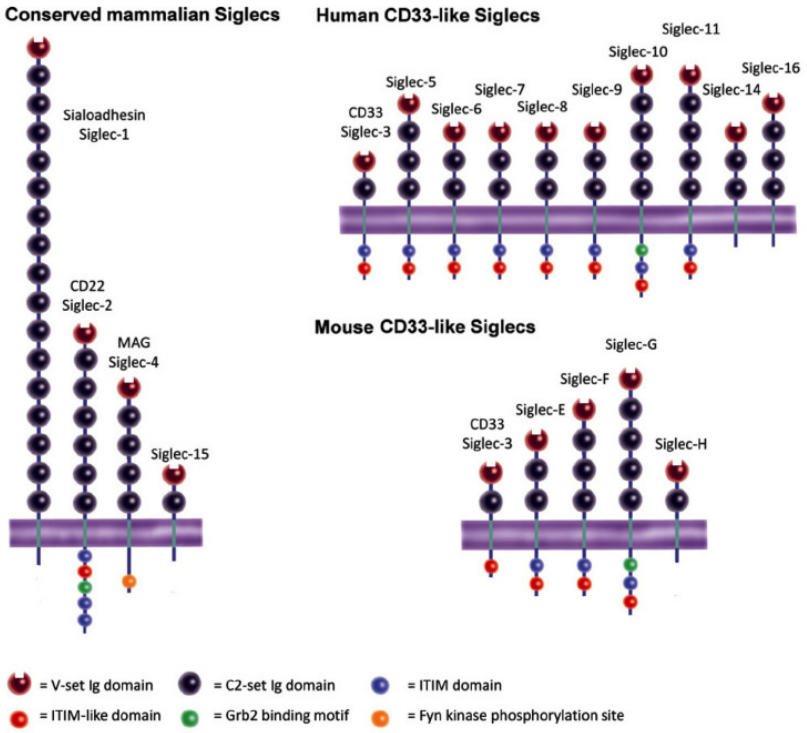

Figure 3. Nomenclature and structure of Siglecs in humans and mice
Siglecs are single-pass transmembrane proteins that comprise a single sialic acid-binding N-terminal V-set domain, variable numbers of C2-set domains, a transmembrane region and a cytosolic tail. The evolutionary conserved Siglec subgroup is shown on the left; the rapidly evolving CD33-related subgroup is depicted on the right (human CD33-related Siglecs on the top, murine on the bottom). Siglec-12 (now renamed Siglec-XII) is not depicted, as it has lost its lectin activity in humans. C, constant; Grb2, growth factor receptor-bound protein 2; Ig, immunoglobulin; ITIM, immunoreceptor tyrosine-based inhibitory motif; MAG, myelin-associated glycoprotein; N, amino; V, variable. Figure adapted from Jandus *et al.* [139].

Since their classification in 1998, Siglecs have emerged as a novel family of regulatory receptors expressed in a cell-type specific manner, predominantly on immune and haematopoietic cells [52, 135, 139]. With the exception of resting T cells, virtually all cell types in the human and mouse immune system express at least one Siglec family member, with some cells expressing multiple CD33-related Siglecs [139]. The Siglec expression pattern of a given haematopoietic cell can also change during differentiation, with acquisition of specific Siglecs at later developmental stages [140, 141]. In addition, the surface expression and function of several Siglec members have also been shown to be influenced by cytokines, TLR activation, viral and bacterial infections, inflammatory and autoimmune diseases and cancer [139]. Many Siglecs undergo endocytosis upon ligand binding, and most Siglecs have also been shown to contain cytoplasmic tyrosine motifs, commonly found in receptors involved in regulation of cell signalling (Fig. 3) [137]. Many Siglecs contain ITIM (immunoreceptor tyrosine-based inhibitory motif) and ITIM-like motifs in their cytoplasmic

tails and can function as inhibitory receptors by dampening tyrosine kinase-driven signaling pathways. Other Siglecs have no tyrosine motifs, but some contain a positively charged transmembrane spanning region which permits association with adapter proteins such as DNAX-activating protein 12 kDa (DAP-12), an ITAM (immunoreceptor tyrosine-based activation motif)-containing adaptor that can trigger both activating and inhibitory signalling [52, 137]. Taken together, while the functions of the Siglecs are still being elucidated, there is growing evidence that the majority are endocytic receptors that contribute to the regulation of cell signaling (inhibition or activation) in immune cells that mediate innate and adaptive immunity [142]. Furthermore, each Siglec has a unique preference for one or more distinct sialic-acid containing carbohydrates expressed on the same cell (*cis*) or on adjacent cells (*trans*) [52, 143], resulting in cell-specific, unique and non-redundant functions of each family member in defined immune cell types [139].

Sialoadhesin structure

Sn was the first protein to be described of the later defined Siglec family. In the mid-1980s, some macrophage types were found to form rosettes with sheep erythrocytes *in vitro*, which could be abolished by sialidase pretreatment of the erythrocytes [144]. This sialic acid-dependent receptor was therefore first named sheep erythrocyte receptor (SER). Later on, the receptor was purified and shown to be a large protein of 185.000 MW and was renamed Sn [145]. As shown in Figure 3, Sn is the largest member of the Siglec family, displaying a total of 17 extracellular Ig-like domains, which can be subdivided into 16 C2-set domains and an N-terminal V-set domain [146]. It has been postulated that the extracellular region of Sn may function to extend the V-set domain away from the glycocalyx so as to enable interaction with ligands in a *trans* fashion [134, 147]. However, even with the 17 Ig-like domains, extension of Sn above the glycocalyx may not always be guaranteed, as Sn can interact with sialic-acid containing carbohydrates expressed on the same cell (*cis*). A comparison of Sn activity in rat splenic and lymph node (LN) macrophages has indicated that splenic macrophages are masked considerably by endogenous sialylglycoconjugates through *cis*-type interactions, which can be ablated by sialidase treatment, whereas LN macrophages are relatively unaffected by this masking [148]. In addition, the extracellular region of Sn itself contains several potential N-linked glycosylation sites and carries complex N-glycans, which can be sialylated [127, 146] and sialylation of Sn may affect binding of the receptor to its ligands [149]. Sialylation of Sn, and Siglecs in general, by specific glycosyltransferases, may be a common mechanism by which Sn- and Siglec-mediated adhesion is regulated.

The V-set domain of Sn is necessary and sufficient for sialic acid binding and three conserved amino acid residues have been identified that are important for the sialic acid-

binding activity: tryptophan 2, arginine 97 and tryptophan 106 [150-153]. Arginine 97 was shown to be crucially important for binding to sialic acid as it was demonstrated that even a conservative substitution of arginine with lysine resulted in a 10-fold loss in binding affinity [154]. Sn binds preferentially to 5-*N* acetylated-neuraminic acid (Neu5Ac), which is α(2,3)-linked to preceding carbohydrates [146, 155]. Neu5Ac is the most abundant of the mammalian sialic acids, two other common sialic acids Neu5Gc and Neu5Ac9Ac are not recognized by Sn [156]. In common with many other lectins, the glycan binding site of Sn exhibits low millimolar binding affinities for naturally sialylated ligands [154]. It is thought that simultaneous multivalent low-affinity associations will create sufficient high avidity and lead to biologically meaningful interactions of Sn with sialic acids on cells or microbial particles, which is supported by *in vitro* data demonstrating increased binding capacity of Sn with increasing saccharide content [134, 157].

Apart from its large extracellular domain, Sn also contains a transmembrane domain and a short cytoplasmic tail, which is poorly conserved between mammalian species and will be discussed later (see section: sialoadhesin as an endocytic receptor involved in signalling) [127, 158]. So far, no evidence has been found for the existence of soluble Sn variants *in vivo*.

Sialoadhesin expression and regulation

Sn is exclusively expressed on cell subsets of the monocyte/macrophage lineage [52, 156]. In addition, in man [158], mouse [159], rat [160] and pig [161] Sn is most abundantly expressed by macrophage subsets occupying secondary lymphoid tissue (Table 4).

Table 4: Sn expression in secondary lymphoid tissues

Tissue/Cell type	Rat	Human	Mouse	Pig
Marginal metallophilic macrophage (MMM)	+	D.N.E.	+	N.D.
Marginal zone macrophage (MZM)	+	-	+/-	+
Periarteriolar lymphoid sheath (PALS)	+/-	-	-	N.D.
Splenic perifolicular zone macrophage (PFZM)	D.N.E.	+	D.N.E.	D.N.E.
Periarteriolar-associated macrophage (PAM)	D.N.E.	+	D.N.E.	+[1]
Red pulp	+/-	+/-	+/-	-
Lymph node subcapsular sinus	+	+[2]	+	+
Lymph node medulla	+	+[2]	+	+

Key: D.N.E., do not exist; N.D., not described; -, undetected; +/-, low expression; +, expression.
[1] Ellipsoidal vessels associated with Sn expression, possibly equivalent to PAM [161].
[2] Sinusoidal expression throughout LN [162].
Table adapted from O-Neill *et al.* [134]

In all the species studied, macrophages lining the SCS as well as the medulla of the LNs highly express Sn [134]. In rodents, Sn^+ macrophages expressing CD11c have been

observed within the B cell follicle and at the boundary between B and T cell areas (Fig. 4) [10]. In pigs, Sn expression has also been observed at the periphery of follicles and on some follicular cells [161]. It has been suggested these cells might correspond to activated Sn^+ macrophages [10].

A schematic of a LN showing the major zones and sinuses is given in Figure 4. Afferent lymphatics enter the LN tissue at the capsule at the periphery and open into the SCS [163], where just underneath a rim of Sn^+ macrophages is present. Many of these macrophages have a 'head' that projects into the sinus and long 'tail' processes that extend into the underlying follicle, with the 'neck' tightly inserted across the lymphatic lining cell layer. Lymph then further travels through cortical and medullary sinuses directly or percolates through the diffuse tissue and medullary cords to reach the medullary sinuses. All lymph leaves the node through the centrally located efferent lymph vessels. This LN structure shown in Figure 4 is found in most mammals, but the pig, along with the rhinoceros, the dolphin and the elephant, has a specific LN structure that is called inverted [163, 164]. In these species, the tissue lacks a large central medullary area and is preferentially composed of B cell and T cell areas [164]. Parts of the diffuse tissue however are arranged into sinuses and cords in a manner similar to the medullary tissue in other species and a SCS is also present around the diffuse tissue [165]. Afferent lymphatics enter the LN centrally at the capsule at one or more sites and penetrate via the trabeculae deep into B cell and T cell areas [163]. Occasionally, branches may arise from the afferent lymphatics outside the LN and join with a SCS where B cell follicles are located peripherally [165]. Where the trabeculae end, their associated sinuses are continuous with many interstitial spaces, which allows lymph to penetrate into the diffuse tissue [165]. The lymph then filters into the peripheral sinuses, which converge and form several efferent vessels at the periphery of the node [163]. Consequently, the flow of the lymph in pigs is identical to that in LNs of other animals, first reaching the area of the node that is rich in B cell follicles and traversing through T cell and medullary areas before exiting the LN. Sn^+ macrophages have been identified at the subcapsular and medullary sinuses in pigs [161] and thus appear, just like in the other species studied, readily positioned to sample incoming lymph for antigens.

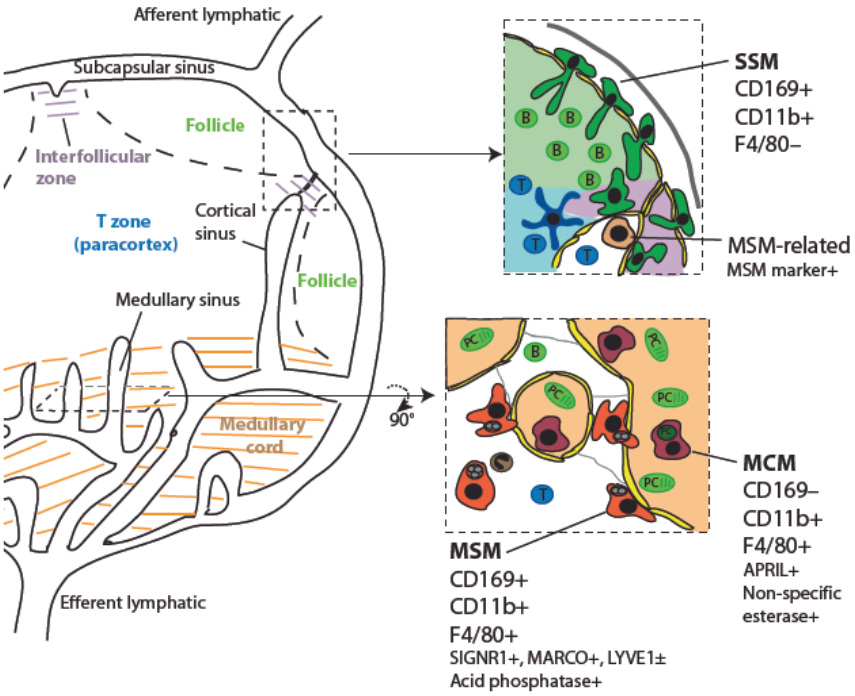

Figure 4. Schematic of the lymph node structure found in most mammals
Boxed regions are enlarged on the right to show more details. Upper box: subcapsular sinus and interfollicular region with B cell follicle shown in green shading, T cell zone in blue and interfollicular region in purple. Lower box: medullary region in cross-section showing medullary cords in light brown. Sinuses are shown in white and sinus-lining lymphatic endothelial cells are yellow. The macrophages are labelled subcapsular sinus macrophages (SSM), medullary sinus macrophages (MSM) and medullary cord macrophages (MCM) and their sialoadhesin (CD169), CD11b and F4/80 phenotype is summarized, although it is not yet established whether the physical segregation of cells with the indicated MSM and MCM marker patterns is as distinct as shown. A DC is shown in dark blue (upper) and a polymorphonuclear cell in brown (lower). Reticular fibers within medullary sinuses are in grey. B, B cell; T, T cell; PC, plasma cell. Figure adapted from Gray and Cyster [166].

Splenic distribution of Sn^+ macrophages varies between species (Fig. 5) [134]. Marginal metallophilic macrophages (MMM), a population that lines the marginal sinus at the periphery of the white pulp in rodents, express high levels of Sn. In rats marginal zone macrophages (MZM) exhibit similar Sn levels to MMM, whereas in mice Sn expression on MZM is relatively lower. Both species demonstrate low levels of Sn expression in red pulp macrophages. With differing splenic architecture in primates, Sn^+ macrophages are located in a compartment found between the red pulp and the marginal zone identified as the perifollicular zone [167]. In the absence of a marginal sinus and MMM in primate spleens, the macrophage sheaths that line capillaries of the perifollicular zone are speculated to be equivalent to the MMM found in rodents. In pigs, splenic Sn expression is observed in the marginal zone and elliptical vessels [161], which may be comparable to the periarterial macrophage sheaths observed in humans. Regardless of their equivalence, both the marginal zone and perifollicular zone environments do at least permit a close contact between Sn^+ macrophages and the circulation and this could be relevant in the context of the physiological function of Sn.

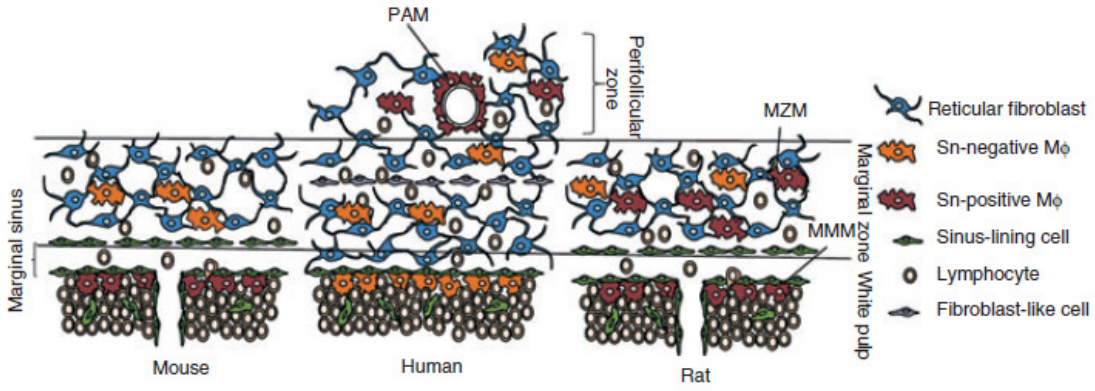

Figure 5. Comparison of the distribution of Sn^+ macrophages in the splenic microenvironment. A framework of reticular fibroblasts (blue) forms the basis of the marginal zone (MZ) and is continuous with the reticular fibroblasts in the red pulp and the sinus-lining cells (green) of the marginal sinus. In rodents, Sn^+ macrophages are observed as a line of cells at the internal border of the white pulp (the so-called marginal metallophilic macrophages (MMM)). The white pulp is separated from the MZ by a marginal sinus, and in the case of the rat spleen, Sn^+ macrophages are observed in the MZ. In humans, no marginal sinus is present and the MZ is devoid of Sn^+ macrophages, but the unique structure known as the perifollicular zone contains Sn^+ macrophages both sporadically and clustered around arteriolae (known as periarteriolar-associated macrophages (PAM)). MZM, marginal zone macrophage. Figure adapted from O-Neill *et al.* [134]

In essence, the LN and the white pulp area of the spleen function as focal centres where microbial particles, antigen-presenting cells (which may carry antigen from the periphery or acquire antigen locally) and their corresponding antigen-specific lymphocytes engage and initiate adaptive immune responses [134]. In the spleen, Sn^+ macrophages are present where blood enters the tissue, while in LN, Sn^+ macrophages are present where lymph enters the tissue. This positioning at the borders of circulating fluids is consistent with the idea that these cells and their Sn have a role in antigen capture/processing and in cell-cell communication. These observations strongly suggest that Sn^+ macrophages may function as mediators of adaptive immunity.

Sn expression can also be found on macrophages residing in non-lymphoid tissues [134]. Both mice and humans express quite high levels of Sn on resident bone marrow macrophages, whereas in rats Sn is apparently absent from this population. Expression of Sn is also observed in the lungs of all species tested [158-160, 168], in the skin of mice [159], in the intestines of mice [159], rats [160] and humans [158], in the brain of mice [159] and humans [158], and in the placenta of the pig [169]. In the thymus, weak Sn expression was found in rats [160] and pigs [170]. In the liver, Sn expression is consistently found on Kupffer cells of all species examined. So far, no evidence exists that Sn is expressed on DCs *in vivo* under normal physiological conditions [156, 171]. Remarkably, a recent study by Gray *et al.* [172] described the acquisition of Sn by IL-17 committed $IL7R\alpha^{hi}$ $CCR6^+$ T cells and natural killer (NK) cells and small numbers of $CD11c^{hi}$ DCs through the acquisition of membrane blebs derived from SCS macrophages. However, the authors stated it cannot be

ruled out that this acquisition was due to tissue preparation. Taken together, under normal physiological conditions, Sn expression is highly regulated and restricted to certain subpopulations of tissue macrophages, whereas it is not found on their precursor cells, the monocytes, nor on DCs.

Certain mediators present in secondary lymphoid tissues have been shown to be crucial for the Sn^+ macrophage population [134]. Colony-stimulating factor 1 (CSF-1) is an important cytokine responsible for macrophage maturation and migration and interference with CSF-1 signalling has been shown to rapidly deplete Sn^+ macrophages in the spleen [173]. A naturally occurring CSF-1 knockout mouse, the *op/op* mouse, has no Sn^+ macrophages in its lymphoid tissues [174]. Upon CSF-1 administration however, the Sn^+ macrophage population can be restored [175]. Together, this strongly suggests a regulatory role of CSF-1 production on Sn^+ macrophage populations. Lymphotoxin (LT) is another mediator shown to be crucial for the Sn^+ macrophages. LT is responsible for generating and maintaining the architecture of secondary lymphoid organs as its knockout causes loss of structural organization in mice [176]. Deficiency of LT-α [177] and LT-β [178] secretion leads to absence of Sn^+ macrophages in lymphoid organs. As B cell depletion affects Sn^+ macrophages in the SCS as well as the marginal zone [179, 180], LT expression by B cells was shown to be directly implicated in this phenomenon [179, 181]. Systemic treatment with LT antagonists replicates the phenotype of B cell depletion, and this leads in the SCS to the replacement of Sn^+ macrophages with a phenotype associated with medullary macrophages [182]. Altogether, these data demonstrate how intrinsic Sn macrophages are to lymphoid tissue and that their emergence is governed by its proper structuring.

Besides homeostatic regulation of Sn expression, inflammatory stimuli can rapidly upregulate Sn expression on subsets of inflammatory macrophages and monocytes. *In vitro*, Sn induction on peripheral blood mononuclear cells (PBMC) and various macrophage subsets isolated from humans [158, 183, 184], mice [185], rats [186, 187] and pigs [188] can be achieved by type I or II interferon (IFN) stimulation. Rat [186, 187], mouse [185] and human [158] Sn induction has also been shown following tumour necrosis factor-α (TNF-α) exposure, and additionally, by ligation of human TLRs involved in viral and bacterial sensing (which also induce IFN-α secretion) [183]. On top of that, Sn expression can be induced *in vitro* on monocyte-derived DC populations after treatment with inactivated human rhinovirus, probably via intracellular RNA sensors that drive type I IFN production [189]. Interestingly, in the case of both mice and pigs an unidentified component of autologous serum has been shown to induce Sn expression [188, 190]. This modulation of expression by circulating factors may well explain the observed changes in Sn expression on macrophage populations following disruption of the blood-brain barrier [191] and afferent lymphatics [192]. The factors

negatively regulating Sn are not as clear. T helper type 2 cytokines in mice [193] and both virus-sensing and bacteria-sensing TLRs in humans [183] have been shown to down-regulate expression, whereas IL-2 and IFN-γ demonstrated some inhibitory effects in rats [194].

In vivo, Sn^+ macrophages have been identified in a variety of inflammatory disease settings (see section: Sn^+ macrophages as immunomodulators in disease settings) which is in line with the regulation of Sn by inflammatory cytokines. Also, the great impact of type I and type II IFN on Sn expression suggest a function in anti-viral activity which is consistent with the observed interaction between Sn and viral particles as well as with a functional role for Sn^+ macrophages in mediating adaptive immune responses (see sections: Sn as a pathogen receptor and Sn^+ macrophages as mediators of innate and adaptive immunity). However, it is less clear how Sn expression can be negatively regulated, and how both up- and downregulation of Sn relate to Sn function *in vivo*.

Sialoadhesin in cell-cell interactions

As mentioned before, it has been proposed that Sn has evolved the unusually large number of 17 Ig domains to extend the sialic acid binding site above the dense glycocalyx to avoid *cis*-inhibition by the abundant sialic acids at the cell surface, thereby permitting *trans* interaction with ligands on other cells [146, 156]. In line with this, Sn displays binding to various cell populations, including cells of the granulocytic lineage [195]. Initially, Sn was defined as a sheep erythrocyte receptor, as Sn^+ macrophages were found to form rosettes with sheep erythrocytes *in vitro* [144]. Later on, Sn was identified as a lymphocyte adhesion molecule involved in the binding of T and B cells, while differential binding to T cells was recorded depending on T cell maturity [196]. In an artificial disease model, Sn^+ macrophages in the liver were observed to form clusters with $CD4^+$ and $CD8^+$ T cells during a successful graft-versus-leukaemia response. Following infusion of anti-Sn antibody, macrophage-T cell cluster formation, along with survival rate, were reduced, suggesting a regulatory role for Sn-Sn ligand interactions [197]. A direct impact of Sn-mediated interaction on lymphocyte behaviour is shown by studies on experimental allergic encephalitis (EAE). Here, it was noted that Tregs in the afflicted tissue displayed increased Sn ligand and that *in vitro*, the absence or blocking of Sn expression on macrophages can lead to efficient proliferation of isolated Tregs [198]. *In vivo*, Sn knock-out mice showed reduced disease severity along with reduced numbers of infiltrating T helper type 1 (Th1) and type 17 (Th17) cells in the central nervous sytem, while higher numbers of Tregs were present [198]. In a more recent study, *in vitro* activation and analysis of all splenic $CD4^+$ T cells showed that an upregulation of Sn ligand expression can also be observed on highly activated $CD4^+$ effector T cells (Teffs), which comprise about 20% of all $CD4^+$ Teffs [199]. Coculture of these activated Teffs with

Sn^+ macrophages or Sn^+ Chinese hamster ovary (CHO) cells resulted in increased cell death of Teffs, again showing a direct impact of Sn-Sn ligand interactions on lymphocyte behaviour. This interaction however might indicate that Sn can perform a dual role in autoimmune diseases. On the one hand, Sn-induced inhibition of Sn ligand-expressing Tregs proliferation can lead to reduced suppression of Teffs and enhanced inflammation as demonstrated in EAE. On the other hand, Sn-induced death of Sn ligand-expressing Teffs could lead to elimination of a hyperactive subset of Teffs and consequently reduced inflammation. The *in vivo* relevance of these observations however still needs to be confirmed. Remarkably, previously described Sn counterreceptors CD43 and P-selectin Glycoprotein Ligand-1 (PSGL-1) [200] were shown to be both dispensable for the Sn-Teffs interaction, leaving the protein carrier of the Sn ligand currently unidentified. In coronary artery disease patients, increased Sn expression on monocytes was related to increased T cell proliferation and pro-inflammatory cytokines secretion [201]. Down-regulation of Sn on the other hand could attenuate proliferation and activation of cocultured $CD4^+$ and $CD8^+$ T cells. Taken all data together, it is clear that Sn-Sn ligand interactions can influence lymphocyte behaviour. Mostly, these interactions appear to be pro-inflammatory, although a recent study suggests interactions might sometimes also be anti-inflammatory.

The observation that Sn can interact with ligands on other cells led to the search for counterreceptors of Sn. The membrane mucin MUC1, expressed on breast cancer cells, was the first counterreceptor identified for Sn, expressed on macrophages infiltrating breast cancer cells [202]. Both MUC1 and the later defined counterreceptor CD43 were shown to bind Sn in a sialic acid-dependent manner, while binding of Sn to membrane receptors via a sialic acid-independent mechanism has also been described [114]. Specific glycoforms of Sn were shown to interact with the cysteine-rich domain of the MR [203, 204] and macrophage galactose-type C-type lectin 1 (MGL1) [205]. Both receptors are lectins that interact with N-glycans on Sn and are expressed by macrophages and DCs. The interaction of Sn with MGL1 is thought to be important to direct cells to the SCS of LN during immune responses [206]. The Sn-glycoform recognized by the MR is mainly expressed on MZM and SCS macrophages and this interaction is thought to be important in the transport of native antigen to follicular zones of the spleen and LNs [203]. Taken together, the interaction of Sn with these lectins may be important to direct antigen (free or cell-associated) to B and T cell zones to enhance antigen-specific immune responses.

Sialoadhesin as a pathogen receptor

Although sialic acids are abundant in all higher organisms, they are less common amongst microorganisms and potential pathogens [156, 207]. However, several important pathogens have evolved various mechanism to synthesize or capture sialic acids and display them on

their surfaces. In line with this several pathogens have already been identified that can be recognized by Sn. So far, human immunodeficiency virus 1 (HIV-1), porcine reproductive and respiratory syndrome virus (PRRSV), *Campylobacter jejuni*, *Neisseria meningitides* and *Trypanosoma cruzi* are known to engage Sn. Due to the strategic positioning of Sn^+ macrophages in the spleen and LNs on the border of lymphocyte rich regions and the entry of blood or lymph, this Sn-dependent recognition can promote pathogen uptake in macrophages which could be important in antigen presentation and immune activation. In line with this, a recent study with a prototypic cytopathic virus (vesicular stomatitis virus) showed that viral replication in Sn^+ macrophages in the splenic marginal zone was necessary to ensure the production of sufficient viral antigen for effective activation of the adaptive immune response [208]. On the other hand, Sn-dependent recognition can also be misused by pathogens to their advantage. In the case of PRRSV, sialylated envelope glycoproteins M/GP_5 mediate binding to Sn on alveolar macrophages leading to subsequent infection [209]. In the case of HIV-1, Sn is misused by the virus to mediate *trans* infection of $CD4^+$ T cells [210]. Taken together, Sn-pathogen interactions can be of benefit both to the host as well as to the pathogen and in which direction the balance shifts will probably be pathogen-specific.

Sialoadhesin as an endocytic receptor involved in signalling

Initially, Sn was described as a non-phagocytic receptor [144], though later on Sn was shown to be involved in the endocytosis of pathogens like PRRSV, *Neisseria meningitides* and *Trypanosoma cruzi*. Also, it was established that Sn-specific mAbs and their antigen binding fragments (Fab) are internalized into porcine alveolar macrophages upon engagement with their receptor via clathrin-mediated endocytosis [103]. Internalization was only partial, as the receptor was still detected at the cell surface at all time points tested, and it was shown that internalized antibodies resided for prolonged times in early endosomes. Together, these data demonstrate the involvement of Sn in internalization processes, yet the signalling motifs involved in its internalization remain to be discovered [103]. As mentioned before, Sn contains only a short, poorly conserved, cytoplasmic domain, devoid of tyrosine-based, di-leucine-based or other known motifs that are implicated in signal transduction and endocytosis (Fig. 6). A study performed by Genini *et al.* however showed that cross-linking of Sn with an antibody resulted in subtle alterations to the MAP kinase and Wnt signalling pathways, suggesting that Sn is able to activate intracellular signalling pathways and thus serves as a signalling molecule [211]. Further research will be needed to clarify the events occurring upon receptor engagement and to identify currently unknown signalling motifs.

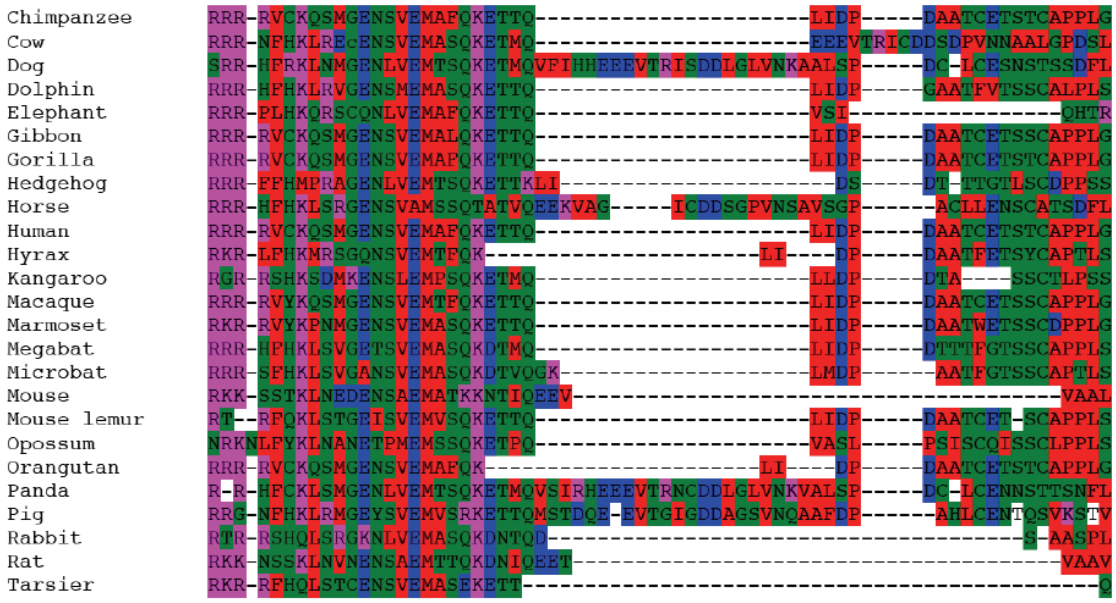

Figure 6. Alignment of the cytoplasmic tails of (predicted) mammalian sialoadhesin.
Clustal W (1.83) multiple sequence alignment of the cytoplasmic domain of (predicted) mammalian Sn, which is poorly conserved between mammalian species. Amino acids (AA) with similar properties are marked with similar colours. Colour code: blue, acidic AA (DE); green, contains: hydroxyl, sulfhydryl or amine group and G (STYHCNGQ); magenta, basic AA without H (RK); red, small AA + hydrophobic AA (including the aromatic AA F and W) (AVFPMILW). Figure adapted from De Baere 2012 [114].

To gain insight in the effect of ligand binding to Sn on macrophage effector functions, a study performed by De Baere *et al.* investigated the effect of antibody binding to porcine Sn (pSn) on macrophage viability, pSn surface expression, reactive oxygen/nitrogen species production, phagocytosis of microspheres, uptake and processing of soluble antigens, MHC I and MHC II cell surface expression and cytokine production [212]. This was done by treatment of porcine primary alveolar macrophages with a pSn-specific mAb in comparison to an isotype-matched control mAb *in vitro*. No significant effect on the effector functions under study were observed, except for a significant reduction of phagocytosis of fluorescent carboxylate-modified beads. Further research is required to assess whether these observations can be of relevance *in vivo* for the clearance of pathogens through phagocytosis after Sn engagement.

Sn$^+$ macrophages as immunomodulators in disease settings

As discussed before, Sn is expressed by macrophage populations located in a variety of tissues, with the highest expression levels present in the secondary lymphoid organs. Also, under inflammatory stimuli Sn is rapidly upregulated on subsets of inflammatory macrophages and monocytes. This regulation by inflammatory cytokines is in line with the identification of Sn$^+$ monocytes and macrophages in a variety of inflammatory disease settings. So far, Sn$^+$ monocytes/macrophages have been found in models of chronic

rejection [213], neointima injury [214], cerebral vasculature injury [215], atherosclerosis [216, 217], rheumatoid arthritis [218, 219], experimental autoimmune uveoretinitis [220], experimental allergic encephalomyelitis [198], systemic lupus erytematosus [221, 222], primary biliary cirrhosis [223], proliferative glomerulonephritis [224, 225] and diabetic nephropathy [226]. As previously discussed, the interaction between Sn and its ligands present on T cells, can alter lymphocyte behaviour, most strikingly shown by interaction of Sn with Tregs in EAE, negatively controlling their expansion and hence autoimmune disease progression [198]. In other mouse models of central nervous system inflammation, experimental autoimmune uveoretinitis [220] and myelin degeneration models [227, 228], Sn knock-out mice exhibit reduced disease severity which correlates with reduced lymphocyte invasion as well. In a rat model of rheumatoid arthritis, accumulation of Sn^+ macrophages into the arthritic synovium and joint space occurs within a day following induction and parallels lymphocyte influx [218, 229, 230]. In addition, treatment with clodronate-laden small unilamellar vesicles reduces overall macrophage burden (including Sn^+ macrophages) and arthritis severity [231], although it is unclear whether this effect resulted specifically from the Sn^+ macrophage subset. Similarly, in humans suffering from glomerulonephritis, analysis of kidney tissue from patients has shown that Sn^+ macrophage accumulation correlated with glomerular injury and that successful glucocorticoid therapy correlated with reduced numbers of glomerular Sn^+ macrophages [225]. As with arthritis, a functional role for Sn^+ macrophages has yet to be established. Until such a role has been established, it remains possible that Sn^+ macrophages are simply byproducts of the inflammatory environment generated in these two diseases. However, taken together, it is clear that Sn^+ macrophages can have a negative impact on disease progression of inflammatory diseases, because of their immune stimulatory role. This makes these Sn^+ macrophages attractive targets to manipulate or eliminate in a cell-directed therapy strategy. A striking example of this approach is a recent targeted delivery of an anti-TNF-α oligonucleotide to Sn^+ macrophages, which resulted in the relief of lupus-like symptoms in mice [130].

In addition to the inflammatory diseases discussed, Sn^+ macrophages have also been found in a variety of cancer models, where they are thought to activate recruited T cells and hence promote a tumoricidal response. In murine graft-versus-leukaemia reactivity models, increased Sn expression in liver and spleen correlated with lymphoma stasis/regression [232-234]. In a rat model of prostate adenocarcinoma, the number of Sn^+ macrophages showed a strong positive correlation with tumour apoptosis and a strong negative correlation with tumor growth [235]. As in mice, rat Sn expression was found to coincide with lymphocyte recruitment. Clinically, increased Sn expression has been recorded in splenic marginal cell lymphoma [162] as well as in macrophage infiltrates of MUC-1-positive breast carcinoma

[202], underscoring the possibility that Sn^+ macrophages may play an important role in the host antitumor immune response.

Taken together, the available data show that Sn knock-out and inhibition diminish the adaptive immune response in the above models. As such, it is tempting to speculate that the purpose of Sn is to somehow promote adaptive immune responses mediated by macrophages [134]. An intriguing example of this might be the development of Guillain-Barré syndrome (GBS). Here, molecular mimicry between *Campylobacter jejuni* sialylated lipooligosaccharides and human nerve gangliosides can trigger the production of cross-reactive antibodies which induce GBS [236]. As *C. jejuni* has been shown to engage Sn, it has been suggested that although *C. jejuni* can be efficiently killed by Sn^+ macrophages, the subsequent enhanced antigen presentation can be the key event leading to the induction of cross-reactive antibodies and ultimately GBS [236].

Sn^+ macrophages involved in haematopoiesis

Initial studies identifying Sn localization at the contact points of resident bone marrow macrophages and erythroblastic islands suggested a role in haematopoiesis [237]. Although the development of Sn knock-out mice did not go on to demonstrate haematopoietic defects [191], a recent study by Chow *et al.* demonstrates that Sn^+ macrophages do have a role to play *in vivo* in erythropoiesis under homeostatic conditions and in disease [238]. Depletion of Sn^+ macrophages (by clodronate liposomes or by the use of the CD169-difteria toxin receptor mouse model) was shown to markedly reduce the number of erythroblasts in bone marrow, although this did not result in overt anemia under homeostatic conditions, probably because of concomitant alterations in red blood cell clearance. However, under conditions of haemolytic anemia, acute blood loss or myeloablation, Sn^+ macrophage depletion significantly impaired erythropoietic recovery. Having demonstrated the role of Sn^+ macrophages in recovery after erythropoietic insufficiency, it was tested subsequently whether macrophage depletion could be beneficial in the context of an overactive erythron in a mouse model of polycythemia vera. Here, it was noticed that Sn^+ macrophage depletion normalized the erythroid compartment, suggesting that erythropoiesis in polycythemia vera remains under the control of macrophages in the bone marrow and splenic microenvironments. These results indicate that Sn^+ macrophages promote late erythroid maturation and that modulation of the macrophage compartment may be a new strategy to treat erythropoietic disorders. These findings also correlate with the functional specialisation of macrophages based on their location. While Sn^+ macrophages play a role in haematopoiesis in the bone marrow, they are involved in the stimulation of adaptive immunity in lymph nodes and spleen.

Sn^+ macrophages as mediators of innate and adaptive immunity

Apart from several disease models where Sn^+ macrophages appear to behave as promoters of the adaptive immune response, several other studies highlight the ability of Sn^+ macrophages to enhance innate and adaptive immunity as well. Innate immune stimulation has so far been shown to depend on type I IFN secretion. In a study by Garcia *et al.*, type I IFN secretion by Sn^+ macrophages upon the local administration of a modified vaccinia virus Ankara vaccine in mice resulted in NK cell accumulation in the subcapsular area of the draining LN and in the activation of the NK cells [239]. In a study performed by Iannacone *et al.*, SCS macrophage secretion of IFN-α in response to vesicular stomatitis virus (VSV) infection prevented viral access to the central nervous system [240]. This demonstrates classical innate immune cell behaviour of pathogen scavenging and release of inflammatory mediators [134]; however, further data illustrate a direct action of Sn^+ macrophages in supporting adaptive immune responses. Junt *et al.* showed that in LNs Sn^+ macrophages capture viral particles (VSV) within minutes after subcutaneous injection, transported them across the SCS floor and directly presented them to migrating B cells in the underlying follicles [241]. Moreover, also particulate antigen (fluorescent particles loaded with the model antigen hen egg lysozyme) and immune complexes are captured and displayed by Sn^+ macrophages to B cells [110, 179, 242]. However, SCS macrophages might not be involved in B cell activation in response to all antigens [10]. For example, although SCS macrophages are targeted by inactivated influenza virus after immunisation, medullary $CD11c^+CD11b^+SIGNR\text{-}1^+$ DCs carry antigen to the follicles and are required for induction of humoral responses [243]. Targeting of influenza virus to SCS macrophages is dependent on mannose-binding lectin [243], whereas targeting of VSV is independent from complement or secreted immunoglobulins [241]. This suggests that antigen opsonisation could have a major effect on how antigen is processed by SCS macrophages.

Sn^+ macrophages can also present lipid antigens in a CD1d dependent manner to invariant natural killer T cells (iNKT cells), leading to iNKT cell activation and population expansion in the LNs [244]. iNKT cells are a specialized subset of classical αβ T lymphocytes, which upon activation rapidly secrete large amounts of cytokines and induce downstream activation of different cell types, including DCs, NK cells, B cells and conventional T cells [244], making them important immune cells involved in coordinating both innate and adaptive immunity. Although purified $CD11c^{hi}$ DCs are also able to activate iNKT cells *in vitro*, they are unable to activate iNKT cells when recovered from draining LNs from immunised animals, suggesting they do not have access to the antigen *in vivo*, again highlighting the prime access to incoming antigen in the lymph of Sn^+ macrophages [244].

The spleen similarly locates Sn^+ macrophages in the vicinity of lymphocyte-rich tissue and flowing fluid (in this case blood rather than lymph) [134]. Interestingly, a recent study using mouse VSV showed that Sn^+ macrophages in the marginal zone of the spleen formed a compartment of enhanced viral replication [208]. This was essential for the induction of adaptive antiviral immune responses and, therefore, for preventing the fatal outcome of infection. As such, the authors suggested that enforced viral replication in MZM is an immunological mechanism that ensures the production of sufficient antigen for effective activation of the adaptive immune response. Also, a recent study by Nikbakht *et al.* showed that Sn^+ macrophages in the spleen are required for the localization of antigen-activated B cells to the follicular perimeter and the subsequent formation of germinal centres by the use of hapten-keyhole limpet hemocyanin (KLH) conjugates [245], again supporting a role for Sn^+ macrophages in the induction of efficient adaptive immune responses.

Sn^+ macrophages have also been implicated in the activation of $CD8^+$ T cells through two potential mechanisms: 1/ antigen (OVA) transfer to $CD8\alpha^+$ DCs in the spleen [100] and 2/ by direct antigen presentation to $CD8^+$ T cells (dead cell-associated antigens and *T. gondii*-derived antigens) [246, 247]. The process by which antigen is transferred to $CD8\alpha^+$ DCs is however still unknown [10] and a possible direct activation role for Sn^+ macrophages has not been eliminated. Cognate interaction between SCS macrophages and $CD8^+$ T cells occurs as these cells are activated, and thus SCS macrophages are probably able to internalise, process and present antigen on MHCI molecules [10]. This however also implicates that Sn^+ macrophages can become a target of $CD8^+$ T cell dependent killing as well. This has been reported in the case of splenic Sn^+ macrophages after infection with *Plasmodium chabaudi* [248] and proposed in the case of SCS macrophages after *Toxoplasma gondii* infection [247].

Subcutaneous vaccination with irradiated necrotic tumour cells activates antigen-specific $CD8^+$ T cells to elicit a protective antitumor immune response [246]. This effect was abolished by selective depletion of Sn^+ macrophages in the LNs. Systemic apoptotic cell administration on the other hand revealed the importance of marginal zone Sn^+ macrophages in debris clearance and maintenance of tolerance. Depletion of macrophages via clodronate liposomes [249] or use of transgenics [250] here resulted in autoimmunity. These findings illustrate once again the importance of Sn^+ macrophages in regulating immune responses.

From the findings that marginal zone and SCS macrophages participate in the induction of an adaptive immune response, targeting antigen to Sn^+ macrophages has been explored as a means of inducing immunity [134]. Targeting OVA antigen to Sn+ macrophages using an anti-Sn antibody delivered intravenously in the presence of an agonist anti-CD40 antibody as

adjuvant, promoted $CD8^+$ T cell activation to a similar extent as does antigen targeting using DEC205 on $CD8\alpha^+$ DCs [10, 100]. The Sn^+ macrophages mediated response is protective against challenge with tumor cells that express OVA. Recently, Kawasaki *et al.* used high-affinity glycan ligand–bearing liposomes to selectively deliver lipid antigens to Sn^+ macrophages via the Sn endocytic pathway [102]. They found that ligand-targeted liposomes were captured by Sn^+ macrophages and potently primed iNKT cells in liver and spleen. These effects occured in an Sn-dependent manner, because no activation was seen with the targeted liposomes in Sn-deficient mice. In pigs, an enhanced humoral immune response and increased reactivity of peripheral blood mononuclear cells was observed after injection of an Sn-specific mouse mAb in comparison to an irrelevant control antibody [104, 161]. In addition, our own research group observed an enhanced humoral immune response to the model antigen human serum albumin (HSA) after Sn-directed targeting of HSA using an Sn-specific mAb [103]. These observations clearly illustrate the importance of Sn^+ macrophages in generating effective immune responses and therefore contribute to the growing interest of Sn^+ macrophages for vaccination strategies.

1.1.4 TARGETING MOLECULES

When speaking of targeting molecules, a distinction should be made between passive and active targeting. Passive targeting is based on the intrinsic properties of the delivery system. A good example of this targeting strategy is the enhanced permeability and retention effect in anti-cancer therapy. Here, macromolecular anticancer drugs administered intravenously escape renal clearance due to their large size, while they can selectively extravasate in tumor tissues due to the abnormal vascular nature present in tumor tissues [251]. For Sn^+ macrophages, one passive targeting strategy has been described by Huang *et al.* [130]. Using a cationic agarose hydrogel, TNF-α anti-sense oligonucleotides were shown to accumulate in Sn^+ macrophages in the spleen. In a mouse model of systemic lupus erythrematosus this resulted in significant amelioration of lupus symptoms. For vaccination strategies in general, passive targeting is also widely employed, with the largest interest going towards nanoparticles (NPs) as delivery vehicles for vaccines; the most prominent examples being virus-like particles (VLPs), liposomes, immune-stimulating complexes (ISCOMs), polymeric NPs and non-degradable NPs [7] (Fig. 7).

Using NPs to deliver antigens, the efficiency of uptake by antigen-presenting cells in comparison to soluble antigen alone can be significantly increased [254]. A further increase in uptake by APCs can however be achieved through active targeting, where cell-surface receptor-specific ligands are attached to the NPs or used on their own to actively target antigens to specific APC subsets. These ligands can be natural ligands like CD40 ligand that

binds to CD40 on APC. Also glycans are explored as targeting vehicles for antigen delivery, as many surface receptors used as address labels for APCs are glycan-binding proteins (lectins). A limitation to the use of ligands of lectins however is the overlap in the ligand specificity of these receptors, and the degree to which they are expressed on single types of APCs [185]. For example, mannose containing glycans recognized by the MR and the Lewis X structures recognized by DC-SIGN are also recognized by other C-type lectins expressed on DCs and macrophages [255]. However, in-silico aided design strategies can be employed to develop ligands with high affinity and specificity. To target Sn^+ macrophages Nycholat *et al.* developed such a high affinity ligand that is an analogue of the natural ligand and which is capable of targeting liposomes specifically to Sn^+ macrophages *in vivo* [256]. Also, Kawasaki *et al.* used high-affinity glycan ligand–bearing liposomes to selectively deliver lipid antigens to Sn^+ macrophages via the Sn endocytic pathway [102]. They found that ligand-targeted liposomes were captured by Sn^+ macrophages and potently primed iNKT cells in liver and spleen.

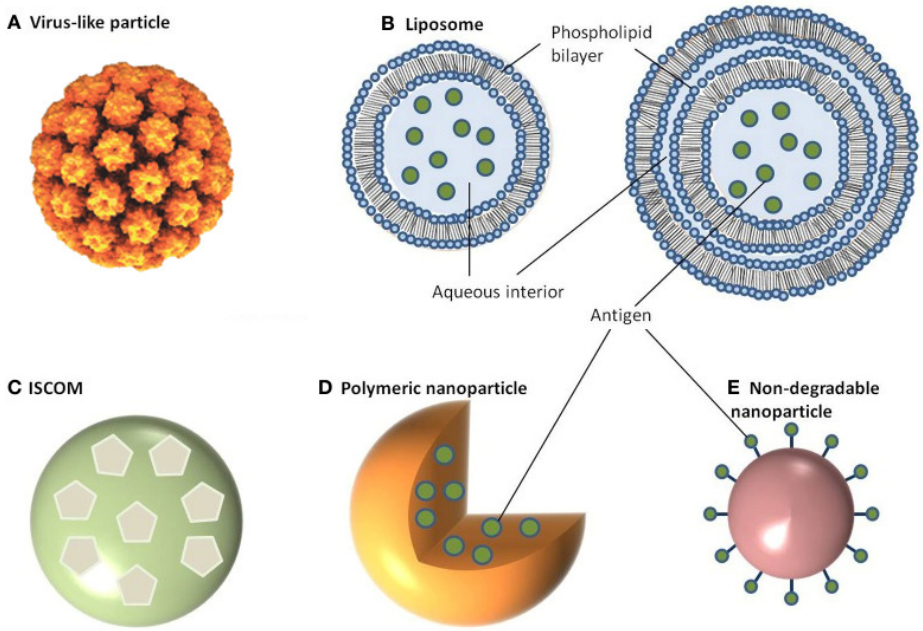

Figure 7. Schematic representation of different nanoparticle delivery systems.
(A) Virus-like particles (VLPs) are viral proteins that self-assemble into structures resembling the conformation of the native virus but devoid of viral genetic material [252]. VLPs can be produced either without modification or by genetically engineering the viral capsid subunit by bioconjugation of the viral capsid subunit with antigenic peptides or other ligands or by site-directed mutagenesis of the intact VLP to create a functional scaffold for multivalent surface presentation of antigens [253]. (B) Liposomes are self-assembling like VLPs but consist of a phospholipid bilayer shell with an aqueous core. (C) Immune-stimulating complexes ISCOMs are colloidal saponin containing micelles of around 40nm that can be used as self-adjuvanting vaccine delivery systems. (D) Polymeric nanoparticles are biodegradable and can be prepared from a range of polymers (e.g. PLGA) to create a vesicle which can either accommodate or display antigens. The release kinetics of incorporated antigens can be controlled by compositional changes to the copolymer. (E) Non-degradable nanoparticles are typically comprised of a gold, carbon or silica shell on which surface antigens can be covalently attached. Figure adapted from Gregory *et al.* [7].

Although cell-surface receptor-specific natural ligands can be employed to target APC, The most widely used targeting molecules are antibodies (Table 3), as their inherent specificity and high affinity for their target makes them ideal candidates for this purpose. In addition, antibodies can be rather easily purified using standard chromatography techniques (protein G, protein A), thus representing a major advantage as compared to natural protein ligands. Antigens can be chemically conjugated to the antibodies. Chemical conjugation however has some disadvantages, like potential affinity loss of the antibody and poor control over the number of antigens conjugated which yields a heterogeneous product [257]. Genetic constructs where the DNA sequence of the antigen is genetically fused to the sequence encoding the mAb therefore represents a more elegant approach [59]. As the induction of an effective T cell response requires the addition of an adjuvant to DC-targeting formulations, targeting both antigen and adjuvant to the DCs is currently being investigated. To further limit potential off-target effects of the adjuvant, new approaches have emerged that shift the use of NPs from passive targeting to active targeting by coupling receptor-specific mAbs or derivatives (e.g. single chain fragment variable) to the NPs (Table 3 and Figure 8). As such, both antigen and adjuvant can be incorporated into the NPs or displayed on their surface [8, 16].

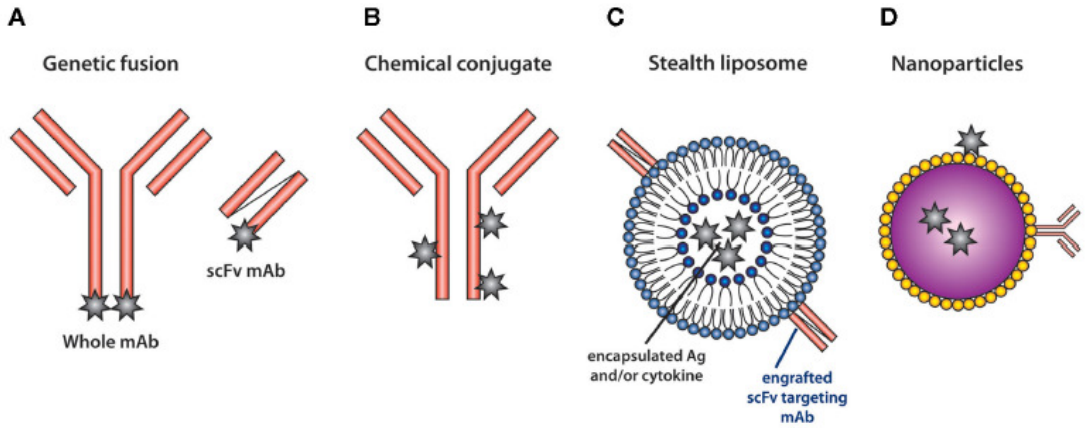

Figure 8. Methods for targeting antigen to APCs *in vivo*
(A) The heavy and light chain of the whole mAb or the single-chain fragments of the variable regions (scFv) are sequenced and genetically engineered to carry antigen (star). Alternatively, (B) antigen can be chemically conjugated to whole mAbs. (C) The scFv or the mAb recognizing APC-specific molecules can also be engrafted into stealth liposomes. The liposomes can carry both antigen and adjuvant. (D) Nanoparticles carrying antigen and/or adjuvant are modified to permit the attachment of APC-specific mAb and have been used to target antigen. Figure adapted from Caminschi *et al.* [16].

For Sn^+ macrophages, our own research group developed immunoconjugates by the cheminal linkage of the model antigen HSA to a pSn-specific mAb. An enhanced HSA-specific humoral immune response was observed in comparison to a non-targeting control immunoconjugate or HSA alone [103]. Also, Backer *et al.* used a mouse Sn-specific mAb to target OVA antigen to Sn^+ macrophages in the presence of an agonist anti-CD40 antibody as adjuvant [100]. $CD8^+$ T cells were activated to a similar extent as by targeting antigen to

CD8α$^+$ DCs using DEC205. The Sn$^+$ macrophages mediated response was protective against challenge with tumor cells that expressed ovalbumin. Finally, Kratzer *et al.* designed fusion proteins of antigen with tandem Ig binding domains of protein G [101]. These fusion proteins were shown to efficiently bind to a range of surface receptor-specific mAbs, including one specific for mouse Sn. Also here, an enhanced OVA-specific CD8$^+$ T cell response was observed by targeting OVA to Sn$^+$ macrophages.

Although most published targeting studies up till now made use of conventional mAbs to target antigens towards APCs (Table 3), antibody engineering approaches and the discovery of 'nanobodies' has created a wealth of Ab derivatives that could be used for targeting approaches as well (Fig. 9). Camelids and sharks produce high-affinity antibodies that are devoid of light chains [258, 259]. Their variable domain (for camelids called 'nanobody') contains the antigen-binding site, which is formed by only 3 complementarity-determining regions (CDRs) instead of 6 in conventional antibodies. The CDR3 region has an extraordinary capacity to form long fingerlike extensions that can extend into cavities on antigens, like e.g. cryptic epitopes present on infectious agents, which are mostly not accessible by conventional mAbs [260]. Also, nanobodies, Fab fragments and single chain fragment variable (scFv) can be engineered into multivalent molecules to increase functional affinity (also called avidity) or in multispecific molecules targeting more than one specific epitope (Fig. 9). An extensive toolbox is thus also available to further optimize the targeting molecule used in the APC-targeting vaccine formulation.

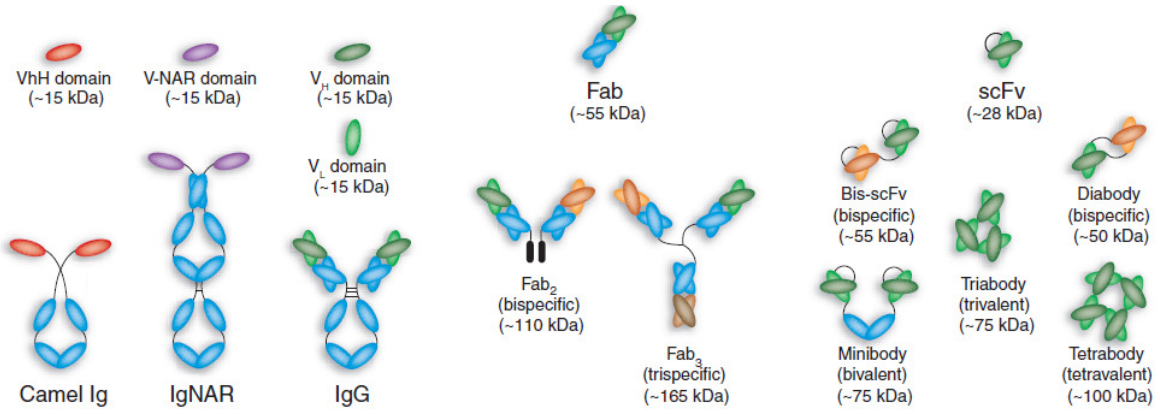

Figure 9. Schematic representation of different antibody formats
A 'classic' IgG molecule (~150kDa) is depicted alongside camelid VhH-Ig and shark Ig-NAR immunoglobulins. Camelid VhH-Ig and shark Ig-NARs are unusual immunoglobulin-like structures comprising a homodimeric pair of two chains of V-like and C-like domains (neither has a light chain), in which the displayed V domains bind their targets independently. Shark Ig-NARs comprise a homodimer of one variable domain (V-NAR) and five C-like constant domains (C-NAR). A variety of antibody fragments are depicted, including Fab, scFv, single-domain VH, VhH and V-NAR and multimeric formats, such as minibodies, bis-scFv, diabodies, triabodies, tetrabodies and chemically conjugated Fab´ multimers (sizes given in kilodaltons are approximate). Figure adapted from Holliger and Hudson [261].

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Chapter

2

Aims of the thesis

Traditionally, vaccines are based on live attenuated or inactivated pathogens. Although successful in many instances, many infectious diseases still exist for which the development of an effective vaccine remains elusive based on these classical approaches (e.g. HIV, malaria, tuberculosis,…). In addition, continuously increasing safety demands for novel vaccine formulations encourage the search for more rational vaccine designs. As such, a wide range of vaccines under development now are based on isolated peptides, proteins or polysaccharides of pathogens, or naked DNA encoding a protective antigen. Although promising, such antigens are often poorly immunogenic and require the addition of an immune-stimulating compound to generate an effective immune response. As a consequence, finding safe, yet efficient ways to boost immunity has become an increasingly important area of research in current-day vaccinology.

To induce an antigen-specific memory immune response, an interaction between antigen-loaded APCs and naive lymphocytes needs to take place in secondary lymphoid tissues. Therefore, one way to improve the efficiency of a vaccine is to improve the efficiency of the uptake of antigen by APCs. As such, targeting antigen to uptake receptors expressed on the surface of APCs is increasingly studied. While most targeting strategies focus on delivery of antigens to different subsets of DCs, Sn^+ macrophages, most abundantly present in spleen and lymph nodes, appear to be strategically placed to capture and process antigens and may thus be an attractive target for vaccination strategies as well. Previously, our research group used a pSn-specific mAb to target the model antigen HSA, which was chemically cross-linked to the mAb, to pSn expressing macrophages. In a porcine model, this resulted in an enhanced immune response to HSA. Although successful, this system implies that the antigen to be targeted is independently produced and purified, which translates into significant optimization for each separate conjugate. Also, chemical coupling to antibodies depends on the presence and distribution of reactive groups, e.g. primary amines on lysine residues, that can be located in or near the antigen-binding region. Upon coupling, this might result in partial or complete loss of the antibody's capacity to bind to the target antigen. In addition, there is limited stoichiometric control because of the large number of reactive groups present in an antibody, leading to a heterogeneous mixture that makes batch to batch consistency hard to effectuate. A first aim of this thesis therefore was to develop a versatile recombinant antibody vector that allows easy production and purification of defined genetic antibody fusion constructs, in order to overcome the problems associated with chemical conjugation of a cargo to an Sn-specific mAb.

A second aim of this thesis was to move away from model antigens and make a fusion construct of our targeting antibody with an antigen derived from an actual pathogen. As such, we selected a linear B cell epitope of PRRSV, known to be a target of virus-neutralizing

antibodies. During the course of an immune response to PRRSV, the induction of virus-neutralizing antibodies is hampered. Also, currently commercially used inactivated vaccines do not succeed in inducing robust virus-neutralizing antibody responses in the pig. To evaluate if targeting a neutralizing epitope towards pSn-expressing macrophages can enhance the induction of epitope-specific and virus-neutralizing antibodies, four copies of the epitope were genetically fused to our recombinant pSn-targeting antibody. These fusion constructs were used to immunize pigs after which a viral challenge was performed.

A third aim of this thesis was to develop a potential immunocastration vaccine candidate. As immunocastration relies on the induction of antibodies against gonadotropin releasing hormone (GnRH), a 10 amino acid peptide, four copies of GnRH were genetically fused to our recombinant Sn-targeting antibody. For the production of recombinant antibodies, the most commenly used expression systems are mammalian cells growing in suspension, so as to enable the rapid scale up of productions. In addition, chemically defined, serum-free media – with no animal-derived components – have become industry standard to further simplify regulatory approval. In this study, we made use of a commercially available suspension adapted CHO cell line to develop cell lines expressing recombinant antibody-GnRH fusion constructs.

Chapter

3

Development of a recombinant antibody to target peptides and proteins to sialoadhesin-expressing macrophages

Karen Ooms, Hanne Van Gorp, Tim Van Gaever, Hans J. Nauwynck and Peter L. Delputte

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Background *Sn^+ monocytes/macrophages have been associated with several diseases like inflammatory and autoimmune disorders as well as viral infections, and they also appear to play a role in the initiation of an adaptive immune response. This makes Sn-expressing cells not only attractive targets for cell-directed therapies, but also an appealing target for vaccination. Furthermore, since Sn was shown to be an endocytic receptor, the conjugation of effector molecules to an Sn-specific ligand should allow intracellular delivery of these conjugates. Previously, we developed functional Sn-specific immunoconjugates that were generated via chemical coupling. Although successful, the system requires significant optimization for each immunoconjugate to be made. To generate a more flexible and controlled system, we developed a recombinant antibody vector allowing the creation of genetic antibody fusion constructs. This paper reports on the characterization of the recombinant antibody and the evaluation of its use for Sn-directed targeting.*

Results *The variable domains of the porcine Sn-specific monoclonal antibody 41D3 were sequenced and cloned in frame with a mouse IgG1 backbone. Transfection of HEK-293T cells with the resulting plasmid led to the secretion of fully assembled IgG into the culture medium. This recombinant antibody rec41D3 was shown to specifically bind to porcine Sn with a comparable affinity as the native monoclonal antibody. In addition, rec41D3 also induced Sn endocytosis in primary macrophages and resided for prolonged times in early/late endosomes. To allow the generation of antibody fusion constructs, a multiple cloning site was introduced at the C-terminus of the heavy chain. Two fusion constructs were generated, one containing a V5 peptide tag and one containing an eGFP molecule. Both constructs were shown to be efficiently produced in HEK-293T cells and easily purified using standard protein G chromatography. In addition, both V5 and eGFP were shown to be co-internalized together with rec41D3 into Sn-expressing primary macrophages.*

Conclusions *A recombinant antibody allowing targeted delivery of peptides and proteins to Sn-expressing macrophages was developed. Production and purification of antibody fusion constructs was possible without major optimization and with batch to batch consistency, confirming the development of a versatile antibody vector to evaluate Sn-directed targeting strategies in a porcine animal model.*

3.1 INTRODUCTION

Sn^+ macrophages have gained increased attention lately because of their unique distribution in lymphoid organs and their redistribution upon immune activation [1]. Situated in the spleen and other secondary lymphoid tissues, Sn^+ macrophages appear to be strategically placed for antigen acquisition and delivery to lymphocytes. Junt et al. showed that in lymph nodes, Sn^+ macrophages capture viral particles (vesicular stomatitis virus) within minutes after subcutaneous injection, transport them across the SCS floor and present them to migrating B cells in the underlying follicles [2]. Moreover, also particulate antigen and immune complexes have been shown to be captured and displayed by Sn^+ macrophages [3-5]. Besides, Sn^+ macrophages present lipid antigens in a CD1d dependent manner to iNKT cells, leading to iNKT cell activation and population expansion [6]. Also, Sn^+ macrophages have been implicated in the activation of $CD8^+$ T cells by either directly presenting antigen to $CD8^+$ T cells [7] or by transferring the antigen to $CD8^+$ DCs in the spleen [8]. Ultimately, several independent research groups showed an enhanced cellular and/or humoral immune response upon Sn-targeted antigen delivery [9-13]. Together, this suggests that Sn^+ macrophages may act as specialized antigen presenting cells involved in the antigen transport chain and contribute to the growing interest in Sn^+ macrophages for vaccination strategies, as recently reviewed by Martinez-Pomares and Gordon [1].

Sn (CD169, Siglec-1) is also present on inflammatory macrophages and activated monocytes [14, 15]. In affected tissue samples of rheumatoid arthritis patients for instance, high expression of Sn was found on inflammatory macrophages [14]. In addition, abundant Sn expression on inflammatory monocytes/macrophages was shown to correlate with disease severity in pathological conditions like multiple sclerosis, atherosclerosis and breast cancer [16-18]. These observations promote the idea of an Sn-directed cell therapy aimed at elimination or immunomodulation of these cells. Recently, targeted delivery of an anti-TNF-α oligonucleotide to Sn^+ macrophages resulted in the relief of lupus-like symptoms in mice [19], further showing the potential of Sn^+ macrophages as a target for immunomodulation. Sn seems thus a promising target for cell-directed therapy, a strategy that is further encouraged by the restricted expression pattern of Sn limiting unwanted side effects. Furthermore, since Sn was suggested to be an endocytic receptor [10, 13], the conjugation of antigens, toxins or drugs to an Sn-specific antibody should allow intracellular delivery of these conjugates.

So far, three systems have been used to selectively target Sn^+ macrophages, namely antibodies, glycan-coated liposomes and cationic agarose hydrogels [9-13, 19, 20]. While the latter was unintentionally identified as an Sn-targeting system [19], the glycans on the glycan-coated liposomes were specifically designed to be ligands with a high specificity and

affinity for Sn [20]. Previously, our research group has made use of an Sn-specific mAb to generate functional Sn-specific immunoconjugates via chemical coupling [10]. Although successful, this system implies, just like the two other described Sn-targeting systems, that the cargo to be targeted is independently produced and purified, which translates into significant optimization for each separate conjugate. Also, chemical coupling to antibodies depends on the presence and distribution of reactive groups, e.g. primary amines on lysine residues, that can be located in or near the antigen-binding region. Upon coupling, this might result in partial or complete loss of the antibody's capacity to bind to the target antigen. In addition, there is limited stoichiometric control because of the large number of reactive groups present in an antibody, leading to a heterogeneous mixture that makes batch to batch consistency hard to effectuate [21]. To overcome these problems associated with the chemical conjugation of a cargo to an Sn-specific mAb, we report here on the development of a versatile recombinant antibody vector that allows easy production and purification of defined genetic antibody fusion constructs. The obtained vector will allow us to generate functional antibody-cargo constructs to evaluate Sn-targeting strategies in a porcine animal model.

3.2 MATERIALS AND METHODS

3.2.1 ETHICS STATEMENT

The experimental procedure for the collection of porcine alveolar macrophages was authorized and supervised by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University.

3.2.2 CELL CULTURE AND MONOCLONAL ANTIBODIES

Porcine alveolar macrophages (PAM) were isolated from 4- to 6-week-old conventional Belgian Landrace pigs as described [36], and cultivated in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1% non-essential amino acids and 1 mM sodium pyruvate. HEK-293T were grown in DMEM medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and 1 mM sodium pyruvate. CHO-K1 cells and CHO-K1 cells stably expressing recombinant pSn [22] were cultivated in F-12 medium supplemented with 10% FBS and 1mM sodium pyruvate. All culture media were supplemented with a mixture of antibiotics and cell cultures were kept in a humidified 5% CO_2 atmosphere at 37°C.

MAb 41D3, directed against pSn [37, 38], and an isotype matched (IgG1) control mAb 13D12, directed against pseudorabies virus glycoprotein gD [23] were purified from hybridoma supernatants using protein G sepharose column chromatography (GE Healthcare), dialyzed to PBS and stored at -70°C until use.

3.2.3 CONSTRUCTION, PRODUCTION AND PURIFICATION OF REC41D3, REC13D12 AND FUSION CONSTRUCTS

The variable domains of mAb 41D3 were sequenced and cloned into the pVITRO1-neo-mcs vector (Invivogen), in frame with a mouse IgG1 backbone (Fusion Antibodies Ltd). For the construction of rec13D12, total RNA from 10^7 hybridoma cells was isolated (RNeasy® mini kit, Qiagen) and cDNA was synthesized by RT-PCR (Superscript® III First-Strand Synthesis System, Life technologies). DNA sequences of mAb 13D12 VH and VL were obtained based on the protocol described by the Mouse Ig-Primer Set of Novagen®. Subsequently, the variable domains of rec41D3 in pVITRO1-neo-mcs were exchanged by the variable domains of mAb 13D12, yielding a plasmid encoding rec13D12.

To construct both rec41D3- and rec13D12-V5, a $(G_4S)_2$-V5 DNA sequence was introduced at the 3' end of the heavy chain sequence using forward primer 5'-AAACGATCG*GGCGGGGGAGGCTCAGGGGGAGGCGGGAGC*GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGGCGGCCGCATGAACGCGTAAA-3' and reverse primer 5'-TTTACGCGTTCATGCGGCCGCCGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACC*GCTCCCGCCTCCCCCTGAGCCTCCCCCGCC*CGATCGTTT-3'. To construct both rec41D3- and rec13D12-eGFP, a $(G_4S)_4$ DNA sequence was introduced first at the 3' end of the heavy chain sequence using forward primer 5'-AAACGATCG*GGCGGGGGAGGCTCCGGGGGAGGCGGGTCTGGAGGCGGGGGAAGTGGCGGGGGAGGCTCA*GCGGCCGCAAA-3' and reverse primer 5'-TTTGCGGCCGC*TGAGCCTCCCCCGCCACTTCCCCCGCCTCCAGACCCGCCTCCCCCGGAGCCTCCCCCGCC*CGATCGTTT-3', followed by the eGFP sequence of pCeMM CTAP(SG) (GenBank Accession number EF467048) amplified by PCR using forward primer 5'- AAAGCGGCCGCAATGGTGAGCAAGGGCGAGGAG-3' and reverse primer 5'-AAAGGCCGGCCTTACTTGTACAGCTCGTCCAT-3'. Underlined sequences represent enzyme restriction sites, sequences in italics represent linker DNA sequences.

For production of all recombinant antibodies and fusion constructs, HEK-293T cells were transiently transfected using calcium phosphate. Transfected cells were cultured in DMEM supplemented with 10% IgG depleted, heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and a mixture of antibiotics in a humidified 5% CO_2 atmosphere at 37°C.

Culture supernatant was collected and IgG were purified from the supernatant using standard protein G sepharose chromatography following the manufacturer's instructions (GE Healthcare). Fractions of the eluate containing the purified protein were pooled and the buffer was exchanged to PBS by dialysis. Purified protein was stored at -70°C until use. Yields of recombinant antibodies and fusion constructs from HEK293-T supernatant were ± 500µg/l for rec41D3, rec13D12 and V5 fusion constructs and ± 250µg/l for eGFP fusion constructs.

3.2.4 SDS-PAGE, COOMASSIE BLUE STAINING AND WESTERN BLOT ANALYSIS OF REC41D3 AND FUSION CONSTRUCTS

Samples of purified proteins were mixed with (non-) reducing Laemmli buffer, boiled for 5 min and subjected to SDS-PAGE (6% non-reducing gel, 10% reducing gel) using a BioRad Mini Protean 3 system. For Coomassie Blue staining, the SDS-PAGE gel was incubated successively in Ultra Pure water, Imperial™ protein staining solution (Thermo Scientific) and Ultra Pure water as destaining solution. Alternatively, for Western blot analysis, proteins were transferred from the SDS-PAGE gel to a PVDF membrane (Membrane Hybond-P, GE Healthcare) via Western blotting (BioRad Mini Trans Blot). The membrane was blocked overnight in PBS + 0.1% Tween 20 + 5% skimmed milk. Detection of recombinant antibodies was performed by subsequent incubation of the blot with peroxidase-labeled polyclonal goat anti-mouse antibodies (Dako), followed by visualization using enhanced chemiluminiscence (ECL; GE Healthcare). Alternatively, rec41D3-V5 protein was detected using peroxidase-labeled anti-V5 antibodies (Life technologies) and rec41D3-GFP protein was detected using a recombinant rabbit monoclonal GFP-specific antibody (ABfinity™, Life technologies) and peroxidase-labeled polyclonal goat anti-rabbit antibodies (Dako), followed by ECL visualization.

3.2.5 SURFACE PLASMON RESONANCE

The generation of a soluble Fc-tagged pSn has been described before [24]. For surface plasmon resonance experiments, a pSn4D-Fc protein was used containing a single point mutation in the first pSn Ig-like domain, since production levels of this protein are significantly higher. mAb 41D3 has been shown to equally bind to this protein [24]. After production and purification, pSn4D-Fc was coupled to an activated CM5 chip to 300 response units (RU). mAb 41D3 (300, 108, 39, 14 nM) or rec41D3 (250, 90, 32, 12 nM) diluted in HBS-EP buffer was injected (30µl/min) for 2 min, followed by dissociation for 5 min. Surface regeneration was performed with 10 mM NaOH for 60 s followed by a recovery phase. The sensorgrams were fitted to a bivalent model accounting for mass-transfer effects.

3.2.6 IMMUNOFLUORESCENCE STAININGS

To assess pSn specificity, CHO cells or CHO cells expressing pSn (CHO-pSn) were seeded on poly-L-lysine (Sigma) coated coverslips and incubated with mAb 41D3, rec41D3 or isotype-matched control antibodies mAb 13D12 and rec13D12 for 1h at 4°C. Cells were then fixed with 4% (w/v) paraformaldehyde (Sigma) in PBS, permeabilized using 0.5% (w/v) saponin (Sigma) in PBS and stained with FITC-labelled goat-anti-mouse IgG to visualize antibodies bound to the cell.

To evaluate pSn endocytosis of rec41D3 in comparison to mAb 41D3, PAM were incubated with 2µg/200µl purified antibodies and cells were fixed with 4% paraformaldehyde at the indicated time points. Afterwards, cells were permeabilized using 0.5% saponin and stained with FITC-labelled goat-anti-mouse IgG to visualize antibodies bound to and internalized in the cells. As a control, cells were fixed (time 0) and incubated with the respective antibodies afterwards. For double immunofluorescence staining with markers of the endosomal compartments, the respective primary antibodies for each endosomal compartment followed by appropriate Texas Red-labelled secondary antibodies was used for their visualization. The primary antibodies used were an affinity purified goat pAb (sc-6414; Santa Cruz Biotechnology), a rabbit pAb (ab32815; Abcam) and a rabbit pAb (sc-5570; Santa Cruz Biotechnology), for early endosome antigen 1 (EEA1), cation-independent mannose-6-phosphate receptor (CI-M6P) and lysosome-associated membrane protein 1 (Lamp1) respectively. Cell nuclei were visualized using Hoechst 33342 (Life Technologies).

To evaluate pSn endocytosis of rec41D3 fusion constructs, CHO-pSn and PAM were incubated with 2µg/200µl purified rec41D3-V5, rec41D3-GFP or their respective isotype controls rec13D12-V5 and rec13D12-eGFP for 1h at 37°C. Cells were then fixed with 4% paraformaldehyde, permeabilized using 0.5% saponin and stained with AF594-labelled goat-anti-mouse IgG1 in addition to FITC-labelled mouse anti-V5 (IgG2a) or rabbit anti-GFP in combination with FITC-labelled goat-anti-rabbit polyclonal antibodies respectively. Cell nuclei were visualized using Hoechst 33342 (Life Technologies).

3.2.7 CONFOCAL LASER SCANNING MICROSCOPY

Z-section images of samples were acquired using a Leica TCS SPE-II laser scanning spectral confocal system (Leica Microsystems GmbH) linked to a Leica DM2500 microscope (Leica Microsystems GmbH). Image acquisition was done using the Leica LAS AF confocal software package and analysis of colocalization was done using CoLocalizer Pro [39]. For the colocalization analysis between Sn and the endosomal markers, the Sn-positive plasma membrane was excluded from the analysis and only internalized Sn was considered.

3.3 RESULTS

3.3.1 DEVELOPMENT OF A SIALOADHESIN-SPECIFIC RECOMBINANT ANTIBODY, REC41D3

To allow production and purification of genetic fusion proteins of an Sn-specific mAb and other peptides/proteins, a recombinant Sn-specific mAb was made. mAb 41D3 was previously described to selectively bind pSn and to be internalized into pSn-expressing macrophages [10]. The variable domains of mAb 41D3 were sequenced and cloned in frame with a mouse IgG1 backbone. The IgG1 backbone was modified so that the resulting plasmid p41D3 contains a C-terminal heavy chain multiple cloning site, allowing removal of the antibody's heavy chain stop codon and insertion of protein encoding sequences (Fig. 1A).

Transfection of HEK-293T cells with plasmid p41D3 led to the secretion of a fully assembled IgG into the culture medium of the same size as mAb 41D3, as shown by Western blot analysis of cell culture supernatants using polyclonal antibodies specific for mouse immunoglobulins (Fig. 1B). Recombinant antibodies were purified from cell culture medium using protein G chromatography and dialyzed to PBS. To assess the purity of rec41D3 after purification, protein samples were resolved via SDS-PAGE under non-reducing and reducing conditions and Coomassie Blue staining was performed (Fig. 1C). The presence of a single band under non-reducing conditions and the presence of two bands under reducing conditions consistent with the sizes of the heavy and light chains of an Ig showed that protein G purification yields pure rec41D3. Western blot analysis further confirmed that the bands present were antibody light and heavy chain fragments (Fig. 1D).

Immunofluorescence stainings of CHO cells expressing recombinant pSn (CHO-pSn, [22]) were performed to assess the specificity of rec41D3 for pSn (Fig. 1E). With rec41D3, a bright surface staining, similar to that of the native mAb 41D3, was seen only on CHO-pSn cells, but not on wild type CHO cells, indicating specific pSn recognition of rec41D3. Control stainings were performed with isotype matched irrelevant mAb 13D12 (gD of pseudorabies virus, [23]), and its recombinant form rec13D12. Development, production and purification of rec13D12 was identical to the procedures used for rec41D3.

Since the final goal is to use rec41D3 as a targeting molecule for pSn, the affinity of rec41D3 for pSn is highly important. Therefore, its affinity was determined using Biacore. As a target, a soluble form of the pSn receptor, consisting of the first 4 N-terminal Ig-like domains of pSn fused to a human IgG Fc (pSn4D-Fc), was coated on the affinity chips. Previously, mAb 41D3 was shown to bind to this pSn4D-Fc [24]. As seen in Figure 1F, both mAb 41D3 and

rec41D3 bound with high affinity to pSn4D-Fc, their equilibrium dissociation constants (K_D) were determined to be 35 and 250 pM respectively.

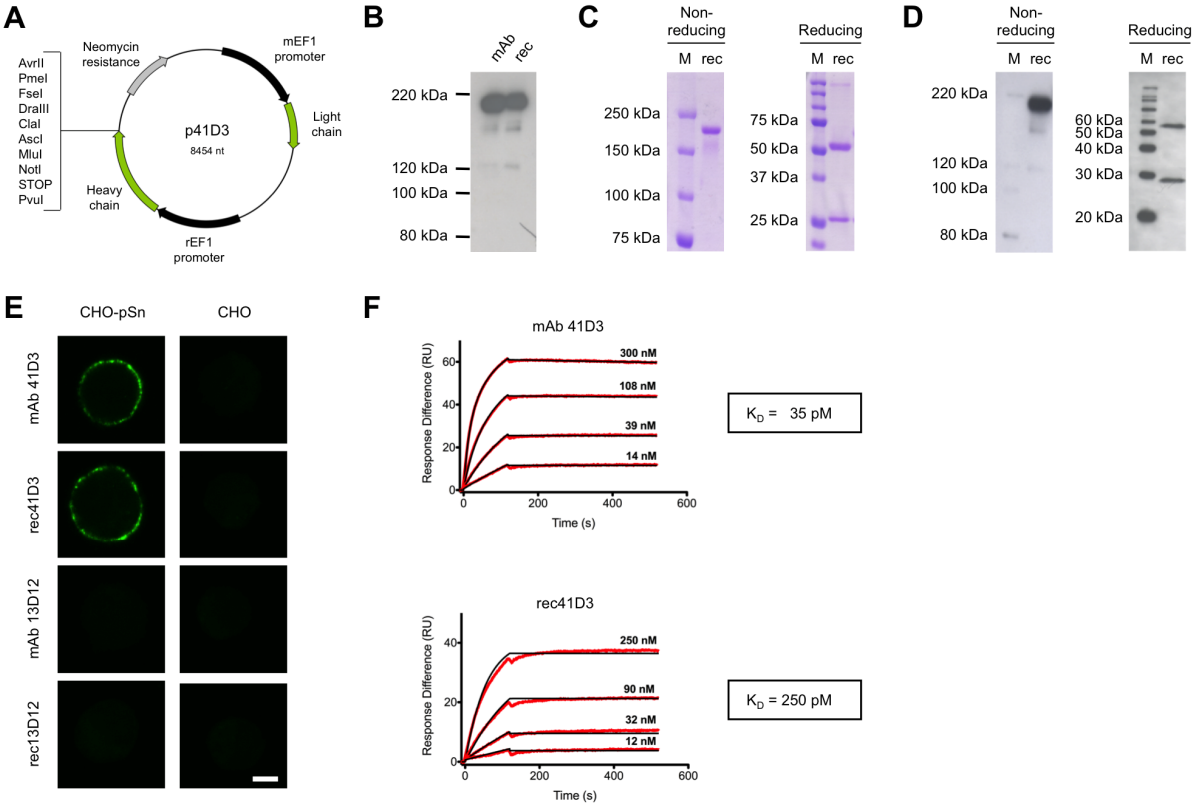

Figure 1. Development, production and purification of a pSn-specific recombinant antibody, rec41D3
(A) Vector map of p41D3 (B) Non-reducing Western blot analysis of hybridoma supernatant of mAb 41D3 compared to p41D3-transfected HEK-293T cell supernatant using polyclonal antibodies specific for mouse immunoglobulins (C) SDS-PAGE of protein G purified transfected cell supernatants to evaluate rec41D3 purity (D) Western blot analysis of purified rec41D3 using polyclonal antibodies specific for mouse immunoglobulins (E) Confocal microscopical analysis of CHO cells and recombinant pSn expressing CHO cells (CHO-pSn) incubated with mAb 41D3, rec41D3 or isotype matched controls mAb 13D12 and rec13D12 at 4°C for 1 hour. Cells were fixed and stained with FITC-labelled goat-anti-mouse IgG. Images represent a single confocal z-section through the middle of the cell. Scale bar: 5µm (F) Kinetic profile of the pSn-41D3/pSn-rec41D3 interaction by Surface Plasmon Resonance measurements. Immobilized pSn4D-Fc was flowed with rising concentrations of mAb 41D3 or rec41D3, after which injection was halted and dissociation was monitored during 5 minutes. Sensorgrams were fitted to a bivalent model and an approximation of the equilibrium dissociation constant was calculated. M, protein marker; mAb, mAb 41D3; rec, rec41D3; KD, equilibrium dissociation constant.

3.3.2 REC41D3 INDUCES pSN ENDOCYTOSIS IN PRIMARY MACROPHAGES

As our ultimate goal is to target pSn^+ macrophages *in vivo*, we studied the capacity of rec41D3 to bind pSn and induce its internalization in *in vitro* cultivated primary cells. Primary PAM were isolated and incubated with the recombinant antibody for different time periods, after which they were fixed and stained to visualize membrane-bound and internalized antibodies. As for mAb 41D3, a clear membrane staining was observed at time zero, while

with increasing time, pSn-positive endocytic vesicles became readily apparent (Fig. 2A). Also, at early time points, endocytic vesicles of both antibodies were mainly present in the vicinity of the plasma membrane, while with increasing time endocytosed pSn was also localized closer to the perinuclear region. Similar to mAb 41D3-induced pSn endocytosis, rec41D3-induced pSn endocytosis is only partial, as confocal microscopical analysis showed that a clear membrane staining remains visible besides the endocytic vesicles. As a control, PAM were incubated with irrelevant, isotype matched mAb 13D12 and rec13D12. No cell staining was observed with these antibodies (data not shown).

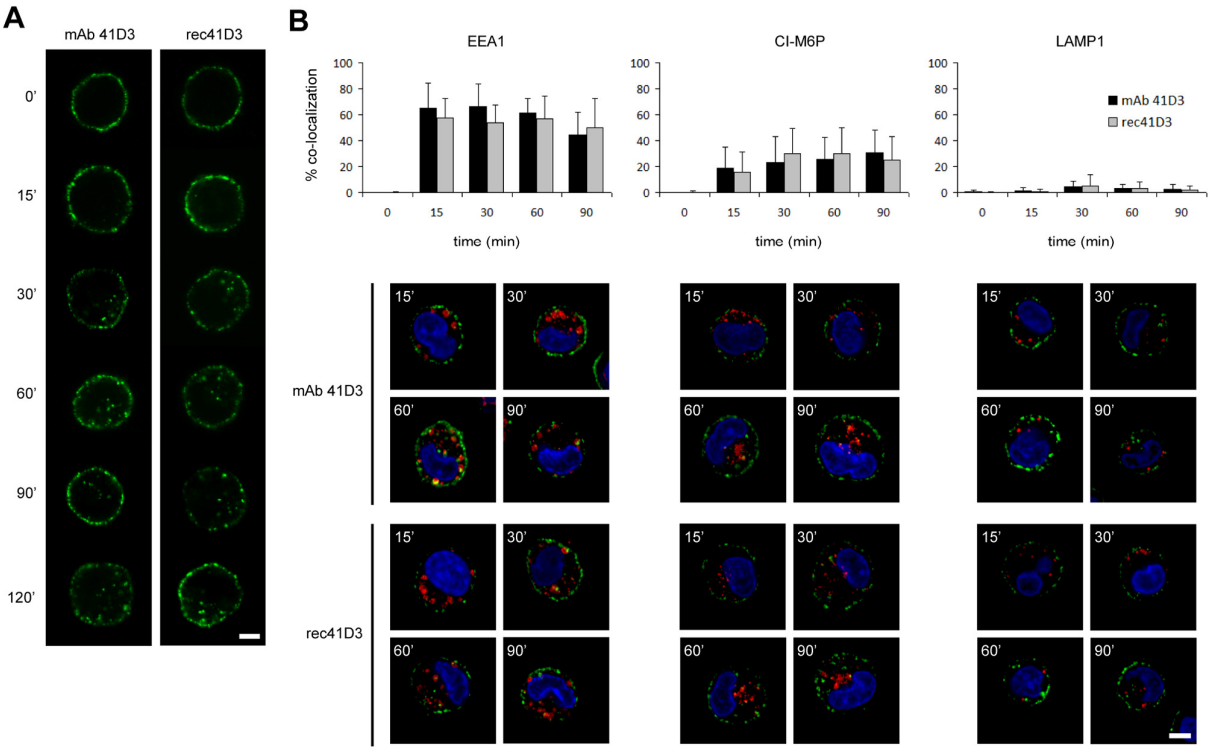

Figure 2. Analysis of rec41D3-induced pSn endocytosis and analysis of co-localization between internalized antibody and endo/lysosomal compartments
(A) Confocal microscopical analysis of mAb 41D3- and rec41D3-induced pSn internalization in primary macrophages. Cells were incubated for the indicated time periods with either antibody at 37°C, fixed, permeabilized and stained with FITC-labelled goat-anti-mouse IgG. Images represent a single confocal z-section through the middle of the cell. (B) Analysis of co-localization between internalized mAb 41D3 or rec41D3 (green) and early endosomes (EEA1), late endosomes (CI-M6P) or lysosomes (Lamp1) (red) in primary macrophages. Co-localization was calculated from confocal z-sections of 25 randomly selected cells of 2 independent experiments at the indicated time points. Data represent the means ± standard deviations. Representative images of macrophages that were incubated for 60 minutes at 37°C with either antibody are shown as overlays of the green and red signal with a yellow colour indicating co-localization. Scale bar: 5µm

In a previous study, we have shown that mAb 41D3 resides for prolonged times in early endosomes [10]. To analyze the intracellular localization of internalized rec41D3 in comparison to mAb 41D3, double immunofluorescence stainings were performed with EEA1, CI-M6P or Lamp1, markers for early endosomes, late endosomes and lysosomes

respectively. For both antibodies, the majority of internalized antibody was localized to early endosomes (around 60%, Fig. 2B), while the remainder was localized to late endosomes. Occasionally, a very limited number of internalized antibodies were localized in a lysosomal compartment. These results show that rec41D3 follows an endocytic pathway similar to mAb 41D3 and resides for prolonged times in early/late endosomes.

3.3.3 REC41D3 TARGETS ITS CARGO V5 AS WELL AS eGFP TOWARDS pSN^+ CELLS

The previous results clearly show that rec41D3 can be used to target Sn-expressing macrophages. To be able to evaluate targeting of a cargo, we aimed at generating functional antibody fusion constructs in which a cargo is coupled to the C-terminus of the heavy chain of the antibody. During the generation of the rec41D3 plasmid, a multiple cloning site was introduced at the C-terminus to facilitate this. Also, a flexible glycine-serine (GS) linker [25] was introduced between the C-terminal Fc part and the cargo linked to rec41D3. This minimizes the risk that unwanted interactions occur between rec41D3 and its cargo, which could result in non-functional antibody and/or cargo. Two fusion constructs were generated: one construct containing a $(G_4S)_2$ linker fused to a V5 peptide tag (rec41D3-V5), the other one containing a $(G_4S)_4$ linker fused to eGFP (rec41D3-eGFP). The GS linker used to generate rec41D3-eGFP is long, to ensure eGFP has the opportunity to fold into a functional protein. HEK-293T cell transfection followed by protein G purification of the supernatant clearly predominantly yields intact fusion proteins as shown by SDS-PAGE (Fig. 3A). Under non-reducing conditions a single band was visible for both fusion proteins, shifted in size in comparison with unmodified rec41D3. Under reducing conditions, it became clear that the antibody's light chains remained unchanged, while the heavy chains were shifted in size, showing the acquisition of extra protein sequences. The presence of V5 or eGFP at the heavy chains of rec41D3 was further confirmed by Western blot analysis (Fig. 3B). These results indicate that rec41D3-V5 contains a V5 peptide tag, while rec41D3-eGFP contains an eGFP molecule.

To further analyze whether or not rec41D3-V5 and rec41D3-eGFP carry their cargo towards pSn^+ cells and induce internalization into the cells, we incubated CHO-pSn and PAM with the fusion constructs for one hour at 37°C. As shown in Figure 3C, both V5 and eGFP were co-internalized together with rec41D3 in both cell types. As a control, CHO-pSn and PAM were incubated with irrelevant, isotype matched rec13D12 fusion constructs rec13D12-V5 and rec13D12-eGFP. No staining was observed with these constructs (data not shown). In conclusion, these data show that rec41D3 allows the targeting of peptides and/or proteins towards pSn^+ cells.

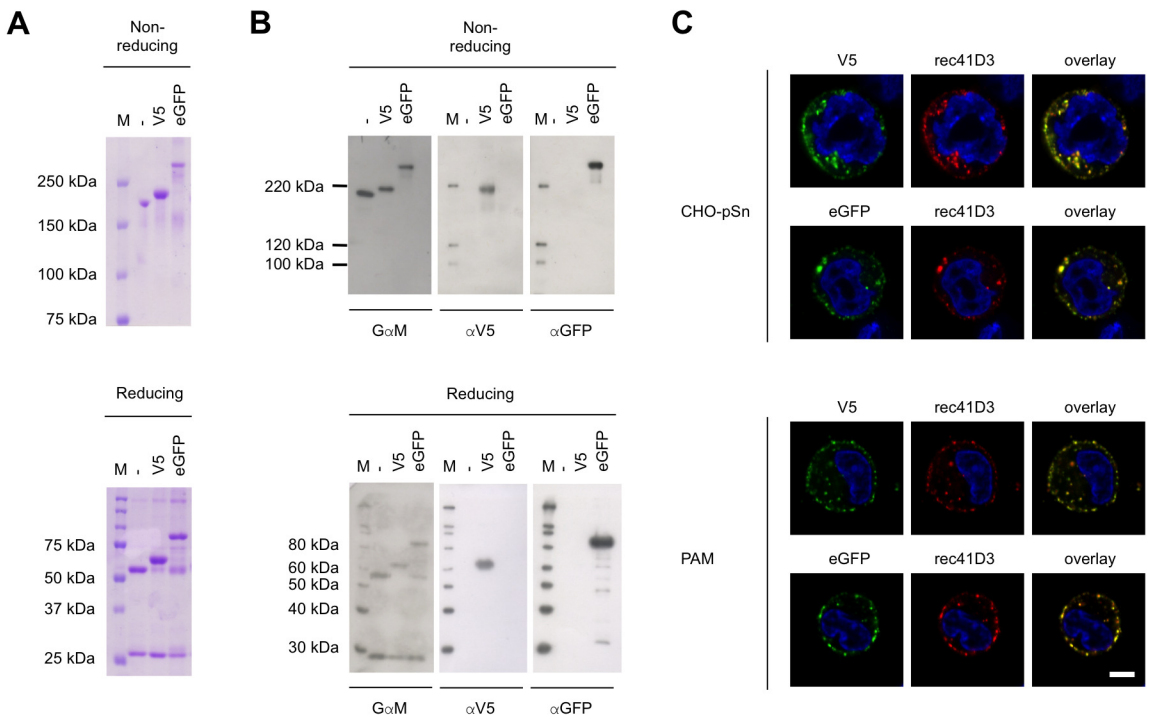

Figure 3. Development of fusion constructs rec41D3-V5 and rec41D3-eGFP and analysis of their internalization into pSn-expressing cells
(A) SDS-PAGE analysis of protein G purified rec41D3-V5 and rec41D3-eGFP constructs in comparison to unmodified rec41D3 (B) Western blot analysis of rec41D3-V5 and rec41D3-eGFP samples in comparison to unmodified rec41D3 using mouse immunoglobulin-specific goat polyclonal antibodies (GαM), a V5-specific mAb (αV5) and an eGFP-specific mAb (αGFP). (C) Confocal microscopical analysis of rec41D3-V5 and rec41D3-eGFP internalization in CHO-pSn cells and primary macrophages (PAM). Cells were incubated with rec41D3-V5 or rec41D3-eGFP for 1 hour at 37°C, fixed, permeabilized and stained with AF594-labelled goat-anti-mouse IgG1 in addition to FITC-labelled mouse anti-V5 (IgG2a) or rabbit anti-GFP in combination with FITC-labelled goat-anti-rabbit polyclonal antibodies respectively. Since the rec41D3-eGFP fluorescence was weak, the signal was enhanced to obtain good microscopical images. Images represent a single confocal z-section through the middle of the cell. M, protein marker; -, rec41D3; V5, rec41D3-V5; eGFP, rec41D3-eGFP. Scale bar: 5µm.

3.4 DISCUSSION

Monoclonal antibodies and their derivatives are currently the fastest growing class of therapeutic molecules [26]. Their inherent promise to minimize side effects by selectively targeting specific target cells has fuelled their development, leading to several FDA-approved antibody therapeutics so far and many more in the pipeline. Although unmodified mAbs proved their worth, the conjugation of effector molecules (like toxins, drugs, radionuclides,…) to mAbs broadened their therapeutic potential, especially in the domain of cancer therapeutics. Besides cancer, other diseases could also benefit from antibody-directed therapies, the only prerequisite being the identification of a receptor exclusively expressed on those immune cells involved in the induction of pathology. In this respect, Siglecs are compelling candidates for therapy, as they display very restricted expression patterns on

subsets of immune cells and may regulate immune cell functions. Furthermore, siglecs are endocytic receptors allowing therapeutic agents conjugated to a mAb to be carried efficiently into the cell [27, 28].

Sn or Siglec-1 is expressed on cells of the monocyte/macrophage lineage, notably on subsets of resident tissue macrophages and inflammatory monocytes/macrophages [15, 27]. A recent report describes the expression of Sn on human mature DCs treated with LPS *in vitro* [29], suggesting that Sn may be present on mature DCs during inflammation *in vivo* as well. Not only have Sn^+ monocytes/macrophages been described in several diseases like inflammatory and autoimmune disorders as well as viral infections, they also appear to play a role in the initiation of an adaptive immune response as recently shown by different independent research groups and nicely reviewed by Martinez-Pomares and Gordon [1]. Together, this makes these Sn^+ cells not only attractive targets for cell-directed therapies, but also an appealing target for vaccination. In our previous study, we developed immunoconjugates by the chemical linkage of the model antigen HSA or a toxin to the pSn-specific mAb 41D3 [10]. Although these immunoconjugates proved efficient for boosting immune responses and killing pSn-expressing cells respectively, the chemical linkage of the cargo to a targeting antibody has many disadvantages. First of all, chemical coupling procedures rely on the presence and distribution of reactive groups, like e.g. primary amines on lysine residues, that can be located in or near the antigen-binding region, which upon coupling might result in partial or complete loss of the antibody's affinity for the target antigen. Secondly, because of the large number of reactive groups present in antibody molecules, a typical distribution can be observed of zero to eight molecules per antibody [30, 31], resulting in high variation of the final conjugate. This variation is unwanted, as it may lead to a heterogeneous mixture of components with distinct affinities, stabilities, pharmacokinetics, efficacies, and safety profiles [21]. Moreover, chemical coupling implies that both antibody and cargo to be linked are independently produced and purified, which represents a significant challenge, especially when the cargo is also a biologic. To circumvent these problems, we opted to generate a recombinant form of the pSn-specific mAb 41D3. As shown in this study, this recombinant antibody displays a comparable affinity for pSn compared to the native mAb. In addition, the recombinant mAb also induces pSn endocytosis in primary macrophages, a feature important to allow functionality of antibody-cargo constructs. As protein sequences are attached to the C-terminus of the antibody's heavy chain, they are less likely to hinder antigen binding by the variable immunoglobulin domains. In addition, each heavy chain will contain only one cargo fused to the C-terminal end. This will result in an antibody with 2 cargos in a defined position and a high intra and inter batch consistency. Furthermore, we could purify the antibody-cargo fusion proteins

using standard protein G chromatography, which represents a major advantage compared to chemical coupling in which purification is needed for both cargo and antibody before, as well as after chemical conjugation.

In this study we managed to make genetic fusion constructs of a peptide or a protein linked to our recombinant mAb. Obviously, the recombinant antibody vector does not allow to make genetic fusion constructs with chemical compounds. For vaccination strategies however, this limitation is not expected to pose any problems, as most antigens used in vaccines are protein based. One challenge however would be to ensure correct folding of the antigen upon genetic fusion to the antibody and to maintain this fold during purification procedures. Similarly, immunotoxins can be made using the Sn targeting vector. Although the production of immunotoxins in eukaryotic cells has been limited due to potential toxicity to the producing cells, several independent research groups have reported on the successful production of immunotoxins in mammalian cell lines, including HEK-293T [32-34]. In case a specific application would require the chemical linkage to an antibody, e.g. when vaccines are based on glyco-epitopes, a recombinant mAb has some major advantages. It allows addition of specific amino acid modifications to the antibody, which will result in site-specific incorporation of drug molecules through chemical linkage yielding batch to batch consistency of antibody-drug conjugates. Examples of such already implemented modifications are the THIOMAB™ technology of Genentech Inc [35] or the methodology of Axup et al. [21].

As our future plans include the use of the developed recombinant antibody to target antigens towards pSn-expressing macrophages *in vivo*, one might be concerned about the immunogenicity of mouse antibodies in pigs. Poderoso *et al.* previously used mouse mAbs as surrogate antigens in pigs to evaluate the role of Sn in the induction of humoral responses and noticed an enhanced anti-mouse antibody response in comparison with a non-targeting isotype control mAb [11]. The induction of anti-mouse antibodies was however low after primary injection of the mAb, only after a booster vaccination antibody titres rose significantly. Previously, we have observed an enhanced anti-HSA antibody response after a single dose vaccination of HSA coupled to mAb 41D3 without adverse clinical effects [10]. Therefore, in our future experiments, we will use a single dose of rec41D3-antigen to evaluate the protective efficacy of antigen targeting to pSn. If further experiments confirm the applicability of this targeting technology, ‘porcinization’ of the recombinant antibody will be examined to enable prime-booster vaccination schedules.

3.5 CONCLUSIONS

A recombinant antibody that targets Sn was developed. In addition, we constructed a vector that allows the genetic linkage of a protein cargo in a defined position at the C-terminus of both heavy chains of a fully assembled antibody. This vector was shown to be versatile, as both a peptide and a more complex, larger protein could be fused. Furthermore, production and purification of the antibody fusion constructs did not require major optimization. In comparison to other Sn targeting strategies (glycan-coated liposomes and cationic hydrogels), the one-step production of the final carrier-cargo product together with the high specificity and affinity of the recombinant antibody may be considered an advantage for drug development. Future research will mainly focus on the development of functional antibody-antigen fusions allowing the evaluation of Sn-directed vaccination strategies.

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Chapter

4

Targeting to sialoadhesin as a vaccination strategy

4.1

Evaluation of viral peptide targeting to porcine sialoadhesin using a porcine reproductive and respiratory syndrome virus vaccination-challenge model

Karen Ooms, Hanne Van Gorp, Sara Botti, Tim Van Gaever, Peter L. Delputte and Hans J. Nauwynck

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Targeting antigens to professional antigen presenting cells resident at the sites where effective immune responses are generated is a promising vaccination strategy. As such, targeting Sn^+ macrophages, abundantly present in spleen and lymph nodes where they appear to be strategically placed for antigen capture and processing, is recently gaining increased attention. Previously, we have shown that humoral immune responses to the model antigen human serum albumin can be enhanced by using a porcine Sn-specific monoclonal antibody to target the model antigen to Sn-expressing macrophages. To date however, no studies have been performed to evaluate whether Sn-targeted delivery of a pathogen-derived antigen can enhance the pathogen-specific immune response. Therefore, we selected a linear epitope on glycoprotein 4 of PRRSV, which is known to be a target of virus-neutralizing antibodies. This paper reports on the targeted delivery of this viral peptide to porcine Sn-expressing macrophages and the evaluation of the subsequent immune response in a vaccination-challenge set-up.

Four copies of the selected PRRSV epitope were genetically fused to a previously developed porcine Sn-targeting recombinant antibody or an irrelevant isotype control. Fusion proteins were shown to be efficiently purified from HEK293T cell supernatants and subsequently, only Sn-specific fusion proteins were shown to bind to and to be internalized into Sn-expressing cells. Subsequent immunizations with a single dose of the fusion proteins showed that peptide-specific immune responses and neutralizing antibody responses after PRRSV challenge were enhanced in animals receiving a single 500µg intramuscular dose of the Sn-targeting fusion protein, although correlations between the two read-outs were hard to effectuate. Furthermore, a minor beneficial effect on viral clearance was observed. Together, these data show that viral peptide targeting to porcine Sn-expressing macrophages can improve the anti-viral immune response, although more research will be needed to optimise vaccine design.

4.1.1 INTRODUCTION

Traditionally, vaccines are based on inactivated pathogens, live attenuated pathogens or pathogen-derived toxins [1, 2]. Although successful in many instances, there may be significant drawbacks of these approaches, including the risk of reversion to virulence, unwanted host reactions such as inflammation or the induction of an autoimmune response. In addition, manufacturing constraints may occur with microorganisms that are hard to cultivate *in vitro*. For veterinary vaccines, also the need to discriminate between infected and vaccinated animals contributes to the growing interest in a more sophisticated vaccine design [3]. Starting from the exclusion of specific protein antigens in vaccine formulations, research has evolved towards the development of true subunit vaccines, containing only fragments of a pathogen. Going even further, the selection of a minimal, protective and immunogenic region of a protein antigen has led to the development of epitope-based peptide vaccines, allowing a precise direction of immune responses. This reductionist approach, although challenging, can result in vaccines that are more immunologically defined and with a better safety profile compared to vaccines generated by traditional empirical approaches.

Although promising, peptides are traditionally poorly immunogenic on their own [4]. Therefore, there is a clear need for potent immunostimulatory adjuvants. In addition, these adjuvants also need to be safe. An alternative to this approach is making the peptide-based vaccines self-adjuvanting by the use of nanoparticular carriers [4] or by targeting the peptides directly to APCs. While most targeting strategies aim at delivery of the antigens to DCs [5-7], Sn^+ macrophages, among other situated in spleen and lymph nodes, appear to be strategically placed for antigen capture and processing and may be an attractive target for vaccination strategies as well [8]. Previously, we used a pSn-specific mAb to target the model antigen HSA, which was chemically cross-linked to the mAb, to pSn-expressing macrophages. In a porcine model, this resulted in an enhanced immune response to HSA [9]. To circumvent the drawbacks associated with the chemical linkage of antigens, we recently developed a pSn-specific recombinant antibody [10]. This antibody allows targeted delivery of peptides or proteins towards pSn-expressing macrophages and thus represents an elegant tool to evaluate Sn targeting as a vaccination strategy.

PRRSV is a single-stranded, positive sense RNA virus that belongs to the family of the Arteriviridae, order Nidovirales [11]. PRRSV is the causative agent of porcine reproductive and respiratory syndrome, a disease present in the majority of swine-producing countries around the world, which causes major economic losses [12, 13]. Although protective PRRSV immunity is a complex matter, it has been shown that sufficiently high titres of virus-

neutralizing (VN) antibodies in serum can offer *in vivo* protection against PRRSV [14, 15]. Currently used inactivated vaccines however, do not induce VN antibodies and are of limited efficacy at best [16, 17]. To test if targeting to Sn increases the induction of VN antibodies, we selected an epitope on glycoprotein 4 (GP4) of the European Lelystad virus (LV) strain which is known to be a target for VN mAbs in continuous cell lines as well as in porcine alveolar macrophages [18, 19]. In addition, during the course of an infection pigs produce antibodies against this linear epitope which are able to neutralize the virus *in vitro* [20]. The GP4 epitope was genetically linked to the pSn-specific recombinant antibody rec41D3 and the resulting product was used to immunize pigs, evaluate the induction of peptide-specific antibodies and assess the efficacy upon a viral challenge.

4.1.2 MATERIALS AND METHODS

4.1.2.1 ETHICS STATEMENT

Animal experiments were approved and supervised by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University. The named institution approved the experiments and provided a permit for this study (Permit Numbers: EC 2011/105 and 2012/038). The experimental procedure for the collection of porcine alveolar macrophages was authorized and supervised by the named institution as well.

4.1.2.2 CELLS AND VIRUS

Primary macrophages were isolated by bronchoalveolar lavage from four- to six-week-old conventional Belgian Landrace pigs as described before [21], and cultivated in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1% non-essential amino acids and 1 mM sodium pyruvate. HEK293T were grown in DMEM medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and 1 mM sodium pyruvate. CHO-K1 cells and CHO-K1 cells stably expressing recombinant pSn (CHO-pSn) [22] were cultivated in F-12 medium supplemented with 10% FBS and 1 mM sodium pyruvate. All culture media were supplemented with a mixture of antibiotics and cell cultures were kept in a humidified 5% CO_2 atmosphere at 37°C.

The PRRSV LV strain was propagated in primary macrophages that were derived from gnotobiotic piglets. The fifth passage of infected cell culture supernatant was purified by ultracentrifugation as previously described by Vanhee et al. (2009).

4.1.2.3 CONSTRUCTION, PRODUCTION AND PURIFICATION OF REC41D3- AND REC13D12-GP4

Construction of the plasmids encoding pSn-specific rec41D3 and isotype matched control rec13D12, directed against pseudorabies virus glycoprotein gD [23], was described previously [10]. To generate both rec41D3- and rec13D12-GP4 plasmids, a $(G_4S)_2$-GP4 DNA sequence was synthesized (Genscript Inc.) and introduced at the 3' end of the heavy chain sequence by restriction enzyme digestion and ligation. Sequencing confirmed the fusion constructs were correctly assembled. HEK293T cells were transiently transfected with the plasmids using calcium phosphate and cultured in DMEM supplemented with 10% IgG depleted, heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and a mixture of antibiotics in a humidified 5% CO_2 atmosphere at 37°C. Culture supernatant was collected and recombinant IgG were purified from the supernatant using standard protein G sepharose chromatography following the manufacturer's instructions (GE Healthcare). Fractions of the eluate containing the purified protein were pooled and the buffer was exchanged to DPBS (Life Technologies) by dialysis. Purified protein was stored at -70°C until use.

4.1.2.4 SDS-PAGE, COOMASSIE BLUE STAINING AND WESTERN BLOT ANALYSIS OF REC41D3- AND REC13D12-GP4

Samples of purified proteins were mixed with (non-)reducing Laemmli buffer, boiled for 5 min and subjected to SDS-PAGE (6% non-reducing gel, 10% reducing gel) using a BioRad Mini Protean 3 system. For Coomassie Blue staining, the SDS-PAGE gel was incubated successively in Ultra Pure water, Imperial™ protein staining solution (Thermo Scientific) and Ultra Pure water as destaining solution. Alternatively, for Western blot analysis, proteins were transferred from the SDS-PAGE gel to a PVDF membrane (Membrane Hybond-P, GE Healthcare) via Western blotting (BioRad Mini Trans Blot). The membrane was blocked overnight in PBS + 0.1% Tween-20 + 5% skimmed milk. Detection of recombinant antibodies was performed by subsequent incubation of the blot with peroxidase-labelled polyclonal goat anti-mouse antibodies (Dako), followed by visualization using enhanced chemiluminescence (ECL; GE Healthcare). Alternatively, GP4 peptides were detected using the mouse mAb XVI11C (IgG2a, [18]) and peroxidase-labeled rabbit anti-mouse IgG2a antibodies (Life Technologies), followed by ECL visualization.

4.1.2.5 IMMUNOFLUORESCENCE STAINING AND CONFOCAL LASER SCANNING MICROSCOPY

To evaluate pSn binding and endocytosis CHO-pSn and primary macrophages were incubated with 2µg/200µl purified rec41D3-GP4 or its isotype control rec13D12-GP4 for one

hour at 37°C. Cells were then fixed with 4% (w/v) paraformaldehyde (Sigma), permeabilized using 0.5% (w/v) saponin (Sigma) and stained with FITC-labelled goat anti-mouse IgG1 in addition to mAb XVI11C in combination with AF594-labelled goat anti-mouse IgG2a antibodies respectively. Cell nuclei were visualized using Hoechst 33342 (Life Technologies). Z-section images of samples were acquired using a Leica TCS SPE-II laser scanning spectral confocal system (Leica Microsystems GmbH) linked to a Leica DM2500 microscope (Leica Microsystems GmbH). Image acquisition was done using the Leica LAS AF confocal software package.

4.1.2.6 EXPERIMENTAL DESIGN OF ANIMAL STUDIES

All piglets were derived from a PRRS-negative farm and their PRRSV seronegative status was confirmed by immunoperoxidase monolayer assay (IPMA) as previously described [24]. The animals were housed in isolation units with HEPA-filtered air and kept during seven days to allow adaptation to the new conditions.

Dose experiment with 50µg or 500µg rec41D3-GP4

In a first preliminary experiment, nine piglets were randomly assigned to three treatment groups. All three groups received one intramuscular (i.m.) injection at six weeks of age. A first group served as mock-vaccinated control group and received an injection of 500µl PBS. The second group received an injection of 50µg rec41D3-GP4 in 500µl PBS. The third group received an injection of 500µg rec41D3-GP4 in 500µl PBS. Seven weeks after the i.m. injection, all pigs were challenged by intranasal inoculation of 10^6 $TCID_{50}$/ml LV as previously described [25]. Blood was taken weekly after vaccination and post challenge. An extra blood sample was taken at five days post challenge when peak viremia was expected. Serum was collected and stored at -70°C. Serum samples for peptide-specific and VN antibody detection were inactivated by incubation during 30 min at 56°C prior to freezing.

Vaccination experiment with rec41D3-GP4 in comparison to rec13D12-GP4

In a second experiment, 30 piglets were randomly assigned to five treatment groups. All treatment groups received one i.m. injection at six weeks of age. A first group (PBS) served as mock-vaccinated control group and received an injection of 500µl PBS. The other treatment groups received an injection of either 150µg rec13D12-GP4, 150µg rec41D3-GP4, 500µg rec13D12-GP4 or 500µg rec41D3-GP4 in 500µl PBS. Four weeks after the i.m. injection, all pigs were challenged by intranasal inoculation of 10^6 $TCID_{50}$/ml LV. Blood was taken weekly after vaccination and at days 0, 1, 3, 5, 7, 10, 14, 21, 28 and 35 post challenge. At the start of the experiment, one animal died due to a hemolytic *E. coli* infection. No other pigs showed clinical signs of *E. coli* infection, but as a preventive measure, all animals

received a three day antibiotic treatment (Baytril®). Further on in the experiment, a second animal died during blood taking. This resulted in a group size of five animals instead of six for the 150µg rec13D12-GP4 and the 500µg rec41D3-GP4 groups. Serum was collected and stored at -70°C. Serum samples for peptide-specific and VN antibody detection were inactivated by incubation during 30 min at 56°C prior to freezing.

4.1.2.7 GP4 PEPTIDE-SPECIFIC ELISA

GP4 peptide for use in ELISA was synthesized by JPT Peptide Technologies as a biotinylated peptide (BioTide). Antibodies against the GP4 peptide were detected by ELISA as previously described [20] with the following modifications. Two-fold serial dilutions of serum samples (starting from 1/100 diluted) were incubated on the peptide-coated plates, followed by washing and incubation with peroxidase-conjugated goat anti-swine IgG antibodies (Abcam) or goat anti-swine IgM antibodies (AbD serotec). For IgG antibody titre determination, the optical density at 450nm (OD450) was measured and OD450 values obtained were expressed relative to the OD450 value obtained with a serum sample of the same animal at the time of vaccination (day 0). If this OD450 sample/negative was more than 2 it was considered a specific signal. Subsequently, antibody titres were determined as the reciprocal of the highest dilution for which an OD450 sample/negative of 2 or more was observed. As serum background levels were higher and more variable in the IgM peptide-specific ELISA, another approach for antibody titre determination was used. Here, the mean of all OD450 values of all PBS mock-vaccinated animals of all time points before challenge was calculated together with the standard deviation (for each serum dilution). If OD450 values obtained were $\geq$ the mean calculated + 3 times the standard deviation, it was considered a specific signal. Subsequently, antibody titres were determined as the reciprocal of the highest dilution for which an OD450 value was obtained $\geq$ the mean calculated + 3 times the standard deviation.

4.1.2.8 SINGLE REPLICATION CYCLE VIRUS-NEUTRALIZATION TEST ON PRIMARY MACROPHAGES

Single replication virus-neutralization test on primary macrophages was essentially performed as described before [20]. Two-fold serial dilutions of sera in PBS were mixed with equal volumes of LV virus resulting in a final titre of $10^{5.5}$ $TCID_{50}$/ml. PBS without serum was included as mock condition. Virus-antibody mixtures were incubated for one hour at 37°C and transferred to a 96-well plate (100µl/well) with primary macrophages (10^5 cells/well) that were cultivated during 72h prior to use. The inoculum was removed after one hour and replaced by medium, after which the cells were further incubated for another 10h, fixed by

drying, and stored at -20°C. The cells were stained for PRRSV infection with mAb 13E2 [26] against the nucleocapsid protein of PRRSV and peroxidase-conjugated goat anti-mouse polyclonal antibodies (Dako), followed by development with 3-amino-9-ethylcarbazole. The number of infected cells in each well was counted in three fields at 200x magnification, and expressed relative (%) to the mean number of infected cells for all mock conditions. VN antibody titres were determined as the reciprocal of the highest dilution that resulted in more than 90% reduction of infected cells.

4.1.2.9 VIRUS TITRATION AND RT-qPCR

Virus titres in serum were determined by virus titration on primary macrophages using standard procedures [24], followed by immunoperoxidase staining with mAb 13E2 against the nucleocapsid protein of PRRSV [26].
RNA was extracted with the QIAamp® Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany, Cod. 51906) according to the manufacturer's recommendations, and finally eluted in 60 µl kit elution buffer. The commercially available TaqMan PRRSV Reagents (Applied Biosystems/Ambion, Cod. 4405547) for PRRSV detection was used according to the manufacturer's recommendations. Real time PCR assays were carried out in a final volume of 12.5 µl including 4 µl of purified RNA as template and run on a 7900HT Fast Real-Time PCR System (Applied Biosystems).

4.1.2.10 STATISTICAL ANALYSIS

Statistical analysis was performed by Kruskall-Wallis test, followed by Dunn's multiple comparisons test to determine differences between area under the curves (AUC). AUC were calculated for each test with its respective detection limit as baseline value. Differences were considered significant if the overall P-value was below 0.05 (**) or 0.1 (*). All statistical analyses were performed using GraphPad Prism version 5.01.

4.1.3 RESULTS

4.1.3.1 CONSTRUCTION AND CHARACTERIZATION OF REC41D3-GP4 AND REC13D12-GP4 FUSION PROTEINS

We previously described the development of a pSn-specific recombinant antibody rec41D3 and an irrelevant isotype control rec13D12 [10]. In the present study, we generated fusion constructs of rec41D3 and rec13D12 containing four repeats of a known neutralizing epitope of GP4 of PRRSV, separated from each other by a short spacer and separated from the antibodies by a GS linker. The amino acid sequence following the antibodies' heavy chains is shown in Figure 1A.

HEK293T cell transfection followed by protein G purification of the supernatant clearly yielded intact fusion proteins as shown by SDS-PAGE (Fig. 1B). Under non-reducing conditions, a shift in size compared to unmodified rec41D3 and rec13D12 was visible for both fusion proteins. Under reducing conditions, the antibody light chains remained unchanged, while the heavy chains have shifted in size, showing the acquisition of extra protein sequences. The presence of the GP4 peptide at the heavy chains of rec41D3 and rec13D12 was further confirmed by Western blot analysis using mAb- and peptide-specific antibodies (Fig. 1C).

To further analyze whether rec41D3-GP4 is able to bind to pSn-expressing cells and is internalized afterwards, CHO cells expressing pSn (CHO-pSn) and primary macrophages were incubated with the fusion protein for one hour at 37°C. As shown in Figure 1D, the GP4 peptide was co-internalized together with rec41D3 in both cell types. As a control, CHO-pSn and macrophages were incubated with irrelevant, isotype matched rec13D12-GP4 fusion proteins. No binding and internalization was observed with this protein. Together, these data show that rec41D3-GP4 and rec13D12-GP4 proteins remain functional and both contain the GP4 sequences at the C-terminus of the heavy chain.

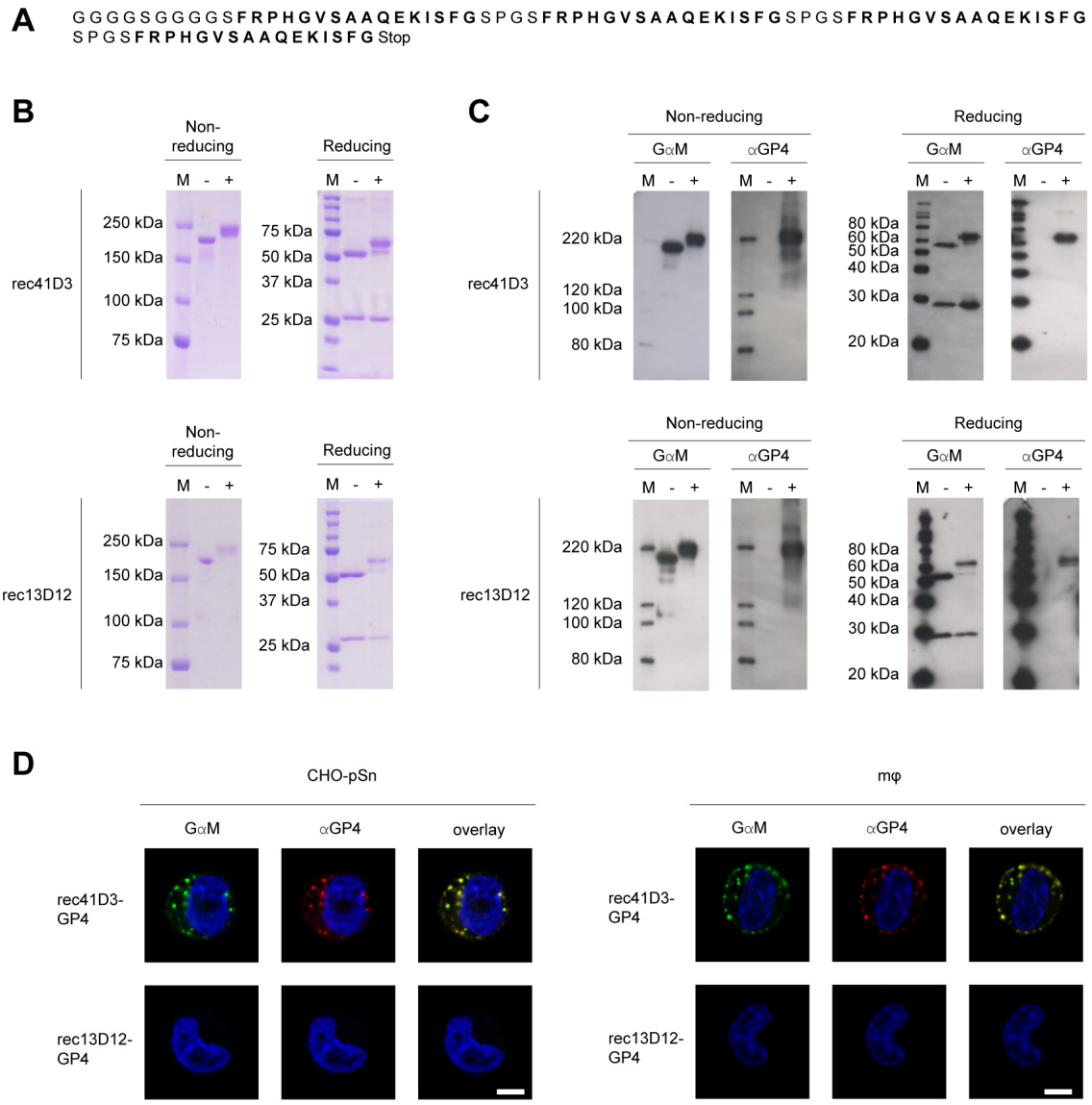

Figure 1. Development of fusion proteins rec41D3-GP4 and rec13D12-GP4 and analysis of their internalization into pSn-expressing cells.
(A) Primary amino acid sequence following the C-terminus of the antibodies' heavy chains. GP4 peptides are indicated in bold (B) SDS-PAGE analysis of protein G purified rec41D3-GP4 and rec13D12-GP4 constructs in comparison to unmodified rec41D3 and rec13D12 respectively (C) Western blot analysis of rec41D3-GP4 and rec13D12-GP4 samples in comparison to unmodified rec41D3 and rec13D12 respectively using mouse immunoglobulin-specific goat polyclonal antibodies (GαM) or a GP4-specific mAb (αGP4). (D) Confocal microscopical analysis of rec41D3-GP4 internalization in CHO-pSn cells and primary macrophages (mφ) in comparison to its irrelevant control rec13D12-GP4. Cells were incubated with rec41D3-GP4 or rec13D12-GP4 for one hour at 37°C, fixed, permeabilized and stained with FITC-labelled goat anti-mouse IgG1 in addition to mouse anti-GP4 (IgG2a) in combination with AF594-labelled goat anti-mouse IgG2a. Images represent a single confocal z-section through the middle of the cell. M, protein marker; -, antibody without GP4; +, antibody with GP4. Scale bar: 5µm.

4.1.3.2 VACCINATION DOSE EXPERIMENT WITH REC41D3-GP4

To determine the optimal dose to administer in a large vaccination experiment, a preliminary experiment was performed with nine pigs randomly assigned to three treatment groups. The first group served as a mock-vaccinated control group and received one i.m. injection of 500µl PBS. The second group received 50µg rec41D3-GP4 in PBS and the third group 500µg in PBS. All pigs were challenged with LV seven weeks post vaccination.

GP4 peptide-specific IgG response

Antibodies against the GP4 peptide were detected by ELISA. As shown in Figure 2A, none of the animals showed detectable levels of peptide-specific antibodies in their serum after vaccination. Upon challenge however, a clear difference was noticed between the three treatments. Two weeks post challenge, all animals that received a 500µg dose of rec41D3-GP4 showed high titres of peptide-specific antibodies, which remained as high during the rest of the experiment. For the 50µg treatment group, one pig responded as strong as the 500µg group starting from two weeks post challenge, one pig responded weakly at two weeks post challenge and its antibody titre rose during the rest of the experiment, while the last pig only showed GP4 peptide-specific antibodies at four weeks post challenge. For the PBS treatment group, peptide-specific antibodies were only detected in two out of three pigs at four weeks post challenge. These observations are reflected in the analysis of the AUC as shown in Figure 2A.

Virus neutralization

To evaluate the ability of the vaccine to inhibit virus replication *in vitro*, serum samples were used in a single replication virus-neutralization test with LV on primary macrophages. As shown in Figure 2B, no VN antibodies were detected after vaccination except for one animal in the 50µg group at three weeks post vaccination. After viral challenge however, VN antibodies appeared at two weeks post vaccination in all 50µg or 500µg vaccinated animals, with the highest titres in the 500µg group. VN antibodies could not be detected in the PBS vaccinated animals at any time point tested. These results led to a significant difference in AUC between the mock-vaccinated animals and the group that received the 500µg dose of rec41D3-GP4 (Fig. 2B).

Viremia

All animals in all groups showed viremia after challenge (Fig. 2C). Viral clearance from blood however was observed in the 500µg group at one week post challenge for two animals and at two weeks post challenge for the third animal. In the 50µg group, one animal showed viral clearance after one week, another after three weeks and the third animal only after four

weeks. In the PBS group, all animals showed viral clearance as well, though only at three weeks post challenge.

Overall, it is clear that a single dose of rec41D3-GP4 has beneficial effects on the induction of antibodies against the GP4 neutralizing epitope after challenge, as well as on the induction of neutralizing antibodies after challenge. In addition, a reduction of the duration of viremia was noted in all animals which received a 500µg dose of rec41D3-GP4, but not in the group with a 50µg dose.

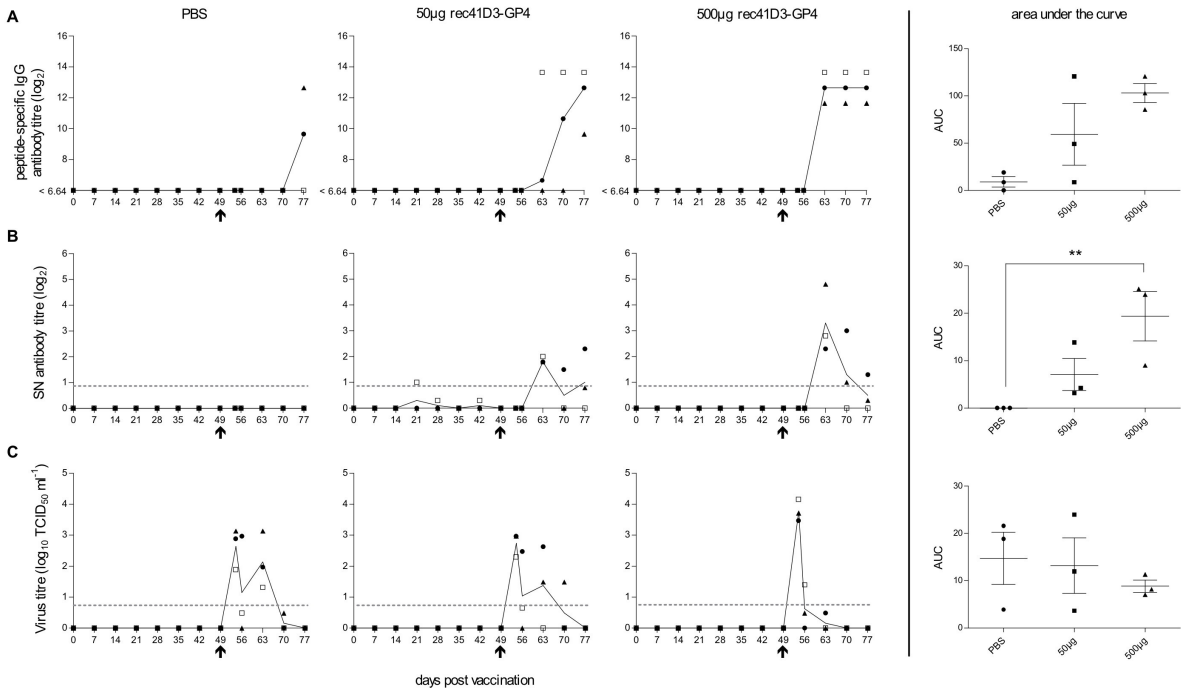

Figure 2. Analysis of serum antibody responses and viremia after vaccination with either 50µg or 500µg rec41D3-GP4 or PBS as mock-control.
Peptide-specific IgG antibody titres (log2) (A), serum neutralizing antibody titres (log2) (B) and virus titres (log10 TCID50 ml-1) (C) together with their respective AUC are shown. Symbols represent individual animals and lines represent median (A) or mean titres (B,C) calculated on all animals present in each group. Bars indicate mean AUC ± SEM for each group. The dotted line represents the limit of detection. Data represent means of at least two independent experiments. ↑ = challenge, * = P < 0.1, ** = P < 0.05.

4.1.3.3 VACCINATION EXPERIMENT WITH REC41D3-GP4 IN COMPARISON TO THE NON-TARGETING CONSTRUCT REC13D12-GP4

As it is clear that the 500µg rec41D3-GP4 dose outperformed the 50µg dose in the previous experiment, this dose was selected for a larger vaccination experiment. As the 50µg dose also had some beneficial effects in some pigs, but showed variation, a low dose of 150µg rec41D3-GP4 was added in this experiment. To be able to assign the beneficial effects to pSn targeting, animals receiving a 150µg or 500µg dose of the non-targeting rec13D12-GP4 control construct were included in the experiment as well.

GP4 peptide-specific response

As shown in Figure 3A, no animals had detectable levels of peptide-specific IgG antibodies after vaccination. After challenge however, a clear difference was noticed between the different treatment groups. Animals that received a 500µg rec41D3-GP4 dose responded the earliest starting from 10 days post challenge, followed by the 500µg rec13D12-GP4 and the 150µg rec41D3-GP4 group at two weeks post challenge. Animals that received a 150µg rec13D12-GP4 dose or the PBS mock-control responded the latest, starting from three weeks post challenge. At four weeks post challenge, all treatment groups reached comparable serum antibody titres. When the AUC values were analysed, it was clear that although differences between groups were not statistically significant, a trend is present towards the best response with the highest rec41D3-GP4 dosage.

Peptide-specific IgM antibody titres were analysed as well. As shown in Figure 3B, all treatment groups displayed an IgM response that arose earlier than the IgG response and lasted for two to three weeks. No significant differences between the AUC of different treatment groups were observed.

Virus neutralization

As shown in Figure 3C, no VN antibodies were detected after vaccination. After viral challenge however, VN antibodies were detected in all treatment groups except for the PBS mock-control. Antibody titres and the number of animals responding were however low in most treatment groups except for the 500µg rec41D3-GP4 group. Overall, the VN antibody response appears to be biphasic, with a rise in antibody titre until two weeks post challenge, a drop at three weeks post challenge and another rise starting from four weeks post challenge. When AUC values were analysed, a significant difference was noted between mock-vaccinated animals and the 500µg rec41D3-GP4 group.

Viremia and viral load

All animals in all groups showed viremia after challenge (Fig. 3D). For the 150µg rec41D3-GP4 group, viral clearance from blood was observed in five out of six animals at seven days post inoculation. This resulted in a significant difference in AUC with the 500µg non-targeting control group. Although this was the only statistically significant difference, it is to be noted that the pSn-targeting doses overall showed lower AUC than the non-targeting controls. For the 500µg rec41D3-GP4 group, a similar virus titre as the group receiving the 150µg dose was expected based on our preliminary dose experiment, but however could not be repeated in this experiment. As viral titres overall were low, it was decided to include a PCR analysis of serum samples to determine the viral load (RNA copies ml-1 serum, Fig. 3D). Here, the two groups having the smallest AUC values were the 150µg and 500µg pSn-targeting groups, though differences were not statistically significant.

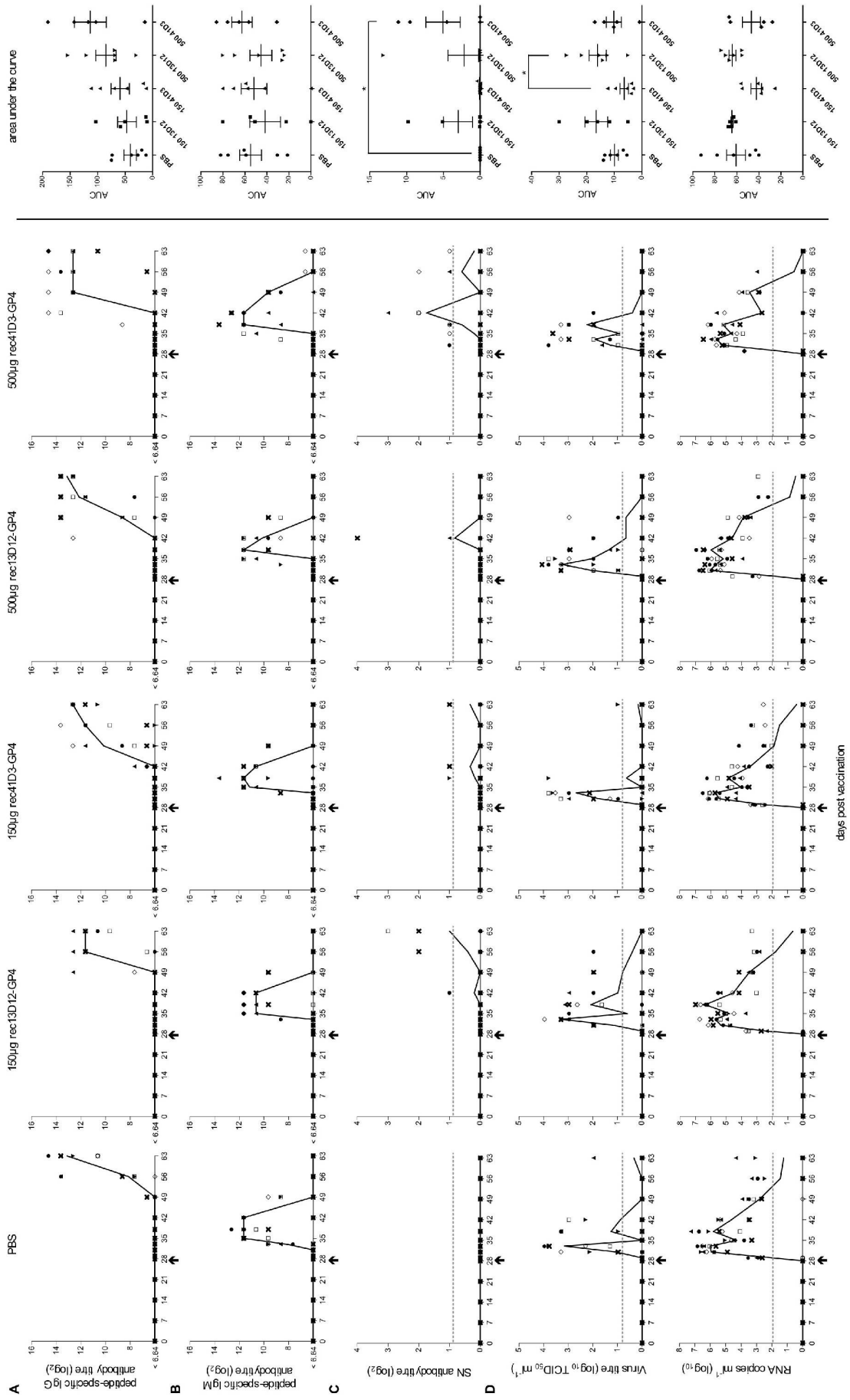
PBS
150µg rec13D12-GP4
150µg rec41D3-GP4
500µg rec13D12-GP4
500µg rec41D3-GP4
A
peptide-specific IgG antibody titre (log2)
B
peptide-specific IgM antibody titre (log2)
C
SN antibody titre (log2)
D
Virus titre (log10 TCID50 ml-1)
RNA copies ml-1 (log10)
days post vaccination
area under the curve
AUC
PBS
150 13D12
150 41D3
500 13D12
500 41D3

Figure 3. Analysis of serum antibody responses, viremia and viral load after vaccination with either 150µg or 500µg rec41D3-GP4, 150µg or 500µg of the non-targeting control rec13D12-GP4 or PBS as mock-control.
Peptide-specific IgG antibody titres (log2) (A), peptide-specific IgM antibody titres (log2) (B), serum neutralizing antibody titres (log2) (C), and virus titres (log10 TCID50 ml-1) and viral load (log10 RNA copies ml-1) (D) together with their respective AUC are shown. Symbols represent individual animals and lines represent median (A,B) or mean titres (C,D) calculated on all animals present in each group. Bars indicate mean AUC ± SEM for each group. The dotted line represents the limit of detection. ↑ = challenge, * = $P < 0.1$, ** = $P < 0.05$.

4.1.4 DISCUSSION

Sn-expressing macrophages are gaining interest as targets for vaccination strategies, as they may act as specialized antigen presenting cells involved in the antigen transport chain, as recently reviewed by Martinez-Pomares and Gordon [8]. Like DCs, Sn-expressing macrophages have been shown to be able to prime naive lymphocytes [27, 28]. In addition, their location in spleen and lymph nodes at the borders of circulating fluids, makes them ideal target candidates to achieve a high antigen load at the sites where effective immune responses are generated. Also, the restricted expression pattern of Sn can prevent removal of antigen by irrelevant cells not involved in antigen presentation. This can be a major asset, as the most promising DC receptor targets to induce humoral immunity appear to be those that show the most selective expression pattern [7]. Indeed, targeting antigen to Clec9A-expressing DCs does not seem to require the addition of an adjuvant to induce antibody responses, while targeting to DEC-205 does [29-32]. One proposed explanation for this phenomenon is that absence of removal of antibody-antigen complexes by irrelevant cells leads to a sustained antigen presentation which promotes the generation of follicular helper T cells driving B cell germinal centre development, while ensuring antigen is available to B cells as well as the antigen processing DCs [7]. Targeting antigens to Sn-expressing macrophages might in a similar way represent a way of inducing immunity without the need to add strong adjuvants to the vaccine formulation. Previously, we have made use of a pSn-specific mAb to target the model antigen HSA to pSn-expressing macrophages, which resulted in an enhanced antibody response to HSA after a single, non-adjuvanted injection [9]. To date however, no studies have been performed in which an actual pathogen-derived antigen is targeted to the Sn-expressing macrophages and the immune response is evaluated in a challenge model. Hence we selected a linear epitope on GP4 of PRRSV, known to be a target of virus-neutralizing antibodies [18]. Four copies of the epitope where genetically fused to a previously developed pSn-targeting recombinant antibody [10]. The incorporation of four copies of the epitope at each heavy chain was chosen over one, as it has been shown previously that peptide repeats can enhance peptide-specific immune

responses [33, 34]. The resulting fusion proteins were subsequently used to immunize pigs, after which a viral challenge was performed.

Upon vaccination, no peptide-specific IgG responses could be detected. After viral challenge however, it was clear that the IgG response against this specific peptide developed more rapidly compared to an infection in non-immunized animals. Clearly, some level of B cell priming had occurred. This is of interest, as it has been shown that targeting antigen towards most DC receptors in the absence of adjuvant induces tolerance to recall antigen [35, 36]. In agreement with targeting antigen to Clec9A on DCs however [7], targeting antigen to Sn in the absence of adjuvant seems to be able to prime the immune system for robust antibody responses to recall antigen. However, we cannot ignore the observation that a high dose of the non-targeting control construct accelerated the onset of the peptide-specific antibodies after viral challenge as well. Although we cannot state a definite reason for this phenomenon, irrelevant mAb might engage with Fc receptors on antigen presenting cells, including Sn-expressing macrophages in the lymph nodes. Immune-complexes have been shown before to be captured by Sn-expressing macrophages and to be subsequently delivered to lymph node B cells [37]. Nevertheless, the B cell priming observed seemed to be the most efficient with the highest pSn-specific targeting dose administered, as IgG titres in this group rose most rapidly. Although we cannot exclude the possibility that other antigen presenting cells were involved in the priming of the immune system, a beneficial effect of Sn-directed targeting cannot be neglected. This observation is in agreement with previous reports showing that antigen delivery to Sn-expressing macrophages enhances the humoral immune response and drives B cell affinity maturation [9, 38-40].

The observation that no peptide-specific response was generated after vaccination however, was rather disappointing, as we previously showed that a single, non-adjuvanted injection of HSA linked to an antibody generated a HSA-specific antibody response [9]. Of course, generation of antibodies towards a peptide represents a bigger challenge compared to generation of antibodies towards an entire protein. Firstly, an entire protein contains T-cell epitopes besides B-cell epitopes, which provide the necessary T-cell stimulation for efficient antibody induction. Our reasoning however was that the recombinant antibody would be a source of these necessary T-cell epitopes. In the future, it might be interesting to include an additional epitope, more precisely a PADRE epitope which is engineered to bind most common HLA-DR molecules with high affinity and acts as a powerful immunogen [41]. Few studies have however been performed using a PADRE epitope in pigs, so the question if similar efficiencies are reached in pigs remains to be elucidated. As more information on swine SLA molecules is however being continuously generated, the development of a swine-specific PADRE epitope might not be too far away in the future. Apart from T-cell help, also

the accessibility of the B cell epitope for B-cell receptors is of great importance. Although we showed *in vitro* that the B-cell epitope is accessible for a mAb recognizing this epitope, we cannot conclude that the conformation *in vivo* allows engagement with B-cell receptors. Clearly, more research is needed to clarify the obtained results in this study and to enhance the induction of a peptide-specific IgG response after immunization.

Besides the peptide-specific antibody response, also the serum neutralizing response was monitored. A significant difference was observed between PBS mock-vaccinated animals and animals that received a high dose of the pSn-targeting construct. This rapid neutralizing antibody response observed after challenge clearly shows that some level of boosting immune responses immediately after challenge had occurred. Also, this rapid neutralizing antibody response might account for the initial reduction of viremia observed immediately after challenge. Unfortunately however, after an initial rise of neutralizing antibody titre in the first two weeks after challenge, a drop in antibody titre is observed at three weeks post-challenge, which might be attributed to suppression of neutralizing antibody responses by viral replication. In this respect, it might be interesting to perform a booster vaccination before viral challenge, so as to be able to limit viral replication to a much lower level. A booster vaccination might be able to generate effective immune responses before challenge. In addition, it might also be interesting to study the effect of the addition of adjuvant to the vaccine formulation, as co-administration of maturation stimuli could also enhance the induction of effective immune responses before challenge.

Although the difference in serum neutralizing response between PBS and targeting construct vaccinated animals was significant in both animal experiments, no clear correlation could be drawn between the peptide-specific response and the virus neutralizing response. In our first experiment, peak neutralization coincides with the highest obtained peptide-specific IgG antibody titre, but later on this antibody titre remains as high while the neutralizing activity declines. In our second animal experiment, one animal showed a high neutralizing activity at day 42, while no peptide-specific IgG response could be monitored. Therefore, it was decided to examine the peptide-specific IgM response as well, but no significant differences between groups could be detected. The animal with the highest neutralizing antibody response in particular did have detectable levels of peptide-specific IgM antibodies in its serum, but two other animals of the same group showed higher peptide-specific IgM antibodies while not showing any PRRSV-neutralizing response. Also, no differences could be observed between groups in IPMA antibody titres (data not shown), which was to be expected as IPMA assays mostly detect antibodies against the nucleocapsid protein [42]. Together, these data show that an increase in virus neutralizing activity after viral challenge is obtained by administering a single 500µg dose of our pSn-targeting construct, but it is

difficult to correlate this with the presence of GP4 peptide-specific antibodies. Given the complex nature of PRRSV protective immunity however, the underlying cause of this lack of correlation may be diverse. In addition, the lack of neutralizing capacity of the GP4 peptide-specific antibodies *in vitro* is perhaps not surprising. When focussing on only one GP4 epitope, antibody titres might have to be exceptionally high to be able to cover enough GP4 molecules on each virion to inhibit infection. Of interest, we indeed observed *in vitro* that antibodies against our epitope purified from a PRRSV-infected pig were able to inhibit virus infection in a concentration-dependent manner, but that a high antibody concentration (500µg/ml) was necessary to yield a more than 90% reduction of infected cells [20]. The antibody titres obtained in our experiments therefore, although reaching a maximum of 14.64 log_2 in ELISA readouts, are probably still insufficient to efficiently inhibit viral infection of primary macrophages.

Going one step further towards *in vivo* relevance, viremia and viral load were examined. Here, a minor beneficial effect was observed for both 150µg and 500µg pSn-targeting groups. Although differences were not statistically significant, it should be noted that these two groups show the smallest AUC, especially when analysing viral load. Although a full protection was not observed, it is still clear that targeting of the viral epitope to pSn did have some beneficial effects on the anti-viral immune response. More research is clearly needed to find out if the vaccination efficacy can be enhanced.

In conclusion, targeting the GP4 neutralizing epitope towards pSn-expressing macrophages improves the peptide-specific IgG antibody response upon challenge, enhances the virus-neutralizing response and displays a minor beneficial effect on protection against viral challenge. Sn-directed targeting thus remains interesting to explore as a vaccination strategy. Obviously however, more research is needed to find out if vaccine efficacy can be enhanced. For PRRSV in particular, development of rationally designed polypeptide vaccines remains an interesting path to follow, as PRRSV is known to 'fool' immunity for instance by displaying an immunodominant decoy epitope adjacent to a major neutralization epitope and additionally by glycan shielding of that neutralization epitope [43, 44]. The real challenge however will be to identify protective B- and T-cell epitopes of PRRSV which are conserved and thus can confer broad protection against various field strains [17]. Until such a good and encompassing knowledge about PRRSV neutralizing epitopes present on the different viral glycoproteins is known, development of epitope based PRRSV vaccines remains elusive.

4.1.5 ACKNOWLEDGEMENTS

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4.2

Development of a suspension CHO cell line expressing a candidate immunocastration vaccine

Karen Ooms, Tim Van Gaever, Hans J. Nauwynck and Peter L. Delputte

4.2.1 INTRODUCTION

Surgical castration of male piglets is common practice in pig farming to prevent aggressive behaviour and the occurrence of an unwanted meat odour known as boar taint [1]. Animal welfare concerns however, are increasing the pressure on pig producers to stop this practice [2]. Presently, surgical castration is being questioned in the European Union and major European pork chains committed themselves to voluntarily end this practice by 2018 [3]. A number of alternatives have been considered to address the issue, including surgical castration with anaesthesia (local or general) and/or analgesia, immunocastration and raising entire male pigs [4]. Of these alternatives, immunocastration appears to be the most promising strategy, as it improves growth performance in comparison to surgical castrates [5-7] while reducing the aggressive behaviour displayed by entire males.

Immunocastration relies on the induction of antibodies against GnRH which prevent GnRH from binding to its pituitary receptor. This disruption of the hypothalamic-pituitary-gonadal axis disrupts the production of testicular steroids, including androstenone (Fig. 1). Androstenone and skatole are the two primary substances responsible for the occurrence of boar taint when accumulated in porcine adipose tissue. The reduction of skatole, produced in the intestine, by immunocastration is most likely due to enhanced metabolic clearance by the liver after suppressed steroid production, as occurs in surgically castrated pigs [8].

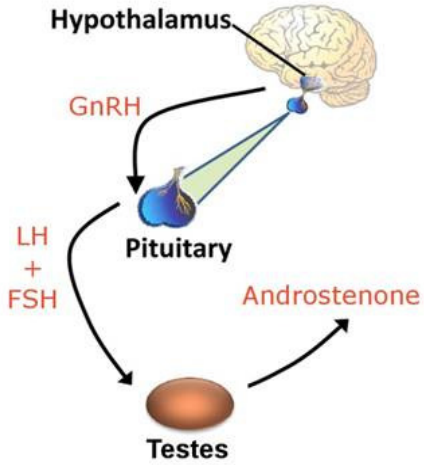

Figure 1. Schematic illustration of the production of androstenone.
Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and stimulates the anterior pituitary to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Stimulation of the testes especially by LH is responsible for the production of androstenone. Figure adapted from [9].

In Europe, Improvac® (Pfizer Animal Health) is a commercially available immunocastration vaccine. It is composed of a GnRH analogue bound to the surface of diphtheria toxin, and combined with a particulate adjuvant, Diethylaminoethyl (DEAE)-Dextran. This vaccine is administered in a prime-boost schedule, at least 4 weeks apart, with the final dosis administered 4-6 weeks before slaughter. Special equipment, the Improvac® vaccinator gun, is required to prevent self-injection, thereby injecting the vaccine in the skin of the pigs.

Tissue reactions due to injection of the vaccine have been observed in 15 to 20% of injection sites [5]. The efficiency of the vaccine in reducing boar taint is high, although in a field study including 319 vaccinated boars, 2,5% and 2% of the vaccinated pigs still had values above the threshold for androstenone and skatole, respectively [4]. Clearly, some room for improvement of the vaccine is left.

The biggest hurdle to overcome in the development of an immunocastration vaccine is the low immunogenicity of GnRH. Being a self antigen, strong immunostimulatory compounds are needed to trigger the development of a GnRH-specific antibody response. An alternative to this approach might be to target the peptide directly to APCs. Indeed, targeting antigen to Clec9A on DCs has been shown to induce antigen-specific antibody responses without the need to add adjuvant to the vaccine formulation [10-12]. One proposed explanation for this phenomenon is the restricted expression pattern of Clec9A [10]. This restricted expression pattern prevents removal of antibody-antigen complexes by irrelevant cells, which leads to a sustained antigen presentation. This sustained antigen presentation then promotes the generation of follicular helper T cells which drive B cell germinal centre development, while ensuring antigen is available to B cells as well as the antigen processing DCs. Targeting antigens to Sn-expressing macrophages might in a similar way represent a way of inducing immunity without the need to add strong adjuvant to the vaccine formulation. Previously, we have shown that antibody responses towards the model antigen HSA can be improved by using a pSn-specific mAb to target HSA towards pSn-expressing macrophages [13]. To circumvent the drawbacks associated with the chemical linkage of antigens, we recently developed a pSn-specific recombinant antibody [14]. This antibody allows targeted delivery of peptides or proteins towards pSn-expressing macrophages and thus represents an elegant tool to evaluate if targeting GnRH towards pSn-expressing macrophages can induce a GnRH-specific antibody response in the vaccinated animals, which ultimately might lead to immunocastration.

Although non-mammalian production systems often result in high yields, mammalian systems are preferred for the production of many therapeutic proteins, especially those that require post-translational modifications such as glycosylation [15, 16]. For recombinant antibodies in particular, CHO and mouse NS0 and Sp2/0 cell lines are the most commonly used expression hosts [17, 18]. To be able to rapidly scale up productions, suspension cell cultures able to grow at high densities are preferred. In addition, chemically defined, serum-free media – with no animal-derived components – have become the industry standard. Invitrogen's Gibco CHO-S cells were the first suspension-adapted CHO cell line derivative available commercially and are accompanied by their own chemically defined, serum-free

medium. In this study, we made use of this CHO-S cell line to develop suspension CHO cell lines expressing recombinant antibody-GnRH fusion constructs.

4.2.2 MATERIALS AND METHODS

4.2.2.1 CELL CULTURE

HEK-293T were grown in DMEM medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and 1 mM sodium pyruvate. CHO-K1 cells and CHO-K1 cells stably expressing recombinant pSn [19] were cultivated in F-12 medium supplemented with 10% FBS and 1mM sodium pyruvate. A mixture of antibiotics was added to both cell cultures and cell cultures were kept in a humidified 5% CO_2 atmosphere at 37°C. Freestyle™ CHO-S cells (Life Technologies) were cultivated in FreestyleTM CHO expression medium supplemented with 8 mM L-glutamine and 50000 units/l of penicillin and 50000 µg/l of streptomycin. Cell cultures were kept in a humidified 8% CO_2 atmosphere at 37°C.

4.2.2.2 PRODUCTION AND PURIFICATION OF REC41D3 AND REC13D12

Construction of the plasmids encoding the pSn-targeting rec41D3 and its irrelevant isotype controle rec13D12 was described previously [14]. HEK-293T cells were transiently transfected with the plasmids using calcium phosphate and cultured in DMEM supplemented with 10% IgG depleted, heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and a mixture of antibiotics in a humidified 5% CO_2 atmosphere at 37°C. Culture supernatant was collected and recombinant IgG were purified from the supernatant using standard protein G sepharose chromatography following the manufacturer's instructions (GE Healthcare). Fractions of the eluate containing the purified protein were pooled and the buffer was exchanged to DPBS (Life Technologies) by dialysis. Purified protein was stored at -70°C until use.

4.2.2.3 DEVELOPMENT OF CHO-S CELLS EXPRESSING REC41D3- OR REC13D12-GNRH

To generate both rec41D3- and rec13D12-GnRH plasmids, a $(G_4S)_2$-GnRH DNA sequence was synthesized (Genscript Inc.) and introduced at the 3' end of the heavy chain sequence of the antibodies by restriction enzyme digestion and ligation. Sequencing confirmed the fusion constructs were correctly assembled. CHO-S cells were transfected according to the manufacturer's instructions (Life Technologies) using the cationic lipid-based Freestyle™

MAX transfection reagent. 24 hours later, 600µg/ml Geneticin (Life Technologies) was added to the culture medium. Standard subculturing of cells was maintained and 7 days later, cells were seeded at 300 cells/well in 96-well plates. Control cells, mock-transfected and equally exposed to 600µg/ml Geneticin, had all died 7 days post mock-transfection. When 96-well plated cells reached confluency, culture supernatant was collected for ELISA screening and cells showing recombinant antibody expression were transferred to 24-well plates and 6-well plates afterwards. When confluency in a 6-well was obtained, cells were single cell cloned in 96-well plates, while the remainder of the cells were stored in liquid nitrogen as back-up. Single cell clones were again screened by ELISA and cultured until confluency in a 6-well was obtained and cells were stored in liquid nitrogen. The 5 most promising clones of each fusion construct were kept in culture and scaled up to a 125 ml shaker flask (VWR international). Cells were not subcultured anymore to allow maximum protein accumulation in the shaker flasks. Cell viability was checked and supernatant was collected daily until all cells had died. Supernatants were checked on ELISA and western blot to determine the optimal time of harvesting supernatant. Subsequently, the best clone for rec41D3- and rec13D12-GnRH was selected for further production of the fusion constructs.

4.2.2.4 ENZYME-LINKED IMMUNO SORBENT ASSAY (ELISA)

MaxiSorp® 96-well plates (Nunc) were incubated overnight with 0.1µg of goat anti-mouse polyclonal antibodies (H+L, Sigma) in PBS. Plates were subsequently washed and blocked with blocking buffer, containing 1% bovine serum albumin (R&D systems) and 0.05% Tween-20. Ten-fold serial dilutions of supernatants in blocking buffer were subsequently incubated on the coated plates, followed by washing and incubation with peroxidase-labelled polyclonal goat-anti-mouse antibodies (Dako). Plates were washed and developed with a substrate solution (R&D systems), after which the reaction was stopped with 1M H_2SO_4 and the optical density at 450nm (OD450) was measured. All wash steps were performed with PBS with 0.05% Tween-20, and all incubation steps were carried out at room temperature for 1h. To be able to determine expression levels in the supernatants, a standard curve was set up using mouse IgG antibodies (Sigma).

4.2.2.5 PRODUCTION AND PURIFICATION OF REC41D3- AND REC13D12-GNRH

The production of fusion constructs was scaled up by using 850cm² roller bottles (Corning Incorporated) put upright on an orbital shaker, each containing 600ml of cell culture, and kept in a humidified 8% CO_2 atmosphere at 37°C. Cell viability was checked routinely and supernatant was harvested when all cells had died (2-3 weeks). Cell supernatants were

stored at 4°C after addition of 0.2mM Pefabloc® (Sigma-Aldrich) to avoid proteolytic degradation until purification was performed. Fusion proteins were purified from the supernatant using standard protein G sepharose chromatography following the manufacturer's instructions (GE Healthcare). Fractions of the eluate containing the purified protein were pooled and the buffer was exchanged to DPBS (Life Technologies) by dialysis. Purified protein was stored at -70°C until use.

4.2.2.6 IMMUNOFLUORESCENCE STAINING AND CONFOCAL LASER SCANNING MICROSCOPY

To evaluate expression of recombinant antibodies by single cell cloned CHO-S cells, cells were seeded on poly-L-lysine (Sigma) coated coverslips, fixed with 4% (w/v) paraformaldehyde (Sigma), permeabilized using 0.5% (w/v) saponin (Sigma) and stained with FITC-labelled goat-anti-mouse IgG. Cell nuclei were visualized using Hoechst 33342 (Life Technologies).

To evaluate pSn binding and endocytosis, CHO or CHO-pSn cells were seeded on poly-L-lysine (Sigma) coated coverslips and incubated with 2µg/200µl purified rec41D3-GnRH or its isotype control rec13D12-GnRH for 1h at 37°C. Cells were then fixed with 4% (w/v) paraformaldehyde (Sigma), permeabilized using 0.5% (w/v) saponin (Sigma) and stained with AF594-labelled goat-anti-mouse IgG1 in addition to serum from an Improvac® immunocastrated pig in combination with FITC-labelled goat-anti-swine IgG. Cell nuclei were visualized using Hoechst 33342 (Life Technologies).

Z-section images of samples were acquired using a Leica TCS SPE-II laser scanning spectral confocal system (Leica Microsystems GmbH) linked to a Leica DM2500 microscope (Leica Microsystems GmbH). Image acquisition was done using the Leica LAS AF confocal software package.

4.2.2.7 SDS-PAGE, COOMASSIE BLUE STAINING AND WESTERN BLOT ANALYSIS OF REC41D3- AND REC13D12-GNRH

Samples of purified proteins were mixed with (non-)reducing Laemmli buffer, boiled for 5 min and subjected to SDS-PAGE (6% non-reducing gel, 10% reducing gel) using a BioRad Mini Protean 3 system. For Coomassie Blue staining, the SDS-PAGE gel was incubated successively in Ultra Pure water, Imperial™ protein staining solution (Thermo Scientific) and Ultra Pure water as destaining solution. Alternatively, for Western blot analysis, proteins were transferred from the SDS-PAGE gel to a PVDF membrane (Membrane Hybond-P, GE Healthcare) via Western blotting (BioRad Mini Trans Blot). The membrane was blocked

overnight in PBS + 0.1% Tween 20 + 5% skimmed milk. Detection of recombinant antibodies was performed by subsequent incubation of the blot with peroxidase-labeled polyclonal goat anti-mouse antibodies (Dako), followed by visualization using enhanced chemiluminescence (ECL; GE Healthcare). Alternatively, GnRH peptides were detected using serum from an Improvac® immunocastrated pig in combination with peroxidase-labeled goat anti-swine antibodies (Jackson ImmunoResearch), followed by ECL visualization.

4.2.3 RESULTS

We previously described the development of a pSn-specific recombinant antibody rec41D3 and an irrelevant isotype control rec13D12 [14]. In the present study, we generated fusion constructs of rec41D3 and rec13D12 containing four repeats of GnRH, separated from each other by a short spacer and separated from the antibodies by a GS linker. As GnRH contains at its N-terminus an uncommon amino acid pyroglutamic acid, which cannot be encoded as such in the genetic sequence, we chose to encode glutamine, of which pyroglutamic acid is a derivate. As previously shown by Turkstra et al., this N-terminal pyroglutamic acid can be replaced by alanine without negatively affecting immunocastration efficacy [20]. The amino acid sequence following the antibodies' heavy chains is shown in Figure 2A.

Suspension CHO cells were transfected with the rec41D3- or rec13D12-GnRH plasmid and exposed to Geneticin (G418) selection pressure 24 hours later. Transfected cells were subcloned twice as described in the methods section. Single cell clones were screened for high producers by ELISA and finally the best clone for both rec41D3- and rec13D12-GnRH was selected for further production of the fusion constructs. As shown in Figure 2B, both clones clearly showed expression of the recombinant antibody. To scale up productions, CHO-S cells were cultivated in roller bottle flasks put upright on an orbital shaker in a humidified 8% CO_2 atmosphere at 37°C. Obtained yields of the fusion proteins were 500µg/l and 2mg/l for rec41D3- and rec13D12-GnRH respectively.

Protein G purification of cell culture supernatants clearly yielded intact fusion proteins as shown by SDS-PAGE (Fig. 2C). Under non-reducing conditions, a shift in size compared to unmodified rec41D3 and rec13D12 was visible for both fusion proteins. Under reducing conditions, the antibody light chains remained unchanged, while the heavy chains shifted in size, showing the acquisition of extra protein sequences. The presence of the GnRH peptide at the heavy chains of rec41D3 and rec13D12 was further confirmed by Western blot analysis using mAb-specific antibodies and serum from an Improvac® immunocastrated pig (Fig. 2D).

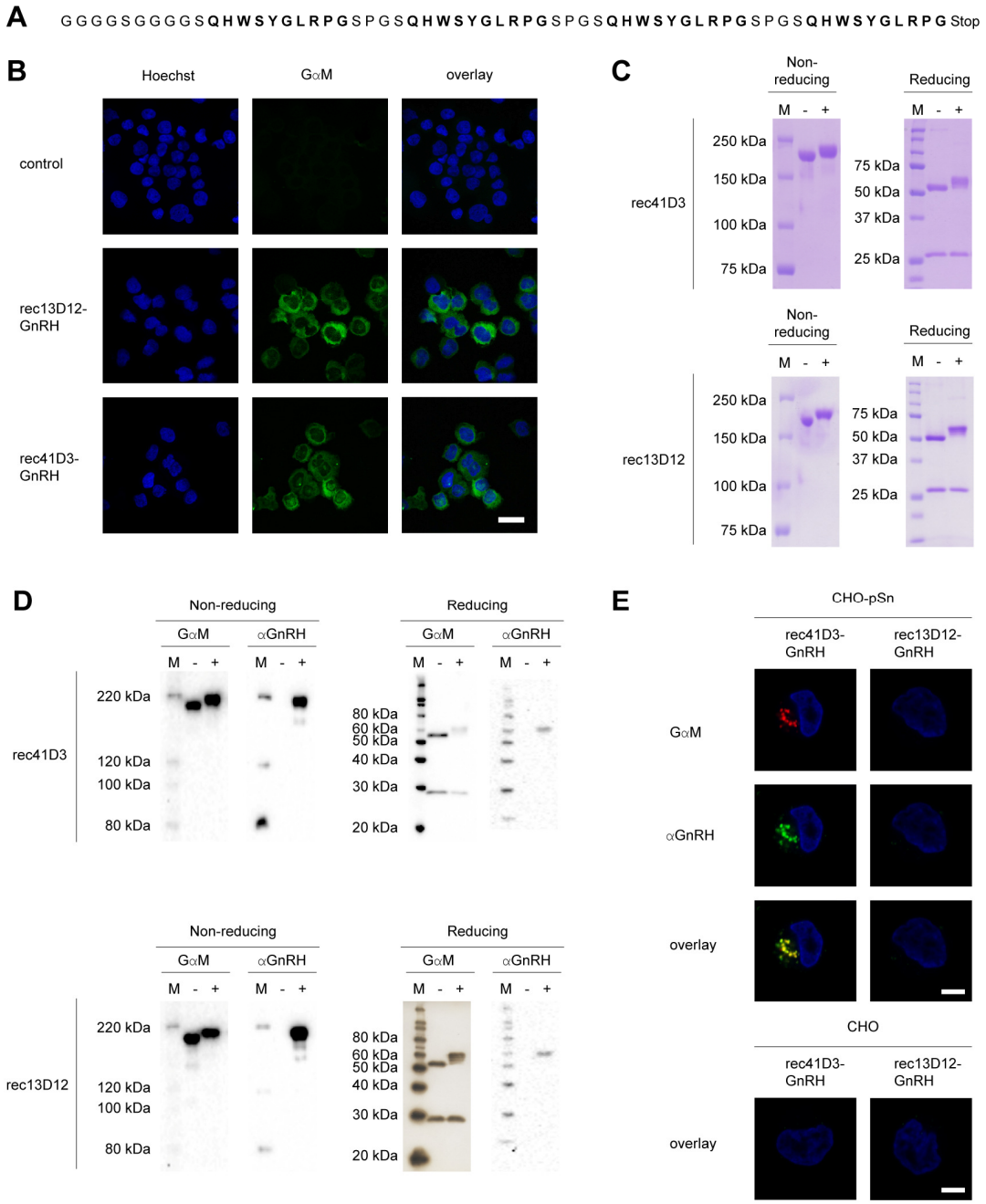

Figure 2. Development of fusion constructs rec41D3-GnRH and rec13D12-GnRH and analysis of their internalization into pSn-expressing cells.
(A) Primary amino acid sequence following the C-terminus of the antibodies' heavy chains. GnRH peptides are indicated in bold. (B) Confocal microscopical analysis of fusion construct expression by single cell cloned CHO-S cells. Cells were plated on poly-L-lysine coated coverslips, fixed, permeabilized and stained with FITC-labelled goat-anti-mouse IgG. Scale bar: 30µm. (C) SDS-PAGE analysis of protein G purified rec41D3-GnRH and rec13D12-GnRH constructs in comparison to unmodified rec41D3 and rec13D12 respectively (D) Western blot analysis of rec41D3-GnRH and rec13D12-GnRH samples in comparison to unmodified rec41D3 and rec13D12 respectively using mouse immunoglobulin-specific goat polyclonal antibodies (GαM) or serum from an Improvac® immunocastrated pig (αGnRH). (E) Confocal microscopical analysis of rec41D3-GnRH internalization in CHO-pSn cells in comparison to its irrelevant control rec13D12-GnRH and in comparison to staining of CHO cells. Cells were incubated with rec41D3-GnRH or rec13D12-GnRH for 1 hour at 37°C, fixed, permeabilized and stained with AF594-labelled goat-anti-mouse IgG1 in addition to serum from an Improvac® immunocastrated pig in combination with FITC-labelled goat-anti-swine IgG. Images represent a single confocal z-section through the middle of the cell. Scale bar: 5µm. M, protein marker; -, antibody without GnRH; +, antibody with GnRH.

To further analyze whether rec41D3-GnRH is able to bind to pSn-expressing cells and is internalized afterwards, CHO cells expressing pSn (CHO-pSn) in comparison to regular CHO cells were incubated with the fusion protein for one hour at 37°C. As shown in Figure 2E, GnRH was co-internalized together with rec41D3 only in CHO-pSn cells. As a control, CHO and CHO-pSn cells were incubated with irrelevant, isotype matched rec13D12-GnRH fusion proteins. No binding and internalization was observed with this construct. Together, these data show that rec41D3-GnRH and rec13D12-GnRH proteins both contain the GnRH sequences at the C-terminus of the heavy chain and can be used in future vaccination experiments.

4.2.4 DISCUSSION

In the present study, Freestyle™ CHO-S cells were used to generate suspension CHO cell lines expressing antibody-GnRH fusion constructs. Both the pSn-targeting construct rec41D3-GnRH and its irrelevant isotype control rec13D12-GnRH were shown to be synthesized as fully assembled fusion proteins by the selected clones and could be purified from cell supernatants using standard protein G chromatography. In addition, rec41D3-GnRH, but not rec13D12-GnRH, binds to and efficiently delivers its cargo to pSn-expressing cells, generating an elegant tool to evaluate pSn-directed GnRH targeting as an immunocastration strategy in future animal experiments.

The production of a stable cell line starting from Freestyle™ CHO-S cells presents itself with a number of advantages. Firstly, CHO cells have been extensively used over the past decades for the production of clinical biopharmaceuticals, what makes it likely that regulatory approval will be more rapid for a novel CHO-produced biopharmaceutical than for one produced in a less well-known cell line [21]. Secondly, its chemically defined serum-free medium avoids regulatory issues concerning the use of animal-derived components in expression media. In addition, the CHO cell line has been adapted for suspension culture, which makes upscaling to larger volumes in for instance fed batch systems further down the line much easier. However, these advantages only hold true if a high-producing cell clone can be selected. Unfortunately, the cell clones generated in our study by Geneticin selection and standard limiting dilution procedures only yield 0.5mg/l for rec41D3-GnRH and 2mg/l for rec13D12-GnRH, which is not sufficient in an industrial setting, as cell lines are nowadays expected to produce at least 1g/l [22]. This lack of success is however perhaps not surprising, as spotting a high-producer clone in a transfected pool of cells has been described as “finding a needle in a haystack” [22]. To generate a stable cell line, exogenous DNA containing the gene of interest has to be integrated into a transcriptionally active site of

the host genome. As the integration is a random event, frequencies of integration at such active sites are very low [23]. Furthermore, many integrations take place at chromosomal sites susceptible to gene silencing [24]. With a limited number of clones screened, there is thus always a great possibility of missing the best candidate. Although a number of optimizations to the procedure used in this study can be considered, such as vector optimization, site-specific integration, the use of the DHFR or GS system instead of antibiotic selection or just the screening of enormous amounts of single cell clones, the procedure will always remain a time-consuming and resource-intensive one. For future product developments, it might therefore be more interesting to perform transient transfections of the CHO cells instead. Transient transfection has also gained increased industrial attention lately, as milligram to gram quantities of proteins are often required in the early development stages of biopharmaceuticals and precious time is lost when waiting for the generation of a stable cell line [25]. Although CHO cells for a long time have not been the host of choice for transient gene expression (TGE), because of the higher yields obtainable with low-cost transfection reagents in HEK293 cells, major effort has been invested on improving TGE in CHO cells, and productivities ranging from 10mg/l to >100mg/l can now be achieved [22]. Of interest however, transient transfection of the CHO-S cells used in this study with our pSn-targeting antibody-GnRH fusion construct using the advised Freestyle™ MAX transfection reagent also only yielded 0.5-1mg/l of secreted antibody (data not shown). As this transfection reagent is expensive, production of sufficient amounts to perform animal experiments would represent an enormous cost. The relatively low-cost polyethylenimine (PEI) reagent has recently been successfully used to perform transient transfections of CHO cells with high productivity, and might thus represent a better alternative for future projects [26].

In conclusion, CHO cell lines were developed that express a pSn-targeting antibody-GnRH fusion construct or its irrelevant isotype control. The obtained production levels of the developed CHO cell lines is however not very high. Nevertheless, antibody-fusion proteins can be easily purified from the selected clone supernatants and a high purity is obtained. In addition, only purified pSn-targeting antibody-GnRH fusion proteins, and not their irrelevant controls, bind to and are internalized into pSn-expressing cells. These proteins can therefore be used to evaluate pSn-directed GnRH targeting as an immunocastration strategy in future animal experiments.

4.2.5 ACKNOWLEDGEMENTS

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Chapter

5

General discussion

In current-day vaccinology it has become clear that some pathogens require a more rational vaccine design, as traditional inactivation or attenuation procedures sometimes fail to yield an effective and/or safe vaccine. As such, a wide range of vaccines under development now are based on isolated peptides, proteins or polysaccharides of pathogens, or naked DNA encoding a protective antigen [1]. These reductionist approaches can result in vaccines that are more immunologically defined and display a better safety profile than vaccines based on traditional approaches. One hurdle to overcome however, is the limited immunogenicity of the isolated antigens used. As such, targeting antigens directly to highly specialized antigen-presenting cells is gaining increased attention. In this thesis, targeting antigens to macrophages via the receptor sialoadhesin (Sn; CD169; Siglec-1) was studied. This APC subset is abundantly present in spleen and lymph nodes, where they are strategically localized for antigen acquisition and processing [2]. In this part of the dissertation, the main results obtained in this thesis will be summarized and discussed in a broader context, with a main focus on future vaccine development.

DEVELOPMENT OF A RECOMBINANT ANTIBODY TO TARGET PEPTIDES AND PROTEINS TO SN^+ MACROPHAGES

In Chapter 3 of this dissertation, a recombinant antibody rec41D3 allowing targeted delivery of peptides and proteins to Sn-expressing (Sn^+) macrophages was developed and compared to the native monoclonal antibody 41D3. Rec41D3 was shown to specifically bind to porcine Sn with a comparable, high affinity as native 41D3. In addition, rec41D3 induced partial Sn endocytosis in primary macrophages and internalized antibody resided for prolonged times in early/late endosomes, just like native 41D3. Furthermore, genetic fusion constructs of the antibody with a peptide or a protein could be easily developed and the fusion proteins were produced by HEK293T cells and purified from the supernatant by standard protein G chromatography. Lastly, the peptide and protein fused to rec41D3 were shown to be co-internalized together with rec41D3 into primary macrophages. For vaccine development based on targeting antigens to Sn^+ macrophages, some important findings are described in the above sentences. First of all, a high specificity and affinity of the targeting molecule enhances the probability of an efficient delivery of the antigen to the APC to be targeted. In addition, the one-step production process of the antibody-antigen fusion construct can be considered a major advantage for practical reasons, as the antigen does not have to be produced and purified on its own, followed by chemical linkage to the antibody, but can benefit from the easy purification process based on the antibody's binding capacity for protein G. Of course, proper folding of the antigen upon genetic fusion will still have to be ensured for each antigen.

Upon engagement of rec41D3 with Sn, partial endocytosis is observed in primary macrophages, while a substantial amount of rec41D3 remains visible at the cell surface. For the generation of humoral immune responses, this partial endocytosis might be of benefit. As it is necessary that antigen-specific B cells encounter their cognate antigen in order to expand and develop into antibody-forming cells [3, 4], rapid endocytosis of all antigen by the APCs might prevent this interaction from happening. Whether the observed partial endocytosis is due to the necessity of a high avidity before endocytosis can proceed or due to recycling of Sn to the macrophage surface with non-processed or partially processed antibody, or both, is unclear at this stage, but in any case, antigen would be available at the macrophage surface to be encountered by antigen-specific B cells. As Sn^+ macrophages are abundantly present in close vicinity to B cells in secondary lymphoid tissue, this concentration of antigen by Sn^+ macrophages in these tissues might thus be an important asset. Sn^+ subcapsular sinus (SCS) macrophages, which are localized at the SCS of lymph nodes, are known to be important for the capture of specific antigens and the subsequent display of these antigens to the underlying B cell follicles, as shown in Figure 1 [5-7].

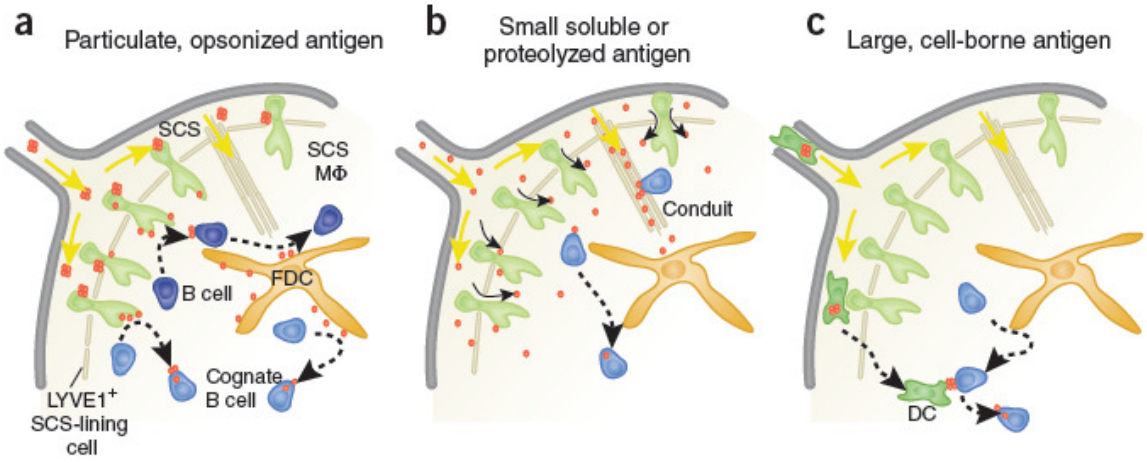

Figure 1. Multiple modes for the encounter of lymph node follicular B cells with antigen.
(**a**) Particulate antigens (such as immune complexes and viral particles) are captured and displayed by SCS macrophages and are then encountered directly by cognate B cells or are transported by noncognate B cells to follicular dendritic cells (FDCs) for later encounter with cognate B cells. (**b**) Soluble antigens of small hydrodynamic radius (in some cases released from large antigens by proteolysis) access the follicle via gaps in the SCS lining or via conduits and are then encountered by cognate B cells. (**c**) Antigens too large to enter passively into lymphatic vessels are carried into the follicle-proximal cortex inside DCs and are then recycled to the DC cell surface to enable encounter by cognate B cells. Figure adapted from Jason G Cyster [7].

As seen from Figure 1a, SCS macrophages capture antigens by their 'heads' protruding into the sinus, and transport it via their 'neck' tightly inserted across the lymphatic lining cell layer towards their long 'tail' processes that extend into the underlying follicle. The precise nature of this antigen movement however, has not been resolved up till now. Two models are proposed where antigen is either transported by translocation or transcytosis, as shown in

Figure 2. Either of these two models proposed probably also shows how rec41D3 travels upon engagement with Sn. If transcytosis occurs, this might be due to recycling of Sn itself or can be initiated by the neonatal Fc receptor (FcRn), which is known to bind internalized antibodies in non-degradative endosomes to transport it back to the cell surface [8].

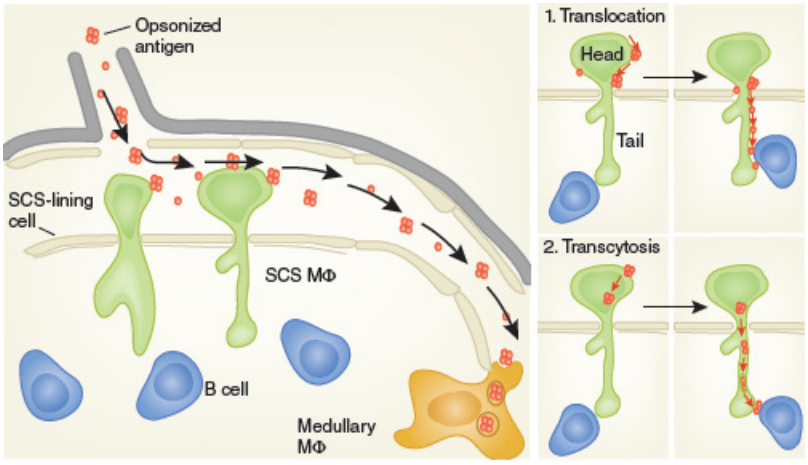

Figure 2. Models for the transport of antigen from the sinus to the follicular surface of the subcapsular sinus (SCS) macrophage.
Particles in the lymph percolate through the SCS and some are captured by unknown receptors on the SCS macrophage, whereas excess material reaches medullary sinuses and is phagocytosed and degraded by medullary macrophages. In model 1 (top right), particulate antigen is retained on the SCS macrophage surface, undergoes fragmentation and moves unidirectionally on the cell surface by unknown mechanisms from the SCS to the follicle, where it is displayed to migrating B cells. In model 2 (bottom right), the particulate antigen is endocytosed into vesicles, transported directionally within the cell and then returned to the cell surface for display. Over a period of hours, antigen that is not removed by migrating B cells is probably degraded by the SCS macrophage. Figure adapted from Jason G Cyster [7].

Once the antigen enters the follicle, it is displayed to migrating B cells. Another question remaining however is whether a very high affinity of the targeting antibody for Sn is the best choice in this respect. B cells need to be able to process the antigen to allow presentation via their MHC molecules in order to obtain the necessary T cell help for the induction of antibody production. If the antigen is too strongly attached to the macrophage, this process might not be able to proceed efficiently. The question if B cells can acquire antigen that is targeted to Sn^+ macrophages by rec41D3 will be an important question to resolve. Similarly, in DC targeting studies, one is still trying to resolve the question how B cells acquire their antigen [3]. Is it antigen not bound to DCs, attached to the DC before endocytosis or taken up by DCs that is rerouted to the DC surface [3]? APC targeting strategies for vaccination would thus surely benefit from finding an answer to these questions.

Interestingly, antibodies endocytosed by Sn on primary macrophages reside for prolonged times in early endosomes, a cell compartment where loading of antigen onto MHCI molecules can occur, a process described as cross-presentation [9]. A recent study by Chatterjee *et al.* targeted identical antigens to either CD40, the mannose receptor or DEC-

205 by the use of respective mAbs and they found an inverse relationship between internalization or antigen degradation and the amount of cross-presentation [10]. Antigens destined for more degradative late endosomes were poorly cross-presented relative to the same antigens targeted to early endosomes, an effect that was independent of the amount of antigen internalized. This observation is also in agreement with reports describing an enhanced $CD8^+$ T cell proliferation after antigen targeting towards Sn by the use of Sn-specific mAbs [11, 12]. This can be of particular interest for vaccination strategies aiming at elimination of antigen-bearing cells, e.g. cells that harbour intracellular pathogens or tumour cells that express tumour-specific antigens.

Although early endosomes are the major compartment where antibodies reside after Sn internalization, around 30% of internalized antibodies can be detected in late endosomes, suggesting acidification of the early endosomes and progression through the endo-lysosomal pathway. Here, antigen loading on MHCII molecules can occur which can stimulate the activation of $CD4^+$ T cells. Interestingly, liposomes coated by Sn-specific sugar ligands are rapidly endocytosed into Sn-expressing cells and predominantly accumulate in lysosomes [13]. This might suggest a differential processing of antigen targeted to Sn depending on the delivery method of the antigen and/or the size of the particles presented. As an explanation, the authors claim that Sn is a recycling receptor and that antibodies bound to Sn are less likely to detach from Sn in early endosomes than sugar-coated liposomes. While antibodies bound to Sn might thus recycle together with Sn to the cell surface, sugar-coated liposomes would detach from Sn in the early endosomes and progress towards lysosomal compartments. Further research will have to confirm this. Especially, experiments with sugar-coated liposomes on primary macrophages could be informative, as previous studies made use of mouse Sn transduced CHO cells. Nevertheless, the theory of Sn recycling to the cell surface with antibody still bound to it might be an important feature to keep in mind for the development of antibody-antigen fusion constructs. While possibly beneficial for the interaction with B cells and the induction of $CD8^+$ T cell immunity, the induction of $CD4^+$ T cell immunity can be impaired. If detachment of the antigen from Sn in early endosomes is needed to induce robust $CD4^+$ T cell responses, adding a cleavable linker between the targeting antibody and the antigen might be interesting to pursue. Cleavable linkers are intensively studied for tumour targeting purposes where toxins are linked to tumour targeting antibodies, as in these studies the toxin should not detach from the antibody before it is internalized into the tumour cells to reduce off-target effects of the toxin [14]. Results obtained from these studies could potentially be applied to rec41D3-antigen conjugates as well to achieve separation of the antigen from the antibody once internalized into Sn^+ macrophages.

EVALUATION OF VIRAL PEPTIDE TARGETING TO pSN USING A PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS VACCINATION-CHALLENGE MODEL

In Chapter 4.1 of this dissertation, we evaluated the induced immune response upon targeted delivery of a viral peptide to porcine Sn^+ macrophages. The viral peptide was a linear B cell epitope of glycoprotein 4 (GP4) of PRRSV, which is known to be a target of virus-neutralizing antibodies. Four copies of the selected PRRSV epitope were genetically fused to the Sn-targeting recombinant antibody rec41D3 or to an irrelevant isotype control rec13D12. Fusion proteins were shown to be efficiently purified from HEK293T cell supernatants and subsequently, only Sn-specific fusion proteins were shown to bind to and to be internalized into Sn-expressing cells. Immunizations of pigs with the resulting fusion proteins were carried out after which a viral challenge was performed.

Upon vaccination, no peptide-specific IgG responses could be detected. This observation was rather unexpected, as we previously showed that a single, non-adjuvanted injection of HSA linked to an antibody generated a HSA-specific antibody response. Clearly, peptide immunizations are more challenging than protein immunizations, and a number of reasons for the apparent lack of response in our experiments can be thought of. Firstly, appropriate $CD4^+$ T cell epitopes, which are essential for a good immune response, might be lacking from the targeting recombinant antibody, thereby impeding the induction of robust T cell help. The incorporation of a PADRE epitope engineered to bind most SLA molecules with high affinity is an established method to resolve such shortcomings. Secondly, progression through the endo/lysosomal pathway of the fusion protein might be insufficient to yield substantial MHCII presentation of $CD4^+$ T cell epitopes. Also, a longer serum half life of the HSA construct might contribute to the discrepancy with the results obtained with the HSA study. Albumin has been shown to engage with FcRn yielding recycling to the extracellular milieu after initial internalization [8]. The induction of antibody responses upon antigen targeting towards Clec9A on DCs in the absence of adjuvant is also presumed to be due to the long serum half life of the targeting construct [15]. Sustained antigen presentation is believed to encourage development of follicular helper T cells which drive the B cell germinal centre response [3, 15]. A longer serum half life of the HSA targeting construct might thus also contribute to the better antibody response upon primo vaccination. The FcRn however also engages with the Fc parts of antibodies, so it remains to be established whether the HSA construct indeed displays a longer serum half life than the fusion proteins used in this study. Another point to take into consideration is that the conformation of the antibody-antigen construct *in vivo* might hamper engagement of the antigen with B cell receptors, although we did show *in vitro* that the antigen is accessible for epitope-specific monoclonal antibodies. Another possible reason for the lack of peptide specific antibody responses is that no adjuvant was incorporated into the vaccine formulation, which can result in insufficient co-stimulatory

molecules present on the macrophage surface to strongly prime naive $CD4^+$ T cells. On the other hand, if all co-stimulation would be absent, immunology textbooks teach that tolerance to recall antigen should be induced instead of a more profound immune response upon booster vaccination (in our case a viral challenge) [4]. Taken together, it is clear that some priming of the immune response had occurred. A booster vaccination and/or the addition of adjuvant to the vaccine formulation can help pave the way towards the induction of effective immune responses before viral challenge. After all, neutralizing antibody responses were observed soon after viral challenge, and a minor beneficial effect on viral load was established as well.

Interestingly though, the advantage of targeting a PRRSV peptide antigen with rec41D3 to Sn^+ macrophages in comparison to the linkage of the antigen to an irrelevant isotype control antibody was rather modest and largely not statistically significant. Three plausible causes for this observation are evident: 1/ the targeting antibody did not reach its receptor efficiently, 2/ the targeting antibody does not allow efficient transfer of the antigen towards B cells or 3/ the irrelevant isotype control antibody is equally taken up and presented by Sn^+ macrophages. Immunizations were performed intramuscularly. Depending on the size of the injected material, efficient drainage into local lymphatics is established. By whole-body imaging, subcutaneously injected fluorescent microspheres 20-200 nm in diameter have been found to readily gain access to lymphatics, whereas particles 500-2000 nm in diameter are inefficient in reaching draining lymph nodes unless carried by cells [7, 16]. Antibodies, having dimensions smaller than 20 nm [17], should thus theoretically efficiently drain into lymphatics. However, we did not check the drainage *in vivo* so we cannot exclude the possibility of retention of the antibody-antigen fusions at the site of injection. If so, fusion constructs would be preferentially taken up by local dendritic cells and transported to the draining lymph nodes, and consequently, the immune outcomes of both fusion constructs would be comparable. Antigen display by dendritic cells in the absence of DC maturation however most often results in the induction of tolerance to recall antigen [18, 19], adding up to the unlikelihood of this theory. As mentioned before, the high affinity of rec41D3 (pM range, Chapter 3) for Sn, might compromise the transfer of antigen from the Sn^+ macrophages towards B cells. In this respect, it might be interesting to perform an experiment where antibodies with different affinities for Sn are used to target an antigen towards Sn^+ macrophages. Many effective technologies have been developed to rapidly affinity mature antibody molecules [20]. Many are based on display technologies, such as phage and yeast display, and involve the creation of a 'secondary' library through diversification of the V genes, and subsequent selection and screening for higher affinity variants [20, 21]. In our case, such technologies could be employed to create antibodies with

a range of lower affinities for Sn than rec41D3. Of course, lowering the affinity can also compromise efficient targeting, possibly reducing the benefit of a better transfer towards B cells. Lastly, immune-complexes have been shown before to be captured by Sn^+ SCS macrophages and to be subsequently delivered to B cells in the underlying follicles [22]. Capture of the immune-complexes was either due to engagement with Fcγ receptors on the macrophages, engagement with complement receptor CR3 (also called Mac1 or CD11b-CD18) or other unknown receptors. Obviously, both fusion constructs used in our study can be captured by SCS macrophages in a similar way, reducing the benefit of targeting directly to Sn.

Another point to mention, is that the targeting study conducted was never intended to yield an effective PRRSV vaccine. Rather, the vaccination set-up was intended to yield a proof-of-concept for future vaccine developments based on targeting antigens towards Sn^+ macrophages. PRRSV has been extensively studied in our laboratory over the past few years and animal studies with PRRSV have been routinely performed. In addition, a linear epitope was identified on GP4 of PRRSV that is a target for virus-neutralizing antibodies [23, 24]. This epitope thus seemed an ideal candidate to test our targeting vaccine potential in a vaccination-challenge set-up. For the development of an effective PRRSV vaccine however, this epitope has some drawbacks. This epitope has been shown to be part of a highly variable and evolutionary flexible region [25, 26], and as a result, antibodies against this peptide would only show neutralizing activity against homologous virus. Furthermore, the GP4 epitope has been shown to be highly susceptible to antibody-mediated selective pressure *in vitro* [23], and the emergence of antibody-escape mutant viruses has later been shown *in vivo* as well [27]. To analyze if such phenomenon had occurred in our experiment, synthesized ORF4 cDNA of serum samples of day 31, 33, 38 and 49 for the 500µg rec41D3-GP4 group and of day 49 for all other treatment groups were sequenced. All ORF4 sequences obtained were identical to the original challenge virus, except for one animal in the 500µg pSn-targeting group (□, Figure 3, Chapter 4.1) at day 49, which displayed a mutation from serine to leucine in our targeted epitope (FRPHGVLAAQEKISFG), most likely affecting the capacity of antibodies generated against the original sequence to neutralize this mutant virus. The induction of peptide-specific antibodies in this animal was high, and this might thus represent an antibody-escape mutant virus. Nevertheless, at day 56 no virus could be detected in serum samples anymore, showing that at that time other aspects of PRRSV specific immunity are capable of blocking virus replication. This observation does show however that due to the high flexibility of PRRSV, an RNA virus prone to mutations, efficient vaccines should probably focus on inducing antibodies to multiple sites. In addition, when focussing on only one epitope, antibody titres might have to be exceptionally high to be

able to cover enough GP4 molecules on each virion to inhibit infection. This reasoning might explain why antibody titres obtained in our experiments were still insufficient to efficiently inhibit viral infection of primary macrophages. Even when focussing on multiple B cell epitopes however, the question will remain if humoral immunity alone is sufficient to tackle PRRSV and if the induction of additional arms of the immune system will be necessary to provide sufficient immune pressure on the virus.

DEVELOPMENT OF A SUSPENSION CHO CELL LINE EXPRESSING A CANDIDATE IMMUNOCASTRATION VACCINE

In Chapter 4.2 of this dissertation, suspension CHO cell lines were developed expressing fusion constructs of the pSn-targeting recombinant antibody rec41D3 or its irrelevant isotype control rec13D12 with four repeats of a GnRH peptide. Fusion proteins could be easily purified from the selected clone supernatants and high purities were obtained. Furthermore, rec41D3-GnRH was shown to bind to and to be internalized into pSn-expressing cells, while no staining (or weak background staining) was observed with isotype control rec13D12-GnRH. Although production levels of the fusion proteins were low (0.5mg/l for rec41D3-GnRH and 2mg/l for rec13D12-GnRH) compared to yields obtained in industry settings, the fusion proteins obtained could be used in future animal experiments evaluating pSn-directed GnRH targeting as an immunocastration strategy.

Considering the results obtained in our previous study targeting a B cell epitope of PRRSV towards Sn^+ macrophages, a single non-adjuvanted injection of rec41D3-GnRH will probably not yield a significant anti-GnRH antibody response. After all, GnRH is a self-peptide and thus we are basically facing the challenge of inducing an autoimmune response. A prime-boost schedule of the fusion proteins would be more interesting to evaluate, and adjuvant can be co-administered to evaluate the addition of maturation stimuli. Ideally, co-administered adjuvant should direct the immune system towards the induction of strong humoral immune responses, making Alum a valuable candidate [28]. In addition, adjuvant can be physically linked to the targeting antibody, reducing potential harmful effects of systemic adjuvant administration. In the field, administration of the commercially available Improvac® vaccine occasionally results in death of the animal within minutes due to the induction of an anaphylactic shock. A reduction of the overall immunogenicity of such vaccine without compromising efficacy would thus clearly be beneficial.

GnRH is a hormone that is equally present in human beings, highlighting the risks associated with potential self-injections when administering the commercially available Improvac® vaccine. As the antigen dose is lowered considerably in our targeting construct and rec41D3 does not bind to human Sn, the risks associated with self-injections of our vaccine would be

reduced due to the lack of targeting in humans. However, as seen in Chapter 4.1, rec13D12 can induce a priming of the immune response as well, thus potentially inducing a priming of the immune response in humans as well. As the enhancement of the immune response by coupling the antigen to rec13D12 can however be Fcγ receptor mediated, Fab fragments or scFv of rec41D3 and rec13D12 could be engineered starting from our rec41D3 and rec13D12 plasmids to eliminate Fcγ receptor involvement. GnRH can be fused to these fragments and resulting fusion constructs can be used to evaluate Sn-directed targeting of GnRH for immunocastration. In addition, bispecific Fab fragments or scFv could be engineered to include an anti-CD40 moiety for the delivery of costimulation. Furthermore, nanoparticles (NP) could be coated with the Fab fragments or scFv to target the NP towards Sn^+ macrophages. GnRH can be coated onto the NP as well or encapsulated inside the NP or both. Most importantly, NP can carry adjuvant inside, reducing potentially harmful systemic effects. In this way, a species-specific immunocastration vaccine could be developed with both a better safety profile for the animals receiving it as well as for the individual administering it.

FUTURE PERSPECTIVES

To finalize this general discussion, some topics are highlighted which can be of importance for future developments in Sn-targeting research.

Porcine lymph nodes

Porcine lymph nodes have a structure that is called inverted, as discussed in the section 'Sn expression and regulation' of Chapter 1 of this dissertation. Afferent lymphatics enter the lymph node centrally at the capsule at one or more sites and penetrate via the trabeculae deep into B cell and T cell areas [29]. Occasionally, branches may arise from the afferent lymphatics outside the lymph node and join with a SCS where B cell follicles are located peripherally [30]. The lymph penetrates into the diffuse tissue and then filters into the peripheral sinuses, which converge and form several efferent vessels at the periphery of the node [29]. Sn^+ macrophages have been identified at the subcapsular and medullary sinuses in pigs [31] and thus appear, just like in the other species studied, readily positioned to sample incoming lymph for antigens. An important remark however is that in rodents, it has been shown that medullary Sn^+ macrophages fit the classical macrophage definition of being strongly phagocytic cells that rapidly degrade internalized material [7, 32], while SCS Sn^+ macrophages have a low rate of antigen internalization and degradation [7, 32]. SCS macrophages also have a lower rate of depletion by clodronate-containing liposomes that kill cells after internalization than do medullary macrophages, which is also consistent with their different rates of phagocytosis [33]. In rodents, it has also been shown that it are these SCS

macrophages that are responsible for the capture of antigens like virus-sized beads, opsonized phycoerythrin, vesicular stomatitis virus, vaccinia virus and adenovirus particles within minutes of their subcutaneous injection and for each of these antigen types, the cells make the captured material accessible to B cells that migrate over their follicular tail processes (Figure 1a) [5, 7, 22, 34]. It remains to be established in pigs whether medullary and SCS macrophages present the same characteristics as they do in rodents. If so, the question is whether the inverted lymph flow in pigs permits the acquisition of equal amounts of antigen by SCS macrophages as in rodents. On the other hand, our targeting strategy targets antigen towards Sn, present on both SCS and medullary macrophages, and internalization via Sn that we have observed so far is always partial with a slow rate of progression down the endo/lysosomal pathway. In this way, antigen should be available for the surrounding B cells in the medulla of porcine lymph nodes. However, it remains to be established whether Sn internalization is identical in porcine medullary macrophages. Taken together, it would be interesting to study in pigs how our targeting antibody carrying an antigen travels and whether or not antigen is readily made available to B cells. Of course, the feasability of such an experiment is not in the range of a comparable experiment in mice at all. Isolation of porcine medullary and SCS macrophages for further experiments *in vitro* can yield valuable information as well, although significant optimization will be necessary to specifically sort these macrophage subsets.

Antigen handling by Sn^+ macrophages

Although it has been established that (one of) the function(s) of Sn^+ macrophages in spleen and lymph nodes is to sample incoming antigens, the mechanisms by which these macrophages proceed in their antigen handling are not completely understood. Yet, there is a consensus that SCS macrophages and MMM in the spleen function to concentrate antigen. As discussed before, SCS macrophages have been shown to capture diverse types of antigen and to display them to underlying B cells. Cognate B cells can acquire antigen directly from SCS macrophages via their B cell receptor or non-cognate B cells can carry antigen from the macrophages to follicular DCs, where cognate B cells can sample them to acquire their cognate antigen [7]. Although it is tempting to speculate that MMM in the spleen function in a similar way to capture blood-borne antigens and transport them to B cells, this has not been visualized up till now [35]. What has been shown though, is that MMM provide a confined area of viral replication necessary to allow the accumulation of sufficient antigen for the efficient induction of adaptive immunity [36]. Here, it is tempting to speculate that SCS macrophages can function in a similar way and SCS macrophages have indeed been shown to be permissive to viral infection [37, 38]. In the lymph nodes, medullary macrophages also express Sn and these macrophages share some markers and the property of being highly

phagocytic with marginal zone macrophages of the spleen, which however only show low expression levels of Sn [39]. It is envisioned that these two macrophage subsets relate to each other as do SCS macrophages and MMM. Here, no antigen concentration is observed, but instead captured antigen is rapidly degraded and their destruction of pathogens draining from sites of infection should help to prevent systemic spread [40]. Upon pathogen infection, these macrophages can also secrete inflammatory cytokines to ensure further action of the innate immune response [35]. Taken together, marginal zone macrophages and their medullary counterparts appear to resemble the phenotype of a classical macrophage supporting the innate immune response, while SCS macrophages and their MMM counterparts also have a prominent role in the induction of adaptive immunity.

Although the importance of SCS macrophages and MMM for the induction of adaptive immunity appears to be undisputed, the question whether they are solely required for capture and transfer of antigen or whether they play a direct role in the activation of naive T lymphocytes clearly is still a matter of debate, and more research is necessary to clarify this issue. The ability of SCS macrophages and MMM to present antigen in the context of MHC class I is not questioned, as SCS macrophages can be killed by $CD8^+$ T cells during *Toxoplasma* infection [41], while MMM can be killed by $CD8^+$ T cells during *Plasmodium chabaudi* infection [42]. However, it is questioned whether these macrophages express sufficient amounts of co-stimulatory molecules to support naive T cell priming [39], and whether these macrophages only contribute to naive T cell priming by their transport of antigen towards DCs. Studies with subcutaneous vaccinia virus infections in mice showed that 85% of the infected lymph node cells were Sn^+ macrophages, with the majority of these being the SCS macrophages [43]. Antigen-specific $CD8^+$ T cells accumulated in proximity to these macrophages in interfollicular regions, but the authors claim that most of the $CD8^+$ T cells contacts were with DCs, and priming was more successful via DCs [37, 43]. However, CD11c-DTR mice were used to study the antigen-presenting contribution of DCs versus macrophages and many lymph node macrophages express sufficient amounts of CD11c to be ablated by DT treatment of CD11c-DTR mice [39, 44]. Consistent with this, a ~10-fold reduction in vaccinia virus-infected macrophages was noted in the DT-treated CD11c-DTR mice. Only the toxin-resistant fraction of macrophages thus contributes to the results obtained following DT treatment. Similarly, Backer *et al.* attributed the potent $CD8^+$ T cell response observed after antigen targeting to MMM in the spleen to transfer of the antigen to $CD8^+$ DCs [11]. However, DCs were purified from mice by a CD11c MACS procedure for *in vitro* evaluation of T cell activation and thus, it is possible that macrophages were also included in these assays. A study by Asano *et al.* on the other hand showed that subcutaneous vaccination with irradiated tumour cells activated antigen-specific $CD8^+$ T cells

to elicit a protective antitumor immune response and that this effect was abolished by selective depletion of Sn^+ macrophages in the lymph nodes [45]. In this study however, also $CD11c^{hi}$ cells (also expressing Sn) were involved in the antigen presentation to the $CD8^+$ T cells which also expressed CD8α, and it may be argued that these cells actually are Sn^+ $CD8\alpha^+$ DCs, that either express Sn by themselves or gain Sn expression by bleb acquisition from macrophages [39, 46]. This bleb acquisition has been proposed as a mechanism used by macrophages to transport antigen towards DCs (and lymphocytes), although the authors can not rule out the possibility that the blebs arise during tissue preparations [47]. One study that has not been argued against yet is a study by Barral *et al.* showing that Sn^+ macrophages can present lipid antigens in a CD1d dependent manner to iNKT cells, leading to iNKT cell activation and population expansion in the lymph nodes [48]. Although purified $CD11c^{hi}$ DCs were also able to activate iNKT cells *in vitro*, they were unable to activate iNKT cells when recovered from draining lymph nodes from immunised animals, suggesting they do not have access to the antigen *in vivo*. Also, Kawasaki *et al.* used high-affinity glycan ligand–bearing liposomes to selectively deliver lipid antigens to Sn^+ macrophages via the Sn endocytic pathway [49]. They found that ligand-targeted liposomes were captured by Sn^+ macrophages and potently primed iNKT cells in liver and spleen and that these effects occurred in an Sn-dependent manner, as no activation was seen with the targeted liposomes in Sn-deficient mice. As iNKT cells are however a specialized subset of classical αβ T lymphocytes, it has not been definitely established yet whether Sn^+ macrophages are able to directly prime naive $CD8^+$ T cells and thus further research is required to resolve this issue. Also, research is lacking concerning the priming of $CD4^+$ T cells by Sn^+ macrophages, either directly or indirectly. Backer *et al.* showed modest stimulation of $CD4^+$ T cells *in vitro* after *in vivo* administration of OVA conjugated to an Sn-specific mAb and *ex vivo* restimulation of splenocytes with OVA peptide [11]. In our study, the lack of antigen-specific antibody induction upon vaccination might also be attributed to a modest or absent $CD4^+$ T cell stimulation. Clearly, further research is required to find out whether Sn^+ macrophages can stimulate $CD4^+$ T cell responses *in vivo*, either directly or indirectly.

Route of immunization

Although it is clear that there are a number of similarities between lymph node SCS macrophages and splenic MMM (both depend on CSF-1, B cells and lymphotoxin, are prone to viral infection and are situated at the interface of the follicle and sinus), there are also notable distinctions. SCS macrophages readily capture immune complexes, while MMM do not [39]. This may reflect anatomical differences of the tissues but could also relate to intrinsic differences in these cells that match their functions with the types of pathogens and antigens they typically encounter. As such, it might also be interesting to perform a targeting

experiment where antibody-antigen fusion constructs are administered intraveneously, so fusion constructs would predominantly be captured in the spleen. As MMM do not capture immune complexes, differences between targeting construct and non-targeting construct might be more pronounced in such experiment. Also, in pig lymph nodes we are not sure yet whether Sn-targeting fusion constructs are as efficiently transferred to B cells as it might be in rodents due to the inversed lymph flow present. In addition, both SCS and medullary macrophages in lymph nodes share equal expression levels of Sn, while MMM have a significantly higher expression level of Sn compared to marginal zone macrophages. This might lead to a more efficient targeting to the macrophages involved in antigen presentation. However, expression levels remain to be established in pigs, where Sn expression is observed in the marginal zone and elliptical vessels [31], for which the latter may be comparable to the periarterial macrophage sheaths observed in humans.

Both intramuscular and intraveneous antigen delivery will predominantly yield systemic immunity. Although this is of major importance against infections, the induction of mucosal immunity is also gaining increased attention. After all, many pathogens enter the body via mucosal sites and a line of defense at these sites thus obviously represents a major benefit. It is envisioned mucosal immunizations (e.g. oral, pulmonary, sublingual and nasal) will also gain importance because of the possibility to yield needle-free vaccines, which have the advantage that they are easy to use/administer and do not require trained health care workers [50]. As discussed in Chapter 1, Sn expression has been observed in the lungs of rats, mice, humans and pigs and in the intestines of rats, mice and humans (not examined in pigs), making it interesting to evaluate the outcome of differential administration routes of antigen-bearing Sn-targeting construct.

Antigen delivery

In this thesis, a recombinant antibody was developed to target antigens towards Sn^+ macrophages. Antibodies have a high specificity and affinity for their target, which makes them ideal molecules for this purpose. Besides antibodies however, an extensive toolbox of antibody derivates has been developed and a comparison of different targeting moieties for their efficiency of targeting antigen towards Sn^+ macrophages and the resulting immune outcomes would be interesting. In addition, an extensive set of nanoparticles for antigen delivery has been developed and these molecules can be used in targeting research as well by the coating of the targeting moiety upon the nanoparticle. This again broadens the possibilities to target antigen towards Sn^+ macrophages. In addition, some nanoparticles have the advantage that they can carry adjuvant inside, or have adjuvanting properties, which can significantly reduce possible side-effects of systemic adjuvant delivery. Taken together, a large array of antigen delivery tools exists and it would be interesting to

investigate whether these different antigen delivery vehicles can positively influence immune responses upon Sn targeting.

To target or not to target?

DC targeting studies most often aim at reaching a single DC subset in order to drive the immune system towards a specific immune response. For example, $CD8\alpha^+$ DCs in mice are thought to be the DC subset capable of most potently priming $CD8^+$ T cell responses. Targeting antigens towards these $CD8\alpha^+$ DCs by Clec9A however also appears to be an interesting strategy to induce robust humoral immunity as well [3]. The proposed human equivalent of mouse $CD8\alpha^+$ DCs is the $BDCA3^+$ DC subset. This was based on phenotypic characteristics, the expression of particular transcription factors, and the fact that they cross-present antigen to $CD8^+$ T cells [51, 52]. Nevertheless, a recent study reported that $BDCA3^+$ dermal DCs suppressed immune responses by the constitutive production of IL-10 and the induction of regulatory T cells [53]. In addition, studies have reported cross-presentation of various forms of antigen by plasmacytoid DCs [54-56], thought to be mainly involved in innate immune stimulation. These observations might raise the question whether putting labels on different DC subsets is actually possible and whether it is not the environment in which the DCs reside that determines their functional specialisation. Without a doubt, certain differentiated DC subsets will be intrinsically better at performing specific immune steering functions, but (re-)programming of DCs by using an appropriate immune stimulus might yield desired immune responses as efficiently as specifically targeting the antigens towards the 'best suited' DC subset, raising the question: why bother targeting DC subsets? Can it be that it is more interesting to find vaccine formulations that are most efficiently taken up by APCs in general and finding the right stimuli to program them towards the desired immune response? Building on to that idea, Sn^+ macrophages can be important cells to study. They are located in spleen and lymph nodes at the borders of incoming blood and lymph respectively. Having a vaccine composition that efficiently drains into the lymph combined with an Sn-targeting moiety enables capture by Sn^+ macrophages in lymph nodes. Providing the right stimulus on the other hand might trigger these macrophages to secrete cytokines that provide the immune environment to steer the immune response in the wanted direction. Furthermore, Sn^+ SCS macrophages can transfer antigen to cognate B cells or non-cognate B cells that carry the antigen towards follicular DCs where cognate B cells have the opportunity to sample the antigens. Intriguingly, bleb formation by Sn^+ macrophages has been proposed to be a mechanism of antigen transfer towards DCs, which can upon antigen retrieval further drive the immune response [47]. Taking these observations into consideration, Sn^+ macrophages can be ideal candidates for antigen delivery and finding out how they can be best reached and activated is most interesting to pursue.

CONCLUSIVE REMARK

Vaccination is by far the greatest success within the field of immunology to date. Current immunotherapeutic approaches seek to continue this success story by refining the classical vaccine approach [51]. One clear trend is the shift towards more complex but highly controlled vaccine design. To facilitate this, fundamental knowledge on the immunological mechanisms underlying immune responses is necessary. Different APC subsets have been defined and knowledge on how they can be properly reached and activated will provide extremely valuable information for further vaccine design. Ultimately, a full exploitation of the immune system is envisioned which can pave the way for the design of vaccines that are safe and capable of treating chronic infections, cancer and even autoimmune diseases. Sn^+ macrophages are one of these APC subsets that due to their characteristics and anatomical location at the sites where effective immune responses are generated represent an attractive target for antigen delivery. Yet, many questions need to be answered before Sn targeting can be effectively harnessed, especially how these macrophages handle antigen internalized via Sn, how these macrophages exactly interact with other immune cells, and what the best way may be to get them activated to drive the generation of humoral and cellular immunity. Answers to these challenges will however shed light on how to exploit the full potential of this intriguing cell subset for next generation vaccines.

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Chapter

6

Summary / Samenvatting

SUMMARY

Classic strategies for the development of vaccines are attenuation or complete inactivation of pathogens. Although these vaccines prove successful in many instances, many infectious diseases still exist for which the development of an effective vaccine remains elusive based on these traditional approaches (e.g. HIV, malaria, tuberculosis,…). In addition, continuously increasing safety demands for novel vaccine formulations encourage the search for more rational vaccine designs. As such, a wide range of vaccines under development now are based on isolated peptides, proteins or polysaccharides of pathogens, or naked DNA encoding a protective antigen. Although promising, as this can lead to the development of vaccines that are more immunologically defined and safer than many existing vaccines, such antigens are often poorly immunogenic and require the addition of an immune-stimulating compound to generate an effective immune response. As a consequence, finding safe, yet efficient ways to boost immunity has become an increasingly important area of research in current-day vaccinology.

The ultimate goal of a vaccine is to induce an antigen-specific memory immune response. To facilitate this, an interaction between antigen-loaded APCs and naive lymphocytes needs to take place in secondary lymphoid tissues. Therefore, one way to improve the efficiency of a vaccine is to improve the efficiency by which antigen is encountered by APCs. As such, targeting antigen to uptake receptors expressed on the surface of APCs is increasingly studied. As the dogma establishes that DCs are the most efficient APCs stimulating naive lymphocytes, most targeting studies have been focused on DCs and their different subtypes. However, recently also Sn^+ macrophages are gaining increased attention as targets for vaccination strategies as their location in spleen and lymph nodes appears to be ideal for antigen capture and presentation [7]. These macrophages and more precisely the immune stimulatory effect of directly targeting antigens towards them, is the subject of this dissertation.

To start this dissertation, the current knowledge on targeting antigens towards APCs as a vaccination strategy is summarised in **Chapter 1**. In addition, the available literature on Sn and Sn^+ macrophages is reviewed which highlights the importance of this macrophage subset in the stimulation of immune responses. To finalize this chapter, an overview is given of targeting molecules already applied for targeting antigens to APCs and targeting molecules that could be implemented in this research area in the near future.

In **Chapter 2**, the aims of this dissertation are formulated.

Chapter 3 is dedicated to the development of a recombinant antibody that can be used to target peptides or proteins to Sn^+ macrophages. Previous research results of our laboratory

had shown that porcine antibody responses to the model antigen HSA could be enhanced by chemically coupling HSA to an Sn-specific mAb. Although successful, this system implies that the antigen to be targeted is independently produced and purified, which translates into significant optimization for each separate conjugate. Also, chemical coupling to antibodies depends on the presence and distribution of reactive groups, e.g. primary amines on lysine residues, that can be located in or near the antigen-binding region. Upon coupling, this might result in partial or complete loss of the antibody's capacity to bind to the target antigen. In addition, there is limited stoichiometric control because of the large number of reactive groups present in an antibody, leading to a heterogeneous mixture that makes batch to batch consistency hard to effectuate. To overcome these problems associated with chemical conjugation of a cargo to an Sn-specific mAb, a versatile recombinant antibody vector was developed. This recombinant antibody rec41D3 was shown to specifically bind to porcine Sn with a comparable affinity as the native monoclonal antibody. In addition, rec41D3 also induced Sn endocytosis in primary macrophages and resided for prolonged times in early/late endosomes. Subsequently, two fusion proteins were generated, one containing a V5 peptide tag and one containing an eGFP molecule, and were shown to be efficiently produced in HEK-293T cells and easily purified using standard protein G chromatography. Furthermore, both V5 and eGFP were shown to be co-internalized together with rec41D3 into Sn-expressing primary macrophages, confirming the development of a versatile antibody vector to evaluate Sn-directed targeting strategies in a porcine animal model.

In **Chapter 4**, the technology developed in Chapter 3 was applied to generate fusion proteins for PRRSV vaccination and immunocastration.

In **part 4.1**, a linear B cell epitope, present on GP4 of PRRSV and known to be a target of virus-neutralizing antibodies, was selected and introduced at the C-terminus of rec41D3 and its irrelevant isotype control. During the course of an immune response to PRRSV, the induction of virus-neutralizing antibodies is hampered, and thus we wished to evaluate if targeting a neutralizing epitope towards pSn-expressing macrophages could enhance the induction of epitope-specific and virus-neutralizing antibodies. As such, a single dose of the resulting fusion constructs was used to immunize pigs and four weeks later, a viral challenge was performed. Upon vaccination, no peptide-specific IgG responses could be detected. After viral challenge however, it was clear that the IgG response against this specific peptide developed more rapidly compared to an infection in non-immunized animals, with the most pronounced induction of IgG responses in the group that had received a 500µg Sn-targeting fusion construct. Also, neutralizing antibody responses after PRRSV challenge were enhanced in animals receiving a single 500µg intramuscular dose of the Sn-targeting fusion protein, although correlations between the two read-outs were hard to effectuate. Lastly, a

minor beneficial effect on viral clearance was observed. Together, these data show that viral peptide targeting to porcine Sn-expressing macrophages can improve the anti-viral immune response, but also show that more research will be needed to further explore vaccination potential.

In **part 4.2**, a potential immunocastration vaccine candidate was developed. As immunocastration relies on the induction of antibodies against gonadotropin releasing hormone (GnRH), a 10 amino acid peptide, four copies of GnRH were genetically fused to our recombinant Sn-targeting antibody. For the production of recombinant antibodies, the most commonly used expression systems are mammalian cells growing in suspension, so as to enable the rapid scale up of productions. In addition, chemically defined, serum-free media – with no animal-derived components – have become industry standard to further simplify regulatory approval. In this study, we made use of a commercially available suspension adapted CHO cell line to develop cell lines expressing recombinant antibody-GnRH fusion constructs. Subsequently, antibody-fusion proteins could be easily purified from the selected clone supernatants and a high purity was obtained. Furthermore, only Sn-targeting fusion proteins were shown to specifically bind to and to be internalized into Sn-expressing cells. These fusion proteins are therefore ready to be used in future animal experiments to evaluate pSn-directed GnRH targeting as an immunocastration strategy.

Finally, in **Chapter 5**, the main findings of this dissertation are recapitulated and discussed in a broader context, with a main focus on future vaccine development. Combining literature with the results obtained in this thesis results in many questions remaining on the way Sn^+ macrophages capture and handle incoming antigen. In addition, further research is needed to explore ways to most efficiently reach these Sn^+ macrophages *in vivo* and get them activated to drive the generation of humoral and cellular immunity. Answers to these challenges will however shed light on how to exploit the full potential of this intriguing cell subset for next generation vaccines.

SAMENVATTING

Klassieke methoden voor het vervaardigen van vaccins zijn het attenueren of inactiveren van pathogenen. Hoewel deze vaccins in veel gevallen succesvol blijken, bestaan er nog steeds verscheidene infectieuze ziekten waarvoor de ontwikkeling van een beschermend vaccin niet mogelijk bleek tot op heden (vb. HIV, malaria, tuberculosis,…). Bijkomend worden er steeds strengere veiligheidseisen gesteld voor het op de markt brengen van nieuwe vaccins, waardoor er een noodzaak blijkt aan een meer rationeel ontwerp van vaccins. Zo zijn heel wat vaccins in ontwikkeling vandaag gebaseerd op geïsoleerde peptiden, eiwitten of polysacchariden van pathogenen, of op naakt DNA dat codeert voor een beschermend antigeen. Hoewel dit zeer beloftevol lijkt om te komen tot vaccins die beter immunologisch gedefinieerd en veiliger zijn dan vele reeds bestaande vaccins, blijken dergelijke antigenen echter vaak zwak immunogeen en vereisen zij de toevoeging van een adjuvans om tot een beschermende immuunrespons te komen. Bijgevolg krijgt de zoektocht naar veilige, maar efficiënte manieren om immuniteit op te wekken veel aandacht in huidig vaccinatie onderzoek.

Het ultieme doel van een vaccin is het opwekken van een antigeen-specifieke immuunrespons die in staat is te beschermen tegen een toekomstige besmetting met het pathogeen. Om dit te bewerkstelligen, is het noodzakelijk dat antigeen-presenterende cellen (APC) die geladen zijn met het antigeen kunnen in contact komen met naïve lymfocyten in secundaire lymfoïde organen. Een manier om de efficiëntie van een vaccin te verbeteren, kan dan ook zijn om de efficiëntie van de manier waarop APC in contact komen met antigeen te verbeteren. Dit inzicht heeft geleid tot onderzoek waarbij antigeen gestuurd wordt richting het oppervlak van APC door het gebruik van een molecule die een receptor op het oppervlak van de APC herkent. Dendritische cellen worden over het algemeen beschouwd als de meest efficiënte APC en bijgevolg werd ook het meeste onderzoek tot dusver gericht naar het bereiken van dendritische cellen en hun verschillende subtypes. Recent kregen echter ook sialoadhesine-expresserende (Sn^+) macrofagen meer wetenschappelijke aandacht omwille van hun locatie in milt en lymfeknopen waar bloed en lymfe die antigenen bevatten, circuleren. Het sturen van antigenen richting deze macrofagen en de evaluatie van de resulterende immuunrespons, is het onderwerp van deze doctoraatsthesis.

Deze doctoraatsthesis start met een samenvatting van de huidige kennis over het sturen van antigenen richting APC voor vaccinatiedoeleinden (**Hoofdstuk 1**). Bijkomend wordt een overzicht gegeven van de tot op heden beschikbare literatuur over Sn en Sn^+ macrofagen, wat de immuunstimulerende rol van dit celtype in de verf zet. Om het hoofdstuk af te sluiten, wordt tot slot een overzicht gegeven van reeds gebruikte moleculen voor het sturen van

antigenen richting APC en eveneens van moleculen die onderzocht kunnen worden voor deze doeleinden in de toekomst.

In **Hoofdstuk 2** worden de doelstellingen van deze thesis voorgesteld.

Hoofdstuk 3 is voorbehouden voor de ontwikkeling van een recombinant antilichaam (rec41D3) dat gebruikt kan worden voor het sturen van peptiden of eiwitten richting Sn^+ macrofagen. Voorgaand onderzoek aan het laboratorium voor Virologie had reeds aangetoond dat antistof responsen tegen het model antigeen HSA in het varken verbeterd konden worden wanneer HSA chemisch gekoppeld werd aan een Sn-specifiek monoklonaal antilichaam. Dit experiment was succesvol, maar chemische koppeling van een antigeen aan een antilichaam impliceert dat het antigeen eerst afzonderlijk geproduceerd en opgezuiverd dient te worden, wat zich vertaalt in aanzienlijke optimalisatie voor elk gewenst conjugaat. Chemische koppeling aan antilichamen is ook afhankelijk van de aanwezigheid en de distributie van reactieve groepen, zoals primaire amines op lysine residuen, die in of nabij het antigeen-bindend domein kunnen gelegen zijn. Na koppeling kan dit mogelijk tot resultaat hebben dat de affiniteit van het antilichaam voor het antigeen gedeeltelijk of volledig verloren gaat. Bijkomend is er ook weinig controle over hoeveel antigenen zullen binden op het antilichaam en op welke locatie van het antilichaam, aangezien heel wat reactieve groepen, en dus mogelijke koppelingsplaatsen, aanwezig zijn. Dit leidt tot een heterogene verzameling van conjugaten, wat het moeilijk maakt gelijkvormigheid in de verschillende producties te verkrijgen. Om aan deze problemen tegemoet te komen werd een recombinante antilichaamvector gemaakt die toelaat eiwitsequenties toe te voegen aan de C-terminus van de zware keten van het antilichaam. Het recombinante antilichaam vertoonde een gelijkaardige affiniteit voor Sn als het natieve antilichaam en induceerde, na binding met Sn, Sn endocytose in primaire macrofagen. De recombinante antilichamen vertoonden, net als het natieve antilichaam, een trage progressie door de endo/lysosomale weg en verbleven meer specifiek voor verlengde tijd aanwezig in vroege endosomen. Bijkomend werd een fusie-eiwit gemaakt van rec41D3 met een V5 peptide tag en een fusie-eiwit van rec41D3 met eGFP. Beide eiwitten werden geproduceerd in het supernatans van HEK293T cellen na transfectie en konden makkelijk opgezuiverd worden aan de hand van proteïne G chromatografie. Bijkomend werd aangetoond dat V5 en eGFP samen met rec41D3 geïnternaliseerd werden in primaire macrofagen. Dit toont aan dat een aanpasbare antilichaamvector gemaakt werd die gebruikt kan worden om Sn-gerichte vaccinatiestrategieën uit te testen in een porcien diermodel.

In **Hoofdstuk 4** van deze thesis werd de technologie ontwikkeld in Hoofdstuk 3 toegepast om fusie-eiwitten te maken voor PRRSV vaccinatie en immunocastratie.

In **deel 4.1** werd een lineaire B cel epitoop aanwezig op GP4 van PRRSV geselecteerd en geïntroduceerd aan de C-terminus van rec41D3 en een irrelevante isotype controle. Deze epitoop is een gekend doelwit voor virus-neutralizerende antilichamen. Tijdens de immuunrespons volgend op een PRRSV infectie verloopt deze inductie van virus-neutralizerende antilichamen echter zeer inefficiënt en aldus wensten wij te evalueren of het gericht sturen van deze epitoop richting Sn^+ macrofagen de inductie van epitoop-specifieke en virus-neutralizerende antilichamen kon verbeteren. Een enkele dosis van de fusie-eiwitten werd toegediend aan varkens en vier weken later werden de dieren geïnfecteerd met het PRRSV virus. Na vaccinatie konden echter nog geen peptide-specifieke IgG responsen gedetecteerd worden. Na virale infectie werd echter duidelijk dat de peptide-specifieke immuunrespons zich sneller ontwikkelde in gevaccineerde ten opzichte van niet-gevaccineerde dieren, met het meest uitgesproken effect zichtbaar bij de dieren die een eenmalige 500µg dosis ontvangen hadden van het Sn-gerichte fusie-eiwit. Bijkomend was ook de neutralizerende antilichaamrespons verhoogd na infectie in deze groep, hoewel de correlatie met de peptide-specifieke respons moeilijk te trekken bleek. Tot slot werd ook een matige verbetering van de viremie na infectie vastgesteld in deze groep. Deze data tonen aan dat het sturen van een viraal peptide naar Sn^+ macrofagen de anti-virale immuunrespons kan verbeteren. Deze data tonen echter ook aan dat meer onderzoek nodig zal zijn om het vaccinatiepotentieel van deze vaccinatiestrategie verder uit te pluizen.

In **deel 4.2** werd een kandidaat immunocastratievaccin ontwikkeld. Immunocastratie is gebaseerd op het opwekken van antilichamen tegen gonadotropin releasing hormone (GnRH), wat een peptide is dat bestaat uit 10 aminozuren. Vier herhalingen van dit peptide werden genetisch gekoppeld aan het Sn-gerichte rec41D3 en een irrelevante isotype controle. Voor de productie van recombinante antilichamen zijn cellen van dierlijke afkomst de meest gebruikte expressiesystemen. Bijkomend worden suspensiecellen geprefereerd omwille van de makkelijke opschalingmogelijkheden. Chemisch gedefinieerde media worden gebruikt zodat geen dierlijke componenten in het productiemedium aanwezig zijn. In deze studie maakten wij gebruik van een commercieel beschikbare suspensie CHO cellijn om cellijnen te onwikkelen die de antilichaam-GnRH fusie-eiwitten constitutief tot expressie brengen. Antilichaam-GnRH fusies konden makkelijk opgezuiverd worden uit het supernatans van de geselecteerde clones en een hoge zuiverheid werd verkregen. Bijkomend konden enkel de Sn-gerichte fusie-eiwitten binden met Sn en werden enkel deze eiwitten naar binnen getrokken in Sn-expresserende cellen. Deze fusie-eiwitten kunnen nu verder gebruikt worden in toekomstige vaccinatie-experimenten.

Tot slot worden in **Hoofdstuk 5** de belangrijkste bevindingen van deze thesis samengevat en bediscussieerd in een bredere context, met een focus op toekomstige vaccinontwikkeling.

Een combinatie van de beschikbare literatuur en de resultaten behaald in deze thesis tonen aan dat veel vragen onopgelost blijven over de manier waarop Sn^+ macrofagen inkomend antigeen vangen en verder verwerken. Bijkomend is het duidelijk dat verder onderzoek noodzakelijk is om uit te zoeken wat de beste manier is om deze macrofagen *in vivo* te bereiken en deze te activeren zodat ze de inductie van een humorale en cellulaire immuniteit bewerkstelligen. Het oplossen van deze vragen kan echter leiden naar diepgaand inzicht in het potentieel van dit intrigerend celtype voor de ontwikkeling van een nieuwe generatie vaccins.

Curriculum Vitae

PERSONALIA

Karen Ooms werd geboren op 25 juli 1985 te Vilvoorde. In 2003 beëindigde zij haar secundaire studies wetenschappen-wiskunde aan het Jan-van-Ruusbroeckcollege te Laken. In datzelfde jaar startte zij haar hogere opleiding tot Industrieel Ingenieur aan de Katholieke Hogeschool Sint-Lieven te Gent, waar zij in 2007 het diploma van Industrieel Ingenieur in de Biochemie behaalde met onderscheiding. Nadien zette zij haar studies verder aan de Universiteit Gent en in 2008 behaalde zij het diploma van Master of Molecular Biotechnology, optie Medical Biotechnology, met grote onderscheiding. Vanaf februari 2009 tot juni 2013 beschikte zij over een doctoraatsbeurs in het Laboratorium voor Virologie aan de Faculteit Diergeneeskunde van de Universiteit Gent. Hier verrichte zij onderzoek naar het gericht sturen van antigenen richting macrofagen die de receptor sialoadhesine tot expressie brengen, dit met het oog op het ontwikkelen van een nieuwe generatie vaccins. Dit onderzoek gebeurde onder leiding van Prof. dr. Hans Nauwynck en Prof. dr. ir. Peter Delputte. Het onderzoek werd gefinancierd door het Industrieel Onderzoeksfonds van de Universiteit Gent.

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Saha D., Lefebvre D.J., Ooms K., Huang L., Delputte P.L., Van Doorsselaere J. and Nauwynck H.J. (2012) Single amino acid mutations in the capsid switch the neutralization phenotype of porcine circovirus 2. *Journal of General Virology*, **93**:1548-1555.

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PARTICIPATIONS TO CONFERENCES AND SYMPOSIA

Abstracts

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