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Comparative analysis of the immune responses elicited by native vs. recombinant vaccines against bovine gastrointestinal nematodes

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"La ciencia siempre vale la pena porque sus descubrimientos,
tarde o temprano, siempre se aplican"

Severo Ochoa

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Abbreviations

^{3}HT	^{3}H-thymidine
ADCC	antibody-dependent cell cytotoxicity
AH	anthelmintic
$Al(OH)_3$	aluminum hydroxide
ANCOVA	analysis of covariance
APC	antigen-presenting cells
ASP	activation-associated secreted proteins
BCA	bicinchoninic acid
bmDC	bone marrow-derived dendritic cells
BMGY	buffer minimal glycerol complex medium
BMMY	buffered methanol complex medium
BSA	bovine serum albumin
CD	cluster of differentiation
CLR	c-type lectin receptor
CP	cysteine-protease
CPM	counts per minute
DAB	3,3´-diaminobenzidine
DC	dendritic cells
dd-ASP	*C. oncophora* double-domain-ASP
DTT	dithiothreitol
ELISA	enzyme-Linked immunosorbent assay
EPG	eggs per gram faeces
ES	excretory/secretory material
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FEC	faecal egg counts
FGS	first grazing season
GI	gastrointestinal
GL	globule leukocytes
GM-CSF	granulocyte macrophage colony-stimulating factor
HMW	high molecular weight
HRP	horseradish peroxidase
IAA	iodo acetamide
IECs	intestinal epithelial cells
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cells
L1	first stage larvae
L2	second stage larvae
L3	third stage larvae
L4	fourth stage larvae

L5	fifth stage larvae
LAL	limulus amebocyte lysate
LN	lymph node
LPS	lipopolysaccharide
M1	classically-activated macrophages
M2	alternatively-activated macrophages
MACS	magnetic-activated cell sorting
MEP	metalloendopeptidases
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLs	macrocyclic lactones
MNCs	mononuclear cells
moDC	monocyte-derived dendritic cells
nASP	*O. ostertagi* native ASP
NK	natural killer
NLR	nod-like receptor
NOS	nitric oxide synthase
OPA	*O. ostertagi* polyprotein allergen
OVA	ovalbumin
PAMP	pathogen-associated molecular patterns
pASP	*O. ostertagi* recombinant double-domain-ASP
PBMC	peripheral blood mononuclear cells
pdd-ASP	*C. oncophora* recombinant double-domain-ASP
PI	proliferation index
PRR	pattern-recognition receptors
PS	phosphatidylserine
RELMβ	resistin-like molecule-β
rPMY	recombinant paramyosin
S-S	di-sulphide
SI	stimulation index
TCR	T-cell receptor
TGF	transforming growth factor
T_H	T helper
TLR	toll-like receptor
TLSP	thymic stromal lymphopoietin
Treg	regulatory T-cell

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Introduction

Introduction

1. The parasites: *Ostertagia ostertagi* and *Cooperia oncophora*

Among the different members of the Trichostrongyloidea family, *Ostertagia ostertagi* and *Cooperia oncophora* are the most prevalent ones affecting cattle in temperate climate areas and are essentially present in all grazing cattle herds. Infection with both gastrointestinal (GI) parasites occurs simultaneously in most cases and it is the main cause of parasitic gastroenteritis in cattle. It typically affects young calves during their first grazing season, provoking diarrhoea, weight loss and anorexia that can eventually lead to death in the most severe cases [1]. Besides their high prevalence and importance for animal welfare, infection with *O. ostertagi* and *C. oncophora* cause major economic losses as reflected by a diminished meat and milk production when inappropriate control measures are taken.

1.1 Life cycle and epidemiology

O. ostertagi and *C. oncophora* have a direct life cycle that consist of a parasitic phase in the host and a free-living phase on pasture (Figure 1). Eggs are excreted in faeces by the host and, once in the herbage, first stage larvae (L1) hatch and moult into second stage larvae (L2). The L2 larvae develop into the infectious third stage larvae (L3) without moulting. Under favourable conditions of temperature and humidity, the development from egg to infectious L3 larvae is approximately two weeks. L3 larvae might survive on pasture for several months and even overwinter due to the retention of the protective cuticle. The parasitic phase occurs when the hosts ingest L3-contaminated vegetation. Once ingested, L3 larvae exsheath the cuticle that was retained from previous stages and penetrate the gastric glands (*O. ostertagi*) or the crypts of the small intestine (*C. oncophora*). The infective L3 larvae develop further into fourth and fifth stage larvae (L4, L5), which colonize the lumen and further develop into sexually mature adult males and females. Adult worms will mate and subsequently produce eggs which will be excreted in the faeces closing the cycle (Figure 1). The normal pre-patent period for both parasites is approximately 21 days, but under certain conditions L4 larvae might enter hypobiosis and become arrested in their development (dormant) for a period of up to 6 months [2].

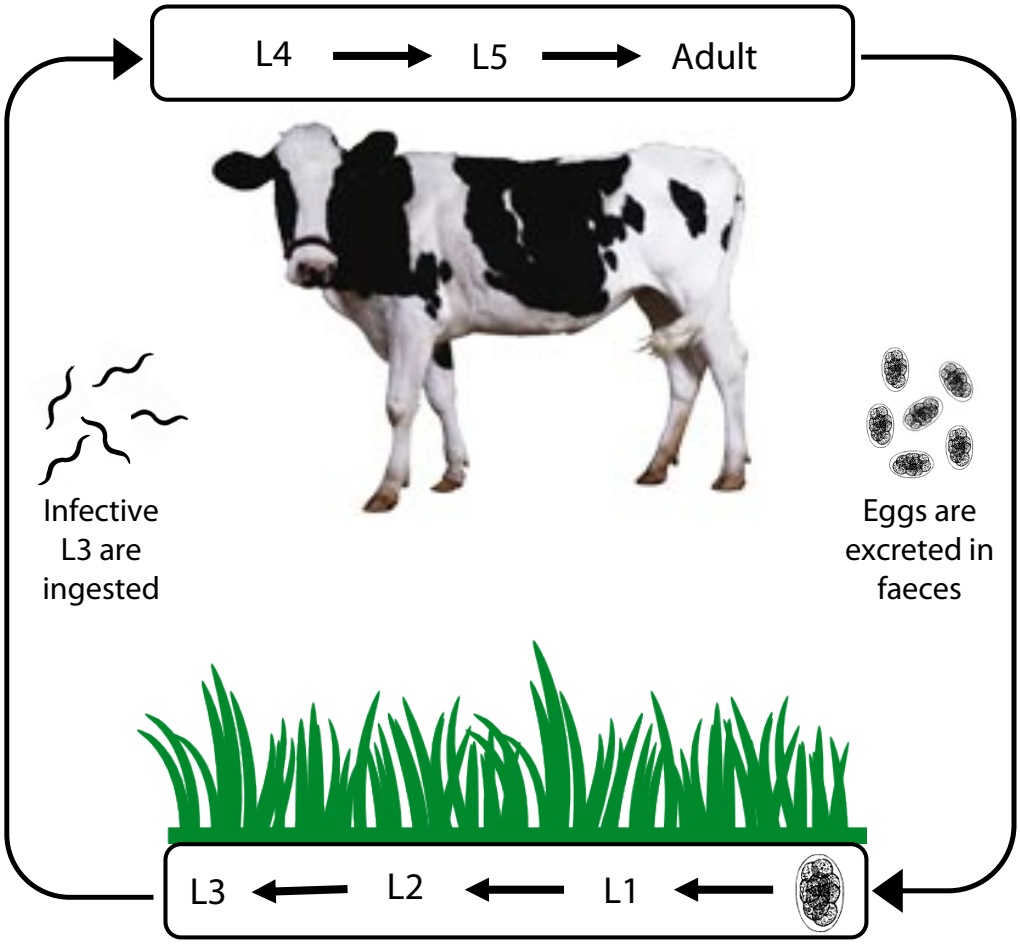

Figure 1. Life cycle of *O. ostertagi* and *C. oncophora*

Remaining *O. ostertagi* and *C. oncophora* L3 larvae from the previous season are ingested from the pasture by the host during a new grazing season, which typically lasts from May-June until September-October in temperate climate areas of the northern hemisphere (Figure 2). These larvae will complete their life cycle inside the host and in a period of 3 weeks new eggs will be deposited on the pasture, which will slowly develop into new infective L3 larvae. As indicated in Figure 2, this development can be affected by temperature, and speeds up from mid-July onwards [3]. This phenomenon, also named the "mid-summer rise", is influenced by the environment and therefore subjected to variability [4].

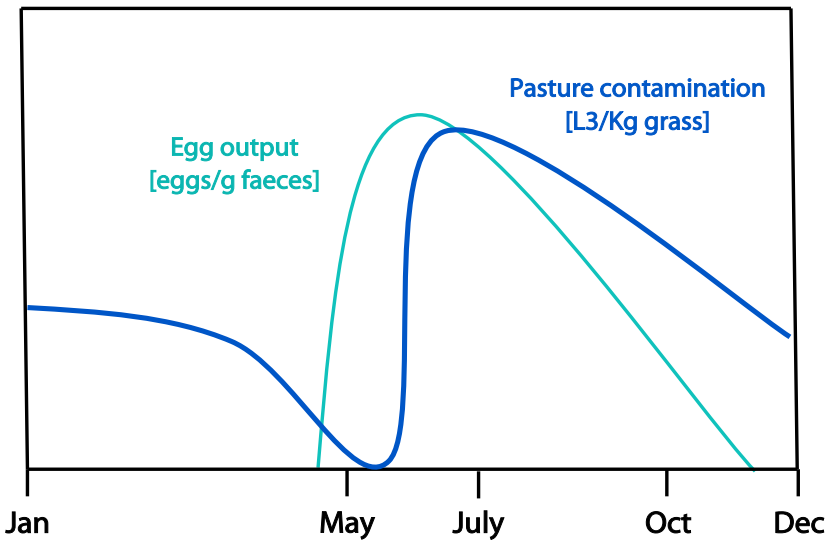

Figure 2. Diagram representing the egg output of cattle and larval contamination of pasture throughout the year [4]

1.2 Clinical signs and pathogenesis

In temperate climate areas, clinical ostertagiosis manifests under two circumstances when the mature larvae emerge from the gastric glands [5]. Type I ostertagiosis occurs in grazing calves shortly after a period of high uptake of infective larvae. Although morbidity is usually high, mortality in these cases is a rare event. In contrast, type II ostertagiosis occurs in yearlings and adult cattle over winter with the emergence of hypobiotic L4 larvae and can cause high mortality rates unless effective treatment is administered. While clinical cooperiosis is rare and infection with *C. oncophora* develops often with subclinical manifestations, symptoms in severe cases are similar to those observed in *O. ostertagi* infections and include loss of appetite, watery diarrhoea, anorexia and dull hair coats [6,7]. The clinical signs during the course of an *O. ostertagi* infection are the result of mucosal damage caused by the growth of larvae in the abomasal gastric glands [8]. Due to gland enlargement and dilation, the epithelial cells are replaced by undifferentiated epithelium, a process that induces an increase in pH levels, a rise in gastrin and the failure to transform pepsinogen into pepsin [9,10]. Recent studies suggest that the rise in gastric pH is likely to be caused by an inhibition of acid secretion by adult larvae excretory/secretory (ES) products [11]. In addition, emergence of adult worms from the gastric gland increases epithelial permeability resulting in high levels of pepsinogen in plasma and protein loss. Altogether, this sequence of events leads to the abovementioned symptoms that characterise bovine ostertagiosis [9,10].

1.3 Economic impact

Infection of cattle with *O. ostertagi* and *C. oncophora* in temperate climate areas is considered a major source of economic losses [12]. If we consider that the cattle industry comprises 89 million cattle in Europe [13] and 111 million cattle in the US and Canada [14], the importance of these infections acquires a greater magnitude and their control becomes essential. In Northern Belgium alone, the estimated loss per year due to GI nematode infections has been estimated at 10 million euro [15]. Amongst all parameters of economic relevance, weight gain and milk production are the most relevant and best documented ones [12]. In terms of weight gain, infection with GI nematodes has the greatest effect on calves during their first grazing season (FGS). Although considered unimportant, it should not be neglected that infections during the second grazing season can be associated with suboptimal production derived from weight losses during the FGS. Besides the evident negative impact of weight loss on milk production, several studies have demonstrated that anthelmintic treatment results in an average increase in milk production of 0.6 Kg/cow per day [12].

1.4 Control strategies

Control measures against *O. ostertagi* and *C. oncophora* infections are generally restricted to young animals during their first grazing season, since older animals tend to develop a naturally acquired immunity from the second grazing season onwards. To date, control of these infections in cattle has relied mainly on the use of anthelmintic (AH) compounds, i.e. benzimidazoles (e.g. albendazole), imidazothiazoles (e.g. levamisole) and macrocyclic lactones (MLs) (e.g. ivermectin). These latter comprise the main drug of choice against GI nematode infections in cattle due to their high efficacy, long-term action and the lack of withdrawal time for milk for the newest generic forms (e.g. pour-on eprinomectin and moxidectin) [16]. Nevertheless, intensive use of AH has important drawbacks. In addition to the associated cost of their use for the farmer, it has been demonstrated that intensive AH treatment interferes with the development of natural immunity [17]. Concern has also been raised due to the increased risk of drug residues in both environment [18,19] and food-chain products due to the intensive use of anthelmintics [20]. Last and most important, resistance to almost every marketed AH (Table 1) against nematode infections in ruminants has developed worldwide over the last decades [21-23]. Together, all these factors have stimulated the search for alternative control strategies.

Table 1. Reported resistance for all marketed anthelmintics (AH) (based on De Graef et. al. 2013)

Anthelmintic class	Generic name	Market release	Reported resistance
Heterocyclic compounds	Phenothiazine	1940	1957
	Piperazine	1954	1966
Benzimidazoles	Thialbendazole	1961	1964
	Cambendazole	1970	1975
	Oxibendazole	1970	1985
	Mebendazole	1972	1975
	Albendazole	1972	1983
	Fenbendazole	1975	1982
	Oxfendazole	1976	1981
	Triclabendazole	1983	1998
Imidazothiazoles and tetrahydropyrimidines	Levamisole	1970	1979
	Pyrantel	1974	1996
	Oxantel	1976	-
	Morantel	1970	1979
Macrocyclic lactones	Abamectin	Late 1970´s	2001
	Ivermectin	1981	1988
	Moxidectin	1991	1995
	Doramectin	1993	2007
	Eprinomectin	1996	2003
Amino-acetonitrile derivative	Monepantel	2009	2014
Spiroindole	Derquantel	2010	-

1.5 Alternative control strategies

Several alternative methods to control GI nematode infections have been tested to date, including the implementation of grazing management strategies such as pasture resting, late turn-out, mowing, reduced livestock density, alternation of grazing species and stock rotation onto clean pastures [24]. However, these methods have some disadvantages since a vast epidemiological knowledge is needed and pasture availability is restricted for many countries, including Belgium.

Another promising alternative method suggested for the control of helminth infections in cattle is maintaining a population in refugia [25]. This method aims to preserve the genes for susceptibility by keeping a proportion of the parasite population unexposed to AH. To do so, targeted selective treatment of individuals that will truly benefit from it instead of whole herd treatment is implemented. This method has proven to be effective in reducing the use of AH while maintaining production rates [26].

Additionally, it has been documented that the predisposition of animals towards an infection is directly influenced by their genetic background [27], and therefore selective breeding of resistant animals has been regarded as a possible alternative approach. However, this method has its downsides including (i) the unavailability of genetic markers to discern susceptibility, (ii) the possibility that selective breeding for resistance to worms might compete with selection against other diseases, and (iii) the possible negative impact on production [27-29].

A fourth alternative solution that has been investigated over the past years is the anti-parasitic potential of natural compounds. Plant products, such as chicory and sainfoin [30,31], have proven their promising potential to control GI nematode infections, although results are only preliminary and further optimisation is required. Other means of biocontrol are the nematode-trapping fungi *Duddingtonia flagrans* and *Monacrosporium thaumasium,* which produce spores capable to resist the GI tract and colonise faeces, where they trap and destroy larvae [32,33]. So far, this approach has shown promising results for control of pasture contamination experimentally [33] and novel delivery systems such as sodium alginate pellets have been tested successfully in the southern hemisphere [32].

A final option to control GI nematode infections is vaccination. The findings, progress and prospects of this alternative will be further laid out in the following sections.

2. Vaccines against gastrointestinal helminths in ruminants

Although first evidences of the use of vaccines dates back to 1000 CE in China, the term vaccine was only proposed in the XVIII century by Edward Jenner after the successful inoculation of material from cowpox (*Variolae vaccinae*) pustules which provided protection against the human smallpox. From the first advances made by Jenner, through the successful vaccination against rabies by Louis Pasteur, vaccines have demonstrated to be a sustainable and essential tool in the control and even eradication of bacterial, viral and, to lesser extent, protozoan infections [34].

In the case of helminths, their complex life cycle, which includes several developmental stages that might occur in a variety of tissues, has challenged the production of protective and cost-effective vaccines. This also applies to gastrointestinal nematodes of ruminants, and substantial efforts have been made to develop effective vaccines against their main nematode species.

2.1 Native vaccines

Vaccination against ruminant GI helminths, either aiming to prevent the infection or to reduce worm fecundity, offer a promising alternative for anthelmintic treatment. Nevertheless, only few vaccines against this type of pathogen have been commercialized over the last decades, mainly derived directly from the whole parasite or its components. Because of the nature of these vaccines, they have been also referred to as "native" vaccines. The first commercialized GI nematode vaccine was produced over 50 years ago by using the **entire attenuated** lungworm *Dictyocaulus viviparus* larvae [35,36]. Oral administration of 1000 x-ray irradiated L3 larvae two times in a 4-week interval resulted in 90-99% protection of cattle. Although not sterilizing, vaccination confers protection for up to 12 months, when booster immunizations are needed to avoid re-infections. The success of this vaccine inspired the production of a commercial irradiated-based vaccine against the sheep lungworm *Dictyocaulus filaria* (Table 2) [37]. In the case of *O. ostertagi* and *C. oncophora,* the first attempts to immunize cattle against the disease with irradiated larvae were unfruitful [38]. Equally unsatisfactory have been the early formulations based on crude somatic extract or excretory/secretory (ES) products of this parasite [39,40].

Despite the promising results with *D. viviparus* irradiated larvae, the failure of this approach in other cases [38,41] deviated the efforts towards the production of vaccines based on gut membrane antigens, somatic antigens or excretory/secretory products (ES). The main advances in the **gut antigen-based** vaccines have been achieved against the sheep blood feeding parasite *Haemonchus contortus*. Vaccination with the gut antigens contortin, H11 and *Haemonchus* galactose-containing glycoprotein complex (H-gal-GP) have proven their protective potential in multiple trials (Table 2) [42-49]. More recently, a commercial native vaccine against *H. contortus* based on the combination of the two gut antigens H11 and H-gal-GP was launched in Australia [50]. Hypothetically, the blood-feeding stages will ingest the antigen-specific antibodies raised after vaccination which will impair the worm´s intestine. In addition, vaccination of calves with gut membrane glycoproteins from *H. contortus* provided high levels of protection against both *H. contortus* and *H. placei* (Table 2) [51]. Also in cattle, Smith et. al. reported moderate protection levels (30-50% reduction in egg output) after vaccination of calves with gut membrane glycoproteins of *O. ostertagi* [52], although no effect on the worm numbers could be observed (Table 2). Surprisingly, cross-protection studies performed in sheep showed that vaccination with these antigens provided protection in animals subsequently challenged with *H. contortus* [52].

In terms of **somatic antigen-based** vaccines, protection (reflected by decrease over 60% in egg output and worm burden) has been achieved through vaccination of lambs with the somatic fraction p26/23 of adult *H. contortus* [53] (Table 2). Also for *H. contortus*, moderate levels of protection (up to 46% and 40% reduction in egg output and worm burden respectively) have been obtained after vaccination of lambs with low molecular weight (LMW) adult somatic antigens [54]. More recently, vaccination with the *H. contortus* adult somatic protein Hc23 isolated provided high levels of protection in lambs [55] (Table 2). In cattle, an attempt has been made to vaccinate animals against *O. ostertagi* with a globin-enriched fraction isolated from adult somatic extracts with highly variable results [56]. While in two of the trials the egg output was reduced in 52 and 63%, in the other two trials no egg count reduction could be observed [56].

Finally, due to their abundant presence at the host-parasite interface and their immunomodulatory properties, **ES products** have been long evaluated as vaccine candidates [57]. Common components found ES products from helminths include proteases, protease inhibitors, lectins and digestive enzymes. Nevertheless, their relative abundance can vary between helminth species and their different developmental stages [57]. In sheep, protection has been achieved after vaccination with cysteine proteases (CPs) and the activation-associated secreted proteins (ASP) Hc15 and Hc24 against *H. contortus* (Table 2) [58,59].

Table 2. Native vaccines against nematodes of ruminants.

Species	Host	Antigen/ Administration route	Reduction eggs/worms (%)	Reference
Dictyocaulus viviparus	Cattle	Irradiated L3 larvae/ oral	90-99/93	[35,36]
Dictyocaulus filaria	Sheep	Irradiated L3 larvae/ oral	97/n.m	[37]
Haemonchus contortus	Sheep	Irradiated L3 larvae/ oral	-/-	[41]
		Contortin/i.m	n.m/78.5	[42]
		CP-enriched membrane proteins / i.m	77/47	[43]
		H11/ i.m	95-99/87-95	[48,49]
		H-gal-GP/ i.m	93/72	[45,47]
		TSBP/ i.m	77/47	[46]
		⊗H11 + H-gal-GP/ s.c	80/n.m	[50]
		Hc15 + Hc24/ s.c	73-99/82-97	[58,59]
		O. ostertagi gut membrane glycoproteins/ i.m	81-97/57-84	[52]
		Somatic fraction p26-23/ i.m-s.c	60/61.6	[53]
		LMW somatic antigens/ i.m-s.c	29.5-46/40	[54]
		Somatic protein Hc23/ i.m-s.c	70-85/67-86	[55]
	Cattle	Gut membrane glycoproteins / i.m	99/s.r	[51]
Haemonchus placei	Cattle	Gut membrane glycoproteins / i.m	97/s.r	[51]
Ostertagia ostertagi	Cattle	Irradiated L3 larvae/ oral	-/-	[38]
		Somatic extract/ i.m	-/-	[39]
		L4 ES material/ i.p	-/-	[40]
		Globin fraction/ i.p-i.m	0-63-/0-28	[56]
		Gut membrane glycoproteins / i.m	30-50/-	[52]
		OPA/ i.m	60/-	[60]
		CP fraction/ i.m	56-80/18	[61-63]
		ASP fraction/ i.m	59-74/47	[63,64]
Cooperia oncophora	Cattle	Irradiated L3 larvae/oral	-/-	[38]
		dd-ASP/ i.m	58,5-91/82	[65]

n.m=not measured, s.r= significant reduction (% not indicated)

i.m=intramuscular, s.c=subcutaneous, i.p=intraperitoneal

* statistically significant

⊗Licensed

Promising results have also been obtained in cattle after immunization with ES products from *O. ostertagi* and *C. oncophora.* Vaccination of calves with *O. ostertagi* polyprotein allergen (OPA) induced a decrease in egg output during a 2-month period together with a reduction in worm length [60]. Similarly, immunization with cysteine protease enriched fractions (ES-thiol) from the ES material of adult worms combined with QuilA adjuvant, but not alum hydroxide ($Al(OH)_3$), resulted in a reduction of 60% in cumulative egg counts in a 2-month period and had a negative effect on worm size [61-63]. Analysis of the composition of the ES-thiol enriched fraction revealed the presence of vitellogenin, metalloproteases, profiling, annexin, translationally controlled tumor protein and additional cytosolic and metabolic proteins [66]. However, the most abundant constituents of this fraction were the activation-associated secreted proteins (ASP) [67]. Although their exact biological function is unknown, ASPs have been proposed to play a role in the transition from the free-living stage to the parasitic stage [68-70]. Additional analysis performed in *O. ostertagi* showed that the ASPs can be located in the reproductive tract of both male and female adult worms, suggesting a role in reproduction [71] Consequently, an enriched *O. ostertagi* ASP fraction was evaluated as vaccine candidate and demonstrated its potential after providing high levels of protection in vaccinated calves, reflected by an egg output reduction of 74% (Table 2) [63]. In this study, an additional ES-thiol cysteine protease (CP)-enriched fraction was tested and provided an 80% reduction in egg output, the highest level of protection ever achieved by vaccination against *O. ostertagi*. However, other proteins were also present in the fraction and therefore it is possible that CPs alone were not fully responsible for protection. Further experiments performed with the ASP-enriched fraction have had consistent results and confirmed once more the protective potential of these proteins against *O. ostertagi* infections in cattle (Table 2) [64].

Similar to *O. ostertagi*, vaccination with native antigens against *C. oncophora* has mainly focused on the use of ASP proteins. The ES products of *C. oncophora* can be sorted in 3 groups based upon their molecular weight, i.e. high, medium and low molecular weight fractions. The medium and low fractions were composed of a complex mix of antigens, while the high molecular weight (HMW) fraction was essentially constituted by pure double-domain ASP (dd-ASP) [72]. To date, only one study has evaluated the potential of these antigens as vaccine candidate against *C. oncophora* infections. Due to the high content on ASPs, the HMW fraction was selected for evaluation in a vaccination trial that comprised both experimental and natural infection. Vaccination with dd-ASP elicited very high levels of protection in animals infected experimentally (91% reduction in egg counts), and provided a 58,5% reduction in egg counts in animals naturally infected during a whole grazing season [65] (Table 2).

2.2 Recombinant vaccines

Although many of the abovementioned native vaccines have successfully provided protection to animals, an expensive and time-consuming process is required for their production, which includes the infection and subsequent slaughter of animals and a cold chain for the vaccine distribution. In addition, the possibility of batch differences cannot be ignored. Consequently, mimicking the protective response induced by native proteins with recombinantly produced antigens is essential for the progress of anti-parasitic vaccines. Although several studies have attempted to produce effective recombinant vaccines against helminths, to date, the only recombinant vaccine available on the market is the one produced in *Escherichia coli* against the cestode *Echinococcus granulosus* in cattle and sheep (Table 4) [73,74]. Other successful although not yet commercialized recombinant cestode vaccines include those against *Taenia saginata* and *Taenia ovis* [73]. Besides these examples, no recombinants have been able to achieve sufficient levels of protection to even be considered for commercialization. The main expression systems that have been utilized for the production of helminth recombinant vaccines are bacterial *(E. coli)*, baculovirus-insect cells, helminths (*C. elegans)* and yeast. Each system has advantages and disadvantages (Table 3) and therefore care must be taken when choosing one or another for recombinant expression of antigens [75,76].

Table 3. Advantages and disadvantages of the main expression systems utilized for the production of recombinant helminth vaccines.

Expression system	Advantages	Disadvantages
Insect cells /Baculovirus	- Secretion of proteins to medium - Proper protein folding - Simple glycosilations present	- Demanding culture conditions - Slow cell growth - Time consuming
Yeast	- Secretion of proteins to medium - Post-translational modifications present - Large yields - Simple media requirements	- High mannose glycosylation - Protein refolding might be required
Bacterial (*E. coli*)	- Simple culture conditions - Large yields - Low cost	- Protein solubility - Post-translational modifications absent
Helminth (*C. elegans)*	-Helminth expression system - Nematode-specific glycans present - Easy to grow and store	- Low level of expression - Although potentially scalable, this has never been done

While a highly effective native vaccine against *D. viviparus* is available, efforts have been made to produce a protective recombinant vaccine that will overcome the high costs and limited shelf life associated with this vaccine. Vaccination of calves with the *E. coli* produced recombinant paramyosin (rPMY) moderately reduced the larvae shedding and worm burden [77] (Table 4). A mixture of CPs against *H. contortus* expressed in *E. coli* was able to induce moderate protection of lambs, which was lower than the one provided by their native counterparts (Table 4) [78]. In addition, *E. coli*–expressed galectins Hco-gal-m and Hco-gal-f from *H. contortus* conferred moderate protection to adult goats [79]. Moreover, a cocktail comprised by the metalloendopeptidases (MEP1, MEP3, MEP4) and aspartyl proteases (PEP1) of the H-Gal-GP fraction of *H. contortus* expressed in baculovirus and *E. coli* respectively, failed to confer protection in sheep (Table 4) [80]. Recently, the gut antigen H11 from *H. contortus* was expressed in *C. elegans* and evaluated in a vaccine trial with unsatisfactory results [81]. On the contrary, a cocktail of 8 proteins identified as ES components from the sheep GI nematode *Teladorsagia circumcincta* were expressed in *E. coli* (n=6) and yeast (n=2) and provided significant levels of protection in lambs after vaccination [82] (Table 4).

Table 4. Recombinant vaccines against nematodes of ruminants.

Species	Host	Antigen/ Administration route	Reduction eggs/worms (%)	Reference
Echinococcus granulosus	Cattle Sheep	EG95/ s.c	n.m/99 n.m/98	73,74
Dictyocaulus viviparus	Cattle	rPMY [1]/ i.m	47-57/31-54	77
Taenia saginata	Cattle	TSA-9 + TSA-18 [1]/ i.m	n.m/90	73
Taenia ovis	Sheep	To45W [1]/ i.m To16 [1]/ i.m ⊗ To18 [1]/ i.m	n.m/75-100 n.m/73 n.m/99	73
Haemonchus contortus	Sheep	CP fraction [1]/ i.m	27/29	78
	Goat	Hco-gal-m/f [1]/ s.c	48/46	79
	Sheep	MEP1[2] + MEP3[2] + MEP4[2] + PEP1[1]/ i.m	-/-	80
	Sheep	H11[3]/ s.c	-/-	81
Teladorsagia circumcincta	Sheep	ES cocktail [1,4]/ s.c	58-70/56-75	82
Ostertagia ostertagi	Cattle	OPA [1]/ i.m	-/-	60
		ASP-1 [2]/ i.m	-/-	83

n.m=not measured, i.m=intramuscular, s.c=subcutaneous, i.p=intraperitoneal

[1]Expressed in *E. coli,* [2]Expressed in baculovirus, [3]Expressed in *C. elegans,* [4]Expressed in yeast
⊗Licensed

The first attempts to obtain a recombinant vaccine against *O. ostertagi* were made by expressing OPA in *E. coli*. Unlike the native form of the antigen, vaccination of calves with OPA could not induce protection or worm size reduction [60]. A common problem of expression in *E. coli* is the low solubility of the proteins [75], which is one of the reasons why the production of OPA in this system failed (Table 4) [60].

To date, only one more attempt has been made to produce a protective recombinant vaccine against *O. ostertagi*. ASP-1, the most abundant one from the ASP-enriched fraction [67], was produced in a baculovirus expression system and used to immunize cattle [83]. Recombinant ASP-1 not only failed to provide protection, it also failed to elicit cross-reactive antibodies against the native-ASP-1, suggesting an aberrant structure in the baculovirus produced recombinant (Table 4) [83]. In addition, no recombinant vaccines have been produced nor tested to date against *C. oncophora* infections in cattle.

Despite these discouraging outcomes for both *O. ostertagi* and *C. oncophora* recombinant vaccines, other expression systems, such as mammalian cells, plants and yeast have not yet been evaluated. Although the mammalian cell expression system is able to produce properly folded proteins, important disadvantages are present when using this tool for commercial production of recombinant vaccines i.e. the high cost of cell maintenance, the low levels of expression, and the slow cell growth [75]. Plants on the other hand can produce glycoproteins that are very homogenous in their N-glycan composition and are highly compliant to engineered adaptations of their N-glycosylation machinery [84]. In addition, they offer a potential for edible vaccines [85]. However, protein re-folding might be required upon production. Finally, the yeast expression system also offers a promising alternative for the production of *O. ostertagi* and *C. oncophora* recombinant vaccines. Yeast grow rapidly in low-cost media, which facilitates massive production of recombinant proteins. Despite their high mannose glycosylation pattern, novel glyco-engineered strains of yeast became available over the past years to circumvent this issue [86].

3. Immunity to gastrointestinal helminths

The immune system consists of a complex network of biological structures and processes whose ultimate goal is the defence of the host against infections. Mounting a proper immune response entails a highly-regulated series of steps namely (i) the immunological recognition of the infection, (ii) the induction of a proper effector response and (iii) the generation of an immunological memory to combat possible re-encounters with the infectious agent [87].

3.1 Lessons learned from gastrointestinal helminth infection models

The front line of defence against an infectious agent consists of the host´s physical and chemical barriers i.e. the skin and mucosal surfaces and their lysozymes and antimicrobial proteins present in the respiratory and genital tracts, and most important in our case, the gastrointestinal tract [88]. The gastrointestinal mucosa comprises the first barrier to gut-invading nematodes such as *O. ostertagi* and *C. oncophora*. The most important component of this layer are mucins secreted by goblet cells, and alterations on its composition can influence the course of an infection. For example, mice lacking mucin 5AC, which production increases during the course of a *Trichuris muris* infection, are unable to clear the parasite [89]. Together with an increase in mucus flow, higher smooth muscle contractility favours expulsion of parasites in a phenomenon known as "weep and sweep" [90]. Another important function of goblet cells in the gut is the production of the resistin-like molecule-β (RELMβ), a protein with direct anti-parasitic activity through its interference with chemotactic sensors present on helminths [91]. In regions like the stomach, changes in pH levels and the presence of hydrolytic enzymes create a hostile environment which also helps avoiding establishment of the parasite. Likewise, the intestine counts on the presence of lysolipids and defensins [87,92].

Once the parasite is able to overcome this initial physical and chemical barrier, it will become in contact with the sentinel cells of the innate immune system present in the epithelium and the lamina propria, such as intestinal epithelial cells (IEC) and dendritic cells (DCs). Upon encounter with the parasite and its products, IEC are capable of alerting the immune system through the production of the alarmins IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) [93]. Both IL-25 and IL-33 have been shown to be important in the induction of protective responses through their direct action on type-2 innate lymphoid cells (ILC2) and mast cells. Administration of IL-33 during *T. muris* infection can accelerate the parasite´s expulsion [94], while studies performed in IL-25 deficient mice

revealed that IL-25 is capable of limiting intestinal inflammation [95]. Interestingly, mast cells are both targets and source of alarmins [96,97], and their depletion in mice can impact the development of protective immunity against *Heligmosomoides polygyrus* and *T. muris* infections [97]. On the other hand, upon activation by alarmins, ILC2 release IL-5 and IL-13, which are involved in eosinophil activation and tissue repair respectively. Both cytokines are also released by T cells and therefore their role in helminth infections will be further discussed below.

Dendritic cells (DCs) comprise, together with macrophages and B cells, the so-called antigen presenting cells (APCs). APCs detect pathogen-associated molecular patterns (PAMPs) present in nematodes through their pattern-recognition receptors (PRRs). In contrast to viruses and bacteria, helminths do not seem to have a uniform PAMP array. Most of the gastrointestinal helminth PAMPs described to date belong to ES products and include the glycoprotein fraction from *Nippostrongylus brasiliensis* [98], phosphatidylserine (PS) lipids from *Ascaris lumbricoides*, ES-62 glycoprotein from *Acanthoceilonema viteae* [99,100] and calreticulin from *H. polygyrus* [101]. The PRRs involved in the recognition of these PAMPs comprise toll-like receptor 2 (TLR2) for PS lipids [102], TLR4 for ES-62 [100] and scavenger receptor A for calreticulin [101]. Other PRRs that have been proposed to recognise GI helminth PAMPs include mannose-binding receptors, collectins, and C-type lectins (DC-SIGN) [103,104]. Upon recognition of PAMPS, APCs will take up, process and present the nematode antigens to naïve CD4-T that reside in secondary lymphoid organs. Amongst all APCs, DC are considered the most potent APC able to priming naïve T cells in a process that requires the combination of 3 signals [105]. The first one involves the recognition of major histocompatibility complex-bound antigens by the T-cell receptor (TCR). The second signal is delivered when CD28 on the surface of T-cells enganges with CD80/86 on DC. Finally, DC will release specific cytokines that will drive the activation and differentiation of naïve T cells into different effector subsets (Figure 3) [105].

In the case of helminths, the response induced by DC is generally regarded as a T-helper 2 (T_H2) response (Figure 3). There are 4 subtypes of T_H cells and their specific function is determined by the type of expression of different transcription factors and the release of certain cytokines (Figure 3). Upon activation, T_H1 cells secrete pro-inflammatory cytokines (i.e IFNγ) that can activate cytotoxic T-cells, natural killer cells (NK) and macrophages, enabling them to eliminate infectious agents more efficiently [87]. This type of response is usually observed to combat viral or intracellular pathogen infections. However, for most nematode infections T_H1 responses provoke an inflammatory reaction that damages the mucosa and consequently increases the host´s susceptibility. For instance, administration of IFNγ to *N. brasiliensis* infected mice retarded the expulsion of the parasite [106], and studies using the GI mouse model *T. muris* indicate that T_H1 responses

are related to the establishment of chronic infections [107]. In contrast, T_H2 cells are known to be the main effectors involved in a protective immunity against multicellular parasites, most particularly helminths, through the secretion of IL-4, IL-5, IL9 and IL-13 [90]. The mode of action of T_H2 cells is further detailed below. The third and most recently discovered T_H effector subset is comprised of T_H17 cell, which mediate pro-inflammatory processes involved in autoimmunity and host defence against extracellular bacteria through the secretion of mainly IL-17 [87,108]. Some studies have revealed that T_H17 cells might have a similar function as T_H1 cells during helminth infections [109], and susceptibility to *T. circumcincta* in lambs has been correlated with the induction of a T_H17 response [110]

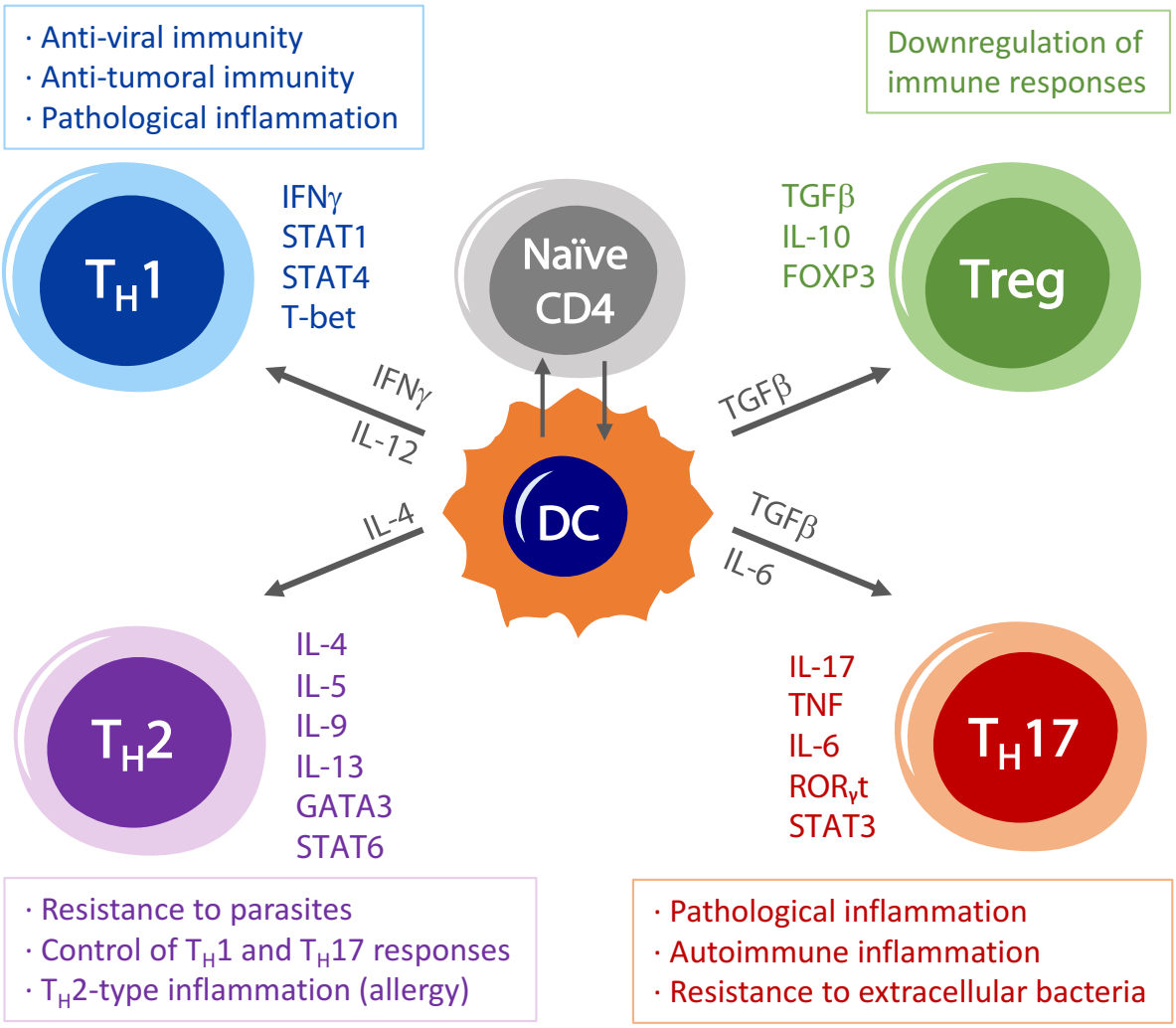

Figure 3. Overview of the cytokines released by dendritic cells (DC) involved in naïve T-helper cell activation and differentiation. Each differentiated subset is characterized by the expression of different transcription factors and cytokines that mediate their specific effector functions.

The last subset is comprised by the regulatory T cells (Treg), whose main role is the regulation of immunity by suppression of T-cell responses. They exert their function through the release of IL-10 and TGFβ and are essential to prevent the development of undesired autoimmunity [87,111]. In helminth infections, they have been suggested to dampen the host immune responses and avoid clearance of the parasite [112,113]. More specifically, studies performed in GI nematode models such as *Strongyloides ratti*, show that depletion of the Treg subsets increased the parasite specific T_H2 response and reduced parasite burden [114]. In addition, in humans it has been suggested that increased exposure to GI helminths promotes strong regulatory immune responses, since children repeatedly exposed to high levels to *A. lumbricoides* and *Trichuris trichuria* display higher levels of IL-10 and TGFβ after *in vitro* stimulation of lymphocytes [115]. During GI helminth infections, T_H2 cells, secrete a broad array of cytokines that will activate several effector cells (Figure 4). When successful, their joint action will result in the expulsion of parasites. The cytokines IL-13 and IL-4 play a pivotal role, since they can increase epithelial turnover, globlet cell differentiation and smooth muscle contractility through the ligation of IL-4R on the surface of IEC [107,116]. Their depletion in several models of GI helminth infection, such as *T. muris* [117], *H. polygyrus* [118] and *N. brasiliensis* [119,120], demonstrated their crucial role in parasite expulsion. Together with their cell and mucus repair functions, IL-13 and IL-4 recruit and activate alternatively-activated macrophages (M2) [121,122] and activate and induce B-cells for the production of IgE [90]. Unlike classical activated macrophages (M1), which interact with T_H1 cells and exert phagocytic activities, M2 macrophages are non-phagocytic and have been observed in multiple helminth infections i.e *H. polygyrus*, *N. brasiliensis* and *A. suum* [123]. They release the enzyme arginase, which intervenes in wound repair and increases smooth muscle contraction in the intestine to facilitate parasite expulsion [124]. For instance, depletion of macrophages during *N. brasiliensis* infection impairs muscle contractility and hence expulsion of the parasite [122]. Moreover, identical effects could be observed after inhibition of the IL-4 receptor alpha chain, which is involved in the signalling of both IL-13 and IL-4 [125].

Other important cytokines involved in the response against GI helminths are IL-5 and IL-9, whose secretion by T_H2 cells together with IL-13 induces the recruitment and activation of eosinophils, basophils and mast cells (Figure 4). A delay in *T. spiralis* expulsion can be observed in IL-5 deficient mice, who also display poor eosinophil infiltration in the mucosa [126], and similar effects could be observed in *T. muris* infected mice after administration of anti-IL-9 antibodies [127]. In both studies, the impaired parasite expulsion was related to an attenuated muscle contractility, highlighting the importance of these cytokines in a protective immune response against helminths.

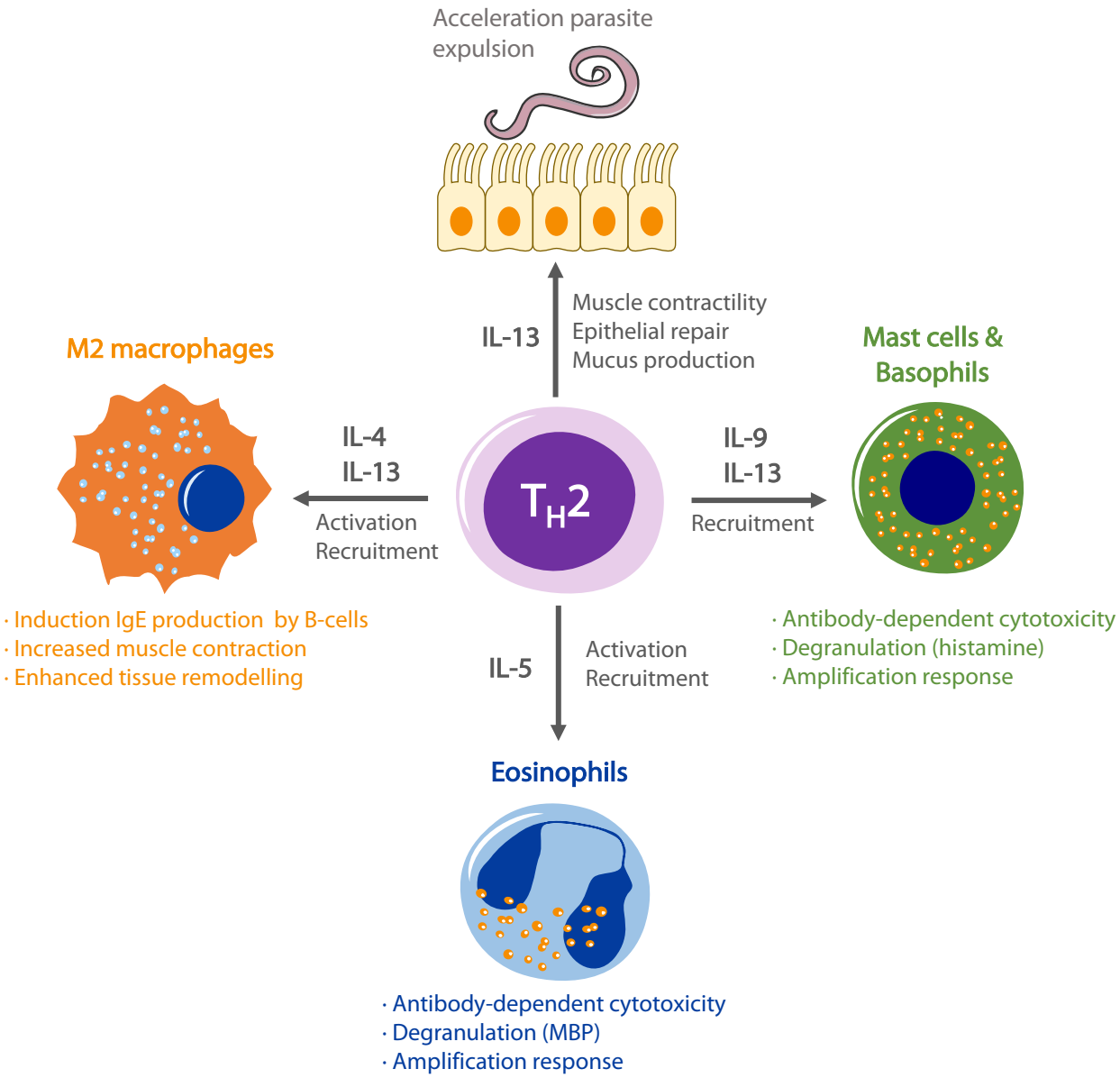

Figure 4. Common T-helper 2 (T_H2) protective response to intestinal helminths

Moreover, crosslinking of the high affinity receptor FcRεI present in eosinophils, basophils and mast cells via antigen-bound IgE induces degranulation [128] with the release of cytotoxic compounds (major basic protein (MBP)), immunomodulatory (histamine) and recruiting factors to amplify the response and help clear the infection [87,90] (Figure 4).. In addition to its cytotoxic role, MBP released by eosinophils promotes mast cell-degranulation in an IgE independent manner [129]. Despite their broad array of effector functions, *in vivo* depletion of these cell subsets does not seem to affect the proper development of a T_H2 response against helminths and their clearance [90], and therefore it is not clear to what extent these cells are essential in the defence against helminths.

Nevertheless, some studies indicate that higher susceptibility could be observed in primary infections of mice with *Strongyloides stercolaris* after eosinophil depletion [130] and with *Trichinella spiralis* after mast cell depletion [131].

Finally, helminth infections promote B cell class switching. The overall importance of antibody production during helminth infections (reviewed in[132]) has been demonstrated in B-cell deficient mice, where increased parasite burdens can be observed after experimental infection with *T. muris* [133] and *H. polygyrus* [134,135] amongst others. Moreover, several studies have demonstrated that protective immunity against *A. suum* [136], *S. ratti* [137], *T. muris* [133], *N. brasiliensis* [138], and *H. polygyrus* [118,135,138] can be passively transferred to naïve animals using immune serum[132]. However, the exact mechanisms by which B-cell produced antibodies contribute to the immunity against helminths are not fully understood. Several studies suggest that antibody-mediated cytotoxicity of immune cells in the gut mucosa and/or the blockage of enzymes released by the worms involved in larval migration and feeding contribute to protection [132]. For instance, it has been demonstrated that antibodies not only bind to parasitic structures and ES products of *T. spiralis*, but also are able to inhibit moulting and contribute to larval expulsion [139]. Although the mediation of IgE in the killing of *T. spiralis* L1 larvae has been demonstrated [140], its role in achieving protection against most of helminth infections seems less pivotal. On the contrary, IgA, IgM and specially IgG, stand out as the isotypes able to provide the most effective immunity [132]. For example, IgM has been correlated with the expulsion of filarial parasites [141] and passive transfer of monoclonal IgM specific for *Brugia malayi* confers protection after infection [142]. Moreover, passive protection against *T. spiralis* can be achieved with specific monoclonal IgA [143,144] and IgG [139,145]. Lastly, IgG antibodies provide the most effective protective immunity against *H. polygyrus* [134] .

3.2 Immunity to ruminant GI helminths

As previously depicted, immunity to helminths has been generally regarded as a type-2 response, essentially due to the observations made in murine models of infection. Although parasite-specific antibodies are detected after infection with ruminant GI helminths, limited information is available for most species on the specific immune responses occurring during the course of an infection.

Infection of lambs with *H. contortus* induces an increase in abomasal pH which facilitates the parasite´s egg production [146,147]. During infection, both a rapid and a delayed rejection of the infection can be observed. The first one has been associated with exclusion of larvae from the tissue by globule leukocytes [148], while the delayed rejection of *H. contortus* has been related to the infiltration of larvae in the abomasal tissues

accompanied by an increase in CD4-T cells, γδ-T cells, B cells and eosinophils [149]. Depletion of CD4 but not CD8-T cells in *H. contortus*-resistant sheep abolished protection towards infection together with a reduction in mucosal mast cells and eosinophils [50]. In addition, it has been shown that *H. contortus*-resistant breeds are able to produce higher levels of the type-2 cytokines IL4, IL13 and IL-5 [150]. Likewise, mast cells and eosinophils numbers are increased during *T. cincumcincta* infection [151] and transcriptomic analysis of abomasal lymph node cells of infected sheep revealed augmented levels of IL-4, IL-13, IL-5, IL-10 and TGFβ [152].

Despite these examples, immunity to some GI helminths of ruminants does not seem to follow the clear-cut profile of a type-2 response. This is especially the case for *O. ostertagi* immune responses, which will be detailed below.

Natural immunity to O. ostertagi

Natural immunity against *O. ostertagi* infections is generally weak and develops slowly, hence animals tend to remain susceptible until at least 2 years of age, when acquired immunity starts to be more evident [153,154]. Even then, the presence of low numbers of larvae in animals after several grazing seasons indicates that achieving sterilising immunity is unusual and improbable [155]. Although not sterilizing, protective immunity against *O. ostertagi* reduces the pathophysiological reaction caused by the establishment of larvae in the gastric glands and directly affects pasture contamination by impairing the egg production by female worms. In addition, morphological changes in the worms (i.e stunting of adult worms) and a reduction in their number are observed after primary infections [154,156].

It has been suggested that the weak and delayed immunity observed in *O. ostertagi* infections is caused by the suboptimal antigenic presentation that occurs in the abomasum in comparison with other gut regions i.e. the small intestine, where immunity against *C. oncophora* infections develops faster. However, the observation of a massive increase in size of the abomasal LNs due to lymphocyte proliferation argues against this hypothesis [154,157]. Together with this increase in the size of the abomasal LNs, immune responses against an *O. ostertagi* infection are typically characterized by mucosal eosinophilia and mastocytosis [155,158]. The increase in the abomasal LNs has been associated to the parasite-specific proliferation of lymphocytes, particularly of B cells, γδ-T cells and natural-killer (NK) cells [157,159,160].

Transcriptomic analysis of the abomasal mucosa performed by Mihi et. al. revealed an upregulation of *CMA1* and *NCR1*, expressed by γδ-T cells and NK cells respectively [158]. Other upregulated genes observed in this study include the T_H1-related cytotoxic factors

granulysin (*GNLY*), granzyme B (*GZMB*) and perforin (*PRF1*), which are known to be produced by globule leucocytes, cytotoxic T-cells and NK cells [64]. Further microarray studies by Li et al. performed in the abomasal mucosa after *O. ostertagi* primary and subsequent challenge infections demonstrate the upregulation of genes related to the complement system and the formation and transepithelial transport of IgA and IgM antibodies [161]. The complement system is regarded as part of the innate immune system and it is comprised by numerous plasma proteins that react with one another to mediate pathogen opsonisation and inflammatory reactions [87]. The complement system involvement in early responses against several pathogens, including parasitic helminths, and its down-regulation by parasites as evasion mechanism has been described [103,162,163].

In terms of cytokine production, early studies by Canals et al. on the cytokine profile of abomasal LNs after primary infection revealed a mixed T_H1/T_H2 response characterized by an upregulation of the IL-4, IL-10 and IFNγ expression levels [164]. Similar observations were made by Almeria et al. in the lamina propria [165]. Additional studies have shown that, although only an increase of T_H2 cytokines could be observed in the LNs, the abovementioned mixed T_H1/T_H2 expression profile is conserved in the abomasal mucosa [166]. More recent studies aimed to elucidate and compare early and late cytokine dynamics in the abomasal mucosa after an *O. ostertagi* infection [158]. The broader panel of cytokines used in this particular study allowed the observation of a mixed T_H1/T_H2 response after single and trickle infection mainly characterized by the upregulation of IFNγ, IL-18, IL-4, IL-9, IL-10 and IL-13, together with the T_H17-type cytokine IL-17. Importantly, the major changes in cytokine expression coincided with the emergence of the larvae from the gastric glands [158].

Humoral immunity against *O. ostertagi* infections is characterized by the presence of increased levels of IgG1, IgG2, IgM, IgA, and lymphatic IgE [167,168]. Their implication in protection has been suggested due to the inverse correlation between antibody levels and worm number and the fast increase in *O. ostertagi*-specific antibodies in second year grazers [167,169]. Interestingly, it has been suggested that T_H2-associated IgG1, antibodies, which rise fast and are the most abundant amongst all antibody types, are indicative of the presence of an infection. On the contrary, T_H1-type IgG2 antibodies are suggested to be correlated with a protective immune response [170]. In line with this, several studies have demonstrated the direct and/or indirect implications of antibodies against gastrointestinal nematode infections over the years [132]. This protective activity would potentially be achieved through antibody-dependent cellular activation of immune cells, or by interfering with larval invasion, migration and feeding processes [132]. Nevertheless, the exact mode of action of antibodies during the course of an *O. ostertagi* infection remains to be elucidated.

Natural immunity to C. oncophora

In contrast with the vast amount of studies aimed to elucidate the immune responses to *O. ostertagi* infections, information on the development of immunity against *C. oncophora* has been somewhat limited as this parasite is often regarded as a mild pathogen. Initial studies revealed an increase in IgA and IgG2 containing cells in the mucosa during primary and secondary infection [170]. Additionally, a series of papers by Kanobana et. al. aimed to further describe the immunological events that occur during the course of a *C. oncophora* infection [171-174]. The high variability in the egg output and worm counts detected in calves after primary infection with *C. oncophora* allows the distinction of 3 animal responder phenotypes termed high, intermediate and low [174]. Although no differences could be detected in peripheral blood lymphocyte and eosinophil counts, intermediate responders showed higher levels of *C. oncophora*-specific serum IgG1 and IgA after primary and challenge infection which correlated negatively with the parasitological parameters [172,174]. In addition, the expulsion of larvae is correlated with an increase in mucosal eosinophils and IgA levels [173]. As previously hypothesized for *O. ostertagi* infections, this observation suggests a role for antibodies in protection against *C. oncophora* [174]. Analysis of the cellular responses revealed a higher proportion of CD4-T cells in blood and LNs but not in the lamina propria of infected animals, and the generation of memory B cells which were rapidly recruited to the gut upon challenge infection [172,174]. Moreover, the increased expression in B cells of the T-cell co-stimulatory protein CD86 suggests that interactions between CD86 and T-cells might be involved in the development of protective immunity against *C. oncophora* [172]. Additional studies revealed two peaks of eosinophil influx at the site of infection which coincided with a primary and secondary infection. It was therefore proposed that the first increase in eosinophils would be a mere consequence of early inflammatory events while the second wave, which concurred with a more pronounced increase in CD4-Tcells, was T-cell dependent and is involved in the effector mechanisms against an infection with *C. oncophora* [171].

Finally, microarray analysis performed by Li and Gasbarre on small intestinal tissue after a single infection showed the upregulation of the T_H2-type cytokines IL-4, IL-5 and IL-13 [175]. Additional transcriptomic analysis of the small intestine after primary and secondary infections with *C. oncophora* revealed the upregulation of the vitamin D receptor and inducible nitric oxide synthase (NOS) only during secondary infections, suggesting a role in acquired resistance [176]. Vitamin D seems to favour the production of T_H2-type cytokines while NOS is considered essential in host defence due to its cytotoxic and immunoregulatory properties [176,177]. More recently, additional mechanisms of resistance

against *C. oncophora* have been indentify which include the upregulation of mucin 12 (MUC12), alkaline phosphatase (ALPI) and 3 lysozymes [178]. These later have been hypothesized to maintain mucosal inflammation that contributes with resistance [178], while ALPI is a known mucosal defence factor [179].

Vaccine-induced immune responses

As described in the previous sections, several attempts with more or less success have been made to develop protective vaccines against the bovine gastrointestinal nematodes *O. ostertagi* and *C. oncophora*. The reduction of egg output and the impact on worm counts and size observed for some of the previous studies are a direct consequence of the immune response elicited by vaccination, and therefore a thorough analysis of the vaccine-induced immunity can help unravel the key to the success of a vaccine.

Important parameters measured after vaccination are antigen-specific antibody levels. The presence of antibodies is a sign of response by the host to the vaccine. Moreover, in many cases, the antigen-specific antibody production is directly correlated with parasitological parameters that define protection. However, in many other cases antibody levels and protection are not correlated.

In the context of *O. ostertagi* vaccination, the first attempts made with cysteine protease (CP)-enriched fractions induced a significant increase in antigen-specific IgG1 and IgG2 antibodies in the abomasal mucosa, which had a negative, although weak, correlation with worm size and egg output [61]. Intramuscular vaccination with ES-thiol fractions adjuvanted with QuilA and $Al(OH)_3$ raised specific IgG1 and IgG2 antibodies in the abomasum, while IgA could only be detected in animals vaccinated with ES-thiol+QuilA [62]. Note that, unlike ES-thiol+QuilA, the formulation adjuvanted with $Al(OH)_3$ did not provide protection to animals despite raising antigen-specific antibodies. The exact mechanisms by which both adjuvants act are however not completely understood. $Al(OH)_3$ is regarded type-2 response adjuvant and it is believed to exert a reservoir function, from which antigen is released slowly for a prolonged period of time [180]. Saponin-based adjuvants such as QuilA exert a more cytolytic function and are associated with mixed type-1/type-2 responses [181]. Abomasal levels of IgG1 and IgG2 were also significantly elevated in animals vaccinated intramuscularly with OPA and were negatively correlated with worm size and egg output [60]. Intramuscular immunisation of animals with the ASP fraction enriched from the ES-thiol products raised ASP-specific IgG1 and IgG2 antibodies both in serum and mucosa [64]. Although no significant changes could be observed in the number of immune cells in the abomasum, the amount of globule leukocytes (GLs) was high. GLs are immune cells that mainly reside in the small intestine

and the stomach [182] and have been previously associated with resistance to *O. ostertagi* and other helminths [183-186]. Although the exact functions of GLs are not fully understood, a recent study in mice by Vogel et al. suggests that GLs are associated with a decreased mucosal inflammation and therefore have a role controlling helminth-induced immunopathology [187]. Transcriptomic analysis of the abomasal mucosa revealed the upregulation of granulysin and granzyme B gene expression, which in addition were negatively correlated with worm counts and egg output and positively correlated with the IgE receptor subunit. Granulysin and granzyme B are known pro-apoptotic molecules mainly produced by NK cells and cytotoxic T-cells. Moreover, histological analysis showed the co-localisation of granulysin in the granules of GLs [64].

The vaccine-induced immunity after vaccination with *C. oncophora* ASP products has also been evaluated in one study. Van Meulder et al. observed that ASP-specific levels of IgG1, IgG2 and IgA were elevated in serum, while only IgG1 was significantly higher in the mucosa [188]. In animals vaccinated intramuscularly, $\alpha\beta$-T cells, $\gamma\delta$-T cells and B cells from the mesenteric lymph nodes proliferated upon in-vitro re-stimulation with ASP. Correlation analysis revealed that IgG1 levels correlate negatively with egg counts, while proliferation of $\gamma\delta$-T cells was positively correlated with the proportion of immature larval stages [188].

4. Conclusion

O. ostertagi and *C. oncophora* remain the most common GI nematodes in cattle in temperate climate areas, where they pose a major constrain on animal welfare and economy. The emerging resistance of the worms to the current anthelmintic control methods urges the search for sustainable and effective alternatives. Vaccines have been put forward as the most sustainable and cost-effective method to control multiple infectious diseases, and several attempts have been made to develop protective vaccines against GI nematodes in ruminants.

Overall, the success of a vaccine depends on its capacity to activate the correct arm of the immune system in the host, which can be monitored throughout the process. A deeper comprehension of the immunological processes that are provoked by vaccination and correlate with protection will aid in the development of effective vaccines against GI nematodes.

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Objectives

Objectives

O. ostertagi and *C. oncophora* pose a major constraint on animal welfare and production. The emerging resistance to the current anthelmintic control methods urges the development of sustainable alternatives such as vaccines. Unfortunately, most of the recombinantly produced vaccine antigens against GI nematodes fail to provide protection when compared to their native counterparts. Therefore, elucidating and comprehending the vaccine-induced immune responses that correlate with protection is of great importance in the development of effective vaccines.

Overall, the **main goal** of the present thesis was to unravel the immune responses associated with protection following vaccination with ASP-based vaccines against the gastrointestinal nematodes *O. ostertagi* (Chapter 2) and *C. oncophora* (Chapter 3). More specifically, this work aimed to analyze and compare the effect of antigen (native vs. recombinant) and adjuvant on the cellular and humoral responses in both cattle and mice.

In addition, the responses elicited by *O. ostertagi* and *C. oncophora* antigens (native vs. recombinant) and adjuvant in bovine dendritic cells was studied (Chapter 4). These antigen-presenting cells are one of the first immune cells encountering the vaccine antigens following vaccination. Therefore, their way of activation could define the subsequent immune responses ultimately leading to protection.

2

Host protective ASP-based vaccine against the parasitic nematode *Ostertagia ostertagi* triggers NK cell activation and mixed IgG1/IgG2 response

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Host protective ASP-based vaccine against the parasitic nematode *O. ostertagi* triggers NK cell activation and mixed IgG1/IgG2 responses

1. Introduction

Helminth infections pose a massive burden on human and animal health worldwide. Despite the widespread development of drug resistant worms, anthelmintic treatment still remains the main method to control these infections [1,2]. Vaccination strategies, either targeting the reduction in adult worm numbers present in the host or the reduction of worm fecundity, offer a promising alternative for anthelmintic treatment [3,4]. Nevertheless, hitherto only few vaccines against this type of pathogens are available. Two of the commercially available vaccines target the cattle and sheep lungworms *Dictyocaulus viviparus* [5,6] and *Dictyocaulus filaria* [7], respectively, and are based on whole irradiated larvae of these worms. Recently a promising vaccine against the blood feeding nematode *Haemonchus contortus* in sheep, based on native antigens isolated from adult worms, was commercialized[8]. However, these examples of vaccines are exceptions. Due to the complex life cycle of helminths, there are many practical issues and high costs involved in the production of high quantities of these vaccines. Therefore, mimicking the protective response by recombinant antigens would provide a major breakthrough in parasite vaccine development. Although this approach has already proven successful for the production of protective vaccines against the cestodes *Taenia saginata* and *Echinococcus granulosus* [9], and the nematode *Teladorsagia circumcincta* [10], it has been unsuccessful in many other cases [11]. In recent years, our research group has developed an experimental vaccine against the mucus dwelling abomasal nematode *Ostertagia ostertagi* in cattle [12-18], which is based on activation-associated secreted proteins (ASP). Intramuscular immunization of cattle with the native ASP (nASP) in combination with QuilA adjuvant raises an effective immune response, resulting in a significant reduction in faecal worm egg shedding of 56-74% during a two-month period [17]. A reduction in worm fecundity is typically the first manifestation of immunity against this parasite. Such decrease can significantly affect pasture infection levels and prevent parasitic gastroenteritis. A similar protective response is however not observed when the native antigen is replaced by a recombinant version produced in insect cells [14]. Furthermore, replacing the QuilA adjuvant by $Al(OH)_3$ has also shown to completely abolish the protective effect of the native antigen [16], indicating that both the antigen and the adjuvant are essential to achieve protection. Understanding how immunity in animals, vaccinated with the nASP-QuilA vaccine, is orchestrated might help to identify the essential features that are needed to induce protection, information which is crucial to direct future recombinant expression work.

Previous research has shown that potential effector mechanisms involved in the vaccine-induced protection are antigen-specific IgG1 and IgG2 antibodies in the abomasal mucosa and increased levels of granule exocytosis, involving the local release of granulysin and granzyme B [18]. Information on the upstream mechanisms triggered by the vaccine and how these are influenced by antigen and adjuvant is still missing. Therefore, the overall aim of the present study was to analyse and compare the effect of both antigen (native vs recombinant) and adjuvant (QuilA vs $Al(OH)_3$) on the cellular and humoral vaccine-induced immune responses.

2. Materials & Methods

Native and recombinant antigen production

For the production of nASP, helminth-naive calves were infected with 200,000 *O. ostertagi* infective larvae (L3) and euthanized 3 weeks later to collect adult worms from the abomasum. Adult worms were cultured, and excretory-secretory proteins collected from the culture supernatant as previously described [17]. The ASP sub-fraction was purified from total adult ES material by thiol-sepharose chromatography, followed by anion exchange chromatography [17]. The protein profile of the obtained material was checked by separation on a 10% SDS-PAGE gel under reducing/denaturing conditions and visualized by Coomassie blue staining. Reduction of nASP was achieved by incubating nASP with 7.5 mM dithiothreitol (DTT) for 15 minutes at 60°C. Afterwards, the nASP was incubated in iodo acetamide (IAA) at 37°C for an additional 30 minutes followed by a dialysis against 150 mM phosphate-buffered saline (PBS). Recombinant ASP was produced in *Pichia pastoris* as previously described [19] and will be referred to as pASP.

Immunization experiments in cattle

All animal experiments were conducted in accordance with the E.U. Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical approvals to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2009/105, EC 2011/183). Two vaccination studies were carried out in cattle, essentially as previously described [13,16]. The aim of study 1 was to analyse and compare the humoral responses induced by the native and recombinant antigens in combination with QuilA.

Twenty-one male crossbreed Holstein calves (6 to 8 months of age) were randomly divided over three groups of 7 animals (QuilA control, pASP+QuilA and nASP+QuilA). A second study was performed to analyse and compare the cellular responses induced by the different versions of the vaccine, i.e. nASP+QuilA, pASP+QuilA, nASP+$Al(OH)_3$, in comparison to control animals vaccinated with PBS. For this study, a Holstein crossbreed population of 16 male helminth-free calves (6 to 8 months of age) was randomly divided into four groups of four animals (PBS control, nASP+QuilA, pASP+QuilA and nASP+$Al(OH)_3$). For both studies, all animals were immunized three times intramuscularly in the neck with a 3-week interval. Control animals received either 750 µg of QuilA (study 1) or 1 ml of PBS (study 2) per immunization, while the animals of the nASP+QuilA, pASP+QuilA and nASP+$Al(OH)_3$ groups received 30 µg of antigen in combination with either 750 µg of QuilA or an equal volume of $Al(OH)_3$ adjuvants (both from Superfos Biosector, Denmark) per immunization. All animals were challenged with a trickle infection of 25,000 L3 larvae (1000 L3/day; 5 days/week, during 5 weeks), which started at the day of the third immunization. Calves from study 1 were euthanized 3 weeks after the last infection, while the animals from study 2 were euthanized immediately after the last infection. Parasitological parameters (i.e. FEC and worm counts) were analysed as described in previous trials [13,16]. Additionally, in study 2, abomasal LNs, abomasal epithelium and lamina propria were also isolated at time of necropsy.

Immunization experiments in mice

All animal experiments were conducted in accordance with the E.U. Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical approvals to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2014/33 and EC 2014/104). Thirty-six 8-week-old C57BL/6N mice (Harlan laboratories) were divided into 6 groups of 6 animals each and immunized three times in a 3-week interval intramuscularly in the thigh muscle. Control groups received 20 µg of QuilA or $Al(OH)_3$ (same volume as used in the antigen-vaccinated group) per immunization. Groups that included antigen in the vaccine formulation received either 5 µg of nASP, pASP or reduced-nASP in combination with 20 µg of QuilA adjuvant, while the last group received 5 µg of nASP in combination with an equal volume of $Al(OH)_3$ per immunization. At time of necropsy (one week after the third immunization), spleens and blood from the retro-orbital vein were collected for cellular and humoral analyses.

Isolation of mononuclear cells

For cattle, PBMCs were isolated weekly from blood by Lymphoprep (Nycomed Pharma) gradient centrifugation. Lymph node MCs were isolated from the draining LNs of the abomasum by homogenization through mechanical disruption of the tissue followed by a Lymphoprep gradient centrifugation. Mucosal MCs were isolated by removing the mucus from the abomasum and separating the mucosa from the submucosa. The mucosa was then cut into pieces of approximately 1 cm and extensively washed in Calcium and Magnesium free (CMF) HBSS (Invitrogen) containing 2 mM DTT (Biosolve). The DTT was removed by washing the tissue with CMF HBSS, after which the tissue was incubated for 30 minutes at 37°C in CMF HBSS containing 5mM EDTA (Invitrogen), while gently stirring. The cells in the supernatant were collected and washed with CMF HBSS. The remaining tissue was washed with CMF HBSS and afterwards minced into small pieces of approximately 2 mm and digested with 2 mg/ml collagenase type-I (Invitrogen) for 1 hour at 37°C, while gently stirring. The cells in the supernatant were collected and washed with CMF HBSS and added to the cell pellet that was obtained earlier (see above). All cells were resuspended in 40% Percoll (GE Healthcare) and layered over a 62% Percoll layer. After centrifugation, all mononuclear cell fractions were isolated, washed and counted prior to cell culture or flow cytometric analysis. To isolate MCs from mice, spleens were removed, mechanically disrupted and homogenized, and passed through a 70 µm cell strainer (BD Biosciences). Erythrocytes were lysed with ACK lysing buffer (Invitrogen) and remaining cells were washed and counted prior to cell culture or flow cytometry analysis.

Antibodies and Flow Cytometry

Cells were labelled in PBS containing 1% Bovine Serum Albumin (BSA), 0.1% Na-azide (both from Sigma-Aldrich) and the antibodies at the concentration recommended by the supplier. After an initial incubation of 20 minutes, the cells were washed and, whenever necessary, subsequently stained with fluorescently labelled secondary antibodies at the concentration recommended by the supplier. The cells were then incubated for 20 minutes prior to washing and resuspension in PBS, and immediately analysed using either FACS Canto or FACS Aria III flow cytometers (BD Biosciences). Non-viable cells were excluded of the analysis based on their propidium iodide (Molecular Probes) uptake.

Primary antibodies used in cattle were: non-labeled CD3 (MM1A, IgG1), TCRγδ (GB21A, IgG2b), CD21 (BAQ15A, IgM) (all from VMRD) and Alexa Fluor 488-labeled CD335 (AKS1, IgG1, AbD Serotec). Secondary antibodies used were: goat anti-mouse IgG1-FITC

(Santa Cruz Biotechnology), goat anti-mouse IgG2a-APC (Invitrogen), rat anti-mouse IgG1-APC (BD Biosciences), rat anti-mouse IgG2b-FITC (Southern Biotech), rat anti-mouse IgM-APCCy7 (Biolegend) and goat anti-mouse IgG2a-PE (Invitrogen). Antibodies used for mice were: CD3-PECy7 (clone 145-2C11), CD4-Biotin (clone RM4-5) and CD8a-PECy7 (clone 53-6.7) from eBioscience, and CD3-APC (clone 145-2C11), CD335-AlexaFluor647 (clone 29A1.4), CD19-Biotin (clone 1D3) and TCRγδ-FITC (clone GL3) from BD Biosciences. Biotin-conjugated antibodies were fluorescently labelled using streptavidin-APC-eFluor780 (eBioscience).

Proliferation assays

The MCs collected from mice and cattle were used in proliferation assays using either ^{3}H-thymidine incorporation or PKH (Sigma-Aldrich) fluorescence intensity reduction as a read-out. For ^{3}HT uptake experiments, 2.5x10^{5} cells were loaded per well in a 96-well round bottom plate (Thermo Scientific) in 200 µl of complete medium composed of RPMI 1640 + GlutaMAX (Invitrogen) supplemented with 50 µg/ml Gentamycin (Invitrogen), 50 µM β-mercaptoethanol (Sigma-Aldrich) and 10% fetal calf serum (Moregate). Each well was either stimulated with medium alone, 5 µg/ml nASP, 5 µg/ml pASP or 1 µg/ml ConA (Sigma-Aldrich), which served as positive control. Each condition was performed in triplicate. After 4 days of culture (cattle) or 5 days of culture (mice), cells were pulsed with 1 µCi ^{3}HT (Perkin Elmer). After an additional 18 hours of culture, cells were harvested and analysed with a 1450 Microbeta β-scintillation counter (Perkin Elmer). Results are shown as stimulation index (SI), which is the ratio of the counts per minute of cells cultured with the vaccine antigen and the counts per minute of cells cultured with medium alone, or as counts per minute (CPM). For the PKH experiments, cells were labelled with PKH26 (Sigma-Aldrich) according to the manufacturer's instructions with the only difference that PKH26 was used at a dilution of 1/125. After labelling, a small fraction of the cells was used for flow cytometric analysis to determine the starting intensity of PKH. The rest of the cells were seeded at 2.5x10^{5} cells/200 µl RPMI complete medium (cattle) or at 5x10^{5} cells/200µl RPMI complete medium (mice) in 96-well round bottom plates, either stimulated with medium alone, 5 µg/ml nASP or 5 µg/ml pASP. After 5 days of culture, the cells were harvested, stained with monoclonal antibodies and analysed by flow cytometry. ModFit LT software (Verity Software House) was used to calculate the proliferation index for the different cell populations, based on the PKH data.

In order to measure cytokine production during the proliferation of bovine PBMCs, blood was collected 2 weeks after the second vaccination in study 2 and PBMCs were cultured in 24-well plates (BD Bioscience) at 1x10^{6} cells/ml in RPMI complete medium and

re-stimulated *in vitro* with medium alone or 5 µg/ml of nASP for the nASP+QuilA, nASP+Al(OH)$_3$ and PBS groups, or pASP for the pASP+QuilA group. After 5 days of culture, supernatants were harvested and used on an ELISA. IFNγ and IL4 capture monoclonal antibodies were coated in carbonate buffer (pH 9.6) on 96-well Maxisorp (Nunc) plates at a concentration of 2 and 4µg/ml, respectively. Plates were blocked with 2% BSA in PBS for one hour at room temperature. Afterwards, 50 µl of supernatant was administered to each well, and medium with a known concentration of either bovine IFNγ or IL4 was used as a standard. All conditions were performed in duplicate. Biotinylated monoclonal antibodies raised against IFNγ or IL4 (kindly provided by Prof. Jayne Hope, The Roslin Institute, UK) were added at a concentration of 2µg/ml. Streptavidin-horseradish-peroxidase (Sigma-Aldrich) was used as a conjugate. O-phenylenediamine (Sigma-Aldrich) 0.1% in citrate buffer (pH 5.0) served as substrate. Optical density was measured at 492 nm. OD values were converted to concentrations using Deltasoft JV software (Deltasoft).

Histology and cell counts

Abomasal tissue samples from the calves used in study 2 were collected and used for histochemical and immunostainings as previously described [18]. Globule leukocytes and mast cells were identified using Sirius Red staining (Polysciences inc.) and a Toluidine Blue staining (Sigma-Aldrich), respectively. The primary antibodies used for immunostainings were rabbit polyclonal anti-human CD3 (Dako), rabbit polyclonal anti-human CD20 (Thermo Scientific) and mouse IgG1 anti-human MAC387 (AbD Serotec). Secondary antibodies used were goat anti-rabbit IgG-biotin (Dako) or Goat anti-mouse IgG-biotin (Dako), diluted in PBS 2% BSA, according to the manufacturer's instructions. Afterwards, sections were stained with the peroxidase-streptavidine complex (Dakocytomation A/S), diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich) and H_2O_2 (Calbiochem) followed by counterstaining with haematoxylin. All sections were mounted in synthetic medium DPX prior to analysis. B cells (CD20), T cells (CD3) and macrophages/monocytes (MAC387) were quantified by taking 4 random pictures per tissue slide at 200x magnification and calculating the number of cells per mm^2 tissue surface. Quantification of globule leucocytes and mast cells was carried out by counting the number of Sirius Red or Toluidine blue positive cells, respectively, present in 8 random fields of view per tissue slide at 400-x magnification. Cell counts were conducted for all animals of each group of vaccinated and infected animals. The results are expressed as cells/mm^2.

ELISA

For cattle, the systemic and abomasal IgG1, IgG2, and IgA levels against *O. ostertagi* nASP were determined by ELISA. nASP was coated in 96-well Maxisorp plates (Nunc) at a concentration of 0.5 µg/ml in carbonate buffer (pH 9.6). After 1 h blocking with 2% BSA-PBS at room temperature, either 100 µg mucus extract in PBS or 100 µl of a 1/200 serum dilution were administered in duplicate and incubated for 1h. After washing 3 times with 0.05% Tween 20-PBS, sheep anti-bovine IgG1, IgG2 and IgA (AbD Serotec) coupled to HRP were used as conjugates (dilution 1/500). O-phenylenediamine 0.1 % in citrate buffer (pH 5.0) served as substrate. Optical density was measured at 492 nm. Antibody responses in mice were measured by coating 96-well Maxisorp plates with nASP at a concentration of 5 µg/ml in carbonate buffer (pH 9.6). Following blocking with 2% BSA-PBS, 100 µl of a 1/200 serum dilution (were tested in duplicate. After washing 3 times with 0.05% Tween 20-PBS, rabbit anti-mouse IgG1, IgG2a (dilution 1/2000) and IgG2b (dilution 1/1000)(Sigma-Aldrich) and rat anti-mouse IgE (dilution 1/1000), all coupled to HRP, were used as conjugates and 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in ABTS buffer (Roche) was used as substrate. Optical density was measured at 405nm.

An inhibition ELISA assay was subsequently designed to evaluate the specificity of the antibodies raised in cattle. Serum samples from the nASP+QuilA vaccinated animals from study 1 were collected at one week after the final vaccination and used in an inhibition ELISA assay. Serum samples from all animals were pooled, diluted 1/200 and subsequently incubated for 1 h at room temperature with different concentrations of either nASP or pASP, ranging from 0 up to 500 pmol/ml, before being analysed by ELISA as described above.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism software. A non-parametric Kruskal-Wallis test was used to determine significant differences in parasitological parameters, cytokine production, antibody responses, cattle ^{3}HT assays and cell frequencies between vaccinated and control groups. For the cattle ^{3}HT assays, an additional ANCOVA (SPSS, IBM SPSS statistics version 23.0) was used to test correlation between weeks with no significant results. To determine significant differences in proliferation between the various cell subsets in the cattle abomasal MCs and mice spleen MCs, non-parametric Wilcoxon test was used. Finally, a non-parametric Friedman test was used to determine the statistical significance of the ^{3}HT assays performed in mice where more than two groups were involved. A *P*-value of ≤ 0.05 was considered significant.

3. Results

Vaccination with nASP+QuilA, but not pASP+QuilA or nASP+Al(OH)$_3$, reduces worm egg production while increasing IgG1 and IgG2 antibody levels.

Animals vaccinated in study 1 with the nASP+QuilA vaccine showed a significant ($p \leq 0.05$) reduction of 59% in cumulative egg output compared to the control vaccinated group. This confirms our previous findings [17]. In contrast, no reduction of faecal egg counts (FEC) was observed following vaccination with pASP+QuilA (Figure 1A). Similar to the observations made in study 1, animals from study 2 vaccinated with nASP+QuilA vaccine showed a non-significant reduction of 42% in cumulative FEC compared with the control vaccinated group, whereas no reduction of FEC was observed in the pASP+QuilA and nASP+Al(OH)$_3$ vaccinated groups (Figure 1B). For both studies, vaccination had no effect on worm counts (data not shown).

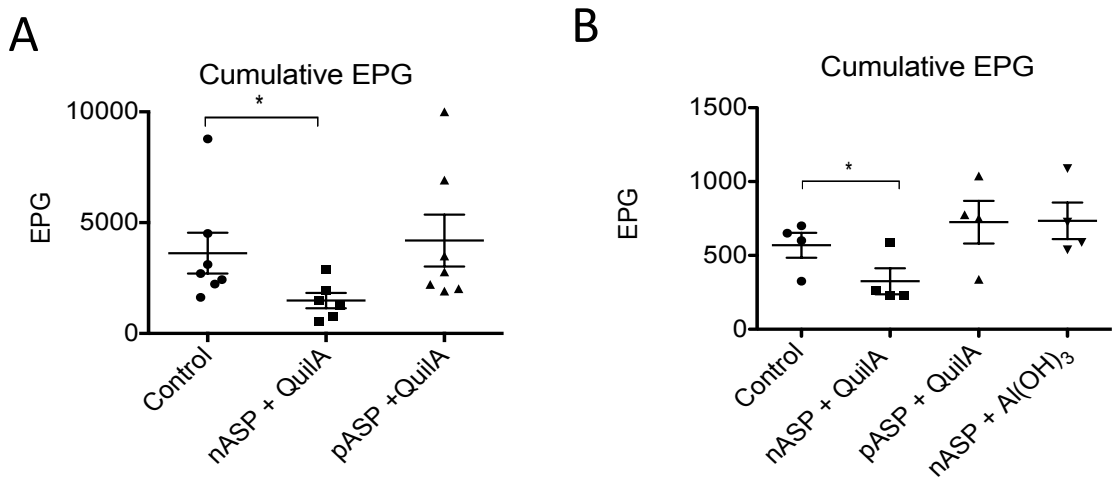

Figure 1. Parasitological parameters. Faecal egg counts (FEC) were determined 3 times each week during the trickle infection until the time of necropsy, and expressed as number of eggs per gram faeces (EPG). For study 1 (A) and study 2 (B), mean cumulative FEC are shown for each animal in each group ± SEM. Statistically significant differences compared to control vaccinated animals are indicated with * ($p \leq 0.05$),

Vaccination with the nASP+QuilA vaccine in study 1 resulted in a significant ($p \leq 0.05$) increase of nASP-specific IgG1 and IgG2 levels in both serum and abomasal mucus samples compared to QuilA control animals (Figure 2A-B). Vaccination with the pASP+QuilA vaccine resulted in a significant ($p \leq 0.05$) increase of nASP-specific IgG1 levels in serum, whereas no significant changes were observed for cross-reactive systemic IgG2 and mucosal IgG1 and IgG2 levels (Figure 2A-B).

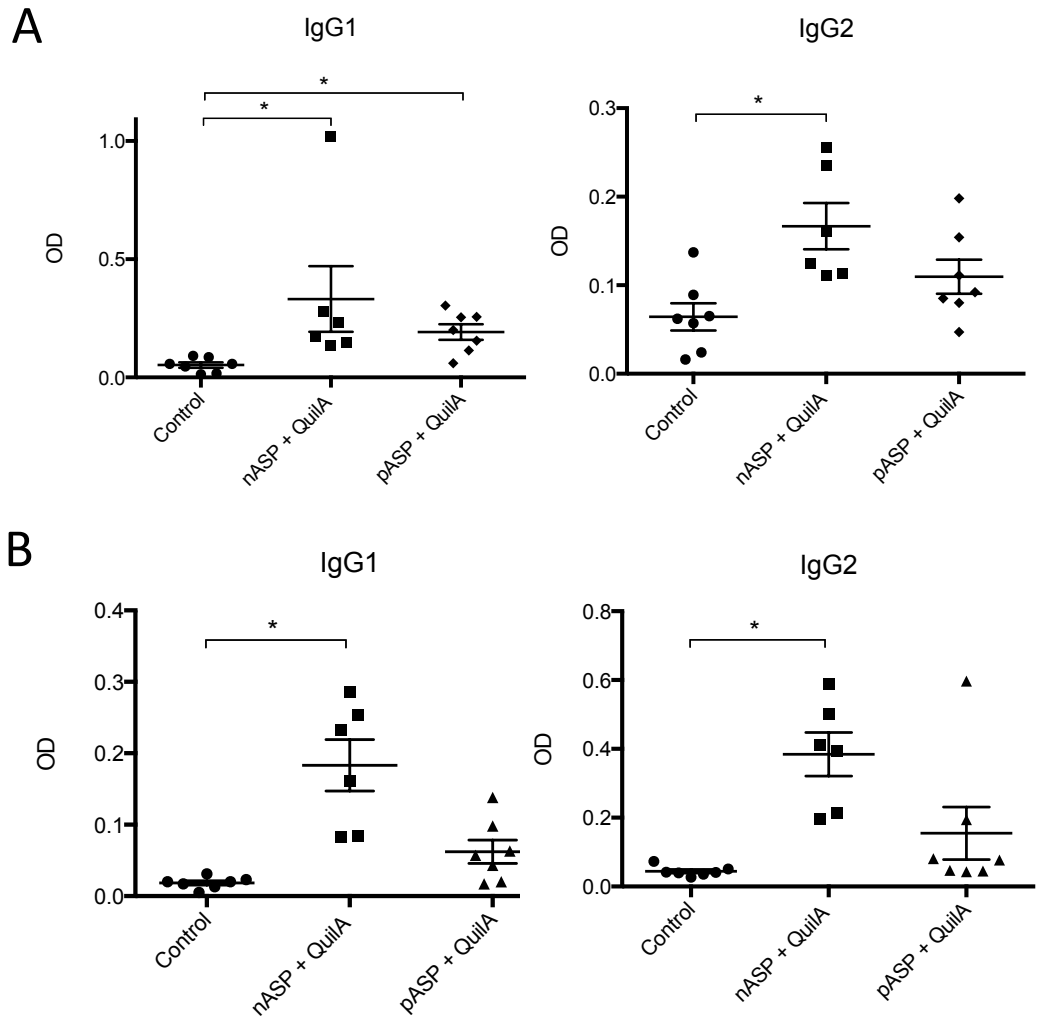

Figure 2. Detection of nASP-specific IgG1 and IgG2 antibodies in blood and mucosa after vaccination of cattle with nASP+QuilA and pASP+QuilA In cattle study 1, (A) serum and (B) abomasal samples were collected during the vaccination period and after subsequent infection, respectively, from all animals. Samples were used for the detection of nASP-specific IgG1 and IgG2 type antibodies through ELISA. The graphs show the individual and mean OD's for each individual animal within each group ± SEM. Statistically significant differences are indicated with * $p \leq 0.05$.

For study 2, nASP+QuilA vaccinated animals had increased levels of nASP-specific IgG1 in both serum and mucosa, whereas this effect was less pronounced for the pASP+QuilA group and completely absent in the nASP+$Al(OH)_3$ group (Figure 3A-B). In contrast to the first study, vaccination had a smaller impact on the amount of nASP-specific IgG2 antibodies, both systemically and in the mucosa. Vaccine-induced IgA's were not detectable in the serum and there were no significant increases in mucosal nASP-specific IgA levels in both studies (data not shown).

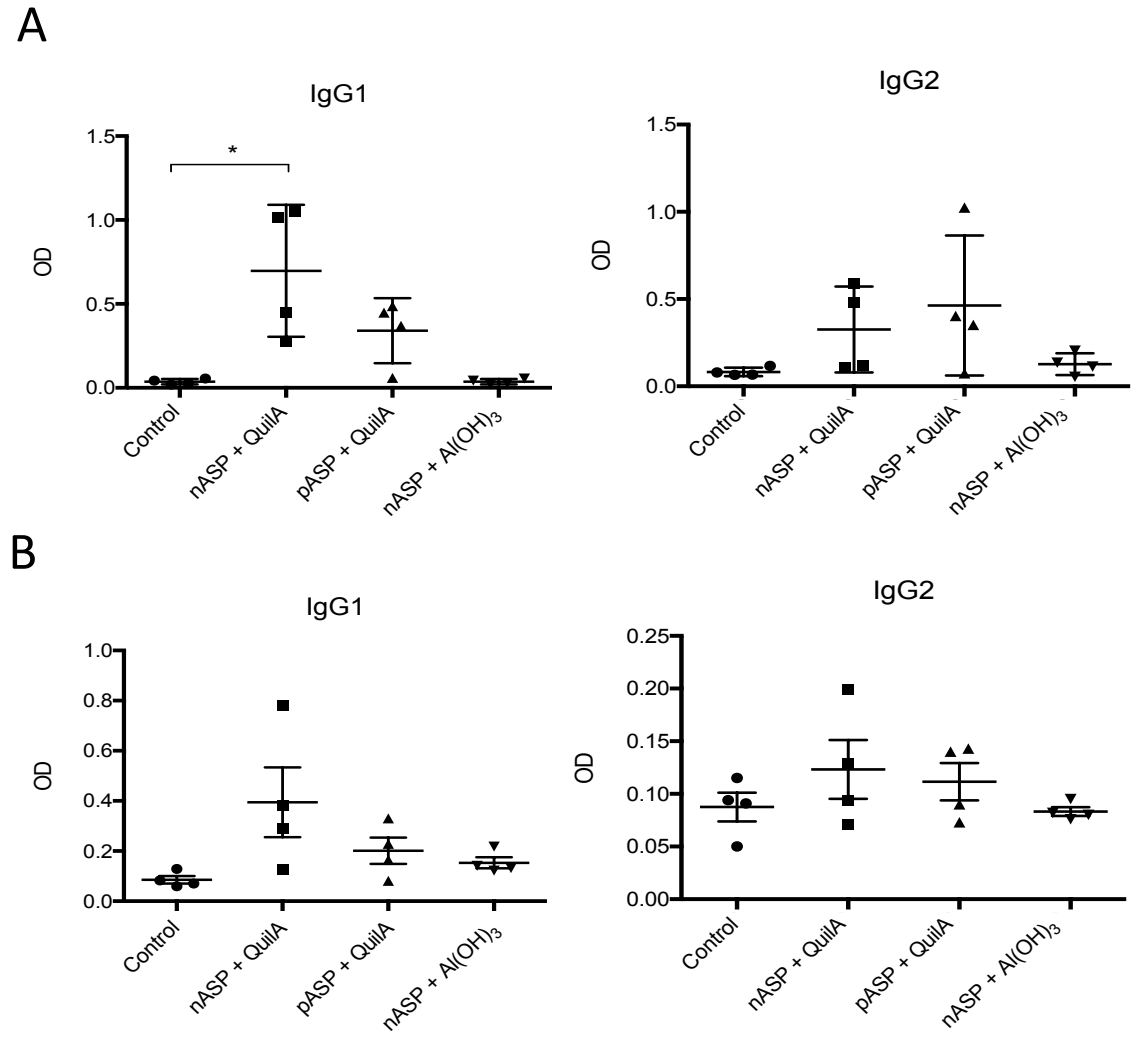

Figure 3. nASP-specific IgG1 and IgG2 antibodies observed in blood and mucosa after vaccination with nASP+QuilA, pASP+QuilA, and nASP+Al(OH)$_3$. In cattle study 2, (A) serum and (B) abomasal samples were collected from all animals and used for the detection of nASP specific IgG1 and IgG2 type antibodies through ELISA. The graphs show the individual and mean (± SEM) OD's for each group. Statistically significant differences are indicated with * ($p \leq 0.05$).

Antibodies raised by the native and the recombinant vaccines differ in their binding properties

To evaluate the specificity of the antibodies raised by the nASP+QuilA vaccine, an inhibition ELISA was performed with serum samples collected one week after the last vaccination from nASP+QuilA vaccinated animals. As shown in Figure 4A-B, pre-incubation of the serum with pASP failed to completely inhibit the binding of the IgG1 and IgG2 antibodies to nASP. In contrast, pre-incubation with nASP almost completely inhibited IgG1 and IgG2 binding to nASP.

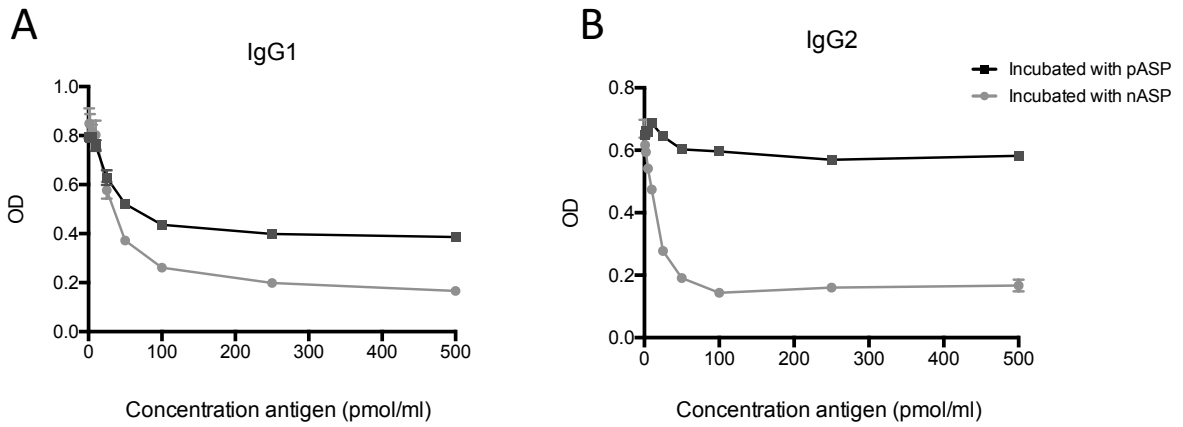

Figure 4. Comparison of the interaction between antibodies raised against nASP with the native and recombinant ASP. (A) IgG1 and (B) IgG2 antibodies from calves vaccinated with nASP+QuilA were evaluated in an inhibition ELISA. Graphs were generated from pooled serum samples in duplicate, where each point indicates the mean OD.

Vaccination of cattle with nASP+QuilA induces memory-like NK cells

In cattle study 2, peripheral blood mononuclear cells (PBMCs) were isolated on a weekly basis and used for phenotypical and functional analysis to measure antigen-specific proliferation *in vitro*. No vaccination-induced changes were observed in the frequencies of αβ-T cells, γδ-T cells, B cells or NK cells in the PBMC fraction (Figure 5A-D). However, when PBMCs were re-stimulated *in vitro* with the vaccine antigens, nASP for the nASP+QuilA and nASP+Al(OH)$_3$ vaccinated animals and pASP for the pASP+QuilA, antigen-specific proliferation was mainly found in the nASP+QuilA group (Figure 6A). Proliferation was the highest in the nASP+QuilA vaccinated animals and became significantly different from control animals on weeks 2 and 4 after the first vaccination ($p \leq 0.05$), with a decline thereafter (Figure 6A). In the pASP+QuilA vaccinated group, antigen-specific proliferation only became significantly different from control animals one week after the first booster vaccination ($p \leq 0.05$) (Figure 3A), but also here, a decline in proliferation was observed afterwards. Proliferation in the nASP+Al(OH)$_3$ vaccinated group (Figure 6A) and in control animals (data not shown) was not significantly different throughout the whole vaccination period. To determine whether *in vitro* re-stimulated PBMCs produced specific cytokines linked to either a Th1 or a Th2 type immune response, IFNγ levels were measured in culture supernatants produced by PBMCs isolated at week 5 and stimulated either with medium alone or with 5 μg/ml of the vaccine antigen for five days. IFNγ levels were significantly higher in the culture supernatant of the cells from the

nASP+QuilA group ($p \leq 0.05$), whereas no effect was observed for the other groups (Figure 6B). There was no detectable IL-4 production after *in vitro* culture of PBMCs isolated from any group of animals with medium nor with the vaccine antigens (data not shown).

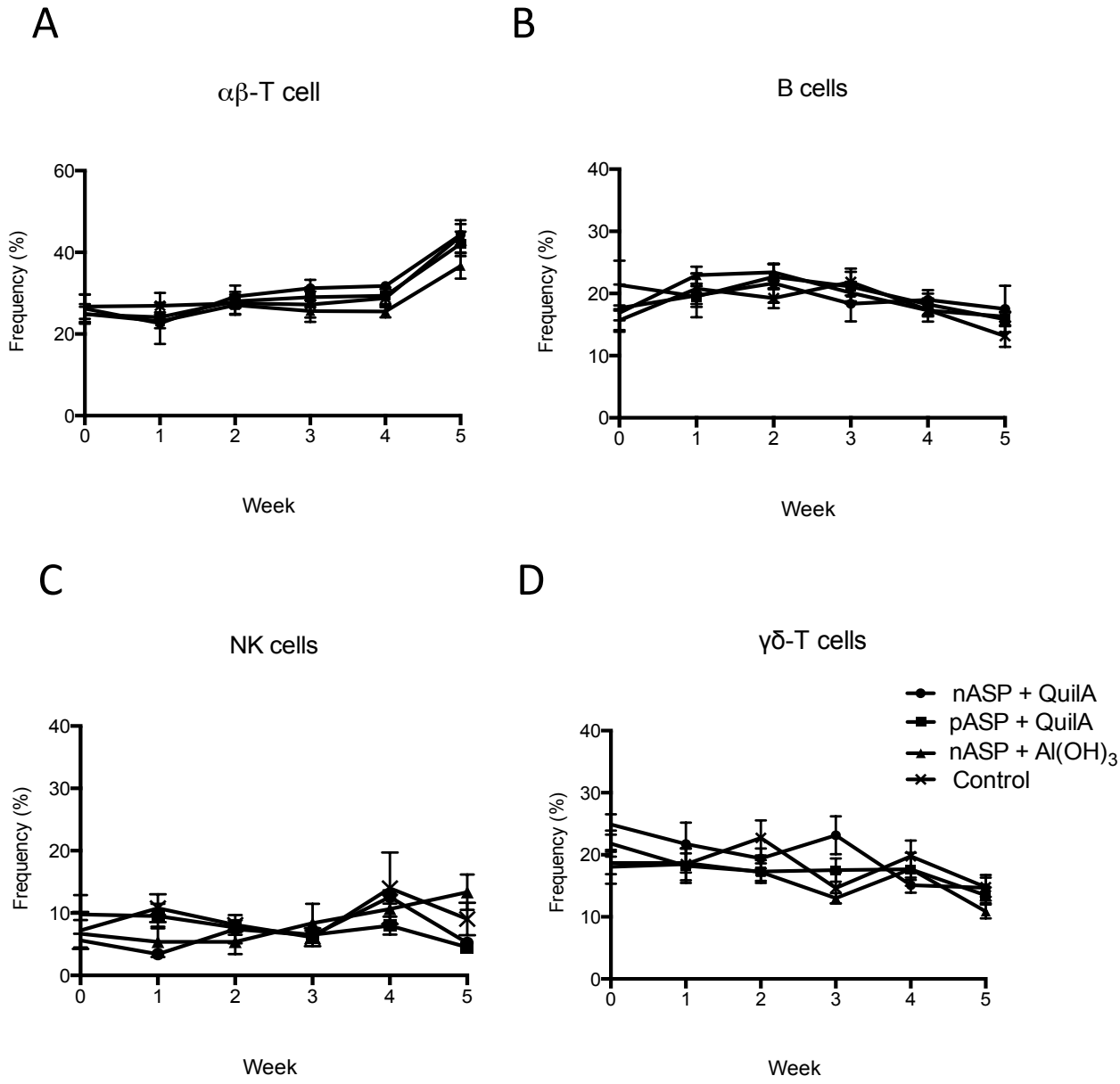

Figure 5. Kinetics of the different immune cells during the vaccination period. In cattle study 2, PBMCs were isolated from each animal weekly during the vaccination period and the cells were stained with monoclonal antibodies to determine the frequencies of (A) αβ–T cells, (B) B cells, (C) NK cells and (D) γδ-T cells.

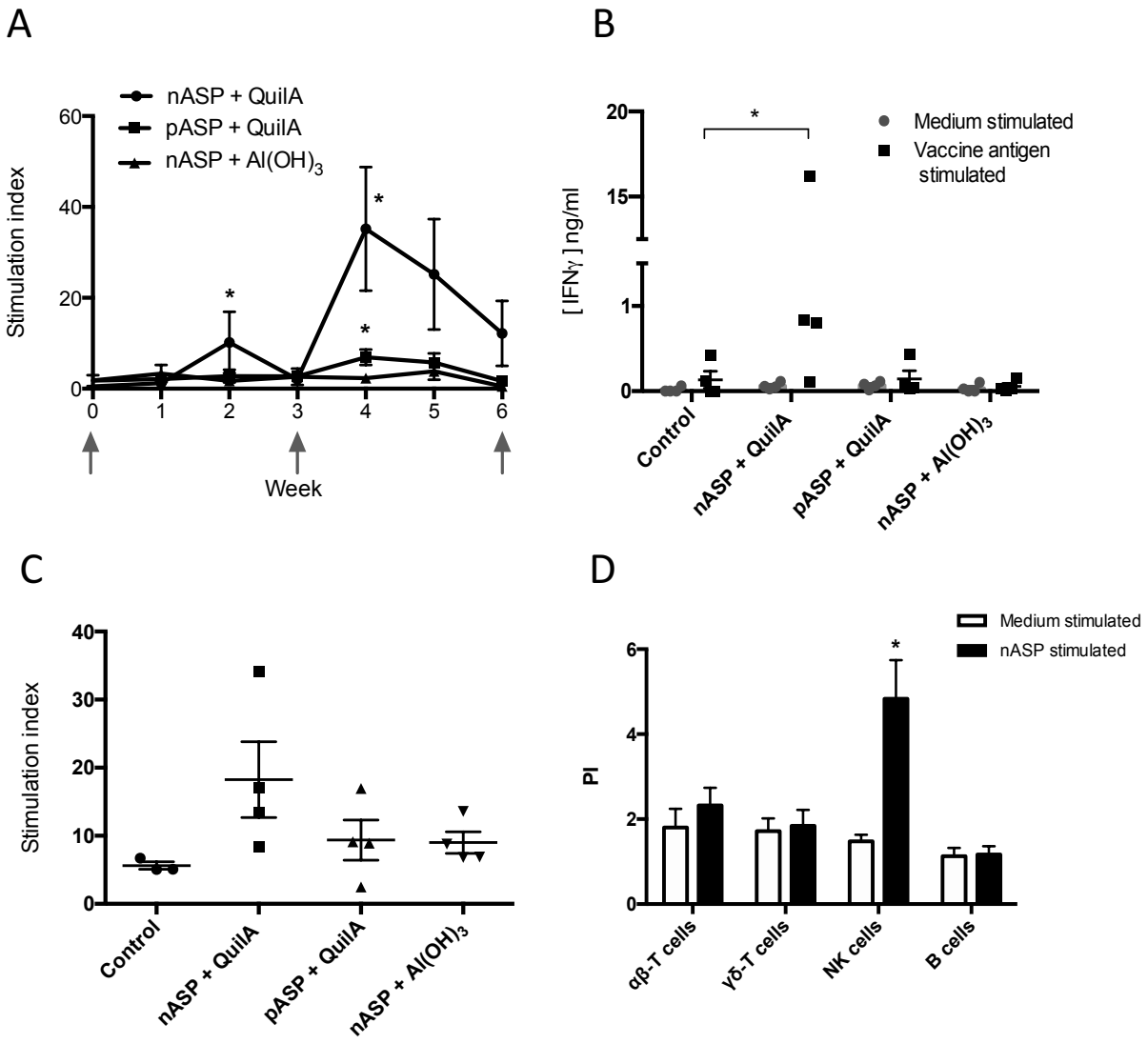

Figure 6. Vaccination with nASP+QuilA triggers systemic nASP-specific IFNγ producing cells and proliferation of NK cells in the mucosa.

(A) During cattle study 2, peripheral blood mononuclear cells (PBMCs) were isolated weekly during the vaccination period and re-stimulated with either medium alone or the antigen used in the vaccine formulation. The arrows indicate the time points at which the animals were vaccinated. ^{3}H-thymidine (^{3}HT) uptake was used as a measure of proliferation. The graph shows the mean stimulation index ± SEM for all vaccinated groups. (B) Five weeks after the first vaccination, PBMCs from nASP+QuilA, pASP+QuilA and nASP+Al(OH)$_3$ groups were stimulated for 5 days with 5 µg/ml nASP or pASP. IFNγ production of these cells was determined in the supernatants using an ELISA. The graph shows the mean concentration of IFNγ for each group ± SEM. (C) At time of necropsy, after 5 weeks of infection following the last vaccination, abomasal mononuclear cells (MCs) were isolated, re-stimulated, and their proliferative capacity was measured as for the PBMCs. Mean stimulation index ± SEM is shown for all groups. (D) Additional assays with PKH were performed on abomasal lymph nodes (LNs) to identify the proliferative populations of the nASP+QuilA vaccinated group. After flow cytometric analysis, the proliferation results were calculated and presented as proliferation index. Statistically significant differences for all graphs are indicated with * $p \leq 0.05$.

Next, to characterize the cellular immune response in the abomasum following vaccination and infection, abomasal lymph nodes (LNs) from all animals were obtained at the time of necropsy (i.e. five weeks after the onset of the trickle infection) for phenotypical analysis and proliferation assays. No statistical differences were found in the frequencies of αβ-T cells, γδ-T cells, B cells or NK cells in any vaccine protocol (Figure 7A-D).

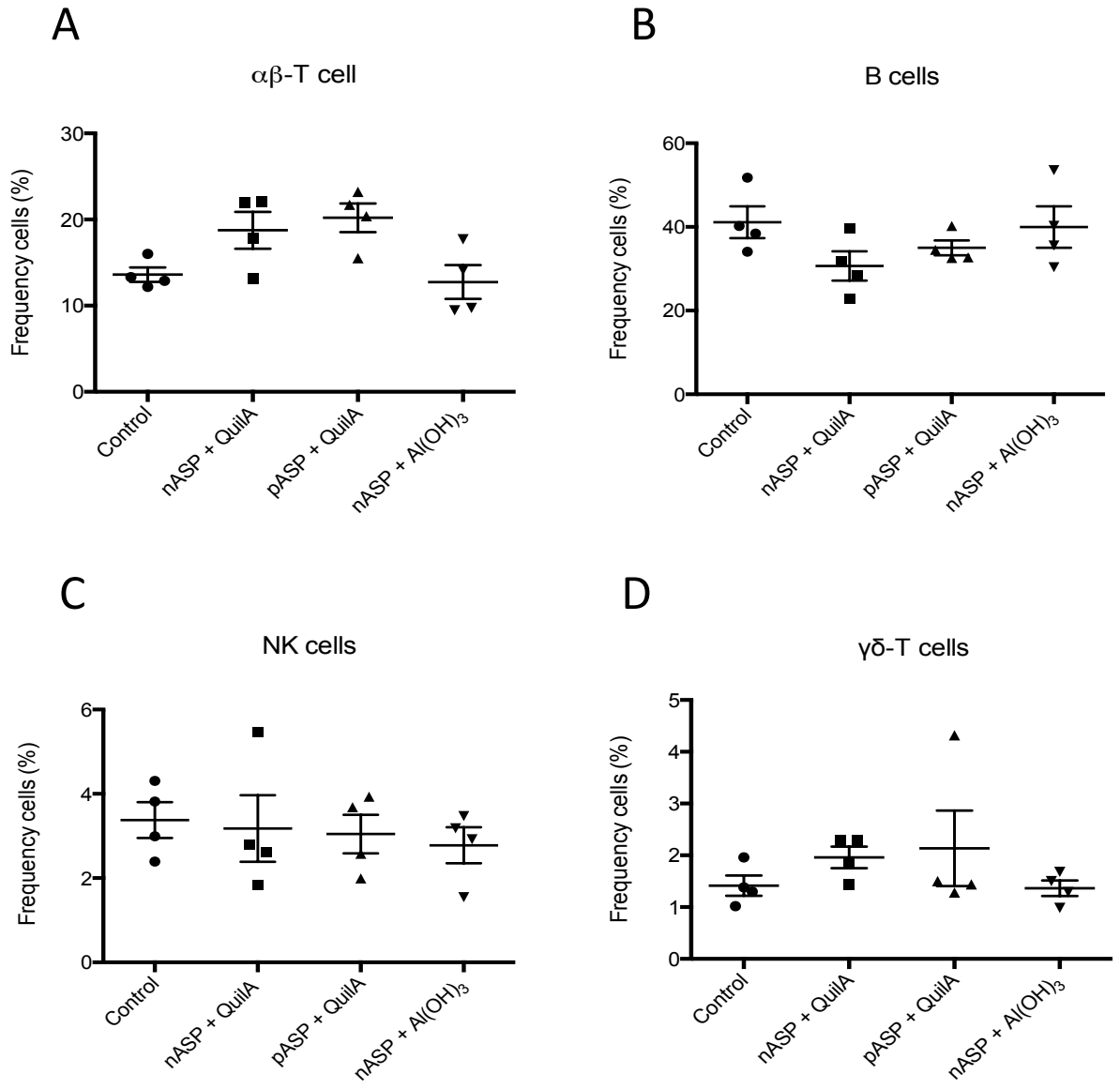

Figure 7. Phenotypical analysis of abomasal LN lymphocytes following vaccination and infection. Lymphocytes from abomasal LNs were isolated from the calves of study II and stained with monoclonal antibodies against CD3, TCRγδ and CD21 and CD335 and analyzed by flow cytometry. The frequencies reflect % of parent population, being the parent population viable single lymphocytes. Individual and mean (± SEM) percentages of (A) αβ–T cells, (B) B cells, (C) NK cells and (D) γδ-T cells for each group are shown.

Similar to the observations made with PBMCs, there was a trend in higher proliferation following re-exposure *in vitro* in the group of animals that was vaccinated with the protective nASP+QuilA vaccine (Figure 6C), although this did not reach statistical significance. These responding cells were subsequently characterized by isolating and labelling the abomasal LN cells of nASP+QuilA vaccinated animals with PKH prior to *in vitro* re-stimulation with the nASP. As shown in Figure 6D, proliferation was mainly detected in the $CD335^+$ cell population, where CD335 expression is typically used to define NK cells.

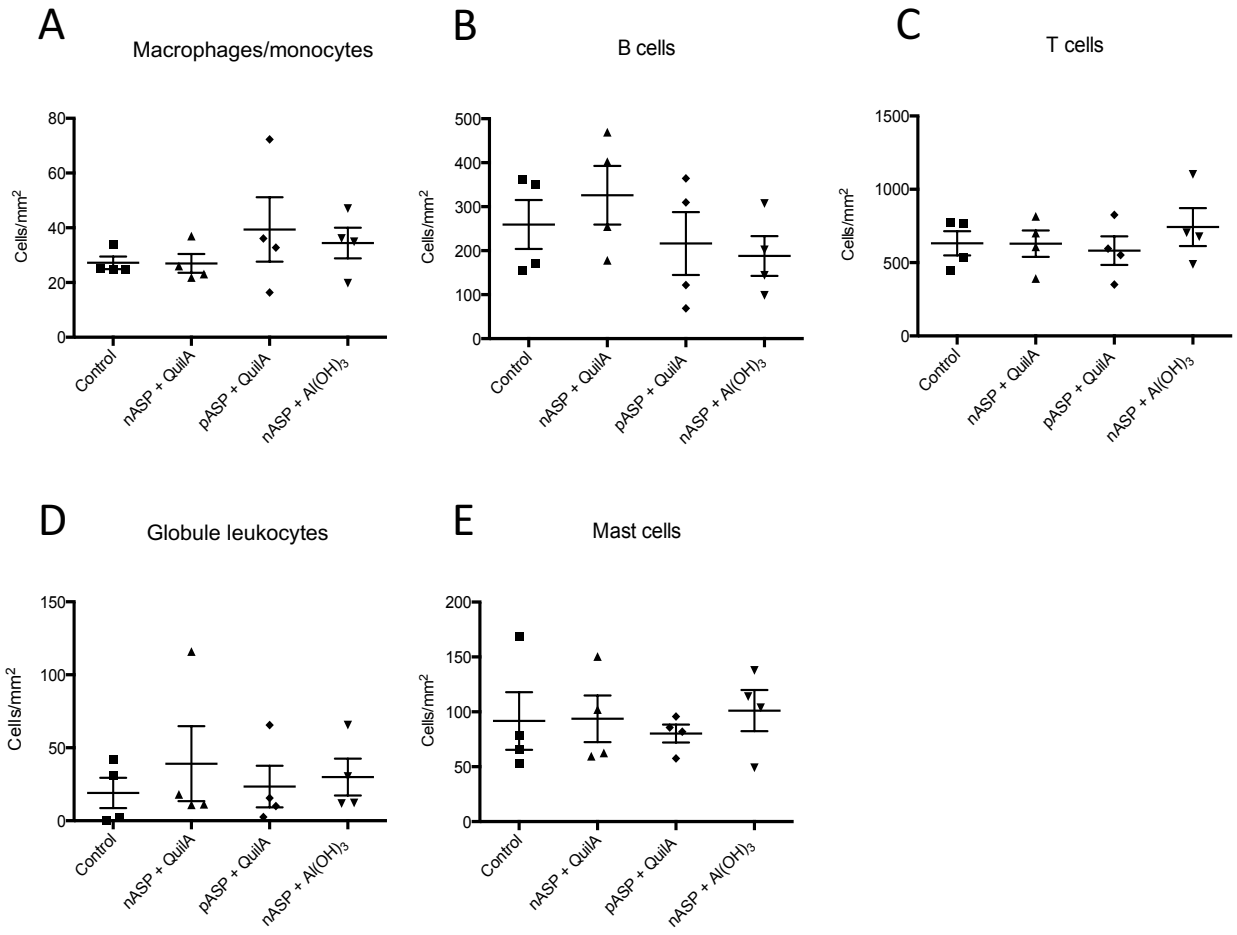

Figure 8. Immune cell counts in bovine abomasal tissue following vaccination and infection. Abomasal tissue samples were collected at the time of necropsy and either stained with antibodies to detect T cells ($CD3^+$), B cells ($CD20^+$) and macrophages/monocytes ($MAC387^+$), or stained with Toluidine Blue or Sirius Red to detect mast cells and globule leukocytes, respectively. The number of (A) macrophages, (B) B cells, (C) T cells, (D) globule leukocytes and (e) mast cells was determined per mm^2 in the mucosa. The graphs show the mean cell counts per mm^2 for the four animals within each group ± SEM.

Finally, immunohistofluorescence and immunohistochemical stainings were conducted on tissue sections from the abomasum. No significant differences in the numbers of T cells, B cells, macrophages/monocytes, mast cells nor globule leukocytes were observed between the vaccinated groups and the control group (Figure 8A-E). In addition, intraepithelial and lamina propria lymphocytes were isolated from the abomasum of all animals at the time of necropsy to determine the frequencies of αβ-T cells, γδ-T cells, B cells and $CD335^+$ cells. No differences were observed in the frequencies of these cells between the different vaccinated groups (Figure 9A-D).

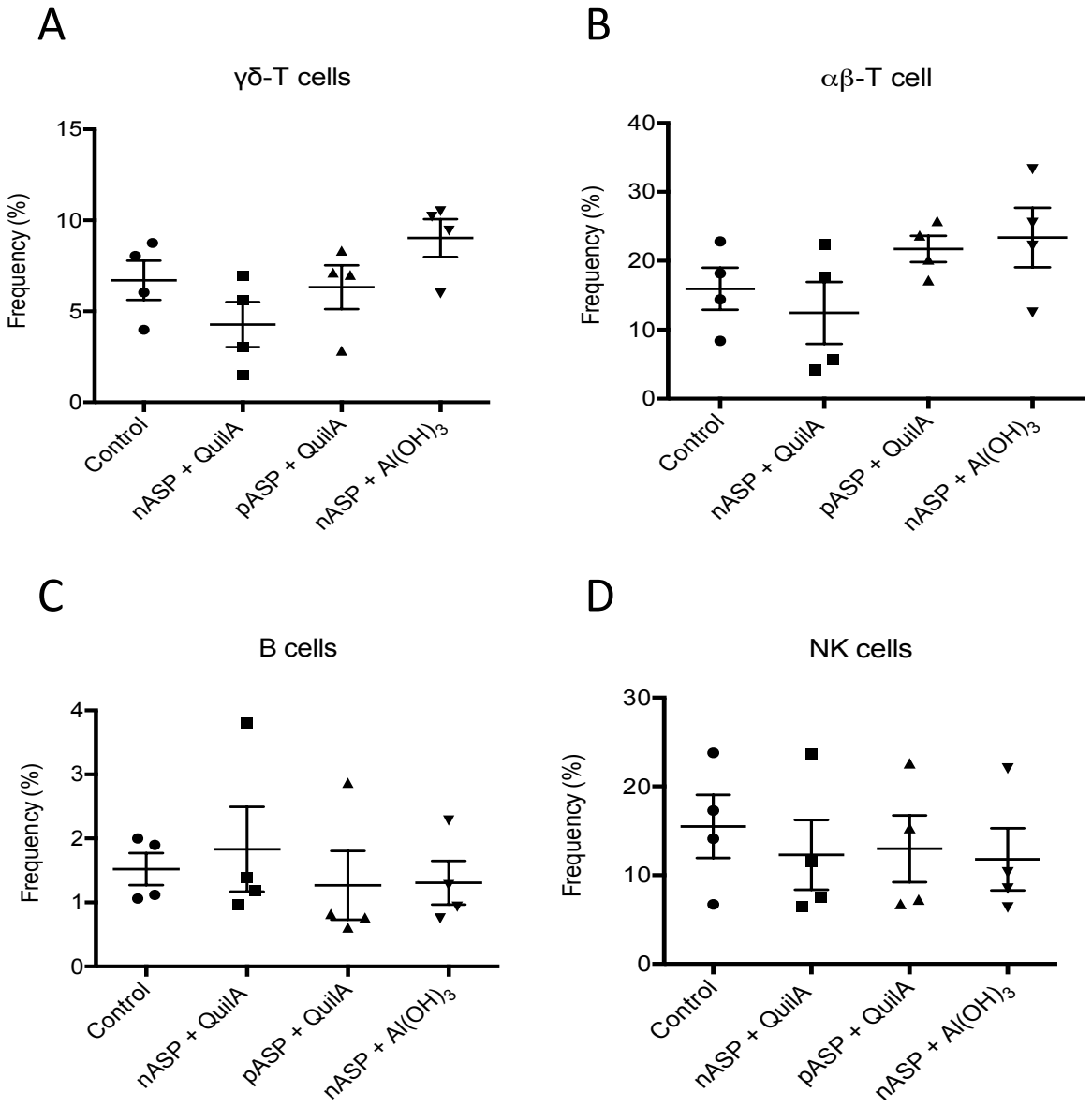

Figure 9. Phenotypical analysis of intra epithelial and lamina propria lymphocytes isolated from the abomasum following vaccination and infection. Abomasal intra-epithelial and lamina propria lymphocytes were isolated and stained with monoclonal antibodies against CD3, TCRγδ, CD21 and CD335 and analyzed by flow cytometry. The frequencies reflect % of parent population, being the parent population viable single lymphocytes. Individual and mean (± SEM) percentages of (a) γδ-T cells, (b) αβ –T cells, (c) B cells and (d) NK cells are shown.

Vaccine-induced immune responses in a murine immunization model are similar to those observed in cattle

Based on the results obtained in cattle, we subsequently investigated whether the observed NK cell response following vaccination with the nASP+QuilA vaccine was unique to cattle or whether this would also be triggered in mice. To characterize the cellular immune response following vaccination in mice, spleen mononuclear cells (MCs) were isolated at time of necropsy, and used for both ^{3}H-thymidine (^{3}HT) and PKH proliferation assays to determine antigen-specific proliferation *in vitro*. As observed in cattle, cells isolated from mice solely vaccinated with either QuilA or $Al(OH)_3$ did not show any proliferation following *in vitro* stimulation with nASP or pASP (Figures 4A and 4E). Although significant proliferation was observed in cells from the pASP+QuilA and nASP+$Al(OH)_3$ vaccinated animals following re-stimulation with the corresponding vaccine antigens ($p \leq 0.05$) (Figures 4B,C,F), the highest proliferation was again observed in the nASP+QuilA group.

Furthermore, exposure of the cells from the pASP+QuilA animals to nASP also resulted in a significant cellular proliferation in comparison to the medium control ($p \leq 0.05$) (Figure 4C). Interestingly, cells from mice vaccinated with the nASP of which the disulphide bonds were reduced did not show any proliferation after re-stimulation with the reduced antigen (Figure 4D). PKH assays were subsequently performed in order to characterize the proliferative cell populations. Confirming the ^{3}HT assays (Figure 4), Figure 5 shows that there was no increased proliferation of cells from the QuilA and $Al(OH)_3$ control groups nor from the reduced-nASP+QuilA group upon *in vitro* stimulation with nASP, pASP or reduced-nASP (Figures 5A, 5D and 5E). On the other hand, B cells, and especially NK and non-T, non-B non-NK cells ($CD3^-/CD21^-/CD335^-$) from nASP+QuilA vaccinated mice proliferated after *in vitro* re-stimulation with nASP compared to cells stimulated with medium alone (Figure 5B). Antigen-specific proliferation of the NK and non-T, non-B non-NK cells ($CD3^-/CD21^-/CD335^-$) following re-stimulation with nASP was also observed with cells from the pASP+QuilA and nASP+$Al(OH)_3$ groups (Figure 5C and 5F), albeit at a lower level compared to the nASP+QuilA group. When re-stimulating cells from the nASP+$Al(OH)_3$ group with nASP, modest levels of γδ-T cell proliferation could also be observed (Figure 5F). Finally, re-stimulation of pASP+QuilA cells with pASP induced antigen-specific NK cells proliferation, which was similar to that observed after nASP stimulation, but again much lower than the levels observed in the nASP+QuilA group (figure 5C).

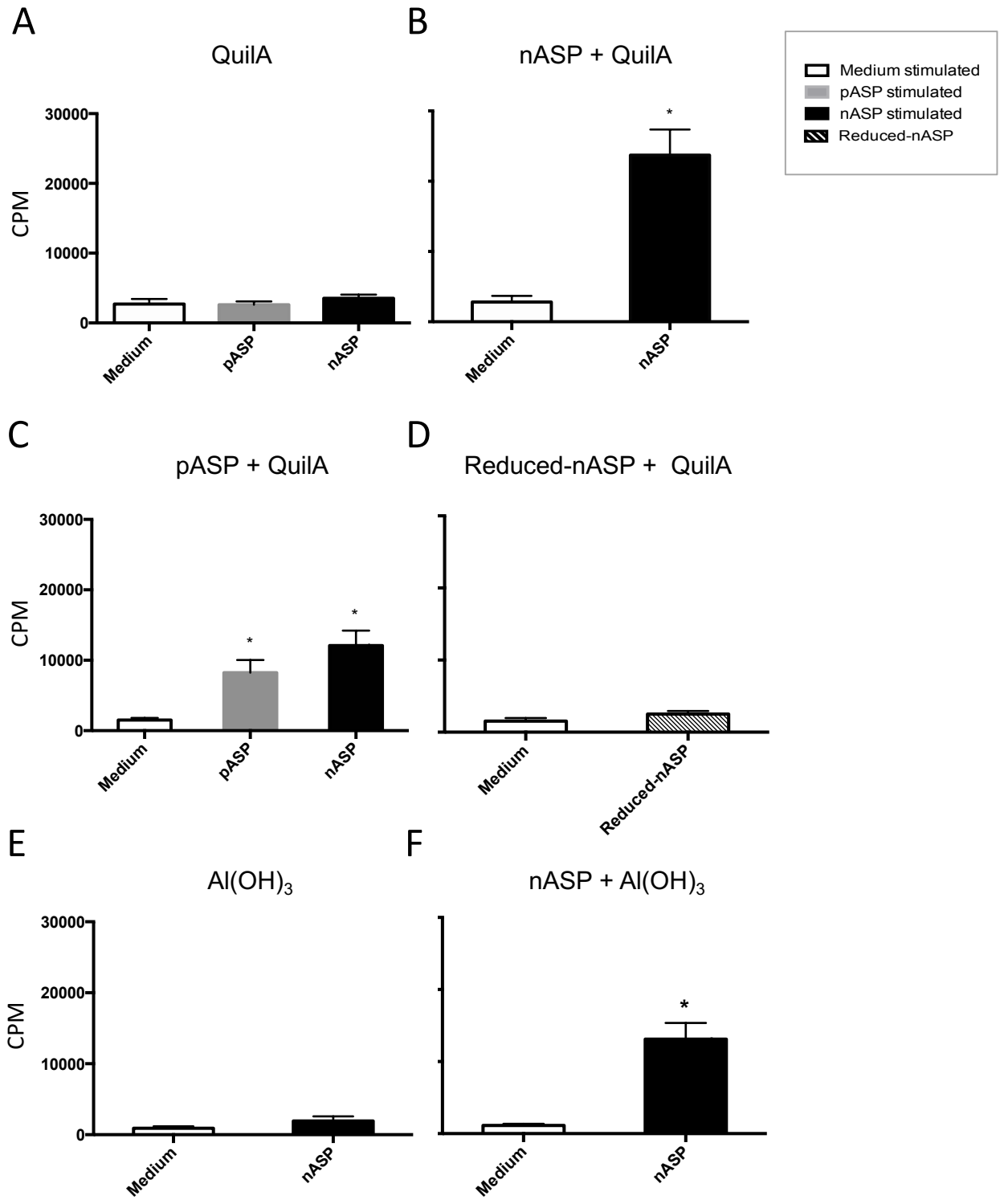

Figure 4. Vaccination of mice with nASP+QuilA induces higher nASP-specific cell proliferation than other vaccine formulations. Mice were immunized three times in a 3-week interval intramuscularly in the thigh muscle and euthanized one week following the last vaccination. Mouse spleen MCs were isolated and re-stimulated with either medium alone, nASP or pASP. ^{3}HT incorporation was used as a measure of total cell proliferation. All graphs show the mean counts per minute (CPM) ± SEM for animals vaccinated with (A) QuilA, (B) nASP+QuilA, (C) pASP+QuilA, (D) Reduced-ASP+QuilA, (E) $Al(OH)_3$ and (F) nASP+$Al(OH)_3$. Statistically significant differences are indicated with *$p < 0.05$.

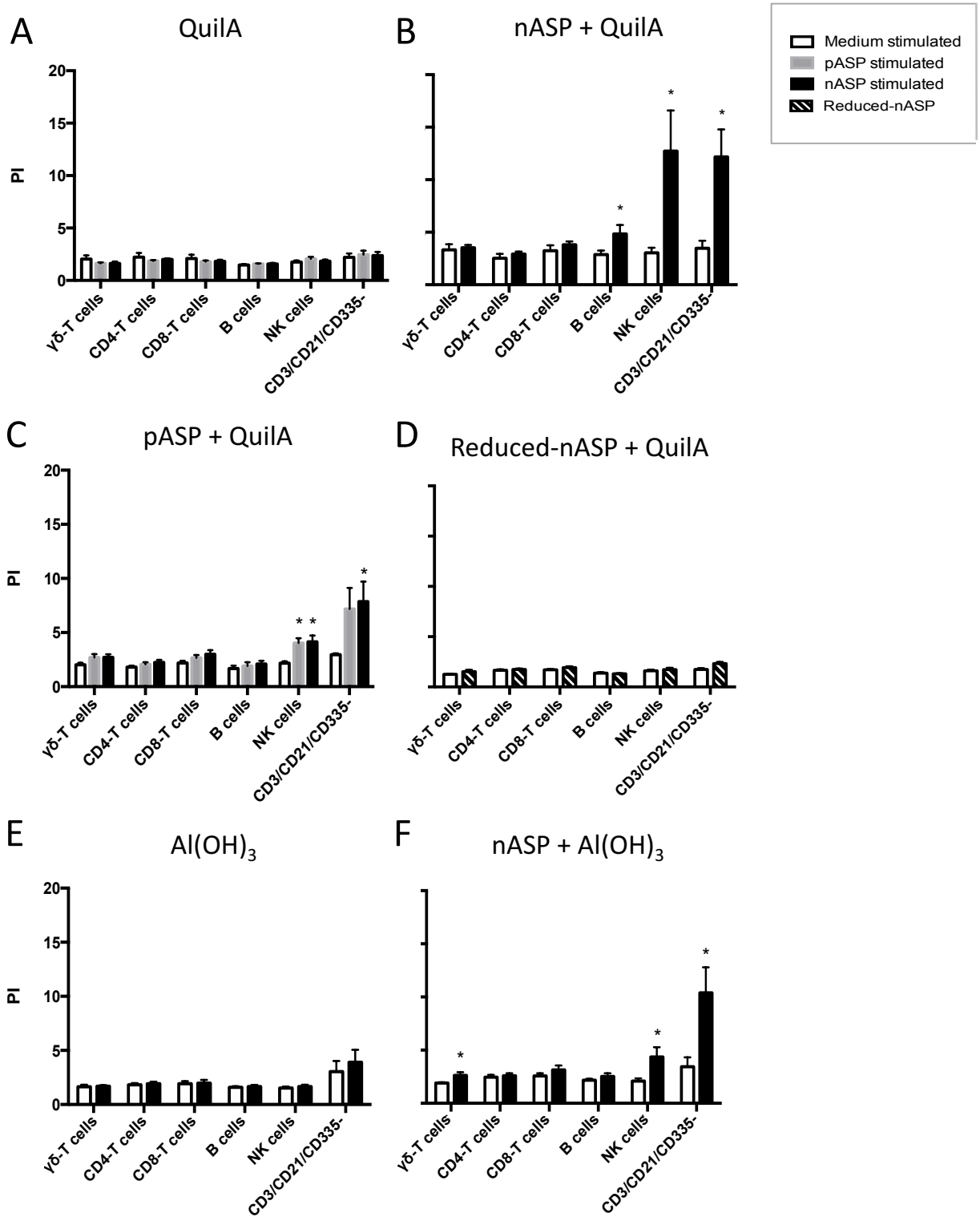

Figure 5. NK cells and $CD3^-/CD21^-/CD335^-$ cells are the main vaccine-induced proliferative cell populations in mice. Mouse spleen MNCs were isolated and re-stimulated for 5 days with either medium alone, nASP or pASP and stained with monoclonal antibodies for further FACS analysis. PKH incorporation to the membrane and its further dilution was used as a measure of cell proliferation. All graphs show the mean proliferation index (PI) ± SEM for animals vaccinated with (A) QuilA, (B) nASP+QuilA, (C) pASP+QuilA, (D) Reduced-ASP+QuilA, (E) $Al(OH)_3$ and (F) nASP+$Al(OH)_3$. Statistical differences are indicated with * $p \leq 0.05$.

At the time of necropsy, all mice were bled from the retro-orbital vein and the serum was used to measure antigen specific IgG1, IgG2a and IgG2b levels by enzyme-linked immunosorbent assay (ELISA), essentially as described for cattle. The levels of nASP-specific IgG1 antibodies were significantly higher in animals vaccinated with nASP+QuilA, pASP+QuilA and nASP+Al(OH)$_3$ ($p \leq 0.01$, $p \leq 0.0001$ and $p \leq 0.05$ respectively) (Figure 6A) compared to their respective controls. No significant increase in antigen-specific IgG1 levels could be detected in the reduced-nASP+QuilA and Al(OH)$_3$ immunized animals. While no significant levels of nASP-specific IgG2a could be detected in any of the groups (data not shown), IgG2b antibodies were significantly higher in animals vaccinated with nASP+QuilA and pASP+QuilA ($p \leq 0.05$ and $p \leq 0.001$ respectively) when compared to the QuilA controls, whereas no significant increase could be observed for the other groups (Figure 6B).

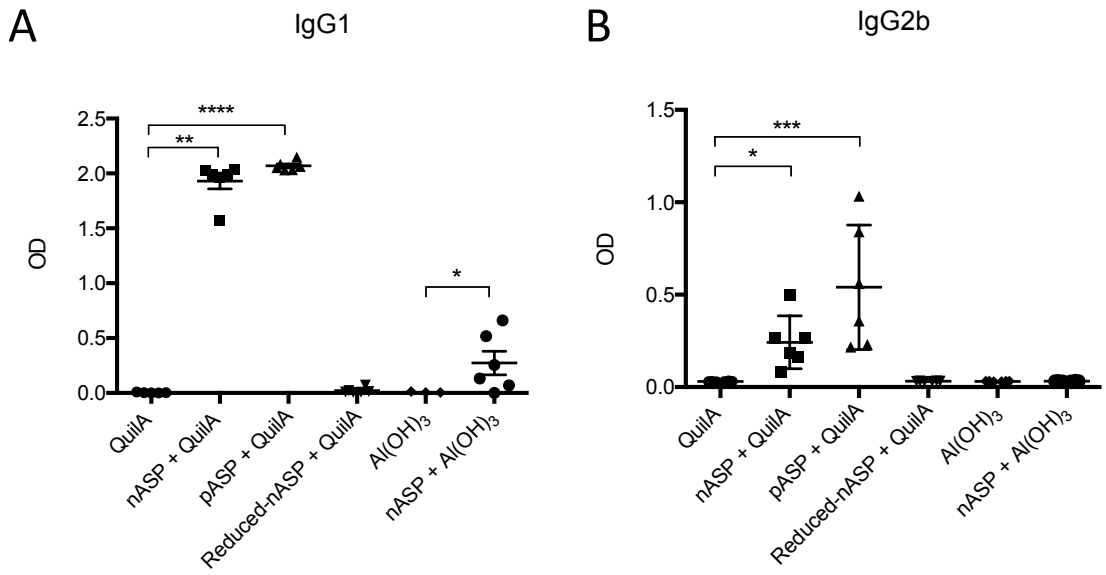

Figure 6. Detection of nASP-specific IgG1 and IgG2b antibodies in blood after vaccination of mice with different vaccine formulations. Mice serum was collected at time of necropsy and used for the detection of nASP specific (A) IgG1 and (B) IgG2b type antibodies through ELISA analysis. The graph shows the OD for each individual animal within each group ± SEM. Statistical differences are indicated with * $p \leq 0.05$, ** $p \leq 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

4. Discussion

NK cells were the major cell population in nASP+QuilA vaccinated and subsequently infected cattle, which proliferated following re-stimulation of MCs from the abomasal draining LN. In addition, in mice also NK cells, together with an unidentified non-T, non-B non-NK cell population proliferated after re-stimulation of spleen MCs. NK cells are large granular lymphocytes that either become directly activated by pathogens or through interaction with other pathogen-activated immune cells. They were originally named after their ability to spontaneously kill transformed, infected or non-self-cells without prior sensitization or activation. Despite their innate origin, there is accumulating evidence that also NK cells can develop long-lived and highly specific memory to a variety of antigens [20-22]. Previous studies also indicate that NK cells are likely to be involved in the natural immune response in cattle following nematode infection. It was already shown that NK cells are important in the proliferation observed in the abomasal lymph nodes during the course of an infection[23,24]. More recently, an antigen-specific proliferation of NK cells was observed in the draining lymph nodes of the small intestine of animals infected with *C. oncophora* [25]. Also, analysis of transcriptional changes by qRT-PCR in the abomasal mucosa following *Ostertagia* infection showed a significant upregulation of the NK cell marker CD335[26] and the expression of granulysin and granzyme, both proteins that are typically produced by NK cells [18].

Furthermore, the flow cytometric analyses performed in this study showed that NK cells represented roughly 15% of the total abomasal lymphocyte population following infection. The observed NK cell activation and IFNγ production following *in vitro* re-stimulation with the native ASP is also in line with previously published data on an ASP (rOv-ASP-1) from the filarial parasite *Onchocerca volvulus.* In a series of papers, it was shown that immunisation with Ov-ASP-1 induced a highly dominant IFN-γ recall response following vaccination, most likely produced by activated NK cells, combined with a mixed IgG1/IgG2 antibody reaction [27-29]. Similar to the *O. ostertagi* ASP, the bioactivity of Ov-ASP-1 was completely lost after denaturing the protein by boiling [27]. It is also interesting to note that secreted protein(s) of the human hookworm *Necator americanus* selectively bind to NK cells and induced IFN-γ production and that this process is dependent on the presence of the cytokines IL-2 and IL-12, likely secreted by activated antigen-presenting cells [30,31]. Whether ASPs are responsible for this activity is currently unknown.

Teixeira-Carvalho et al. (2008) previously hypothesized that the activation of NK cells and IFN-γ production is part of an immune evasion strategy by the worms, in an attempt to down-regulate a protective Th2 response[29]. In case of the *O. ostertagi* vaccine it is

unclear whether the NK cell activation is necessary to obtain protection or, alternatively, whether the protective capacity of the vaccine is based on the generation of antibodies, potentially interfering with the immunomodulatory activity of the ASP. Over the years, several studies have demonstrated that antibodies can provide protective immunity against gastrointestinal helminths in a direct and/or indirect way [32]. This protection would hypothetically be achieved through antibody-dependent activation of immune cells in the intestinal mucosa, or by interfering with enzymes and processes required for larval migration, invasion and feeding. In that context, nASP+QuilA vaccinated cattle had an increased amount of nASP-specific IgG1 and IgG2-type antibodies both in blood and in the abomasal mucus. Interestingly, NK cells express FcγRIII (CD16) at their surface, which can mediate antibody-dependent cytotoxicity through the recognition of sequestered antigens [33]. NK cells are highly abundant in the intraepithelial and lamina propria lymphocyte fractions of the abomasum, creating a high chance for effective contact between their $CD335^+$ receptor and the different parasitic life stages of *O. ostertagi*. Therefore, a possible mechanism of action for NK cells against *O. ostertagi* could be the recognition of antibody-sequestered nASP and subsequent antibody mediated degranulation. Since NK cells are known to express granzyme B, perforin and granulysin, this could be an interesting link with previous observations on the upregulation of granulysin and granzyme B in animals protected by vaccination against *O. ostertagi* [18].

The cellular and humoral responses observed for the protective nASP+QuilA vaccine were markedly higher compared to responses observed for the non-protective nASP+$Al(OH)_3$ vaccine, both in cattle and mice. The exact mechanisms by which both adjuvants act are not completely understood yet. It has been shown that $Al(OH)_3$ exerts a reservoir function, from which antigen is released slowly for a prolonged period of time [34], whereas saponin-based adjuvants such as QuilA exert a more cytolytic function [35]. Despite the fact that $Al(OH)_3$ is regarded as a Th2 type immune response inducing adjuvant, saponin-based adjuvants seem to induce a stronger antibody response against the vaccine antigen than $Al(OH)_3$ [36], supporting the hypothesis of a possible important role for antibodies in the vaccination-induced protection against *O. ostertagi*. Additionally, not only the choice of adjuvant, but also the conformation of the antigen has proven to be essential in triggering a protective immune response. Unfolding the ASP completely abolished its ability to induce both NK cell activation and antibody induction. Together with the lack of protection provided by the recombinant ASP, these observations indicate that protein folding, potentially in combination with N- and/or O-glycans present on the peptide core of the native ASP, is crucial for its immunoreactivity and that these factors could be at the basis of the inability of recombinant ASP in triggering a similar response. This is in line with the results of inhibition ELISA's showing that antibodies raised upon

nASP vaccination preferentially bind nASP over pASP, suggesting the presence of either different and/or additional epitopes.

In conclusion, the outcome of this study indicates that immunization of animals with the protective nASP+QuilA vaccine is associated with antigen-induced proliferation of NK cells and the production of antigen-specific IgG1 and IgG2 antibodies. Replacing either: i) the native antigen by a recombinantly produced version or, ii) the QuilA adjuvant by $Al(OH)_3$ both had a significant impact on the cellular and humoral vaccine-induced responses. Whether NK cells and antibodies are actually essential for conferring protection against an *O. ostertagi* challenge infection remains unknown. Also, the molecular and cellular mechanisms underlying the NK cell activation and the structural elements of the nASP that are crucial in this process require further research.

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3

Comparative analysis of the immune responses induced by native versus recombinant versions of the ASP-based vaccine against the bovine intestinal parasite *Cooperia oncophora*

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Comparative analysis of the immune responses induced by native vs. recombinant versions of the ASP-based vaccine against *C. oncophora*

1. Introduction

The economic impact of gastrointestinal nematode infections in ruminants has been extensively demonstrated over the years [1]. Up to date, control of these infections in livestock relies almost exclusively on the use of anthelminthics, but the increasing spread of anthelmintic resistance worldwide illustrates the need for alternative control strategies [2,3]. Successful vaccination against gastrointestinal nematodes with native and recombinant proteins in cattle and sheep [4-8], demonstrates that protein-based vaccination is a promising alternative to the current control methods [9]. Recently, our group has shown that vaccination with native double-domain activation-associated secreted protein (ndd-ASP) obtained from the excretory-secretory material of the adult stages of the bovine intestinal nematode *Cooperia oncophora* [10] provides protection both in experimental and natural conditions, as indicated by a decrease in the cumulative egg output of 91% and 59%, respectively [7]. Under natural conditions, vaccination of cattle with the ndd-ASP also resulted in a 65% reduction of pasture larval counts and a decrease in 82% of worm counts when compared to the control group [7]. Additional studies on the immune response elicited by vaccination with ndd-ASP have shown an increased antigen-specific antibody production, which correlated negatively with the egg output. Moreover, significant T-cell proliferation was induced in ndd-ASP vaccinated animals after *in vitro* stimulation with the antigen, which was positively correlated with a higher proportion of immature larval stages [11]. Despite these promising results achieved with the native antigen, recombinant production of the antigen would be an absolute requirement for the economic viability of the vaccine [9,12]. Therefore, the objectives of the present study were: (i) to produce and evaluate the protective capacity of a *Pichia*-produced version of the dd-ASP, and (ii) to compare the cellular and humoral responses induced by both native and recombinant versions of the antigen.

2. Materials & Methods

Native and recombinant antigen production

Preparation of the native *C. oncophora* double-domain ASP (ndd-ASP) was carried out as previously described [10]. In short, excretory-secretory material of adult worms was collected and fractionated over a Superdex 200 16/70 size-exclusion chromatography column. The purity of the ndd-ASP fraction, which eluted first from the column, was checked by reducing and non-reducing one-dimensional gel electrophoresis (SDS-PAGE) and visualized by Simply-Blue SafeStain (Invitrogen) staining. Afterwards, the protein band was excised and protein identity was further confirmed by mass spectrometry.

Recombinant double-domain ASP (pdd-ASP) was expressed in *Pichia pastoris* as follows: its coding sequence [10] was PCR amplified and subsequently cloned into the pGEMt-Easy subcloning vector (Promega) according to the manufacturer's instructions. Following transformation into DH5α competent cells (Invitrogen), clone selection on X-gal plates and sequence verification, the pGEMt-Easy-nDD-ASP construct was *Eco*RI-*Xba*I linearized and inserted in the *Pichia* expression vector pPICZα B (Thermo Fischer Scientific). The resulting expression plasmid was used to transform *P. pastoris* strain KM071H (Invitrogen) by electroporation. Afterwards, individual clones growing on minimal plates were isolated and tested for secretion of pdd-ASP by SDS-PAGE followed by Coomassie Brilliant Blue staining and immunoblotting with bovine-anti-ndd-ASP antibodies. A clone expressing pdd-ASP was freshly grown on plate and then used to inoculate a shake flask culture with buffer minimal glycerol complex (BMGY) medium. After 48 hours of growth at 29°C, the cells were pelleted by centrifugation for 15 minutes at 1,000 x *g* followed by resuspension of the cells in BMMY and further growth at 29°C. Every 24 hours, extra methanol (0.5%) was added to the culture and after 96 hours of induction the cells were finally pelleted by centrifugation for 15 minutes at 2,000 x *g*. The cell medium was harvested and filtered over a 0.22 µm membrane, after which the supernatant was concentrated on a Centriprep YM10 (Millipore), dialyzed to 50 mM sodium acetate pH 5.0 and desalted on a HiPrep 26/10 Desalting column (GE Healthcare Bio-Sciences). This fraction was then applied to a Resource-S cation exchange column (GE Healthcare Bio-Sciences AB) equilibrated in 50 mM sodium acetate buffer (pH 5.0) and the bound pdd-ASP was eluted employing a gradient from 0 up to 1 M sodium chloride (NaCl) in the same buffer. Fractions containing pdd-ASP were pooled and buffer-exchanged to PBS by gel filtration on a Sephadex G25-column (GE Healthcare Bio-Sciences).

The protein profile of the obtained material was checked on a 12% reducing one-dimensional electrophoresis (SDS-PAGE). Additionally, ndd- and pdd-ASP proteins were blotted onto a poly-vinylidene fluoride (PVDF) membrane for 1 h at 1mA/cm^2 of gel surface followed by a 1 h blocking step in PBS + 0.5% Tween80 (PBST). The membrane was afterwards incubated with ndd-ASP-specific antibodies (dilution 1/500) for 1 h, followed by extensive wash with PBST and incubation for 1 h with horseradish peroxidase (HRP)-conjugated rabbit anti-bovine IgG1 antibody. 3,3´-diaminobenzidine (DAB) (Sigma) served as substrate. All steps were performed at room temperature.

Immunization experiments in cattle

All animal experiments were conducted in accordance with the EU Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical approvals to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2014/70, EC2015/40). Two vaccination studies were carried out in cattle as previously described [7,11].

The aim of study 1 was to investigate the protective capacity of the recombinant antigen. Twenty-one helminth naïve male crossbreed Holstein calves (6 to 8 months of age) were randomly divided over three groups of 7 animals (QuilA control, ndd-ASP+QuilA and pdd-ASP+QuilA). Subsequently, a second study was performed to analyse and compare the cellular responses induced by the native and recombinant antigens, where twelve helminth naïve female crossbreed Holstein calves (6 to 8 months of age) were randomly divided over three groups of 4 animals (QuilA control, ndd-ASP+QuilA and pdd-ASP+QuilA). For both studies, all animals were immunized three times intramuscularly in the neck with a 3-week interval. Control animals received 750 µg of QuilA (Superfos Biosector), while the animals of the ndd-ASP+QuilA and pdd-ASP+QuilA groups received 30 µg of antigen in combination with 750 µg of QuilA per immunization. All animals were challenged with a trickle infection of 25,000 L3 larvae (1000 L3/day; 5 days/week during 5 weeks), which started on the day of the third immunization, and were then euthanized 3 weeks after the last infection. Parasitological parameters (i.e. faecal egg and worm counts) were monitored as previously described [7,11].

Isolation of peripheral blood mononuclear cells

In Study 2, blood samples were collected weekly from the jugular vein using vacutainer tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep (Nycomed Pharma) gradient centrifugation.

Additionally, mesenteric lymph nodes (LNs) of the small intestine were isolated at time of necropsy, and lymph node mononuclear cells (MCs) were isolated by homogenization through mechanical disruption of the tissue followed by Lymphoprep gradient centrifugation. After centrifugation, all MC fractions were isolated, washed and counted prior to cell culture and flow cytometric analysis.

Flow Cytometry

Cells were labelled in Flow Cytometry Staining Buffer (eBioscience) and all antibodies were used at the concentration recommended by the supplier. After incubation for 20 minutes with primary antibodies, the cells were washed two times prior to staining with fluorescently-labelled secondary antibodies. The cells were then incubated for an additional 20 minutes, washed and resuspended in PBS to be immediately analysed using a FACS Aria III flow cytometer (BD Biosciences). Non-viable cells were excluded from the analysis based on their propidium iodide (Life Technologies) uptake. Primary antibodies used were: non-labeled CD3 (MM1A, IgG1), TCRγδ (GB21A, IgG2b), CD21 (BAQ15A, IgM), CD8 (BAQ111A, IgM) (all from Monoclonal Antibody Center, Washington University), CD4 (CC8, IgG2a) and CD335 (AKS6, IgG2b, kindly provided by Prof. Dr. Anne K. Storset, NMBU, Norway). Secondary antibodies used were: goat anti-mouse IgG1-V450 (BD Biosciences), goat anti-mouse IgG2a-APC (Invitrogen), rat anti-mouse IgG2b-FITC (Southern Biotech) and rat anti-mouse IgM-APC-Cy7 (Biolegend).

Proliferation assays

The PBMCs and MCs collected from blood and mesenteric LNs, respectively, in study 2 were used in proliferation assays using either ^{3}H-thymidine (^{3}HT) incorporation or PKH26 (Sigma-Aldrich) fluorescence intensity reduction as a read-out as previously described [8]. For the ^{3}HT uptake experiments, cells were seeded at 1.25x10^{6} cells/ml in a 96-well round-bottom plate (Thermo Scientific) in complete medium (RPMI 1640 + GlutaMAX (Invitrogen), 50 µg/ml Gentamycin (Invitrogen), 50 µM β-mercaptoethanol (Sigma-Aldrich) and 10% fetal calf serum (Moregate)). Each well was either stimulated with medium alone, 5 µg/ml ndd-ASP, 5 µg/ml pdd-ASP or 1 µg/ml ConA (Sigma-Aldrich). Five days later, cells were pulsed with 1 µCi ^{3}HT (Perkin Elmer). After 18 h, cells were harvested and analysed with a 1450 Microbeta β-scintillation counter (Perkin Elmer). For the PKH26 experiments, PBMCs and mesenteric lymph node MCs were collected one week after the last vaccination and at time of necropsy, respectively, and labelled with PKH26 (Sigma-Aldrich) as previously described [8].

After labelling, cells were seeded at $2.5x10^6$ cells/ml in complete medium in 96-well round-bottom plates, and either stimulated with medium alone, 5 µg/ml ndd-ASP or 5 µg/ml pdd-ASP. After 5 days of culture, the cells were harvested, stained with monoclonal antibodies and analysed by flow cytometry. ModFit LT software (Verity Software House) was used to calculate the proliferation index (PI) for the different cell populations.

To measure cytokine production during the proliferation of bovine PBMCs and mesenteric lymph node MCs, supernatants of the abovementioned cultures were collected at time of harvesting after 5 days of culture. Enzyme-linked immunosorbent assay (ELISA) for detection of IFNγ and IL-4 was performed by coating capture monoclonal antibodies in carbonate buffer (pH 9.6) on 96-well Maxisorp (Nunc) plates at a concentration of 2 µg/ml and 4 µg/ml, respectively. Plates were incubated with blocking buffer (2% bovine serum albumin (BSA) in PBS supplemented with 0.05% Tween20 (Sigma-Aldrich)) for one hour. Afterwards, 25 µl or 50 µl of supernatant (for detection of IFNγ and IL-4, respectively) were placed in each well and blocking buffer was used to adjust the volume to 100 µl per well. Biotinylated monoclonal antibodies raised against IFNγ or IL-4 were added at 2 µg/ml. Streptavidin-horseradish-peroxidase (Sigma-Aldrich) was used as a conjugate and TMB (Thermo Scientific) served as substrate. After incubation of 15-30 min with the substrate, the reaction was stopped by the addition of 0.2M H_2SO_4. Optical density was measured at 450 nm. All antibodies were kindly provided by Prof. Jayne Hope (The Roslin Institute, UK).

Antibody responses

For both studies, serum was collected before the first immunisation and one week after each immunisation. In addition, a 1 cm^2 piece of the small intestine (complete intestinal wall) at 3 meters from the pylorus was collected at time of necropsy. Small intestinal extracts were obtained by incubating ground frozen tissue in PBS for 2 h at 4º while shaking, and protein concentration was determined with a bicinchoninic acid (BCA) assay kit (Pierce). Samples were subsequently centrifuged for 15 minutes at 16000 g and the supernatants collected. The systemic and intestinal IgG1, IgG2 and IgA levels against the *C. oncophora* ndd-ASP were then determined by ELISA. ndd-ASP was coated in 96-well Maxisorp plates (Nunc) at a concentration of 0.5 µg/ml in carbonate buffer (pH 9.6). After 1 h incubation with blocking buffer (2% BSA-PBS-0.05%Tween20), either 100 µg intestinal extract or 100 µl of serum 1/200-diluted in blocking buffer were added in duplicate and incubated for an additional hour. After washing, sheep anti-bovine IgG1, IgG2 and IgA (AbD Serotec) coupled to HRP were used as conjugates (dilution 1/500). 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Roche) served as substrate. Optical density was measured at 405 nm with a reference wavelength at 492 nm.

In addition, inhibition ELISA (iELISA) assays were performed to evaluate and compare the specificity of the antibodies raised against the native and recombinant versions of the dd-ASP. For both studies, pooled small intestinal extracts from the ndd-ASP+QuilA vaccinated animals were incubated at a final concentration of 200 µg/ml for 1 h at room temperature with different concentrations of either ndd-ASP or pdd-ASP, ranging from 0 to 1000 pmol/ml. The pre-incubated samples were afterwards tested in duplicate in a ndd-ASP coated plate as previously described for the conventional ELISA. Finally, an additional iELISA for the IgG1 isotype was performed for study 2 where intestinal extract from ndd-ASP+QuilA vaccinated animals was pre-incubated with pdd-ASP, and tested afterwards on a a ndd-ASP coated plate as described for the conventional ELISA.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism software. A non-parametric Kruskal-Wallis test followed by a Dunn´s multiple comparison test was used to determine significant differences in parasitological parameters, antibody responses and ^{3}HT assays. For these latter, an additional ANCOVA (SPSS, IBM SPSS statistics version 23.0) was used to evaluate the impact of the outcome of one specific week in the results of the consecutive week. To determine significant differences in cytokine production and proliferation between the various cell subsets in the PBMCs and MCs, a non-parametric Mann-Whitney test was used. A *P*-value of ≤ 0.05 was considered significant.

3. Results

Evaluation of a C. oncophora recombinant-ASP protein as potential vaccine candidate

Following expression and purification of the pdd-ASP, the correct size and purity of the protein was verified by SDS-PAGE analysis (Figure 1), showing uniform glycosylation in contrast to its native counterpart which displays more heterogeneity in its N-glycan profile. Afterwards, the protein band was excised and protein identity was further confirmed by mass spectrometry (data not shown). This material was subsequently used in a first study to evaluate the protective capacities of the recombinantly produced *C. oncophora* dd-ASP protein in comparison to the native antigen.

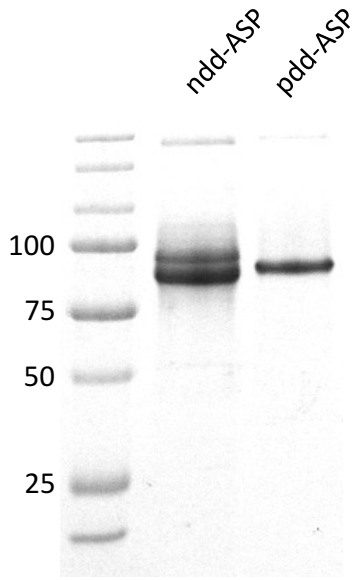

Figure 1. Recombinant dd-ASP. Upon gel filtration, the recombinant material was collected, analysed and compared with the ndd-ASP for protein content in a 12 % SDS-PAGE.

As indicated in Figure 2, animals vaccinated with the ndd-ASP+QuilA vaccine showed a reduction of 99% in cumulative faecal egg counts (cumFEC) and 88% decrease in worm counts compared to the control vaccinated group, confirming previous findings [7]. Lower worm counts were observed following vaccination with pdd-ASP+QuilA, although differences did not reach statistical significance (Figure 2B).

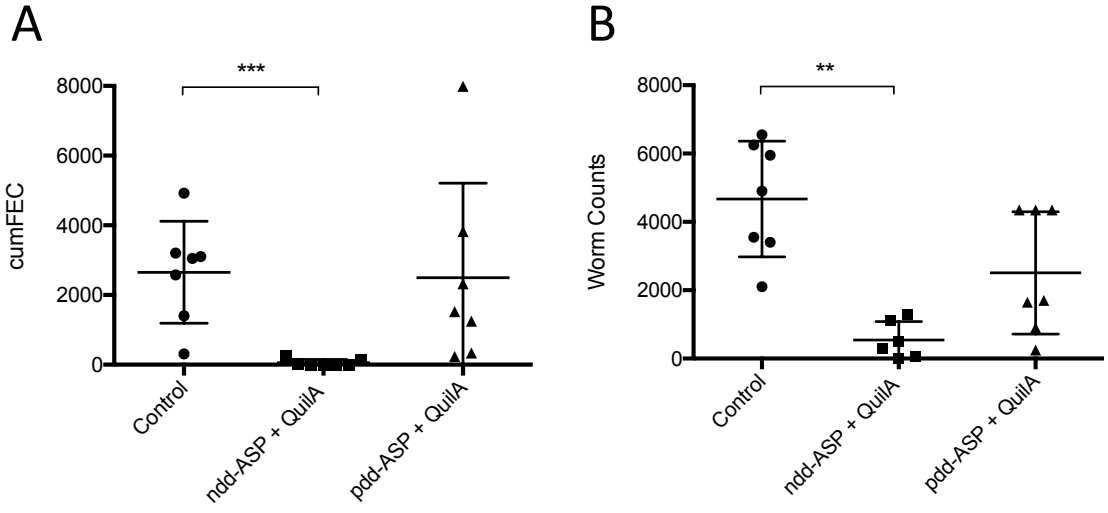

Figure 2. Effect of vaccination with native versus recombinant dd-ASP of *C. oncophora* on egg and worm counts. For animals vaccinated with QuilA alone (Control), ndd-ASP+QuilA and pdd-ASP+QuilA, (A) cumulative number of eggs per gram faeces (cumFEC) and (B) worm counts in Study 1 were determined 3 times each week during the trickle infection and at the time of necropsy, respectively. Graphs show the mean cumFEC or worm counts in each group ± SEM. Statistically significant differences compared to control vaccinated animals are indicated with * ($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$).

Antibody responses after vaccination with native and recombinant versions of the vaccine

Vaccination with both ndd-ASP+QuilA and pdd-ASP+QuilA vaccines resulted in a significant increase of ndd-ASP-specific IgG1 and IgG2 levels in serum compared to QuilA control animals ($p \leq 0.05$, $p \leq 0.01$), while a significant increase in systemic IgA levels could only be detected after vaccination with ndd-ASP+QuilA (Figure 3A). In the small intestinal mucosa, vaccination with ndd-ASP+QuilA and subsequent infection also resulted in a significant increase of ndd-ASP-specific IgA, IgG1 and IgG2 levels ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$), whereas vaccination with pdd-ASP+QuilA only induced a significant increase in ndd-ASP-specific IgG2 level, and higher though not significant levels of IgG1 or IgA (Figure 3B). To determine possible differences in the specificity of antibodies induced by vaccination and subsequent infection with ndd-ASP+QuilA, pooled small intestinal extracts collected at necropsy were subjected to an iELISA. Pre-incubation of the small intestinal extracts with pdd-ASP failed to inhibit the binding of the IgG1, IgG2 and IgA antibodies induced in ndd-ASP+QuilA vaccinated animals, while pre-incubation with ndd-ASP almost completely inhibited IgG1, IgG2 and IgA binding to ndd-ASP (Figure 3C).

Analysis and comparison of the cellular vaccine-induced responses by the native and recombinant vaccines

The aim of the second study was to confirm the results obtained in study one in terms of antibody responses, and to compare the differences in the vaccine-induced cellular responses between both native and recombinant vaccines. To do so, an experimental design similar to study 1 was carried out where animals were challenged post-vaccination and cellular, humoral and parasitological parameters were monitored.

To monitor cellular responses during the vaccination period, PBMCs were isolated on a weekly basis to measure antigen-specific proliferation *in vitro*. Results are shown in Figure 4A as stimulation index (SI) (= counts per minute (CPM) antigen stimulated cells / CPM medium stimulated cells). After *in vitro* restimulation of PBMCs with the vaccine antigens (ndd-ASP for the ndd-ASP+QuilA and Control groups, and pdd-ASP for the pdd-ASP+QuilA) antigen-specific proliferation was found in the ndd-ASP+QuilA group (Figure 4A). Proliferation in the ndd-ASP+QuilA group was the highest and significantly different from control animals on week 4 after the first vaccination ($p \leq 0.01$). From week 5 onwards, cellular proliferation continued to be significantly higher in the ndd-ASP+QuilA group compared to control and pdd-ASP-vaccinated animals ($p \leq 0.01$). One week after the third vaccination (week 7), vaccine antigen-specific proliferation could also be observed in animals vaccinated with pdd-ASP+QuilA (Figure 4A).

Additionally, an ANCOVA test was performed to assess the potential effect of the results of one week in the following week with no significant results.

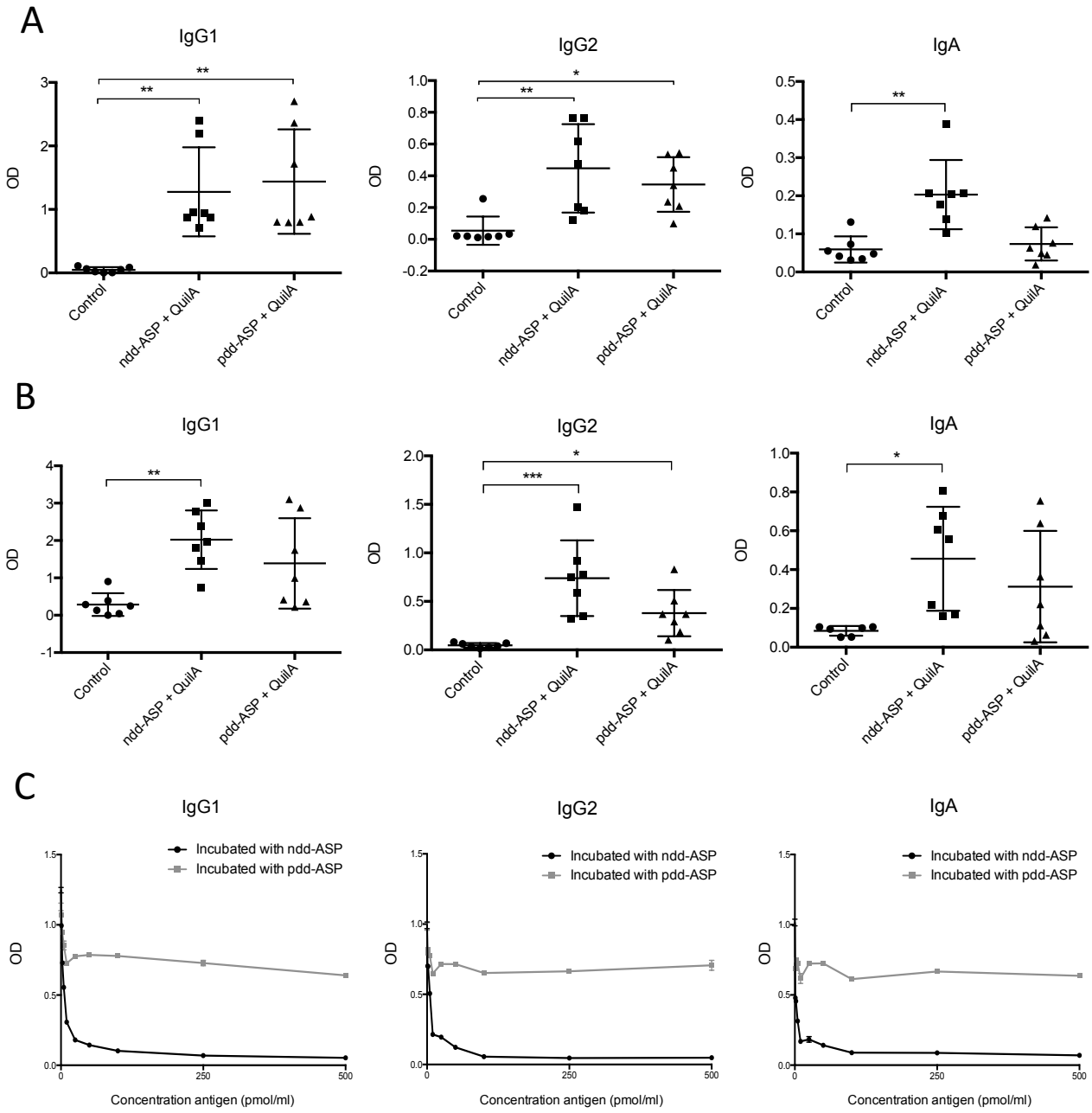

Figure 3. Antibody responses in serum and mucosa after vaccination with the native and recombinant vaccines. In cattle study 1, (A) serum from one week after the second vaccination and (B) mucosal samples after vaccination and subsequent infection were collected from all animals and used for the detection of ndd-ASP-specific IgG1, IgG2 and IgA antibodies through ELISA. The graphs show the individual absorbance values within each group and the mean ± SEM. Statistically significant differences are indicated with * ($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$). (C) Small intestinal extracts from calves vaccinated with ndd-ASP+QuilA were pre-incubated with either ndd-ASP or pdd-ASP and evaluated in an inhibition ELISA for IgG1, IgG2 and IgA isotypes. Graphs were generated from pooled samples analysed in duplicate.

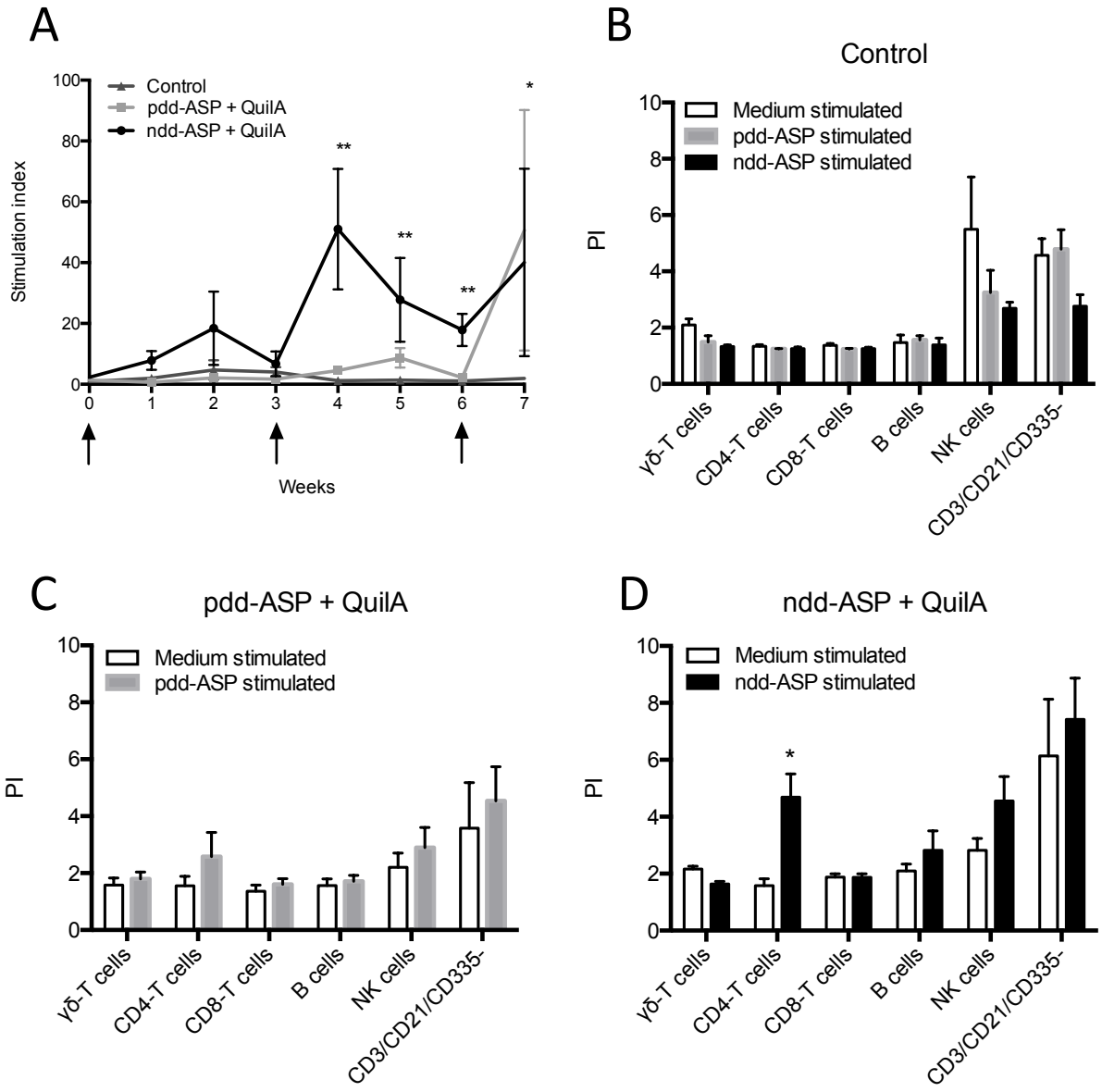

Figure 4. Systemic cellular responses following vaccination with native and recombinant vaccines. (A) During the vaccination period, PBMCs were isolated weekly, re-stimulated with either medium alone or the antigen used in the vaccine formulation, and the proliferation was determined based on ^{3}HT incorporation. The graph shows the mean stimulation index ± SEM for all vaccinated groups (n=4), where the arrows indicate the time points at which the animals were immunised. (B,C,D) Seven weeks after the first vaccination, PBMCs from all groups were stimulated for 5 days with medium or the vaccine antigen, stained with PKH to determine the proliferation with flow cytometry. The mean proliferation indices (PI) ± SEM of (B) Control (n=4), (C) pdd-ASP+QuilA (n=4), and (D) ndd-ASP+QuilA (n=4) vaccinated animals were calculated and compared to those of the control group. Statistically significant differences for all graphs are indicated with * ($p \leq 0.05$), **($p \leq 0.01$).

To determine the phenotype of the antigen-specific PBMCs that proliferated during the vaccination period after *in vitro* stimulation, PBMCs were isolated at week 7, labelled with PKH and cultured either in the presence of medium alone or 5 µg/ml of the vaccine antigen. After 5 days, cells from all groups were harvested and analysed by flow cytometry. Although the thymidine data showed antigen-specific proliferation at week 7 in the pdd-ASP+QuilA group, no proliferating cell population could be identified in the PKH assay (Figure 4B-C). Whether this is due to a population of cells for which no specific markers were included in the PKH assay is currently unclear. On the other hand, significant antigen-specific proliferation of CD4-T cells after *in vitro* stimulation with ndd-ASP was observed in animals vaccinated with ndd-ASP+QuilA when compared to the medium stimulated cells ($p \leq 0.05$) (Figure 4D).

To characterize the phenotype of the cellular response in the small intestine following vaccination and challenge infection, LNs from all animals were collected at time of necropsy and MCs were stimulated *in vitro* as previously described for PBMCs. For all groups, proliferation of γδ-T cells, CD4-Tcells, NK cells and non-T, non-B non-NK cells ($CD3^-/CD21^-/CD335^-$) was significantly higher after re-stimulation with ndd-ASP ($p \leq 0.05$) (Figures 5A-C), suggesting that the challenge infection had an effect on the local immune response. However, proliferation after re-stimulation with ndd-ASP was significantly higher in animals vaccinated with ndd-ASP+QuilA for CD4-T cells and CD8-T cells when compared to the control and pdd-ASP+QuilA groups ($p \leq 0.05$) (Figure 5D). ELISAs performed to detect the presence of either IL-4 or IFNγ in the culture supernatants showed that *in vitro* restimulation with ndd-ASP induced a significantly higher production of both IL-4 and IFNγ in all groups when compared to the medium stimulated ($p \leq 0.05$) (Figures 5E-F).

Identical as in study 1, the systemic and mucosal antibody responses were measured both in a quantitative and qualitative way. The levels of IgG1 and IgG2, but not of IgA ndd-ASP-specific antibodies in serum were significantly higher in animals vaccinated with both ndd-ASP+QuilA and pdd-ASP+QuilA ($p \leq 0.05$) (Figure 6A). In the mucosa, IgG1 levels were significantly higher in ndd-ASP+QuilA vaccinated animals but not in pdd-ASP+QuilA vaccinated animals ($p \leq 0.05$) (Figure 6B). In contrast, animals vaccinated with pdd-ASP+QuilA showed a significant increase in antigen-specific IgG2 and IgA levels ($p \leq 0.01$ and $p \leq 0.05$ respectively). This increase, although not significant, could also be observed in animals vaccinated with ndd-ASP+QuilA (Figure 6B). Finally, iELISAs for IgG1, IgG2 and IgA were performed with intestinal extracts from animals vaccinated with ndd-ASP+QuilA with identical results to those observed in Study 1 (Figure 6C).

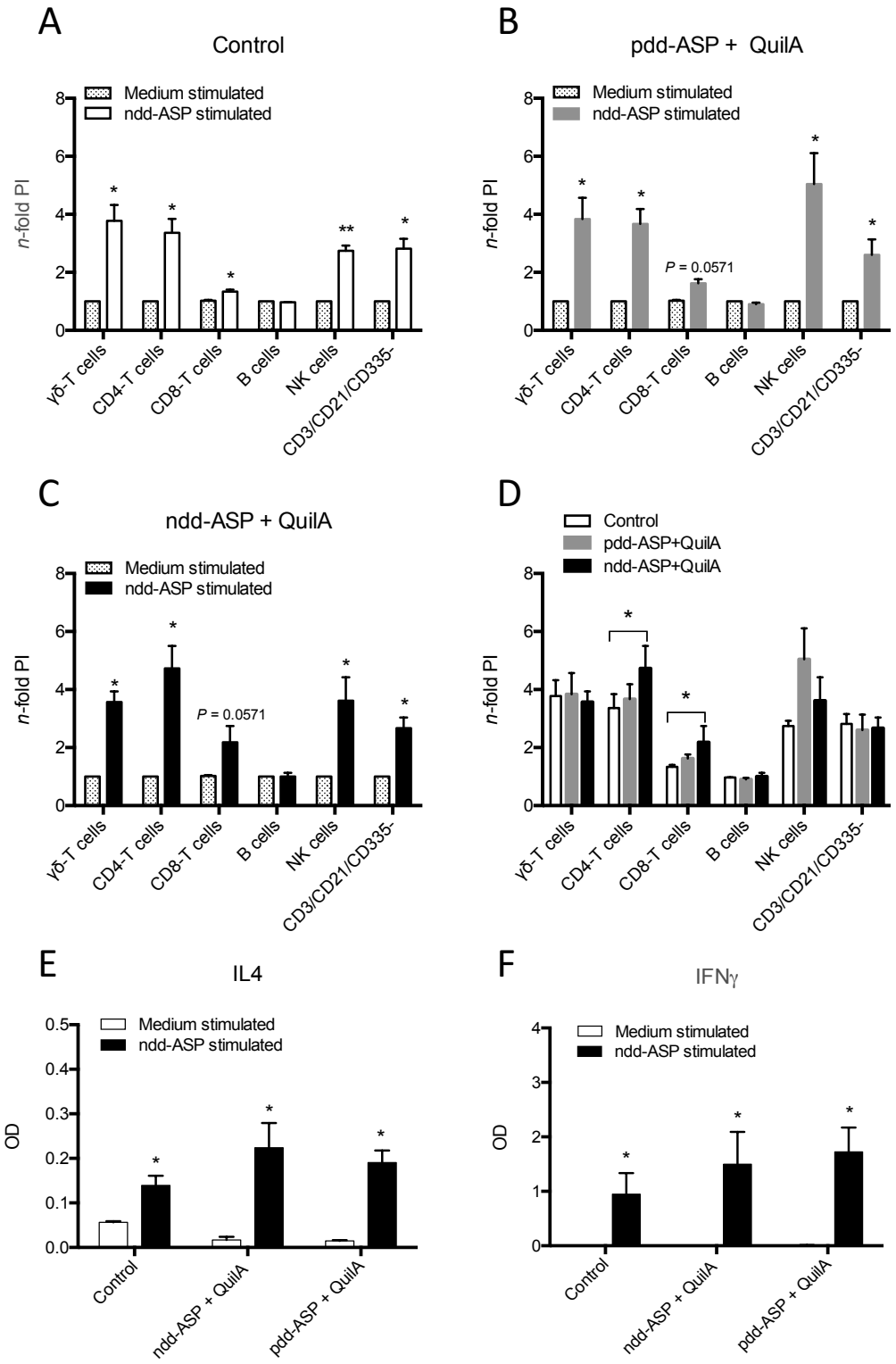

Figure 5. Mucosal cellular responses following vaccination with native and recombinant vaccines. At time of necropsy, after vaccination and 5 weeks of subsequent infection, MNCs were isolated from the small intestinal lymph nodes and their proliferative capacity was evaluated after *in vitro* stimulation with ndd-ASP. Mean n-fold proliferation index (PI) ± SEM is shown individually for (A) Control (n=4), (B) ndd-ASP+QuilA (n=4) and (C) pdd-ASP+QuilA (n=4) vaccinated animals. (D) Additionally, n-fold PI between all groups after ndd-ASP stimulation were compared and are shown as mean ± SEM. Supernatants of the previous cultures were collected and the production of (E) IL-4 and (F) IFNγ was determined. Both graphs show the mean OD of either IL-4 or IFNγ from supernatants of medium-stimulated or antigen-stimulated cells for each group (n=4) ± SEM. Statistically significant differences for all graphs are indicated with *($p \leq 0.05$).

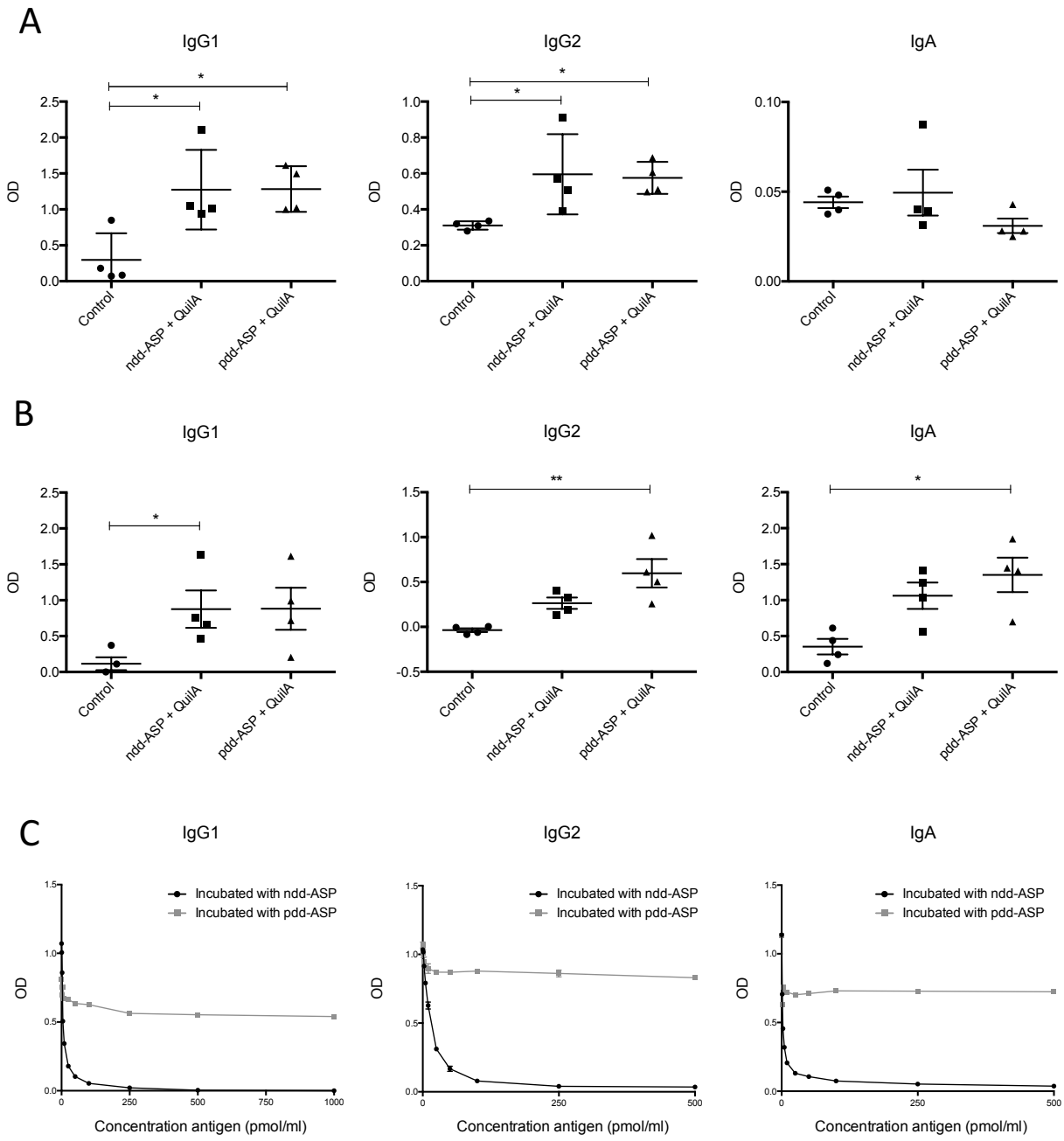

Figure 6. Antibody responses in serum and mucosa after vaccination with the protective and non-protective vaccines. In cattle study 2, (A) serum from one week after the last vaccination and (B) mucosal samples were collected from all animals and used for the detection of ndd-ASP-specific IgG1, IgG2 and IgA antibodies through ELISA. The graphs show the individual absorbance values within each group and the mean ± SEM. Statistically significant differences are indicated with * ($p \leq 0.05$), **($p \leq 0.01$). (C) Small intestinal extracts from animals vaccinated with ndd-ASP+QuilA were pooled, pre-incubated with either ndd-ASP or pdd-ASP and evaluated in an inhibition ELISA for IgG1, IgG2 and IgA isotypes. Graphs were generated from tested samples in duplicate, where each point indicates the mean OD ± SEM.

Finally, in terms of parasitological parameters, no reduction could be observed for animals vaccinated with pdd-ASP+QuilA (Figure 7). On the other hand, 3 out of 4 animals in the ndd-ASP+QuilA group did show a clear reduction in cumEPG (Figure 7A) but, importantly, 1 animal was not protected.

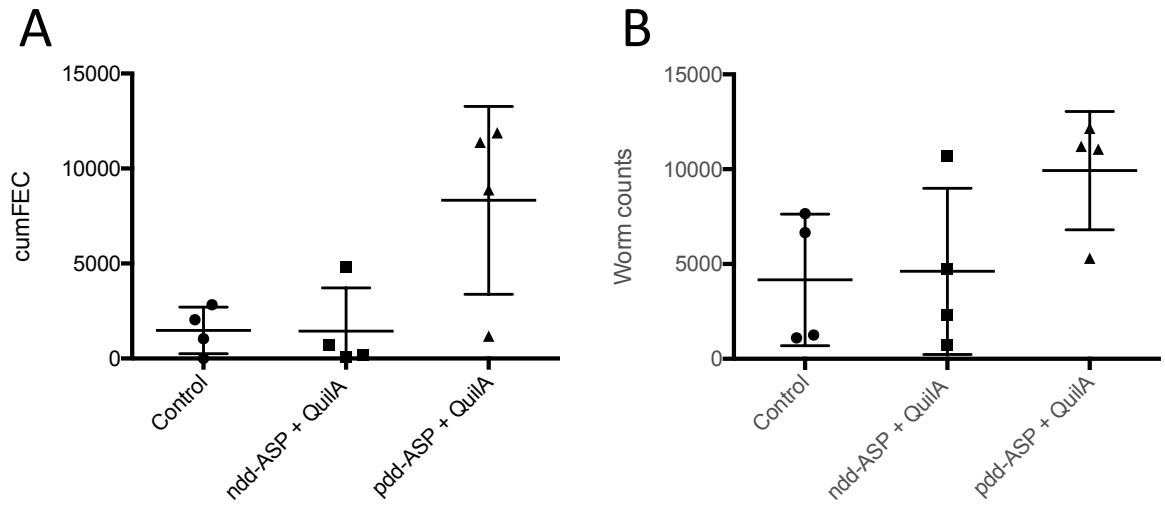

Figure 7. Parasitological parameters. (A) Cumulative number of eggs per gram faeces (cumFEC) and (B) worm counts in study 2 were determined 3 times each week during the trickle infection and at the time of necropsy respectively. Graphs show the mean cumFEC or worm counts for each group ± SEM.

To investigate whether there was a correlation between antibody specificity and protection, an IgG1 iELISA was performed on intestinal extract from the individual animals vaccinated with ndd-ASP+QuilA, in which the extracts were pre-incubated with pdd-ASP and subsequently tested for binding to a ndd-ASP coated plate. Ideally, pdd-ASP would be able to display similar inhibitory capacities towards antibodies as the ndd-ASP, and pre-incubation of antibodies from ndd-ASP-vaccinated animals with pdd-ASP should prevent them from binding the ndd-ASP present on the plate. The results of this assay, as shown in Figure 8A, indicated firstly that pdd-ASP is unable to inhibit antibodies from binding to the ndd-ASP. In addition, results show that there was a variation in the specificity of the antibodies induced in the different animals and that the antibodies present in the non-protected animal showed the lowest specificity toward ndd-ASP (Figure 8B). Such potential relationship was not observed for cellular proliferation or antibody levels (results not shown).

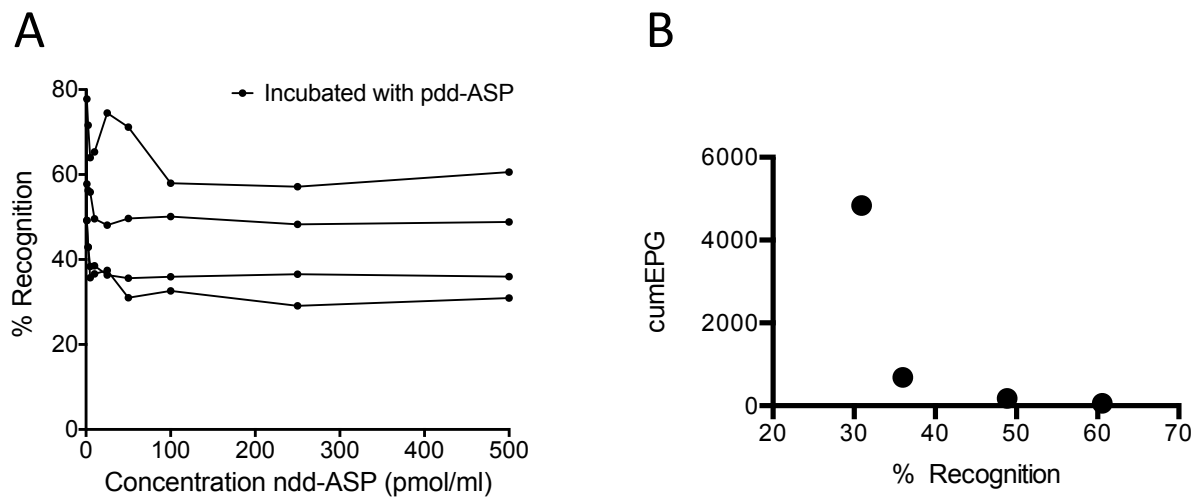

Figure 5. Correlation between protection and antibody specificity. Small intestinal extracts from animals vaccinated with ndd-ASP+QuilA and subsequently infected in study 2 were individually tested in an iELISA by pre-incubation with pdd-ASP and later evaluation in an ndd-ASP coated plate. (A) The graph shows the individual percentage (%) of recognition for each animal. (B) Correlation between cumFEC values and antibody % of recognition in the animals vaccinated with ndd-ASP+QuilA.

4. Discussion

Similar to our previous findings [7], vaccination with the native antigens (ndd-ASP) provided protection against an experimental infection with *C. oncophora*. Inducing a protective response with recombinant antigens has proven to be extremely challenging for most of the anthelmintic vaccines tested to date [8,12]. The present study shows that this is also the case for the double-domain ASP vaccine against *C. oncophora*, as vaccination of calves with a *Pichia*-produced version of the antigen (pdd-ASP) did not confer any protection.

Previous studies on the vaccine-induced immunity with the native antigen against *C. oncophora* revealed the induction of a strong antigen-specific cellular response in the small intestinal mucosa, mainly characterized by the presence of antigen-specific αβ- and γδ-T cells [11]. In line with this, the present study demonstrates that vaccination of calves with ndd-ASP+QuilA, but not with pdd-ASP+QuilA or QuilA alone, resulted in an increased systemic memory response from the second vaccination onwards, which was characterized by the induction of antigen-specific CD4-T cell proliferation.

Although proliferation of almost all cell populations could be detected for all groups in the mesenteric lymph nodes, the magnitude of this response and, more specifically of the CD4-T cell population, was higher in animals vaccinated with the native antigen and subsequently infected. These results not only agree with the findings by Van Meulder et al. (2015), but also resemble the naturally acquired immunity to *C. oncophora* where an augmented CD4-T cell frequency has been observed both systemically and at the site of infection [13].

While immunity against helminth infections has been commonly associated with a T_H2 response, the production of both IL-4 and IFNγ by mesenteric lymphocytes in all groups after re-stimulation with ndd-ASP suggests a combined T_H1/T_H2 type response. The observation of a mixed T_H1/T_H2 response against a helminthic infection is not novel and has been extensively documented for other helminth species such as *Fasciola hepatica* [14], *Trichinella spiralis* [15] and *Ostertagia ostertagi* [16]. In terms of humoral responses, the overall results indicated that both native and recombinant vaccines induce the production of antigen-specific IgG1, IgG2 and IgA antibodies. Nonetheless, inhibition ELISAs for all isotypes showed that the antibodies induced by the ndd-ASP preferentially bind to the ndd-ASP and not to the recombinantly produced version of the antigen (pdd-ASP) and vice versa (data not shown). Whether this difference in antibody specificity is important in conferring protection is still unclear. However, it is interesting to note that the only animal with high cumFEC from the ndd-ASP vaccinated group raised antibodies with the lowest specificity towards ndd-ASP, indicating the existence of a negative correlation between antibody specificity and parasitological parameters. This suggests an important role for antibodies in the protective immune response, an observation that has also been previously made for naturally acquired and vaccine-induced immunity against *C. oncophora* [11,17].

Overall, the observation that both cellular and humoral responses differ between animals vaccinated with either the native or the recombinant form of the dd-ASP antigen are in line with our previous observations in the vaccine-induced immunity against *O. ostertagi* [8]. This differential immune recognition is possibly caused by differences between native and recombinant proteins in terms of protein conformation and/or the associated glycan structures. ASPs belong to the cysteine-rich secretory/antigen 5/pathogenesis-related 1 (CAP) superfamily and have been suggested to play a role in immune evasion, although their exact biological functions remain unknown. As the name itself suggests, proteins belonging to this family are rich in cysteine residues that form several disulphide bridges, resulting in a highly conserved α-β-α-sandwich structure [18]. The exact constitution of these disulphide bonds has only been obtained based on 3D-

crystallographic data from recombinantly expressed material [19,20], and whether these bridges are actually present in the native ASPs remains unclear.

However, what seems evident is that protein folding is essential for mounting a proper immune response, as demonstrated by the abolition of all vaccine-induced immune responses (both cellular and humoral) when vaccinating animals with an unfolded version of native *O. ostertagi* ASP1 protein (Chapter 2). In addition, structural analysis has revealed differences in the glycans present on the native and recombinant versions of the dd-ASP antigen (unpublished data). Potentially these differences influence the immunogenicity of the antigens. Therefore, further studies with non-glycosylated forms of the antigens are necessary to provide further insights on the importance of these glycans.

Taken together, the outcome of the present study shows that vaccination of calves with a protective ndd-ASP+QuilA vaccine, but not with its recombinant counterpart, results in a strong cellular memory response mainly characterized by the presence of antigen-specific CD4-T cells in both blood and intestinal mucosa. Although both ndd-ASP and pdd-ASP-based vaccines induced similar levels of antibodies, the specificity of the antibodies raised by both antigens clearly differed. The possible structural differences that impede the recombinant vaccine to mount a protective immune response remain unknown and form the basis of our current research.

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4

Effect of native and recombinant ASPs on bovine dendritic cell maturation

Effect of native and recombinant ASPs on bovine dendritic cell maturation

1. Introduction

In chapters 2 and 3, the differences in vaccine-induced immunity between the native and recombinant vaccines against *O. ostertagi* and *C. oncophora* were assessed. Briefly, we demonstrated that immunisation with the native antigens, unlike their recombinant counterparts, elicited a stronger cellular response after in vitro re-stimulation characterized by the proliferation of CD4-T cells and NK cells for *C. oncophora* and *O. ostertagi* respectively. This augmented cellular response in native vaccinated animals was accompanied by an increased antibody response in the gastrointestinal mucosa, in particular for IgG1 and IgG2. In addition, antibodies raised by vaccination with the native antigens preferentially bind the native and not the recombinant antigens, suggesting a difference in antibody specificity.

These studies have however focused exclusively in the systemic and mucosal immune responses, without addressing how the upstream events right after intramuscular vaccination might influence the consequent immune responses. As stated in Chapter 1, DC are professional APCs distributed across all lymphoid and non-lymphoid tissues, including the skeletal muscle [1,2]. They take up, process and present antigens to other immune cells (i.e. T cells, B cells and NK cells) that reside in secondary lymphoid organs through the expression of MHC-II and co-stimulatory molecules. In addition, the release of soluble mediators aids to steer and modulate the immune response towards a type-1, type-2, type-17 or regulatory immune response. Antigenic presentation and subsequent activation of T-cells by DCs occurs through the interaction of MHC complexes on the surface of DC and the T-cell receptor (TCR), together with the ligation of the DC co-stimulatory molecules (i.e. CD80, CD86) and the secretion of mediators (i.e IL-4, IL-10) [3]. Similarly, the synergistic actions of contact dependent (i.e CD40/CD80 ligation) and independent signals (i.e. IL-12, IL-18) are required for DC-mediated NK cell activation [4]. In terms of interactions with B cells, it has been demonstrated that DC can retain unprocessed antigens and transfer them to B cells in a process that induces isotype switching by B cells [5].

Based on this, it is conceivable that following vaccination, the differential activation of DC by native and recombinant ASPs could impact the subsequent immune response and hence determine the success or failure of the vaccine. Therefore, the aim of this chapter was to investigate the responses elicited after stimulation of bovine monocyte-derived dendritic cell (moDC) with native and recombinant ASPs from *O. ostertagi* and *C. oncophora*.

2. Materials & Methods

Antigen preparation

Preparation of the native *O. ostertagi* ASP (nASP) and *C. oncophora* double-domain ASP (ndd-ASP) was carried out as previously described [6-9]. In short, the native fractions were purified from total adult ES material by thiol-sepharose chromatography followed by anion exchange chromatography in the case of *O. ostertagi*, while for *C. oncophora* ES was fractionated over a Superdex 200 16/70 size-exclusion chromatography column. The purity of both ASP fractions was checked on a 10% SDS-PAGE gel under reducing conditions and visualized by Coomassie or Simply-Blue SafeStain (Invitrogen) stainings. Recombinant ASP of both species was produced in *Pichia pastoris* as previously described [10] and will be referred to as pASP for *O. ostertagi*, and pdd-ASP for *C. oncophora*.

Bovine monocyte-derived dendritic cell generation and stimulation

All animal experiments were conducted in accordance with the EU Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical approvals to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2012/24). For the generation of monocyte-derived dendritic cells (moDC), venous blood was drained from the vena jugularis of five naïve 6-month old Holstein-Friesian calves using vacutainer tubes (BD Bioscience) and peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep (Nycomed Pharma) gradient centrifugation. PBMCs were subsequently incubated with anti-human CD14 microbeads (Miltenyi Biotec) and positively selected in a magnetic cell sorter (MACS separator, Miltenyi Biotec) according to the manufacturer´s instructions. The purity of the CD14 fraction was evaluated by flow cytometry and was always ≥97%. Monocytes were then seeded at 10^6 cells/ml in flat-bottom plates (Thermo Scientific) and cultured for 7 days in complete medium (RPMI 1640 + GlutaMAX (Invitrogen), 50 µg/ml Gentamycin (Invitrogen), 50 µM β-mercaptoethanol (Sigma-Aldrich) and 10% fetal calf serum (Moregate)) supplemented with 10 ng/ml recombinant porcine GM-CSF (rpGM-CSF) and 1 ng/ml recombinant bovine IL-4 (rbIL-4) (R&D Systems). At day 3, cultures were supplemented with fresh cytokines. At day 7 cells were supplemented with fresh cytokines and stimulated for 48h with the antigens (nASP, pASP, ndd-ASP and pdd-ASP) at 5 µg/ml in the presence or absence of QuilA (5 µg/ml) (Superfos Biosector). LPS (Invivogen) was used as positive control at 1 µg/ml, while medium alone served as negative control.

After stimulation, supernatants were collected for further cytokine analysis and cells were harvested using 200 µl/well Accutase (Innovative Cell Technologies) during 10 minutes and subsequently analyzed by flow cytometry.

Co-stimulatory molecules

Cells were labelled in Flow Cytometry Staining Buffer (eBioscience) and all antibodies were used at the concentration recommended by the supplier. After incubation for 20 minutes with primary antibodies, cells were washed two times prior to staining with fluorescently-labelled secondary antibodies. Cells were then incubated for an additional 20 minutes, washed and resuspended in PBS to be immediately analysed using a FACS Aria III (BD Biosciences) or CytoFLEX flow cytometer (Beckman Coulter). Non-viable cells were excluded of the analysis based on their Sytox Blue (Life Technologies) uptake. Primary antibodies used were: non-labeled CD40 (IL-A156, IgG1), CD86 (IL-A190, IgG1), CD80 (IL-A159, IgG1) and MHC-II (CC158, IgG2a) (all from BioRad). Secondary antibodies used were: goat anti-mouse IgG1-FITC (Santa Cruz Biotech) and goat anti-mouse IgG2a-APC (Invitrogen). Mean fluorescence intensity (MFI) for all parameters was obtained using the FACS Diva (BD Biosciences) or the CytExpert software (Beckman Coulter).

DQ-ovalbumin

DQ-Ovalbumin uptake assay (DQ-OVA, Thermo Fisher Scientific) was used to evaluate the ability to take up antigen of the unstimulated and stimulated moDC. MoDC were split into two plates to be incubated for 2 hours at either 4°C or 37°C with 1 µg/well BODIPY-conjugated DQ-OVA, and the MFI was measured by flow cytometric analysis as described above.

Cytokine ELISA

To measure cytokine production after stimulation of moDC with the different antigens, supernatants of the abovementioned cultures were collected and enzyme-linked immunosorbent assay (ELISA) was performed for detection of IL-4 (antibodies kindly provided by Prof. Jayne Hope, The Roslin Institute, UK), IL-12, (BioRad) and IL-6 (Bovine IL-6 DuoSet, R&D Systems) with the antibody pairs shown in the table below.

Except for IL-6, where manufacturer's instructions were followed strictly, capture monoclonal antibodies were coated overnight at 4°C at different working concentrations in carbonate buffer (pH 9.6) on 96-well Maxisorp (Nunc) plates, followed by incubation with blocking buffer (5% bovine skimmed milk powder (Marvel)) in PBS for one hour. Afterwards, 50 µl of supernatants were placed in each well. Biotinylated monoclonal antibodies were added at the appropriate concentration. Streptavidin-horseradish-peroxidase (Sigma-Aldrich) was used as a conjugate and ABTS (Roche) served as substrate. Optical density was measured at 405 nm.

Table 1. Overview of the antibody pairs used in the cytokine ELISAs.

Antibody	Clone	Working concentration (µg/ml)
IL-4	CC314	4
IL-4 biotin	CC313	2
IL-12	CC301	8
IL-12 biotin	CC326	5

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software. A non-parametric Kruskall Wallis test was used to determine significant differences between all conditions versus medium control for all parameters (DQ-OVA, co-stimulatory molecules and cytokine production). A p-value of ≤ 0.05 was considered significant.

3. Results

Upregulation of co-stimulatory molecules

The mean fluorescence intensity (MFI) of the co-stimulatory molecules CD40, CD86, CD80 and MHC-II was measured in moDC upon stimulation with the native and recombinant ASPs for both *O. ostertagi* (Figure 1) and *C. oncophora* (Figure 2) alone or in combination with QuilA.

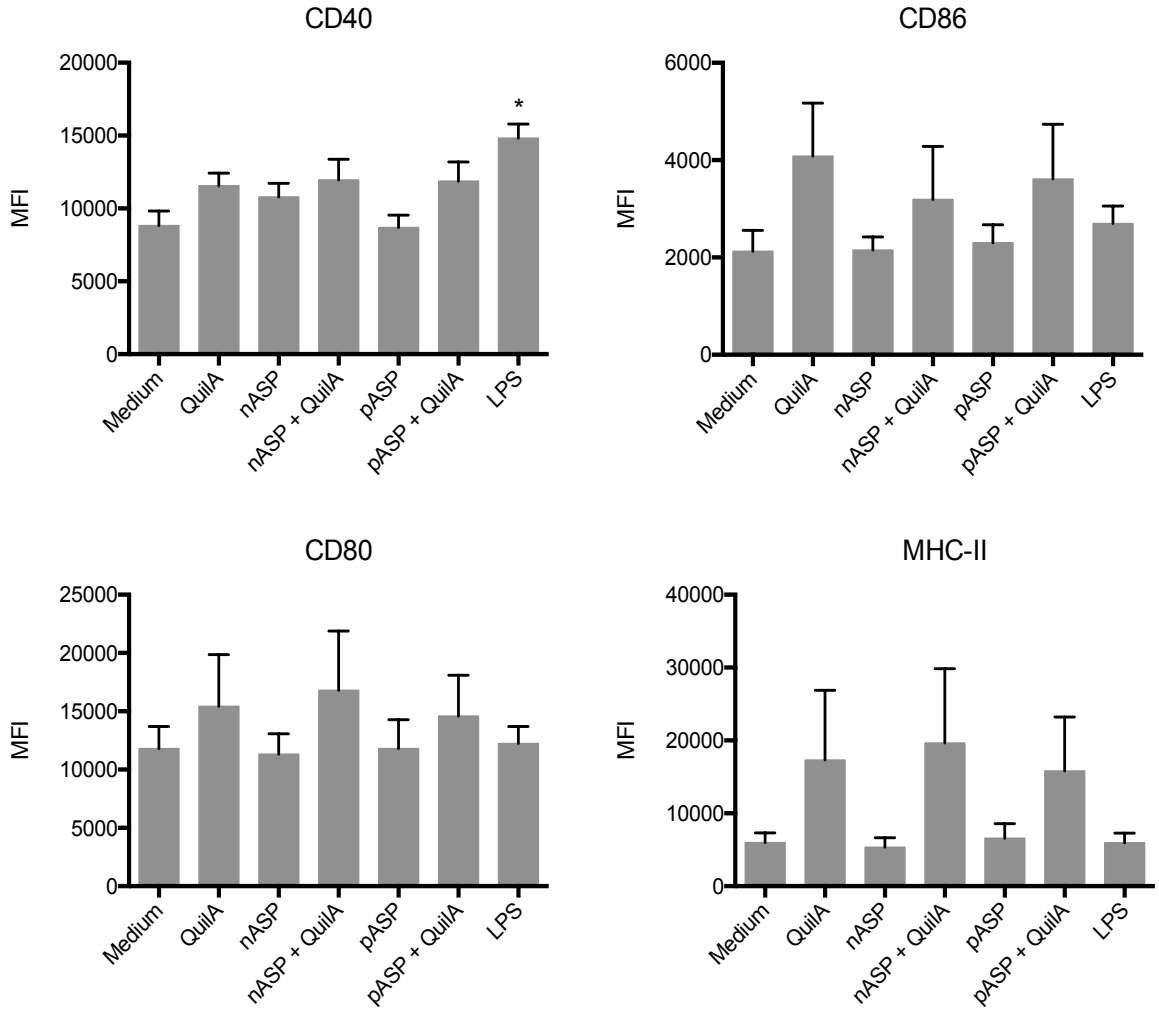

Figure 1. Effect of native and recombinant ASPs from *O. ostertagi* on the expression of costimulatory molecules on the surface of moDC. MoDCs were stimulated with native (nASP) or recombinant (pASP) (5 µg/ml) alone or in combination with QuilA (5 µg/ml) and LPS (1 µg/ml). After 48h, the mean fluorescence intensity (MFI) for CD40, CD86, CD80 and MHC-II was measured. Results are presented as arithmetic mean of the individual values for each animal and condition ± SEM. Statistically significant differences to unstimulated (medium) are indicated with *($P \leq 0.05$).

In general, no significant differences could be detected in the expression of any of the co-stimulatory molecules for all conditions tested. A significant increase in CD40 expression could only be observed after stimulation of moDC with LPS ($p \leq 0.05$) (Figure 1) and ndd-ASP and pdd-ASP in combination with QuilA ($p \leq 0.01$ and $p \leq 0.05$ respectively) (Figure 2). Nevertheless, a general trend towards higher expression of the co-stimulatory molecules CD40, CD86, CD80 and MHC-II can be observed in all the conditions where QuilA was included.

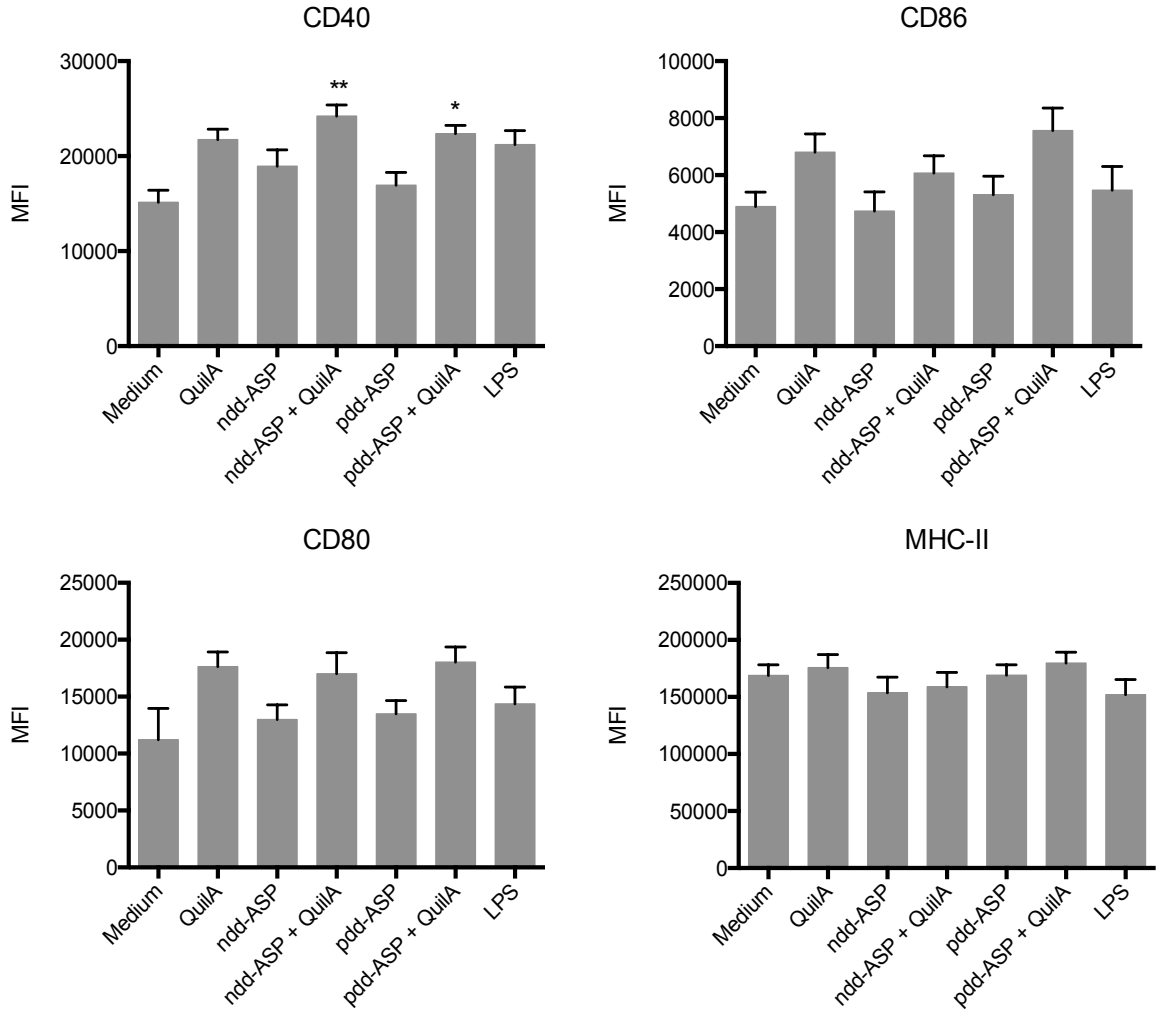

Figure 2. Effect of native and recombinant ASPs from *C. oncophora* on the expression of costimulatory molecules on the surface of moDC. MoDCs were stimulated with ndd-ASP or pdd-ASP (5 µg/ml) alone or in combination with QuilA (5 µg/ml) and LPS (1 µg/ml). After 48h, the mean fluorescence intensity (MFI) for CD40, CD86, CD80 and MHC-II was measured. Results are presented as arithmetic mean ± SEM. Statistically significant differences to unstimulated (medium) are indicated with *($P \leq 0.05$), **($P \leq 0.01$).

Ovalbumin uptake

Immature DC are characterised by their high capacity for antigen uptake, which decreases during maturation. DQ-ovalbumin (DQ-OVA) exhibits green fluorescence upon uptake and proteolytic degradation by immature moDC, and therefore can be used to determine the maturation status of DC after stimulation with the different antigen combinations.

To correct for possible background fluorescence, the MFI of 4°C DQ-OVA-pulsed moDC was subtracted from the MFI of DQ-OVA-pulsed moDC at 37°C. These values are represented in Figure 3, which shows that both for *O. ostertagi* and *C. oncophora* antigens, only the presence of QuilA induced a decrease in DQ-OVA uptake when compared to unstimulated cells. Although not significant, a slight decrease in DQ-OVA uptake could also be observed for moDC stimulated with antigen alone and LPS.

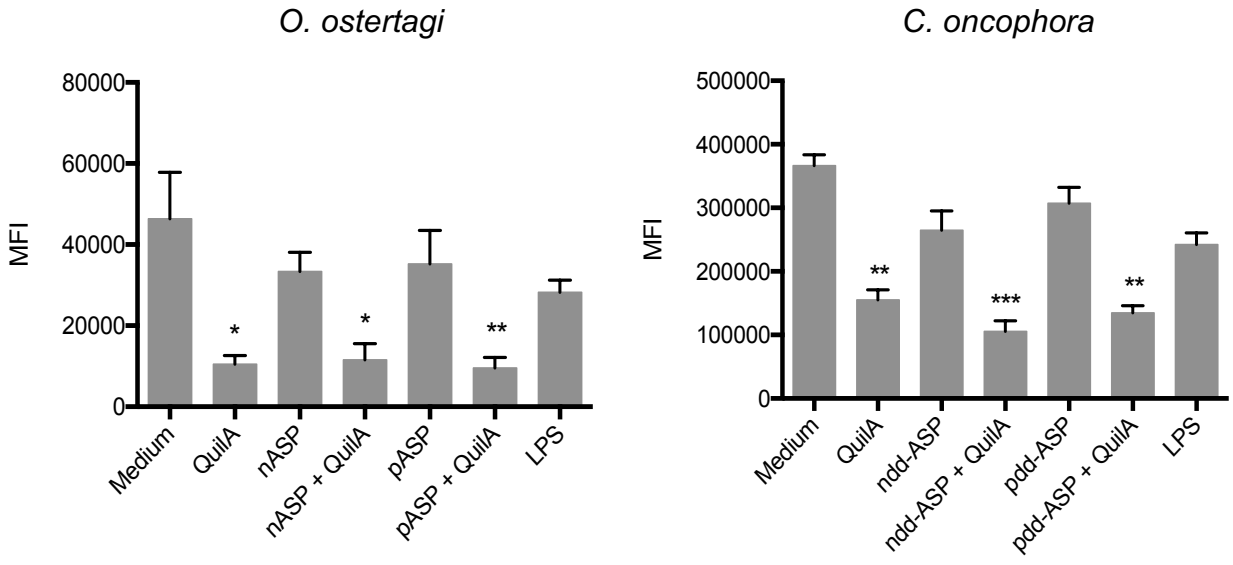

Figure 3. Effect of native and recombinant ASPs from *O. ostertagi* and *C. oncophora* on the maturation status of moDC. MoDCs were stimulated with the native and recombinant ASPs (5 µg/ml) of *O. ostertagi* (left) and *C. oncophora* (right) alone or in combination with QuilA (5 µg/ml) and LPS (1 µg/ml). After 48h, the cells were incubated for and additional 2h with DQ-OVA and the mean fluorescence intensity (MFI) was measured. Results are presented as arithmetic mean ± SEM. Statistically significant differences to unstimulated (medium) are indicated with *($P \leq 0.05$), **($P \leq 0.01$), ***($P \leq 0.001$).

Cytokine production

For *O. ostertagi*, stimulation of moDC with LPS and nASP alone or in combination with QuilA induced the production of significant levels of IL-12 when compared to unstimulated cells ($p \leq 0.001$ and $p \leq 0.05$ respectively) (Figure 4). IL-6 levels in the culture supernatants were also significantly elevated after stimulation with nASP alone and LPS ($p \leq 0.05$ and $p \leq 0.001$ respectively), while no significant increase of IL-4 could be detected for any of the conditions (Figure 4).

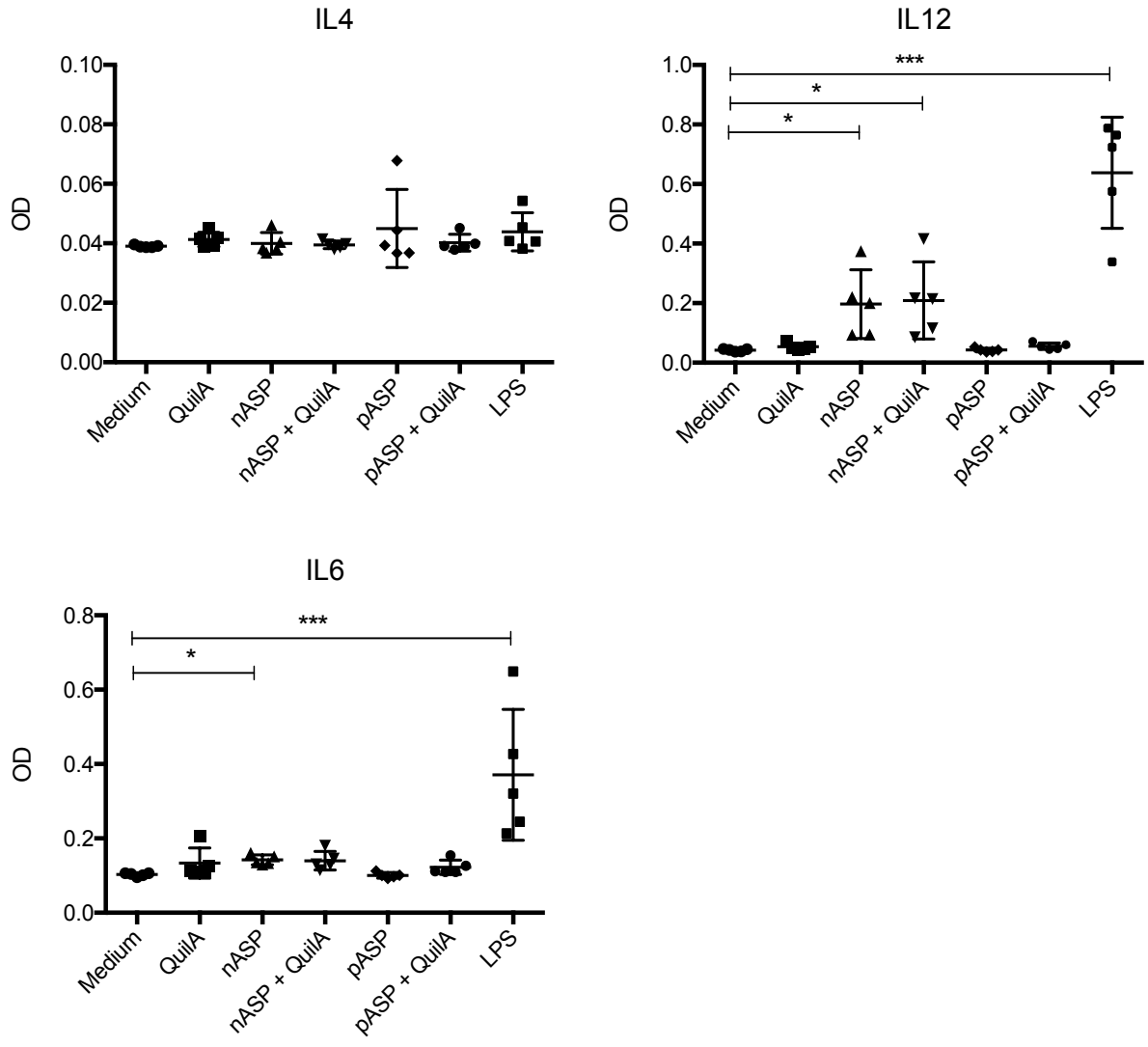

Figure 4. Cytokine production by moDc after stimulation with native and recombinant *O. ostertagi* antigens. MoDCs were stimulated with the nASP and pASP (5 µg/ml) alone or in combination with QuilA (5 µg/ml) and LPS (1 µg/ml). After 48h, the culture supernatants were collected for the detection of IL-4, IL-12 and IL-6. Results are presented as arithmetic mean ± SEM. Statistically significant differences to unstimulated (medium) are indicated with *($P \leq 0.05$), ***($P \leq 0.001$).

Similarly, for *C. oncophora* stimulation of moDC with LPS and ndd-ASP alone or in combination with QuilA induced the production of significant levels of IL-12 when compared to unstimulated cells ($p \leq 0.001$ and $p \leq 0.01$ respectively) (Figure 5). The levels of IL-6 in the culture supernatants were also significantly elevated after stimulation with LPS ($p \leq 0.05$) and, although not significant, a clear increase could be detected after stimulation with ndd-ASP alone or combined with QuilA. No significant increase of IL-4 could be detected for any of the conditions (Figure 5).

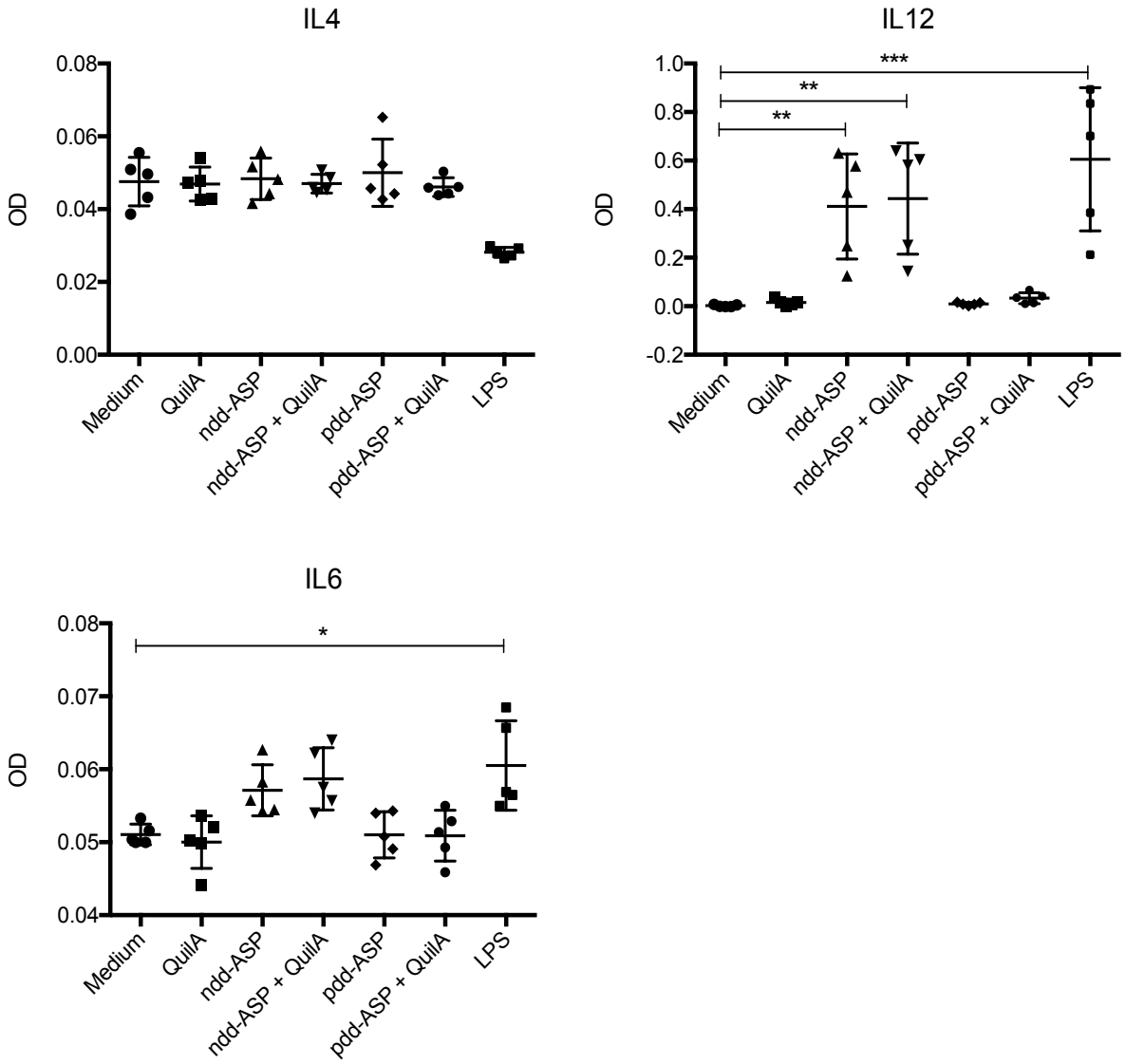

Figure 5. Cytokine production by moDc after stimulation with native and recombinant *C. oncophora* antigens. MoDCs were stimulated with the ndd-ASP and pdd-ASP (5 µg/ml) alone or in combination with QuilA (5 µg/ml) and LPS (1 µg/ml). After 48h, the culture supernatants were collected for the detection of IL-4, IL-12 and IL-6. Results are presented as arithmetic mean ± SEM. Statistically significant differences to unstimulated (medium) are indicated with *($P \leq 0.05$), **($P \leq 0.01$), ***($P \leq 0.001$).

4. Discussion

The goal of the present study was to evaluate the responses of bovine moDC upon stimulation with native and recombinant ASPs from *O. ostertagi* and *C. oncophora* alone or in combination with the adjuvant QuilA. In general, up-regulation of co-stimulatory molecules seemed to be weak and linked to the use of QuilA as stimulant and not to *O. ostertagi* or *C. oncophora* ASPs. Moreover, a mature DC phenotype, as established by the DQ-OVA assays, was also associated with QuilA. These observations agree with previous studies in rodents where stimulation of DC with helminth products induces immunogenic DCs that however fail to display the hallmarks of a mature phenotype i.e. the expression of costimulatory molecules [11]. Similar observations have also been made with bovine DC after stimulation with *Giardia duodenalis* ES [12]. On the contrary, incubation of bone marrow-derived DC (bmDC) with *N. brasiliensis* ES material induced the upregulation of the co-stimulatory molecules CD86 and OX40L [13].

Regarding QuilA, Robson et. al. demonstrated that DC are the principal APC able to prime CD4-T cells upon in vitro stimulation with this adjuvant [14]. In addition, its stimulatory effect on DC has been previously shown in human moDC, which become activated after *in vitro* stimulation with the QuilA subfraction QS-21 [15]. Despite these observations made using *in vitro* studies, little is known about the exact mechanisms by which QuilA and its subfractions stimulate immune cells *in vivo*. Given its saponin nature and demonstrated lytic effects [16], one can speculate that the destabilization of the cell plasma membranes at the site of injection and subsequent cell lysis induced by QuilA creates an inflammatory environment where immune cells will be recruited. Interestingly, during the FACS analysis performed in our *in vitro* experiments, we observed a decrease in viable cells in samples stimulated with QuilA (data not shown). Nevertheless, whether this QuilA induced cell lysis helps in the activation of DC *in vitro* is unclear.

Although up-regulation of co-stimulatory molecules and the acquisition of maturity appears to be linked to QuilA, the detection of IL-6 and IL-12 in culture supernatants relates to the use of native-ASP as stimulant. Interestingly, *Onchocerca volvulus* ASP1 induces IL-12 production by human DC in a CD40-dependent manner [17]. In this context, it is important to note that *in vitro* stimulation of mononuclear cells isolated from native-ASP vaccinated animals induced the secretion of IFNγ, as IL-12 is a pro-inflammatory cytokine which stimulates the production of IFNγ by T cells and NK cells [18,19]. On the other hand, IL-6 is a T_H17-promoting cytokine demonstrated to have a limiting effect on Th2 responses during *H. polygyrus* infections [20] .

Based on these results, the question rises whether the IL-12 production by bovine moDC is exclusively due to the action of native-ASP or if the presence of other components in the native vaccine, such as LPS, might be influencing this response. LPS is a known strong inducer of IL-12 [21]. In this context, a recent study by Hamilton et al. (2016) shows that stimulation of bovine DC with Bacille Calmette Guerin (BCG) induces the expression of MHC-II, CD40 and CD80 and the production of IL-12. In addition, these activated DC are able to activate NK cells *in vitro*, as indicated by the release of IFNγ and the upregulation of the activation marker CD25 [22]. Based on this, we evaluated the potential presence of endotoxin on native-ASP preparations using the limulus amebocyte lysate (LAL) test. High endotoxin levels were detected in several batches of both nASP and ndd-ASP (data not shown), albeit the source of this LPS is unknown. ASPs are obtained by incubating adult worms that are isolated directly from the gut, where the microbiome stands a potential source of LPS. Consequently, it is possible that LPS is co-purified with the native ASPs. Alternatively, ASP samples might become contaminated during the manipulation in the laboratory. One may wonder whether the presence of LPS in the native antigen preparations contributes and to what extent to the immune responses elicited by the native antigens. Studies performed in a mouse model of traumatic skeletal muscle injury revealed that DC are recruited and maturate in the injured muscle tissue [2]. These DC are capable of taking up i.m-injected OVA, and, interestingly, their migration to the local draining LN to initiate a T_H1 response is enhanced in the presence of LPS [18]. In line with this, LPS-enhanced migration of moDC has been observed in mice upon i.m vaccination with Alum-adjuvanted OVA combined with low doses of LPS [23]. Additionally, these DC displayed a higher capacity to induce IFNγ production by T cells in the local draining LNs[23].

Finally, one might question whether moDC are a good model for skeletal muscle DC. In mice, DC have been widely studied and the different subsets with specialized functions that reside in the different tissues and organs are well described [24]. Although many efforts have been made to characterize the different bovine DC subsets, no information is yet available on the skeletal muscle populations. Nevertheless, the phenotypic characterization of skin-draining DC has revealed the presence of 4 different subsets with varying functions [25-28], and therefore it is very likely that the muscle also counts with phenotypically and functionally different subsets of DC. Therefore, using muscle-isolated DC would be ideal to determine the responses observed in muscle upon vaccination, given that moDC might not be representative enough. However, many considerations have to be taken into account when contemplating this approach. First of all, a deep knowledge of the different muscle-resident DC populations is needed. Secondly, a proper isolation method for muscle DC in cattle should be optimize, and care must be taken that

this process does not affect DC functionality in the subsequent assays. Finally, we have the ethical considerations, since it is very likely that the isolation of enough muscle DC requires the sacrifice of animals. For these reasons, to date, moDC are the best alternative available to unravel the effect that the ASP vaccines have on DC.

In summary, the outcome of this study suggests that the presence of QuilA, but not of native or recombinant antigens alone, is essential in the induction of DC maturation. This is reflected in the DC´s inability of taking up DQ-OVA after stimulation with QuilA alone or in combination with any of the antigens. In addition, only native-ASP stimulated moDC released IL-12 and IL-6 while the levels of IL-4 remained low. Of special interest is the release of high levels of IL-12, and whether its induction is due to the action of native-ASP or other components, such as LPS. To further address this hypothesis, studies using LPS-free native ASPs should be performed.

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5

General discussion

General discussion

The main objective of this thesis was to discern the immune responses associated with protection following vaccination with *Ostertagia ostertagi* and *Cooperia oncophora* native and recombinant ASP-based vaccines. The outcome of this thesis demonstrates that, although recombinant-ASPs fail to induce protection, the study and comparison of the immune responses elicited by native and recombinant ASP provided valuable information for the development of efficient recombinant vaccines. In this final chapter, observations of these studies that deserve extended attention, recent ideas and future prospects will be further discussed.

Implications of the vaccine-induced cellular responses

While immunity against helminths has been commonly associated with type-2 responses, a type-1 skewed immune response characterised by antigen-specific proliferation of NK cells and the production of IFNγ is observed upon vaccination of cattle with ***O. ostertagi*** nASP. Moreover, stimulation of bovine DC with native-ASP induced the production of IL-12, a strong NK cell activator. Interestingly, very similar results were obtained in studies on vaccine-induced immunity against *Onchocerca volvulus*. For instance, vaccination with recombinant *O. volvulus* ASP expressed in *E. coli* induces antigen-specific production of IFNγ, most likely by NK cells [1], and stimulation of DC with this antigen leads to the production of IL-12 [2]. Although all these observations suggest a potential conserved mechanism of NK cell activation by ASP, whether this activation occurs in a direct or indirect manner remains to be elucidated. Based on literature, I here propose two non-exclusive modes of NK cell activation (Figure 1). The first one, as laid out in chapter 4, will depend on the interaction between NK cells and helminth antigen-loaded DC [3,4]. DC can express different PRRs which can recognise various GI helminth PAMPs, i.e. *Acanthoceilonema viteae* ES62 recognised by TLR4, *Ascaris lumbricoides* (PS) lipids recognised by TLR2, and *Heligmosomoides polygyrus* calreticulin recognised by class A scavenger receptor. In addition to TLRs, DC also possess C-type lectin receptors (CLR), NOD-like receptors (NLR) and RIG-1 receptors involved in the recognition of a broad array of PAMPs. It is therefore likely that, because ASP is a glycoprotein, more than one receptor is involved in its recognition and uptake. Following antigen uptake in the muscle, DC migrate to secondary lymphoid organs where the direct contact with NK cells, together with the release of soluble factors (i.e IFNγ and IL-12) will result in a reciprocal activation of both cell types.

The second suggested mode of NK cell activation is based on the direct interaction between lymph node-resident NK cells and ASP. For instance, *Necator americanus* ES products, which contain ASP, are able to directly bind NK cells and induce the production of IFNγ in an IL-2/IL-12 dependent manner [5]. These observations suggest that the release of IL-2/IL-12 by DC is necessary for ASP-mediated NK cell activation, and therefore both modes of action might occur simultaneously (Figure 1).

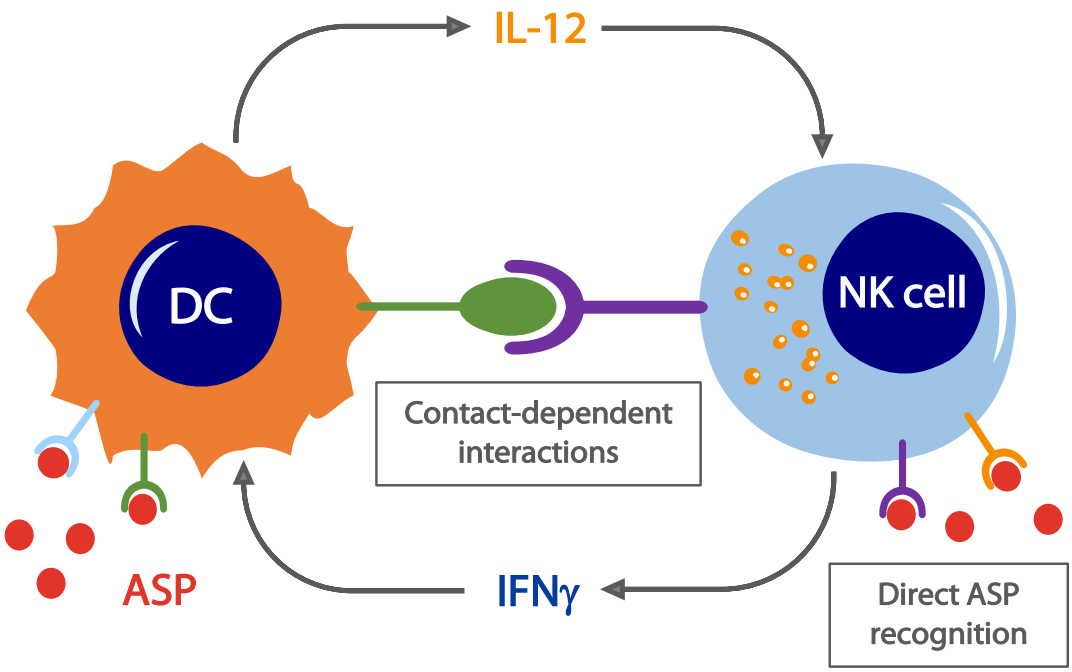

Figure 1: Although NK cells might directly bind to pathogen-derived products, they generally require signals from dendritic cells (DC) for their effector function. On the other hand, DC can become activated upon ligation of parasite-derived products by PRRs and activate NK cells through contact - dependent and soluble signals (i.e. IL-12). In return, NK cells stimulate DC maturation through the release of IFNγ.

All these observations argue for the potential existence of a conserved mechanism in which the interaction of ASP with DC and/or NK cells leads to a type-1 response. Nevertheless, whether this response relates with protection or with an immune evasion strategy by the parasites is still unclear. Assuming this response is related to protection, one should further contemplate how these newly activated NK cells affect the subsequent immune responses and, importantly, the production of highly specific antibodies. In this context, interesting observations have been made in the interaction between B cells and NK cells and how these latter can impact antibody production and specificity.

For instance, IFNγ can enhance antibody production by LPS or antigen-primed B cells [6]. Moreover, NK cells have been shown to stimulate isotype switching by B cells in a contact-dependent manner [7]. In contrast, if the NK response would be the result of an evasion strategy by the worm, it will be interesting to consider the potential direct activation of B cells by ASP. Importantly, B cells are able to produce antigen-specific antibodies in a T cell-independent manner. In this context, the observation of a direct interaction between ASP from *N. americanus* [8] and *O. volvulus* [2] with B cells, together with the direct stimulatory effects of LPS on B cells, is of special interest. In addition, although it has been generally assumed that antibody responses elicited in a T-cell independent manner do not result in the generation of B cell memory, many studies have argued against this paradigm [9].

In contrast to the results obtained for *O. ostertagi*, the induction of ***C. oncophora*** ASP-specific CD4-T cells suggest that the effector mechanisms involved in this vaccine-induced immune response entail a classical DC priming of T cells followed by T-cell dependent B cell activation. Alternatively, as stated in Chapter 4, antigen-loaded DC might be able to also directly transfer unprocessed antigen to B cells and induce isotype switching in a T-dependent manner [10]. In addition, it is important to recall that DC have been shown to prime CD4-T cells upon stimulation with QuilA[11], the adjuvant of choice during all the studies performed in the present thesis.

Finally, both studies revealed the proliferation of a native-ASP-specific non-T/non-B/non-NK cell population. Although the traditional concept of immunological memory has been typically associated with cells expressing antigen receptors (i.e B cells and T cells), recent evidence suggests that several groups of innate cells can respond strongly upon reactivation with antigens. These subsets of memory-like innate cells include macrophages and type-2 innate lymphoid cells (ILC2) [12]. ILC2 are related to resistance against helminth infections, and although their rapid expansion upon restimulation is not mediated by direct contact with antigens, they can proliferate under the influence of cytokines displaying an antigen nonspecific memory [12,13]. It is therefore possible that the cytokines released by other cells (i.e. T cells)[14] during *in vitro* restimulations condition the response of these cells and induce their proliferation. On the contrary, macrophages are able to directly interact with antigens. Importantly, their immune memory, also termed "trained immunity", has been demonstrated during helminth infections. *N. brasiliensis* infection induces long-lived macrophages that damage the parasite and induce protection against reinfection in a T and B-cell independent manner [15]. A thorough study of these innate populations during vaccine-induced immune responses could therefore throw some light on the non-T/non-B/non-NK cell population mystery.

Future approaches

Taking all the above-mentioned information into account, several approaches can be followed in practice to unravel the exact mode of action of both *O. ostertagi* and *C. oncophora* native vaccines on the immune system and the implications on protective immunity. In first instance, it would be interesting to identify which immune cells directly interact with labelled native-ASP. This approach, which includes biotinylation of ASP and can be carried out making use of flow cytometry or immunohistochemistry, has previously been shown to be successful for *O. volvulus* [2] and *N. americanus* [5] ASPs as well as for *S. ratti* heat shock proteins [16]. To determine whether NK cell activation is DC-dependent or, by contrary, ASP alone is sufficient, assays where NK cells are culture with ASP alone or in the presence of DCs can be carried out. Moreover, it should be determined whether *O. ostertagi* and *C. oncophora* native-ASP, or in contrast LPS, are responsible for the IL-12 production by DC. In practice, removal of LPS from protein-containing aqueous solutions can be achieved through the use of affinity matrices. Nevertheless, there is no certainty of how this method affects protein integrity and recovery and its impact on these two aspects should be determined. As an alternative, several studies make use of polymyxin B, an antibiotic commonly used in the clearance of endotoxin contamination in culture media that can be added during the stimulation period [2,5,17]. Secondly, to address the impact of NK cells on antibody production, it will be interesting to perform vaccination studies where antibody levels are determined after vaccination of NK cell deficient mice. In relation to this, preliminary results suggest that NK cells are not required for the induction of nASP-specific antibodies, since these latter are induced in NKp46 conditional knock-out (NK deficient) mice after vaccination with nASP. During these studies however, certain animals showed what seemed an increase in the expression of NKp46 upon *in vitro* stimulation of MC with nASP, and therefore the results were considered inconclusive. Finally, to unravel the identity of the so-called mysterious population new vaccination trials should be performed in which exhaustive transcriptomic analysis upon restimulation of immune cells is included. Due to the limited availability of antibodies for bovine cell markers, I believe that this approach is the most suited to provide information on this matter. Finally, immunization of cattle with *C. oncophora* native-ASP results in higher levels of protection than the one observed for *O. ostertagi*. Because each vaccine induces a different antigen-specific immune cell population, it will be interesting to co-vaccinate animals simultaneously with both *O. ostertagi* and *C. oncophora* native-ASP and assess the potential influence of CD4-T cells on the protective capacities of *O. ostertagi* nASP.

Implications of the vaccine-induced antibody responses

Vaccination with native but not recombinant *O. ostertagi* ASP induced an increase in serum and mucosal IgG1 and IgG2 levels. Furthermore, increased IgG1 and IgG2 antibodies were observed in serum and mucosa after vaccination with both native and recombinant *C. oncophora* ASP. The analysis of the binding properties of antibodies raised by both vaccines showed that antibodies raised by vaccination with the native-ASP are highly specific and preferentially bind to native-ASP. In addition, a potential relationship between antibody specificity and protection was demonstrated. Interestingly, a correlation between antibodies and protection has been previously determined during natural infection with *O. ostertagi* [18]. More specifically, IgG2 antibodies which are commonly associated with type-1 responses correlate with protection despite being less abundant than IgG1[19].

Together with the differences observed for vaccine-induced cellular responses, the findings regarding humoral responses suggest that native and recombinant-ASP from both parasites vary in their structure, and that this difference could lay at the basis of the inability of recombinant ASP to trigger effective responses. However, whether these differences reside in the protein structure or the associated glycans remains to be elucidated. ASPs are members of the cysteine-rich glycoprotein/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) protein family with an α-β-α sandwich structure held together by several di-sulphide (S-S) bridges [20,21]. For ***O. ostertagi***, two ASP molecules have been described to be present in the ES-thiol fraction, i.e ASP-1 and ASP-2, with a molecular weight around 30 kDa and two hybrid N-glycosylations [22,23]. The structure of ASP-1 has been unveiled after structural analysis of a recombinantly-produced version and it is characterized by the formation of a stable dimer maintained through the formation of 6 intermolecular S-S bonds [24] (Figure 2). Moreover ASP-1, unlike any other ASP, displays an additional intramolecular S-S bridge between the N and C-termini of each monomer which provides the protein with an almost-cyclic structure [24] (Figure 2). Because these results were based on the use of recombinant ASP, additional mass spectrometric analysis on the native ASP revealed that certain S-S bridges present in the recombinant-ASP were not detectable in the native-ASP, one of them being responsible of the cyclic structure. On the other hand, ***C. oncophora*** double-domain native ASP contains 24 cysteine residues that form 12 intramolecular SS-bonds. Although no information is yet available on the structure of *C. oncophora* recombinant ASP, it is conceivable that, as for *O. ostertagi*, the presence or absence of certain bonds has a major impact on the protein conformation and hence on its immunogenicity.

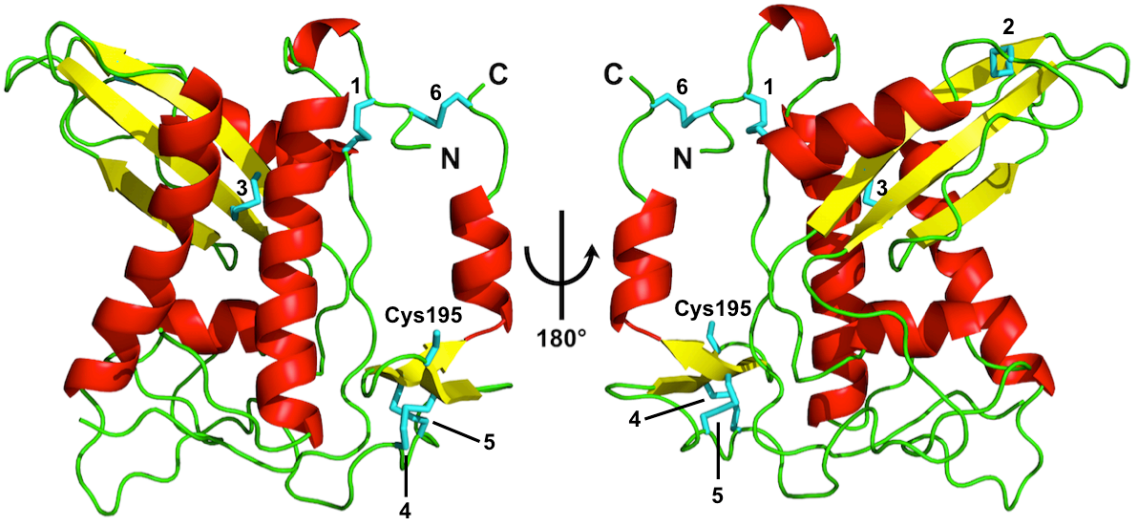

Figure 2: Recombinant ASP1 structure

In terms of glycan structures, both *O. ostertagi* and *C. oncophora* native ASP carry paucimannosidic and hybrid-type N-glycans containing a core α1-6 fucose, whereas the recombinant versions either carry a GalGlcNAcMan5 (pASP) or a Man5 (pdd-ASP) glycoform (Figure 3). It is important to note that the glycans found on native ASP are common amongst nematodes but usually not present in vertebrates, and therefore they could trigger the immune system. Interestingly, the α1-3 fucose present in *H. contortus* gut antigen H11 constitutes an important epitope for IgE [25]. Based on this, preliminary studies have been carried out in our lab that aim to elucidate the importance of glycans in the induction of highly specific antibodies. While deglycosylation of *C. oncophora* native-ASP decreases antibody recognition, removal of the glycans on the recombinant form improved the specificity towards the native-ASP. It is therefore likely that the larger glycans present on the recombinant-ASP are masking certain epitopes.

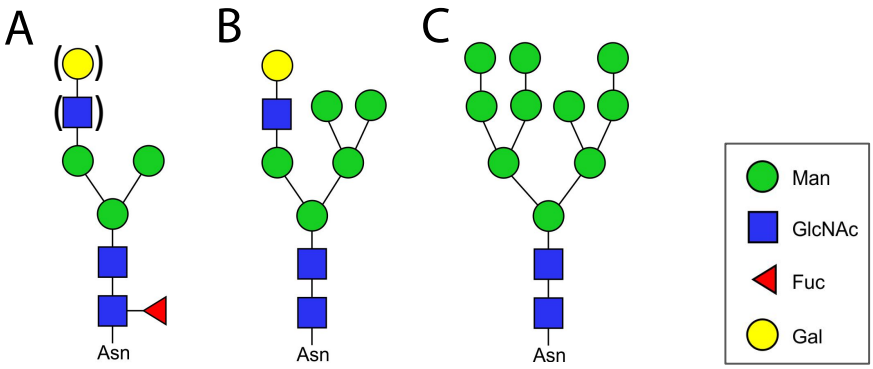

Figure 3: Schematic representation of the N-glycans found on the native (A) and recombinant *O. ostertagi* (B) and *C. oncophora* (C) ASPs.

These findings, together with the abolishment of all immune responses upon vaccination with reduced-ASP (chapter 2) suggest that both protein and glycan structures are essential for the immunogenicity of ASP proteins.

Future approaches

Unravelling the exact structural differences between native and recombinant ASP and the impact of these on immunity will provide essential information that will allow us to speed up the development of effective recombinant vaccines. Based on the information currently available, several approaches can be taken regarding the impact of both protein and glycan structures on the immunogenicity of ASP.

First of all, insights on the structure of recombinant *C. oncophora* ASP are required to unravel the exact differences with the native form. Given that *O. ostertagi* native-ASP lacks certain S-S bonds present in the recombinant version, it would be interesting to replace the cysteine residues responsible for these bonds in the recombinant-ASP through site-directed mutagenesis. A similar approach could be followed for *C. oncophora* if the structural analysis reveals differential bond formation between native and recombinant forms. In terms of glycan structures, the above exposed preliminary results indicate that the glycans present on the native-ASP are most likely part of an epitope. In order to mimic this structure on the recombinant-ASP, two approaches can be used. The first one consists on the expression of ASP in the glycoengineered *P. pastoris* GlycoSwitch strain developed by the group of Prof. Dr. Nico Callewaert (VIB/UGent) [26]. The second will make use of glycoengineered tobacco plants for the expression of ASP [27]. This technology is available in the Laboratory of Nematology of the University of Wageningen (The Nederlands). Taking all these potential adaptations in mind, the more practical method to rapidly screen the newly engineered forms of recombinant ASP would be the competition/inhibition ELISA. The protective potential of the successful candidates can be evaluated in subsequent vaccination trials.

While all the above-mentioned approaches might help to overcome the structural differences between native and recombinant ASP, they do not provide information on the exact epitopes recognised by the host´s immune system. From the vaccination studies with the reduced-nASP form we can assume that the epitope recognised by native-ASP-specific antibodies is conformational and not linear, and therefore a conformational epitope mapping approach would be the best suited in this case.

Finally, in the previous section several methods were proposed to unravel the interactions between the cellular compartment with ASP and the implications in the induced immune responses. The evidence exposed in this section suggests that antibodies are potentially correlated with effective immune responses, and therefore practical means should be taken to further describe their exact involvement in protection. Ideally, it will be interesting to set up cultures with the different developmental stages that reside in the host (i.e from L3 to adult) in the presence of antibodies obtained from immune animals. This will provide an insight on the direct effect of antigen-specific antibodies on worm growth and function. In addition, to address the question whether protection depends upon antibody-dependent cell cytotoxicity (ADCC), assays where both worms and antibodies are cultured in the presence of immune cells should be performed. During such studies, cell sorting techniques can be used to address the implications of each cell population individually. Unfortunately, sequential *in vitro* growth of all developmental stages that reside in the host is not yet feasible. Circumventing this issue on first instance will therefore have a major impact on future research.

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Summary
Samenvatting

Summary

O. ostertagi and *C. oncophora* are the most prevalent gastrointestinal (GI) nematodes of cattle in temperate climate regions which pose a major constrain on animal welfare and production. Infection with both parasites is the main cause of parasitic gastroenteritis in cattle and typically affects young calves during their first grazing season, provoking diarrhoea, weight loss and anorexia that can eventually lead to death. The widespread resistance to the current anthelmintic control methods, urges the development of sustainable alternatives such as vaccines. In that context, our group has developed two protective experimental vaccines against both parasites based on activation associated secreted proteins (ASP) obtained from the excretory/secretory (ES) material of adult worms. Nevertheless, recombinant production of these protective vaccine antigens is an absolute requirement for their commercialization. To date, most of the recombinantly produced vaccines antigens against livestock GI nematodes have unfortunately failed to provide protection when compared to their native counterparts. Elucidating the vaccine-induced immune responses that correlate with protection is of great importance and therefore the overall aim of the present thesis was to unravel the immune responses associated with protection following vaccination with native and recombinant ASP-based vaccines against the gastrointestinal nematodes *O. ostertagi* and *C. oncophora.*

In first instance (**Chapter 2**), we evaluated the protective potential of a *O. ostertagi* recombinant ASP (pASP) produced in the *Pichia pastoris* expression system in cattle. Moreover, the effect of antigen (native (nASP) vs. recombinant) and adjuvant (QuilA vs. $Al(OH)_3$) in the vaccine induced immune responses was investigated in both cattle and mice. Immunization of cattle with the protective nASP+QuilA vaccine was associated with antigen-induced proliferation of natural killer (NK) cells combined with IFNγ secretion and the induction of a mixed IgG1/IgG2 antibody response. This ASP-specific activation and proliferation of NK cells was also observed in mice following the same vaccination regime. However, replacing QuilA by $Al(OH)_3$ or nASP by pASP significantly decreased the capacity of the vaccines to trigger both NK cell activation and antibody responses and failed to induce protection against a challenge infection. In addition, antibodies raised by the native vaccine were highly specifically and preferentially bind to nASP. Finally, reducing the nASP completely abolished its ability to induce NK cell activation and antibody responses upon vaccination, suggesting an important role of protein conformation on the immunostimulatory activity of nASP.

Similarly, in **Chapter 3** we aimed to compare the cellular and humoral mechanisms underlying the vaccine-induced responses by the native (ndd-ASP) and recombinant *C. oncophora* vaccines. Immunization of cattle with the native *C. oncophora* vaccine conferred significant levels of protection after an experimental challenge infection, whereas the recombinant vaccine did not. Moreover, vaccination with ndd-ASP resulted in a higher proliferation of CD4-T cells both systemically and in the small intestinal mucosa when compared with animals vaccinated with the recombinant antigen. In terms of humoral response, although both native and recombinant vaccines induced similar levels of antibodies, animals vaccinated with the native vaccine were able to raise antibodies with greater specificity towards ndd-ASP in comparison with antibodies raised by vaccination with the recombinant vaccine.

In **Chapter 4** we studied the responses elicited by *O. Ostertagi* and *C. oncophora* antigens (native vs. recombinant) and adjuvant in bovine dendritic cells (DC). Stimulation of DC with QuilA induced moderate activation reflected in the increased up-regulation of co-stimulatory molecules and the inability to take up DQ-OVA antigens. In addition, stimulation of DC with native but not with recombinant ASP from both parasites elicited the production of IL-12. Due to the presence of LPS in certain native-ASP batches, the question rises whether the induction of IL12 production is due to the action of the native-ASP or due to other components such as LPS.

Finally, **Chapter 5** presents the discussion and future prospects. While this project provided broad information on the vaccine immune responses that might correlate with protection against *O. ostertagi* and *C. oncophora*, many questions remain to be addressed. For instance, the exact mechanism by which native ASP elicit protective immune responses is yet unknown. Additional information on the interactions of native ASP with different immune cells would help not only to understand the immunological requirements for protection, but also provide essential information on crucial epitopes recognized by the immune system. Simultaneously, it will be essential to perform a detailed analysis of the structural differences between native and recombinant ASP.

Samenvatting

O. ostertagi en *C. oncophora* zijn de meest voorkomende gastro-intestinale (GI) nematoden bij vee in gematigde klimaatregio's, die bovendien een belangrijke beperking vormen voor dierenwelzijn en productie. Infectie met beide parasieten is de belangrijkste oorzaak van parasitaire gastro-enteritis bij runderen en beïnvloedt meestal jonge kalveren tijdens hun eerste weideseizoen. Dit veroorzaakt diarree, gewichtsverlies en anorexia, wat uiteindelijk tot de dood kan leiden. De wijdverspreide resistentie tegen de huidige anthelmintica noodzaakt de ontwikkeling van duurzame alternatieven zoals vaccins. In die context heeft onze groep twee beschermende experimentele vaccins ontwikkeld tegen beide parasieten, gebaseerd op activatie geassocieerde gesecreteerde eiwitten (ASP), verkregen uit het excretie/secretie (ES) materiaal van volwassen wormen. Niettemin is een recombinante productie van deze beschermende vaccin antigenen een absolute vereiste voor hun commercialisering. Tot op heden bieden de meeste van de recombinant geproduceerde vaccin antigenen tegen GI-nematoden bij vee helaas geen bescherming in vergelijking met hun natieve tegenhangers. Het ophelderen van de vaccin-geïnduceerde immuunresponsen die correleren met bescherming is van groot belang. Daarom is het algemene doel van deze thesis het ontrafelen van de immuunresponsen die geassocieerd zijn met bescherming, na vaccinatie met natieve en recombinante ASP-gebaseerde vaccins tegen de gastro-intestinale nematoden *O. ostertagi* en *C. oncophora.*

In eerste instantie **(Hoofdstuk 2),** evalueerden we het beschermende potentieel van *O. Ostertagi* recombinant ASP (pASP) geproduceerd in een *Pichia pastoris* expressiesysteem bij runderen. Bovendien werden de effecten van antigeen (natief (nASP) vs. recombinant) en adjuvans (QuilA vs. Al (OH) $_{3)}$ in de vaccin-geïnduceerde immuunrespons onderzocht bij zowel runderen als muizen. Immunisatie van kalveren met het beschermende nASP + QuilA-vaccin was geassocieerd met een antigeen-geïnduceerde proliferatie van natural killer (NK) cellen, gecombineerd met IFNy secretie en de inductie van een gemengde IgG1/IgG2 antilichaam respons. Deze ASP-specifieke activatie en proliferatie van NK-cellen werd ook waargenomen bij muizen die hetzelfde vaccinatieschema kregen. Echter, het vervangen van QuilA door Al (OH) $_3$ of nASP door pASP verminderde significant de capaciteit van de vaccins om zowel NK-celactivering als antilichaamresponsen te veroorzaken en kon geen bescherming bieden tegen een experimentele infectie. Daarnaast waren antilichamen die door het natieve vaccin werden opgewekt zeer specifiek en bij voorkeur bindend aan nASP.

Ten slotte, door het reduceren van het nASP verminderde het vermogen om NK-celactivering en antilichaamresponsen bij vaccinatie te induceren. Dit laat vermoeden dat eiwitconformatie een belangrijke invloed heeft op de immunostimulerende activiteit van nASP.

Ook in **Hoofdstuk 3** trachtten we de onderliggende cellulaire en humorale mechanismen van de vaccin-geïnduceerde responsen van de natieve (ndd-ASP) en recombinante *C. oncophora* vaccins te vergelijken. Immunisatie van kalveren met het natieve *C. oncophora* vaccin bood significante niveaus van bescherming na een experimentele infectie, in tegenstelling tot het recombinante vaccin. Bovendien resulteerde vaccinatie met ndd-ASP in een hogere proliferatie van CD4-T-cellen, zowel systemisch als in het mucosaal weefsel van de dunne darm, dit in vergelijking met vaccinatie met het recombinante antigen. Wat de humorale respons betreft, hoewel zowel natieve als recombinante vaccins gelijkaardige hoeveelheden antilichamen opwekten, waren dieren die gevaccineerd werden met het natieve vaccin in staat om antilichamen met een grotere specificiteit ten opzichte van het ndd-ASP te produceren, in vergelijking met antilichamen die werden opgewekt door vaccinatie met het recombinante antigeen.

In **Hoofdstuk 4** bestudeerden we de immuunresponsen veroorzaakt door de *O. ostertagi* en *C. oncophora* antigenen (natief vs. recombinant) en adjuvans in bovine dendritische cellen (DC). Stimulatie van DC's met QuilA induceerde een activatie, gekenmerkt door een verhoogde opregulatie van co-stimulerende moleculen en het onvermogen om DQ-OVA antigenen op te nemen. Bovendien leidde stimulatie van DC's met natieve, maar niet met recombinant ASP van beide parasieten tot de productie van IL-12. Echter, door de aanwezigheid van LPS in bepaalde natieve ASP batches, rijst de vraag of de inductie van IL-12 productie te wijten is aan de werking van het natieve ASP of aan andere componenten, zoals LPS.

In Hoofdstuk 5 volgen tot slot de discussie en toekomstperspectieven. Hoewel dit project uitgebreide informatie aangeleverd heeft over de vaccin-geïnduceerde immuunresponsen die mogelijk correleren met bescherming tegen *O. ostertagi* en *C. oncophora,* blijven veel vragen voorlopig onbeantwoord. Bijvoorbeeld, het exacte mechanisme waarmee het natief ASP beschermende immuunresponsen opwekt, is nog onbekend. Aanvullende informatie over de interacties van het natief ASP met verschillende immuuncellen zou niet alleen helpen om de immunologische vereisten voor bescherming te begrijpen, maar ook essentiële informatie verstrekken over cruciale epitopen die door het immuunsysteem herkend worden. Bovendien blijkt het essentieel om een gedetailleerde analyse van de structurele verschillen tussen natief en recombinant ASP uit te voeren.

Word of gratitude

Word of Gratitude

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