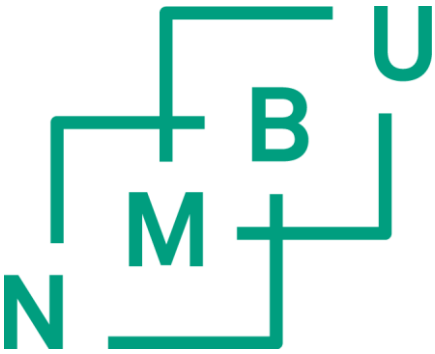
Investigation of the Gut-Associated Lymphoid Tissue in Sheep with emphasis on Natural Killer Cells

Thesis for the degree of *Philosophiae Doctor*

Line Olsen

Adamstuen 2015



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Contents

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Tromsø, April 2015

Line Olsen

Summary

The gut-associated lymphoid tissue (GALT) of sheep has been investigated for decades, and several immune cells receding within these structures have been described. The GALT of sheep has specialized compartments described to contain myeloid and lymphoid cells. The newly developed antibody against ovine NCR1 has been shown to label lymphocytes with conventional attributes of NK cells. However, the precise localization, phenotype and activation of $NCR1^+$ cells in the small and large intestinal lymphoid compartments remain unknown.

Investigations presented in this thesis were done using different types of immuno-labelling on tissue sections and in flow cytometry. The $NCR1^+$ cells were detected in the gut of foetuses from day 70 in the gestation, and these cells resided in compartments where T cells usually are seen; the inter-follicular area, dome and lamina propria. The $NCR1^+$ cells of the ovine foetal gut showed an increasing fraction of c-kit expression in the last period before birth. The localization of $NCR1^+$ cells described in the three papers is stable in foetus, normal unchallenged lambs and during cryptosporidiosis. The studies have revealed two separate phenotypic subtypes, both exhibiting characteristics of conventional NK cells. However, based on the new classification of innate lymphoid cells described in mouse and human, some of the $NCR1^+$ cells could be grouped as a subtype featuring regulatory and less cytotoxic functions, designated ILC22. From the youngest group of foetuses investigated we found $NCR1^+$ cells to proliferate, whereas this fraction of cells decreased to a minimum and were almost absent in the juvenile lambs. During an experimental infection with *Cryptosporidium parvum* we found the $NCR1^+$ cells to be faithful to their compartmentalization and showed an elevation of activation status.

The results presented in this thesis may be useful for comparative considerations and for general understanding of $NCR1^+$ cells in the sheep. It may be a basis for further investigations of the GALT and immune responses in the gut of sheep.

Summary in Norwegian (sammendrag)

Tarmassosiert lymfatisk vev har vært undersøkt i flere tiår, og dette lymfatiske vevet har spesialiserte områder, inneholdende myeloide og lymfoide celler. Det nylige utviklede antistoffet mot sau NCR1 er blitt vist å merke lymfocytter med klassiske karakteristika kjent for dreper(NK) celler. Det er imidlertid ennå ikke blitt beskrevet lokalisasjon, fenotype eller aktiveringsstatus hos $NCR1^+$ celler i tynn- og tykktarmens lymfoide områder hos drøvtyggere.

Undersøkelser som er presentert i denne avhandlingen ble gjort ved hjelp av ulike typer immunmerking på snitt av vev og i væskestrømcytometri. $NCR1^+$ celler ble funnet i tarm hos fostre fra 70 dager i drektigheten og ble funnet i områder hvor T celler vanligvis er lokalisert; interfollikulær områder, dome og lamina propria. $NCR1^+$ celler som ble funnet i sauefosterets tarm viste å ha økende uttrykk av c-kit i den siste perioden før fødsel. Studiene har avslørt to separate fenotypiske subtyper, der begge innehar typiske karakteristiske trekk av klassiske NK celler. Det er imidlertid kommet en ny klassifisering av medfødte lymfoide celler, som kan føre til at noen av de $NCR1^+$ cellene kan bli klassifisert som en subtype vist å være mer regulatorisk og mindre cytotoksisk, kalt ILC22. Vi fant prolifererende $NCR1^+$ celler i de yngste fostrene, der andelen av disse sank til et minimum mot fødsel og var nesten fraværende i unge lam. Lokaliseringen av $NCR1^+$ celler beskrevet i de tre artiklene er stabil i fostre, normale friske lam og i cryptosporidieinfiserte lam. I løpet av en eksperimentell infeksjon med *Cryptosporidium parvum* fant vi at de $NCR1^+$ cellene viste uendret lokalisasjon i tarmen og en økning av aktiveringsstatus.

Resultatene som er presentert i denne avhandlingen kan være nyttige for komparative betraktninger og for å få en generell forståelse av $NCR1^+$ celler i sau. Det kan være et grunnlag for videre undersøkelser hos tarmassosiert vev og immunrespons hos sau.

Abbreviations

Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
CD	cluster of differentiation
cNK	conventional natural killer cell
CP	colon patch
DC	dendritic cell
FAE	follicle-associated epithelium
FcγR	Fc gamma receptor
FFPE	formalin fixated and paraffin embedded
GALT	gut-associated lymphoid tissue
IFA	interfollicular area
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
IPP	ileal Peyer`s patch
JPP	jejunal Peyer`s patch
LTi	lymphoid tissue inducer
MALT	mucosa-associated lymphoid tissue
M cell	membranous/microfold cell
MHC	major histocompatibility complex
NCL	natural cytotoxicity ligand
NCR	natural cytotoxicity receptor
NK	natural killer
PBS	phosphate buffered saline
PP	Peyer`s patch
RORγt	retinoic acid receptor-related orphan receptor gamma
TCR	T cell receptor
TNF	tumour necrosis factor

List of papers

Paper I

Olsen L, Åkesson CP, Boysen P, Aleksandersen M, Press CMcL, Drouet F, Storset AK, Espenes A.

NCR1+ cells appear early and accumulate c-kit+ phenotype during foetal GALT development.

Manuscript.

Paper II

Olsen L, Boysen P, Åkesson CP, Gunnes G, Connelley T, Storset AK, Espenes A.

Characterization of NCR1+ cells residing in lymphoid tissues in the gut of lambs indicates that the majority are NK cells.

Veterinary Research 2013, **44**:109.

Paper III

Olsen L, Åkesson CP, Storset AK, Lacroix-Lamandé S, Boysen P, Metton C, Connelley T, Espenes A, Laurent F and Drouet F.

The early intestinal immune response in experimental neonatal ovine cryptosporidiosis is characterized by an increased frequency of perforin expressing NCR1+ NK cells and by NCR1-CD8+ cell recruitment.

Veterinary Research 2015, **46**:28.

Introduction

Intestinal defence mechanism and barrier function

The main function of the intestine is to break down and absorb ingested nutrients, and at the same time protect the host from pathogens and damaging processes. The intestine constantly needs to discern between innocuous and harmful material, ready to act as soon as it senses danger. For this purpose, the gut has developed a complex system that is comprised of both epithelial cells and immune cells that communicate to be able to suppress responses against harmless antigens, so called oral tolerance, and at the same time being able to initiate inflammation against pathogens trying to take advantage of this unresponsiveness.

The intestinal epithelium is one cell layer thick, and functions as a physical barrier between the external environment and self. Tight junctions between the epithelial cells block uncontrolled passage of substances. In addition, the epithelium produces antimicrobial peptides and mucins that hinder pathogens from passing the barrier. The epithelial cells also transport immunoglobulin (Ig)A from lamina propria plasma cells into the gut lumen to maintain immune exclusion (Brandtzaeg 2009).

Underneath this barely 30 µm thick layer of epithelial cells, is a complex mixture of immune cells. In some segments of the gut these immune cells are organized in defined compartments with discrete functions (Figure 1). The organized lymphoid tissues of the gut together with the draining intestinal lymph nodes are designated the gut-associated lymphoid tissues (GALT). The GALT of the intestines can be divided into inductive and effector sites, where organized lymphoid structures like the Peyer's patches (PPs) functions as induction sites, while the lamina propria and the absorptive epithelium alongside the whole length of the gut is considered an effector site, although these distinctions are not absolute (Brandtzaeg, 2009). In general, an immune response begins with antigen processing and presentation at inductive sites, which results in the stimulation of immune cells, mainly B and T cells. Subsequently, these lymphocytes leave the inductive site and enter the blood to home in the effector site, where they elaborate their specific immune response immediately or persist at site for long periods ready to act promptly should a pathogen intrude. Innate responses are executed on site wherever an insult may arrive.

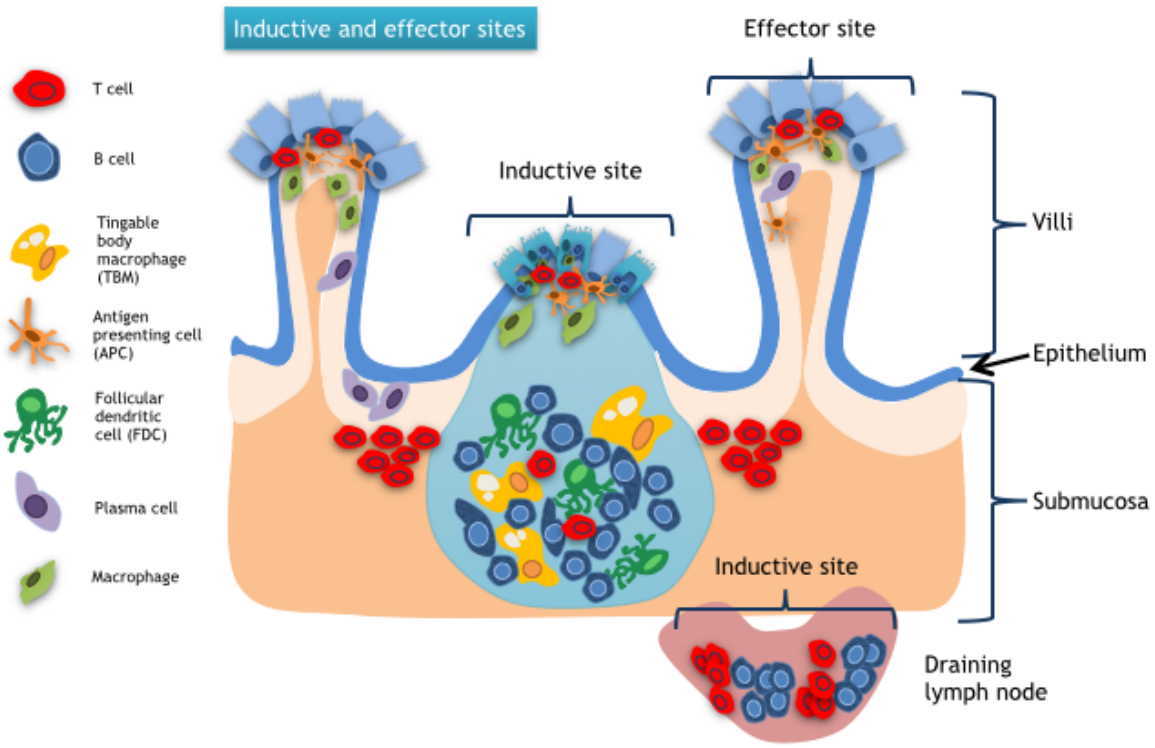

Figure 1. *The inductive and effector sites of PPs and draining lymph node with a selection of immune cells involved in the immune response.*

Gut-associated lymphoid tissue (GALT)

The GALT is comprised of immune cells that are distributed in the lamina propria, between epithelial cells, and in organized lymphoid structures regionally throughout the small and large intestine, indicating the importance of immunity therein (Blum and Pabst, 2007). These organized lymphoid structures, were originally reported already in 1677 by Johann Conrad Peyer as “intestinal glands” in sheep (Peyer, 1677). Almost three hundred years later the avian equivalent, the bursa of Fabricius of chickens, was described (Glick et al., 1956). Similar intestinal lymphoid organs were later found in rabbits, pigs, rodents and humans.

Comparative considerations

Rodent models have been the preferred option for studies of the immune system. However, extrapolating results from studies in rodents directly to humans or any animal species to expand our knowledge and understanding of the immune system might be bigoted. In many aspects, closer similarities exist between large animal species and humans than between rodents and humans. The mice and human intestine differ in anatomy, morphology and function. They are both monogastric species, however the intestinal development and maturation differs already at the fetal stage. The lymphoid tissue of the murine small intestine seem to be underdeveloped at birth and requires microbial stimulation (Moreau and Corthier, 1988) to develop to its mature form. Mice are further the only species having cryptopatches, whereas humans have the less investigated lymphocyte-filled villi (Brandtzaeg et al., 2008). Both species possess PPs and isolated lymphoid follicles in the small intestine. The advantage in the use of veterinary species as models in microbiological and immunological research has been demonstrated, e.g. the ruminant cannulation model is highly valuable for studies on trafficking of immune cells from peripheral tissues to the draining lymph node (Hein and Griebel, 2003) and the results from investigations of prion transport over the intestinal barrier using a loop model in sheep is likely relevant for how prion diseases can be transmitted also in other species (Jeffrey et al., 2006; Åkesson et al., 2012). Ruminant models should be no less valid for general assumptions than experimental rodent species; perhaps to the contrary due to their more relevant size. In addition, living conditions play a role, and studying immunology in larger veterinary species might reveal greater similarities to human, since they are naturally exposed to a plethora of agents in their living environment, which the laboratory animals are not, raised under specific pathogen-free conditions (Boysen et al., 2011). Thus, there is a continuous need for comparative studies, including animal species of veterinary importance. In addition, new knowledge on domestic animals is of course valuable in itself because it is needed to improve animal welfare and production in these species.

Structure and function of sheep GALT

The organized lymphoid tissue of small intestinal Peyer`s patches (PPs) and the lymphoid patches of the colon (CPs), as well as the solitary lymphoid follicles present along the gastrointestinal tract, are the main inductive sites of the gut immune system in the sheep. These sites are responsible for the immune surveillance of the intestinal lumen, and for the generation of immune responses within the mucosa. In lambs, the continuous ileal PP (IPP) is

responsible for the generation of B cells, and is considered a primary lymphoid tissue, unlike the jejunal PPs (JPPs) and CPs, which are recognized as secondary lymphoid tissues (Aleksandersen et al., 1990; Landsverk, 1984; Reynolds and Morris, 1983; Yasuda et al., 2006). As depicted in Figure 2, the PPs and CPs of sheep can be divided into compartments based on morphology, cellular composition, and function (Aleksandersen et al., 1991; Landsverk et al., 1991a; Reynolds and Morris, 1983). Each B cell containing follicle in the submucosa is surrounded by a capsule, except on the luminal side where the follicle protrudes through the muscularis mucosae where it merges with the dome. The dome contains myeloid and lymphoid cells and is covered by the follicle-associated epithelium (FAE), containing microfold (M) cells specialized in transcytosing macromolecules that may be presented to the underlying immune cells. Between the follicles and beneath the lamina muscularis mucosae is an area rich in T cells; the interfollicular T cell area (IFA) (Corpa et al., 2001; Press et al., 1992; Renström et al., 1996). A further compartment is the lamina propria, which is found along the whole length of the gut, and is present both within and beyond the borders of PPs and is regarded mainly as an effector site (Brandtzaeg, 2011).

The segments of the GALT differs in shape and size, where the IPP has large B cell containing follicles that exceed the smaller IFAs, whereas JPPs and CPs have large and wide IFAs compared to their smaller follicles (Figure 2.). The IPP also differ from other GALT structures in that the FAE covering the domes lacks conventional M cells (Landsverk, 1981; Landsverk et al., 1991b; Liebler et al., 1995).

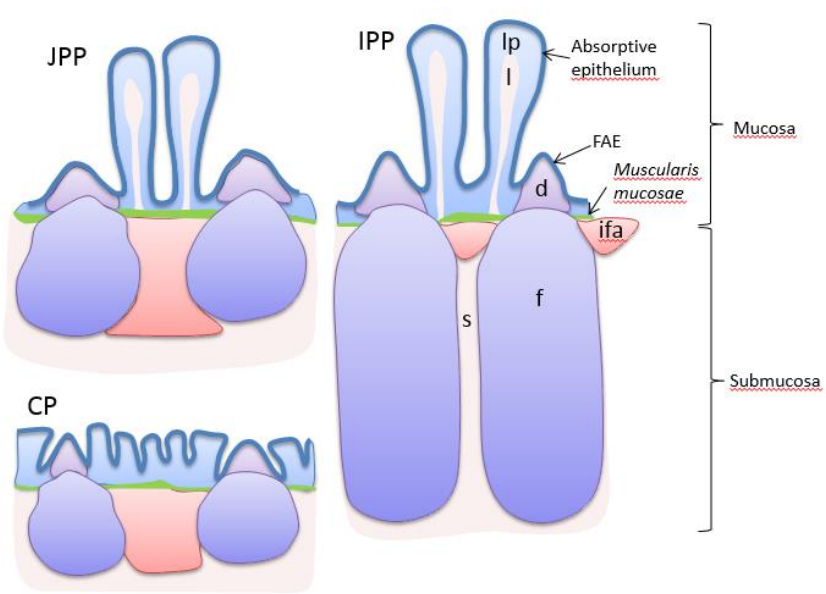

Figure 2. *The GALT includes the jejunal Peyer`s patches (JPP), ileal Peyer`s patch (IPP) and colon patch (CP). The main compartments of the GALT; dome (d), follicle (f), interfollicular area (ifa) are shown. Note the morphological differences between the segments. Lamina propria (lp), lacteal (l), submucosa (s). Made by Caroline Piercey Åkesson.*

GALT development

The intestinal immune system develops prenatally in humans and ruminants, ready to meet the challenges of recognizing and responding appropriately towards normal microflora, food components and pathogens, already at birth. The ovine fetus spend in total 150 days in gestation and is by virtue of a syndesmochorial placentation, isolated from exogenous antigens including maternal antibodies (Brambell, 1970) and even shielded from small molecules during gestation (Boyd et al., 1976). The GALT structure and function vary among species. In humans and ruminants, the jejunal and ileal PPs differ in both structure and function, and the lymphoid patch anlagen are formed in the foetus and do not require the presence of intestinal microbiota for development. In rabbits and rodents, PPs do not fully develop and mature before 2 to 4 weeks after birth, and the development of the PPs in some of these species appears to be entirely dependent on stimulation by the normal intestinal microflora (Pearson et al., 2012; Tizard, 2013).

The development of organized aggregates of lymphoid cells in the intestine of lamb foetuses is known to start as early as 70 days of gestation in the large colon (Aleksandersen et al., 1991), and at around 75 days in the jejunum (Reynolds and Morris, 1983). In the ileum, organized lymphoid tissue is observed at around 97 days of gestation, a few days after the formation of the dome (Nicander et al., 1991). The organization of the PPs starts as an accumulation of lymphocytes beneath some of the high folds of the primordial epithelium that characterize the immature mucous membrane. This leads to the formation of a follicle filled with B cells (Aleksandersen et al., 1991). T cells are present and scattered in the mucosa even before the formation of the follicle, where it later also forms the narrow IFA between the B cell follicles when these are formed (Yasuda et al., 2004). The IPP is the largest constituent of the GALT in lambs and extend from the ileocecal valve and orally 1-2 meters into the distal jejunum. This lymphoid tissue has been shown to involute and almost disappear at 18 months of age (Reynolds and Morris, 1983). The follicles in the adult sheep ileum are reduced in numbers and share morphological features with follicles of the JPPs and large intestinal lymphoid aggregates where the typical IPP once was (Lie et al., 2005).

Induction of intestinal lymphoid tissue formation

Humans and mice have a lineage of hematopoietic cells that is essential for the development of secondary lymphoid organs, named lymphoid tissue inducer (LTi) cells. In mice, these cells colonize the gut already at day 12.5 of gestation (end of second trimester). They do not only migrate to putative sites of lymphoid formation, but will also later participate in the maturation of the FAE. They have also been recognized as influential intestinal immune effector cells in postnatal life. LTi cells express and require the nuclear hormone receptor retinoic acid receptor related orphan receptor (ROR) γt (Montaldo et al., 2014). Other RORγt-dependent cell subtypes of interest in this thesis and with respect to intestinal immune function are cells displaying natural cytotoxicity receptor (NCR) and typically secrete interleukin (IL)-22. These cells function postnatally by maintaining tissue homeostasis at barrier surfaces through interaction with commensal bacterial communities (Spits and Cupedo, 2012; Spits and Di Santo, 2011). Veiga-Fernandes et al. (Veiga-Fernandes et al., 2007) studied the development of intestinal lymphoid tissue in mice, and found a NCR^+ population at around gestation day16 that was speculated to be important in PP organogenesis.

Immune system – innate and adaptive

The immune system is the result of cooperation between the innate and the adaptive responses that together provide effective protection. The cells of the innate immune system monitor the mucosal surfaces and become immediately involved when pathogenic agents have passed the barriers and threaten to cause disease. The innate immune system, with key players as NK cells and dendritic cells (DCs), starts to work with its rapidly responding chemicals and cellular defense mechanisms. The last line of defense is the adaptive lymphocytes commonly comprised of T and B cells, these belong to the slower adaptive response. It is no doubt that the innate and acquired immune systems are closely linked, depend on each other and work together rather than separately (Medzhitov, 2007). Today, the traditional view that innate immunity is nonspecific and lacks memory while the adaptive immunity is characterized by specific antigen recognition and memory is no longer an accurate categorization of the immune system. Recent studies show that even cells of the innate immune system adapt after their first encounter with pathogens and thus may have a memory (Min-Oo et al., 2013), although different from the traditional T and B cell memory.

In this thesis, a special interest of NK cells and their role based on compartmentalization in the intestinal tissue was the initiation of the project.

Natural killer (NK) cells

Natural killer (NK) cells are large granular lymphocytes that can kill a target cell missing self-major histocompatibility complex (MHC) molecules, a common mechanism of virus-infected and tumour cells to evade recognition by adaptive lymphocytes. This targets for direct NK cell-mediated killing (Horst et al., 2011), a characteristic that lead to the "missing self" hypothesis (Ljunggren and Karre, 1990). The mechanisms involving this killing event have later been well documented. The release of cytotoxic granules and subsequent lysis of target cells are characteristics of what today is called "conventional" NK cells, later denoted cNK cells. Natural cytotoxicity occurs when activating signals overrule inhibition and involves the spontaneous release of cytotoxic granules, containing granzyme and perforin. Antibody-dependent cell-mediated cytotoxicity (ADCC) is the cross-linking of Fc gamma receptors (FcγR) on the cNK cell to antibodies specific for the target cell. This may be accompanied by other recognition events between the cNK cell and target cell (Boysen and Storset, 2009).

Research on these killer cells was for a long time focused into their cytotoxic abilities, until their important involvement as an orchestrator of immune responses was discovered (Degli-Esposti and Smyth, 2005; Strowig et al., 2008), such as direct interactions with macrophages (Atochina and Harn, 2005; Haller et al., 2002) or DCs (Lucas et al., 2007). cNK cells also provide an early source of interferon-γ (IFNγ), which is necessary for T helper 1 $CD4^+$ T cell polarization in the lymph nodes (Martin-Fontecha et al., 2004). Another characteristic cytokine produced by cNK cells is the tumor necrosis factor (TNF).

In humans, cNK cells have been defined as $CD3^-$ and TCR^- lymphocytes that express CD56 and most often CD16 (FcγRIIIA) (Hercend and Schmidt, 1988; Trinchieri, 1989). CD16 is an activation receptor mediating recognition of antibody-opsonized targets and its high expression on human cNK cells is associated with strong cytotoxic potential and good IFNγ producing capabilities. However, the absence of the CD16 molecule does not seem to be constant and expression of CD16 can be up-regulated during inflammatory bowel disease in humans (Steel et al., 2011). cNK cells in mice do not express the human CD56, however the murine counterpart NK1.1 has been used. Further investigation of human cNK cells revealed

two distinct subsets; the mature cytotoxic $CD56^{dim}/CD16^{+}$ subset, and the smaller subset of $CD56^{bright}/CD16^{-}$ cells (Chan et al., 2007; Romagnani et al., 2007). The NCR1 (NKp46, CD335) has been proposed to be the most precise pan-species marker for cNK cells (Moretta et al., 2005; Walzer et al., 2007). Ovine NK cells were first described as $CD16^{+}/CD14^{-}$ lymphocytes (Elhmouzi-Younes et al., 2010), and later defined more precisely by the expression of NCR1 (Connelley et al., 2011).

The ovine NK cells were found to reside in similar tissues as in human and mice (Gregoire et al., 2007); blood, spleen, mesenteric and somatic lymph nodes, uterus, lung and intestine (Connelley et al., 2011). NK cells reside in the same areas as DCs and T cells of the lymph nodes of several species. Thus, the cross-talk between NK cells and DCs that have been shown *in vitro* from other species, are likely to occur in these areas. There is still limited data that confirm these interactions *in vivo* and we have limited information beyond the human and murine species.

Innate lymphoid cells (ILCs)

In the past 5 years, it has been an agreement that innate lymphocytes comprise a heterogeneous group of cells composed of individual populations with discrete, yet complementary functions in maintaining homeostasis and providing protection during an innate immune response (Eberl, 2012; Spits et al., 2013; Spits and Di Santo, 2011). We have begun to appreciate that innate lymphoid cells (ILCs) are not confined to cNK cells but encompass a much broader cellular diversity.

Based on their phenotypic and functional characteristics, the ILCs are subdivided into three different groups. Of interest are group 1 and 3 ILCs, where group 1 contain cNK cells, that carry NCR1, produce IFNγ and have a cytotoxic function. The group 3 ILCs contains subsets expressing the transcription factor RORγt, which is crucial for their development and function, whereas the group 1 ILCs, including cNK cells does not require RORγt and is thus of a separate lineage.

NCR1 surface expression exists on cells today defined in groups not developmentally nor functionally related to cNK cells. The first NK-like cell phenotypically similar to cNK cells, was found in mice, and appeared in the small intestinal lamina propria during infection (Cella et al., 2009). These NCR1 expressing lymphoid cells were triggered by acute exposure to IL-

23 to secreted IL-22, IL-26 and leukemia inhibitory factor, which in consequence stimulated epithelial cells to secrete IL-10, proliferate and express a variety of mitogenic and anti-apoptotic molecules. After the established classification of the three groups of ILCs, these $NCR1^+$, IL-22 excreting cells, now called ILC22, are placed in the group 3 ILCs together with LTi cells. ILC22 are considered crucial for the formation of secondary lymphoid organs during embryogenesis, and colitogenic NCR^- ILC3s. Despite certain plasticity between some NCR^+ ILC3 and NCR^+ ILC1 cells, ILCs producing IFNγ are for the time being, classified as group 1 ILCs.

Despite overlapping phenotype between the cNK and ILC22 cells (Table 1.) in their developmental stages, they are now considered separate cell types with independent and nonintersecting developmental pathways (Ahn et al., 2013). Although, the definition of ILC groups in sheep is lacking, with today's knowledge, similarities with man and mouse are present. In addition, the presence of a novel population of $NCR1^+$ cells which also expressed CD3 was recently reported in cattle, and these cells were distinct from conventional NK, T and NKT cells (Connelley et al., 2014).

Table 1 – Markers defined on some human ILCs and their sequential acquisition of markers on differential stages of human cNK cells.

Marker	ILC1 (cNK cells)				ILC2	ILC3	
	Early stage	**Intermediate stage**	**Late stage**	**Mature**	**Nuocytes / Natural helper cells**	**LTi**	**NCR^+ ILC3 (ILC22)**
CD34	+	- (?)	-	-	-	-	-
CD117 (cKit)	+	+	+ / -	-	+	+	+
NCR1	-	+	+	+	-	-	+
CD4	-	-	-	-	-	+	- / +
CD3	-	-	-	-	-	-	-
RORγt	-	-	-	-	-	+	+

ILC, innate lymphoid cell; cNK, conventional Natural Killer; LTi, lymphoid tissue-inducer; NCR, natural cytotoxicity triggering receptor. (Freud et al., 2014; Montaldo et al., 2014; Spits et al., 2013).

NCR1$^+$ cells in the intestine

NK-like cells have long been documented in the intestinal epithelium and lamina propria of mice (Tagliabue et al., 1982) and humans (Leon et al., 2003). NCR1$^+$ cells have been described in the lamina propria, as well as in dome and IFAs of the PPs of both mice and man, whereas few cells were found intraepithelially (Luci et al., 2009). In addition to a subtype characterized as cNK cells, it soon became evident that an NCR1$^+$ cell population found in the gut of both mice and humans are different from those in peripheral blood, spleen and thymus, today designated ILC22, due to their ability to mainly produce IL-22. It has been proposed a relationship between this population and LTi cells, based on their resemblance in both phenotype and localization in the gut (Luci et al., 2009). Both ILC subsets express RORγt and c-kit (CD117). In human gut, most NCR1$^+$ cells have been characterized as cNK cells, as they are not RORγt$^+$ (Tomasello et al., 2012). The concept that the absence or presence of RORγt defines NCR$^+$ cells into ILC group 1 or 3, respectively, is challenged by Vonarbourg et al. They showed that mice ILCs, in addition to upregulatingNCR1, have the ability to lose their RORγt expression during development from LTi cells to ILC22-like cells (Vonarbourg et al., 2010). However, it cannot be excluded that these cells were cultured from a precursor giving rise to previously unknown subtypes. Sawa et al. (Sawa et al., 2010) were unable to replicate these studies, and were therefore favoring today's idea that LTi and ILC22 are separate populations.

Cryptosporidiosis

Cryptosporidium is an intracellular, extracytoplasmatic, apicomplexan parasite of a complicated taxonomy with around 25 species, which can infect a wide variety of vertebrate hosts, including humans (Xiao et al., 2004). After the excystation of the oocytes in the gastrointestinal tract, the sporozoites internalize in the apical host-cell membrane of the epithelial cell to form a vacuole, with an electron-dense "feeding organelle" (Figure 3). From here, they are capable of completing both sexual and asexual cycles within the same host (Figure 4). The infectious load of *Cryptosporidium parvum* may be of considerable importance for the human health (Robertson and Chalmers, 2013).

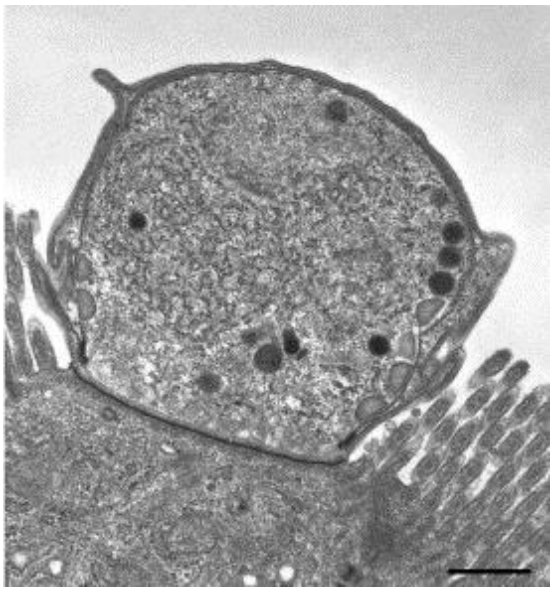

Figure 3. *Electron micrograph (EM) showing a mature macrogamete, attached to the surface epithelium of piglet gut experimentally infected with Cryptosporidium. Source: Tzipori S., Microbes and infection. 2002 Mar; 4(10): 1047–1058. With permission from Elsevier (License nr. 3610231263888).*

The impact on animal health and welfare has also economic consequences; gastro-intestinal parasites in sheep has been estimated in Great Britain to be the most costly disease (Nieuwhof and Bishop, 2005), and in Australia reduced carcass productivity was detected for lambs that tested positive for *Cryptosporidium* or *Giardia* (Sweeny et al., 2011). The worldwide distribution in both wild and domestic ungulates (Martin et al., 2011) and the possibility for healthy individuals to excrete resistant *Cryptosporidium* oocysts into the environment and water supplies makes the disease difficult to control (Meinhardt et al., 1996).

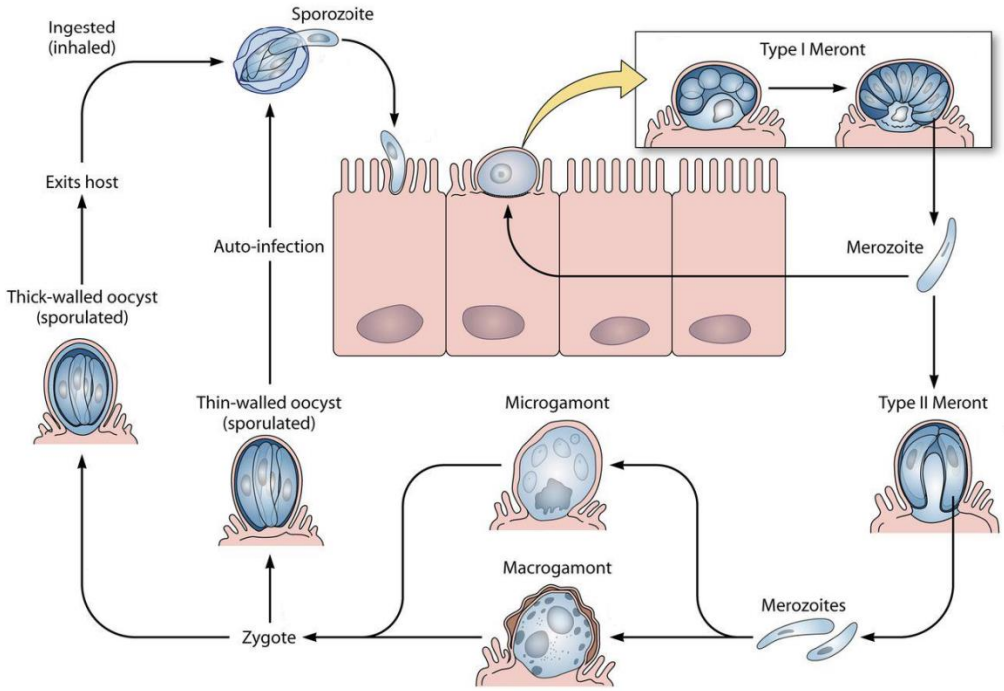

Figure 4. *Schematic representation of the Cryptosporidium parvum life cycle. Source: Maha Bouzid et al. Clin. Microbiol. Rev. 2013;26:115-134. Modified with permission from American Society for Microbiology©.*

As of date there is no tested and accepted vaccine, nor effective specific treatment present against cryptosporidiosis. Some active molecules were effective against cryptosporidiosis, as halofuginone lactate (Naciri et al., 1993), paromomycin (Mancassola et al., 1995) and decoquinate (Mancassola et al., 1997). However, they only prevent or treat the symptoms and decrease the oocyst excretion without eliminating it. The zoonotic nature, animal health and welfare aspects, as well as the economic impact make cryptosporidiosis one of the most important gastro-intestinal diseases in sheep production today.

Pathology of cryptosporidiosis

Intestinal cryptosporidiosis is associated with villus atrophy of variable severity, characterized by blunting and fusion of villi, and by hypertrophy of crypts of Lieberkühn (Angus et al., 1982; Snodgrass et al., 1984; Tzipori et al., 1981). The epithelium will alter appearance to become cuboidal, rounded, or low columnar, and sometimes exfoliate or form irregular projections at tip of villi (Figure 5). All stages of cryptosporidia occupy abundantly the microvillous border on the epithelium, except those in crypts of Lieberkühn. The distribution is typically in the small intestinal segments of the distal jejunum and ileum, although parasites may occur in the cecum and colon. Neutrophils and mixed mononuclear cells infiltrate the lamina propria. In calves, intraepithelial infiltration of T cells has also been documented (Wyatt et al., 1997).

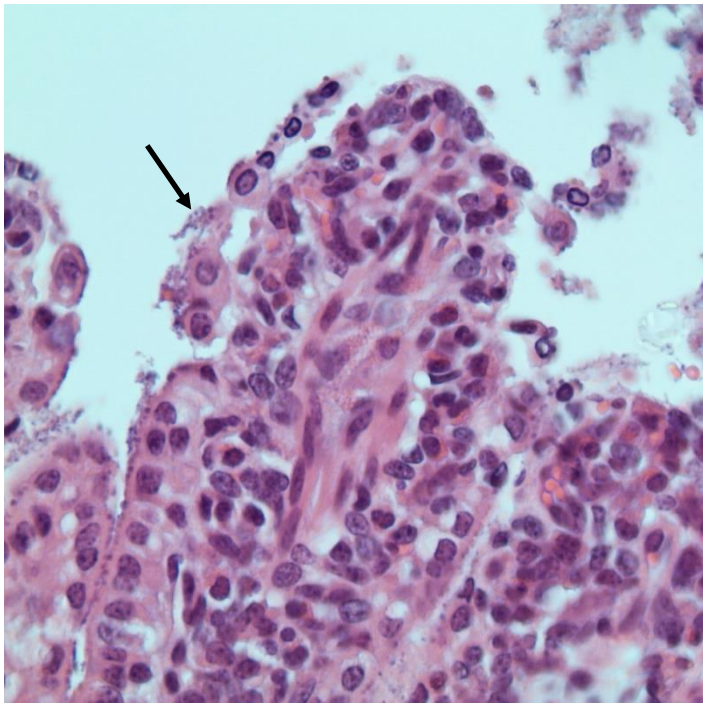

Figure 5. *H&E section from small intestinal villi infected with Cryptosporidium parvum. Note the sloughing of the absorptive epithelium and the inflammatory cell infiltrate in the lamina propria and intraepithelially. Cryptosporidia is seen on the surface of the epithelium (arrow).*

Immunity against *Cryptosporidium parvum*

Studies involving experimental cryptosporidiosis in mice and natural infections in humans show involvement of both innate and adaptive immune responses in the intestine. Severe combined immunodeficiency (SCID) mice, lacking B and T cells, develop chronic inflammation which progressively becomes fatal (McDonald and Bancroft, 1994; McDonald et al., 1994; Ungar et al., 1991). Because the infection may lead to high mortality in immunocompromised humans, especially those infected with HIV and developing AIDS, a particular interest to $CD4^+$ T cells emerged. In an experimental setting, recovery and protection from reinfection have been associated with $CD4^+$ T cell responses starting from the second week post inoculation (McDonald and Bancroft, 1994; McDonald et al., 1992; McDonald et al., 1994). This response was associated with IFNγ production in cattle (Wyatt et al., 1997; Wyatt et al., 2001). Wyatt et al. has conducted several experiments in calves where they found resident $CD8^+$ and $CD4^+$ T cells to accumulate in the small intestinal mucosa, first around epithelial cells and later including lamina propria of the villi and PPs.

In experimental studies done in mice, the innate immunity has proved to be crucial in the early phase of infection (Korbel et al., 2011; Lantier et al., 2013). As cNK cells are central players in the innate immunity as an important source of IFNγ, it is suggested that they are involved in the early reduction of the parasitic load in the gut during cryptosporidiosis. In reality, it has been sprawling results regarding cNK cells` involvement in intestinal cryptosporidiosis. In a mouse model, both cNK cell dependent and independent IFNγ was required for the control of this infection (Barakat et al., 2009; McDonald et al., 2013). Other research groups have failed to prove a potent role of cNK cells in neonatal mice (Lantier et al., 2013).

Aims of the study

Main objective

The main aim of the current work was to provide novel information about ovine NK cell distribution and phenotype during GALT development in the fetus and juvenile lambs under steady state and during infection.

Sub-goals

1. Characterize phenotype of lymphoid tissue residing NCR1^{+} cells (Paper I, II, III).
2. Quantify and study the distribution of NCR1^{+} cells in relation to the GALT morphology in foetuses and lambs (Paper I, II, III).
3. Investigate the relation of NCR1^{+} cells to other cells of the GALT immune system (Paper I, II, III).
4. Investigate the response of NCR1^{+} cells during experimental cryptosporidiosis (Paper III).

The *Summary* of papers provides an overview of the obtained results, and in the *Results* section; the *Material*, *Methodological considerations* and *Results* across the three papers are discussed in a broader context than provided in the individual papers.

Summary of separate papers

Paper I

NCR1+ cells appear early and accumulate c-kit+ phenotype during foetal GALT development

The aim of this study was to describe the amount, distribution and phenotypic characteristics of $NCR1^+$ cells during the development of GALT, from 70 days of gestation until birth. Using multicolor immuno-fluorescence on cryosections, lymphoid cell markers as CD3 and CD79 were used to identify the compartments described for GALT, and the localization of $NCR1^+$ cells were correlated with these structures. The progenitor markers CD34 and c-kit, in addition to the proliferation marker Ki67, were used to investigate possible origin and the stage of development of the $NCR1^+$ cells. In this unique material, we found that $NCR1^+$ cells are present as early as 70 days of gestation, and that they co-localized with $CD3^+$ T cells in the IFAs and domes when these compartments were established. A larger proportion of $NCR1^+$ cells were found to proliferate in the intestinal tissues at the earliest time points investigated, than at late gestation. $NCR1^+$ cells lacked CD34 antigen throughout the material. However, there was a gradual increase in $NCR1^+$ cells co-expressing c-kit, particularly towards the end of gestation. In conclusion, we found $NCR1^+$ lymphocytes to appear early in GALT development of ovine fetuses, and that these cells displayed a phenotype of late developing conventional NK cells and possibly also an ILC22 subpopulation.

Paper II

Characterization of NCR1+ cells residing in lymphoid tissues in the gut of lambs indicates that the majority are NK cells

With the aid of flow cytometry and *in situ* methods using multicolor immune-fluorescence, this study aimed to characterize $NCR1^+$ cells present in the intestinal tissues with respect to CD3 and CD16, and to compare the relative number of $NCR1^+$ cells in the different lymphoid tissues, with focus on GALT. With morphometry, the density of $NCR1^+$ cells was compared between different compartments of the GALT in the small and large intestine of one-month-

old lambs. We found that $NCR1^+$ cells were mainly CD3 negative, meaning they were not a subpopulation of T cells (NKT cells), but NK cells. The relative number of $NCR1^+$ cells was found in GALT in accordance with previous studies performed in calves. In this study, CD16 was found to be high on most $NCR1^+$ cells in the ovine tissues examined. The expression of CD16 on NK cells is usually high on NK cells that have strong IFNγ producing capabilities and high cytotoxic potential. Some discrepancy was found in the investigation of the number of $NCR1^+$ cells comparing flow cytometry and *in situ* investigation. A low proportion of mononuclear cells in the ileal PP were found to be $NCR1^+$ in flow cytometry, whereas a substantial number of such cells on tissue sections were detected *in situ*. This finding can be explained by the abundance of B cells residing in the large follicles of this organ, which causes a dilution effect in flow cytometry. In conclusion, we postulate that the $NCR1^+$ cells found in this study mainly are NK cells due to their high expression of CD16. In addition, wherever NK cells were identified, we also found a close relation to T cells and dendritic cells. This support the theory of the close communication found *in vitro* between these immune cells.

Paper III

The early intestinal immune response in experimental neonatal ovine cryptosporidiosis is characterized by an increased frequency of perforin expressing NCR1+ NK cells and by NCR1-CD8+ cell recruitment.

During an experimental infection with *Cryptosporidium parvum* in neonatal lambs, several lymphocytic parameters were investigated with both flow cytometry and histological tools. The aim of the study was to investigate the early immune response in the different sections of the small intestine and associated lymphoid tissues, with a particular focus on NK cells and $CD8^+$ T lymphocytes. We found typical lesions of enteritis with immune cell recruitment in the small intestine, with lesions peaking at day 6 post inoculation, followed by recuperation. Flow cytometric investigation of $NCR1^+$ cells, revealed no change in proportion between controls and infected, although a slight increase in their absolute numbers were observed in the small intestine of infected lambs. However, no increase was observed on tissue sections. On the other hand, the NK cells were activated in the infected compared to the control, demonstrated by an increase in perforin and CD16 expression. The most conspicuous finding in this paper was the strong increase of the $CD8^+/NCR1^-$ cell population in the intestine. They

seemed to be a part of the T lymphocyte cell population due to their CD3 co-expression. Collectively, this paper demonstrated that activated NK cells, with a high cytotoxic potential, were present very early in the small intestine and were likely involved in the innate response to cryptosporidiosis. At the same time, a possible adaptive immune response as indicated by an early increase in the $CD8^+$ T cell population was present in this material.

Discussion

Materials

Animals

The material used in this study was collected from lambs and lamb foetuses on several occasions. The breeds used are for dual purpose production; producing meat and wool or milk. All procedures were conducted in accordance with the respective countries laws/legislations and regulations controlling experiments using live animals.

The tissues from foetuses (Paper I) is a unique material sampled in the late 80s and the early 90s by colleagues from the faculty (Press et al., 1998; Press et al., 1996; Renström et al., 1996). The foetuses were untreated control specimens from studies made on B cell ontogeny done on Australian Merino and Swiss Alpine x Black Jura breed from Australia and Switzerland, respectively.

For the remaining studies (Paper II and III), lambs were provided from protected and approved research facilities in Norway and France. The Norwegian white and the Norwegian white x Texel (Paper II) breeds from Norway have only one lambing a year, ranging from April-May, highly dependent on daylight length that varies along the longitude of the country (71°8′2″ N - 57°58′46″ S). On the other hand, the sheep from the Institut National de la Recherche Agronomique (INRA) facilities, Préalpes du Sud, has the ability to breed "out of season" which is a huge advantage since analyses like flow cytometry, depending on fresh tissues, can be performed several times a year. For this thesis we have included tissues from foetuses (Paper I), lambs that were less than 2 weeks old (Paper III) and one month old lambs (Paper II).

During the autopsies, a general macroscopic evaluation was done to reveal any diseases related or unrelated to the experiment (Paper II and III). A detailed knowledge of the normal anatomy was important in order to localize subtle changes in the gut wall. For the cryptosporidiosis experiment, exclusion criteria for the non-infected control lambs included lethargy, in addition to potential macro- and/or microscopic pathological findings that could show evidence of the animal having been sick, e.g. diarrhoea, emaciation and pneumonia. Severe clinical cases were euthanized for ethical reasons and excluded.

A wide range of organs were collected during the experiments, including all segments of the gut (Figure 6), somatic and mucosal lymph nodes, tonsils, spleen, thymus, blood and sometimes bone marrow. For the foetuses and the lambs less than a week old the GALT was not always detectable macroscopically and was therefore sometimes not sampled, especially in the colon. The study of foetuses (Paper I) was based on pre-collected, archive material. Thus, flow cytometry was not performed in that study.

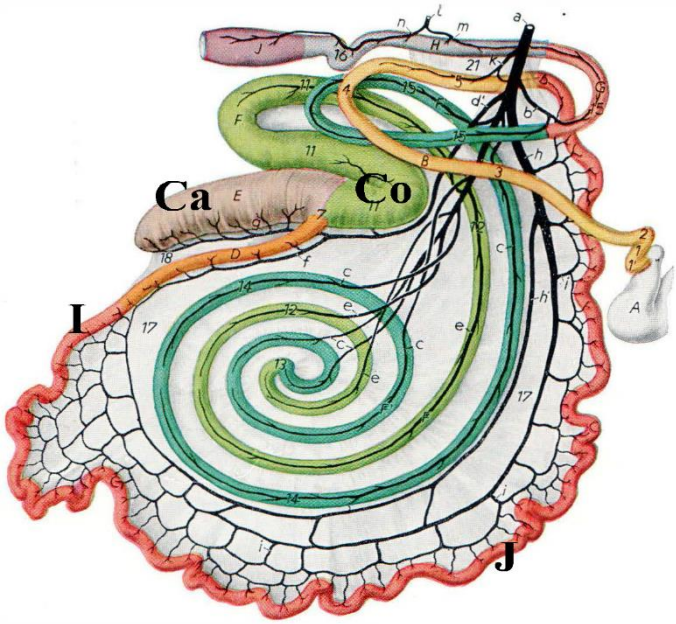

Figure 6. *The anatomy of the ruminant intestinal system. Samples collected from jejunum (J), ileum (I), caecum (Ca) and beginning of ascending colon (Co). Modified from The Viscera of the Domestic Animals by R. Nickel with permission from Springer (License nr. 3607040987952).*

Tissue processing and fixation

Rapid sampling and fixation of gut tissues is important to preserve the fragile mucous membrane that otherwise decay fast post mortem. Within 20 minutes after euthanization, the intestine was opened longitudinally from the mesenteric side, rinsed in phosphate-buffered saline (PBS) and prepared either for cryofixation or formalin fixation. For cryofixation, the intestinal tissues were placed with the mucosal side down on a piece of liver (preferably from the same animal) to protect the mucosa from freezing artefacts. The tissue was submerged in a bath of chlorodifluoromethane (Isceon™) or isopentane that is cooled by liquid nitrogen before wrapping in aluminium foil, coated with a plastic layer to avoid drying and subsequent cracking of the tissue. The samples were stored temporarily in the liquid nitrogen until storage in ultra-freezers at -70-80°C. Cryofixation will preserve epitopes in tissues, which then is available for labelling. However, the tissue morphology is not as well conserved as with formalin fixation, and careful handling during sectioning and staining procedures is needed. It

should be noted that the foetus material of the oldest group included (Paper I) had gone through a thaw-freeze cycle rendering the epithelium somewhat damaged, causing increased autofluorescence in sections from this tissue (see definition on page 33). However, the morphology and reactivity for Abs were still considered appropriate based on comparisons with tissue sections from the other groups.

For formalin fixation and paraffin embedding (FFPE), the intestinal content, if present, was carefully removed by flushing with PBS, and the tissue samples were then immediately submerged into buffered formalin.. Tissues were embedded in paraffin within a week after sampling to prevent overfixation and to give best quality of immunohistochemical staining. Stripping and precise orientation of the tissue was done when embedding in paraffin. FFPE is better for conserving tissue morphology. But one has to be aware that it may cause tissues to shrink, which introduces artefacts, such as widening spaces between cells. This fixative will also cause cross-bindings of the proteins, which might mask or lower the affinity of the epitopes. An appropriate de-masking protocol to optimize the use of buffers with optimal pH value in combination with autoclave or microwave heat-treatment was established for each Ab. Nevertheless, FFPE tissues produced the best possible morphology and longevity of the tissues.

Cells for flow cytometric analysis have to be harvested fresh and the further labelling of cells and analyses need to be done in consecutive steps.

Antibodies

A challenge working with veterinary species is the limited availability of specific markers for the immune system. Abs specific for ovine species have become more commercially available during the last years. In addition, Abs reacting with antigens that are highly conserved across vertebrate species can be used in unconventional animal models. In our studies, the CD3 Ab is a good example of an Ab raised against the human protein with good cross-reactivity between species, including sheep. Still, many Abs employed for detection of ovine immune antigens are produced by research groups in different laboratories and have often limited availability. The use of some Abs is limited by method of analysis. We experience more success in fresh tissue suspensions analysed by flow cytometry, than by tissues processed for *in situ* investigations, as was the case with Abs against perforin and CD16.

Methodological considerations

Several methods were used to study the GALT of lambs and fetuses. To study microscopical structure and cellular composition of tissues, histology (Paper II and III) and immunohistochemistry (Paper III) using FFPE sections, and immuno-fluorescence on cryosections (Paper I, II and III), were applied. Flow cytometry (Paper II and III) was important for the quantification of cell populations and measuring amount of antigen on such populations. Ultrastructural studies were performed with transmission and scanning electron microscopy (Paper III).

Immuno-detection on cells and tissues

Immuno-labelling is a process that enables detection and localization of an antigen to a particular site on a cell, tissue or organ. Tissue sections were labelled using an indirect method (Figure 7). For this approach, the primary Ab binds to its target antigen in the tissue and is visualized by secondary Abs that is conjugated to either a fluorophore or the enzyme peroxidase, and is able to bind to the primary Ab. This method allows several secondary Abs to bind to the primary Ab and in this way lead to an amplification of signal.

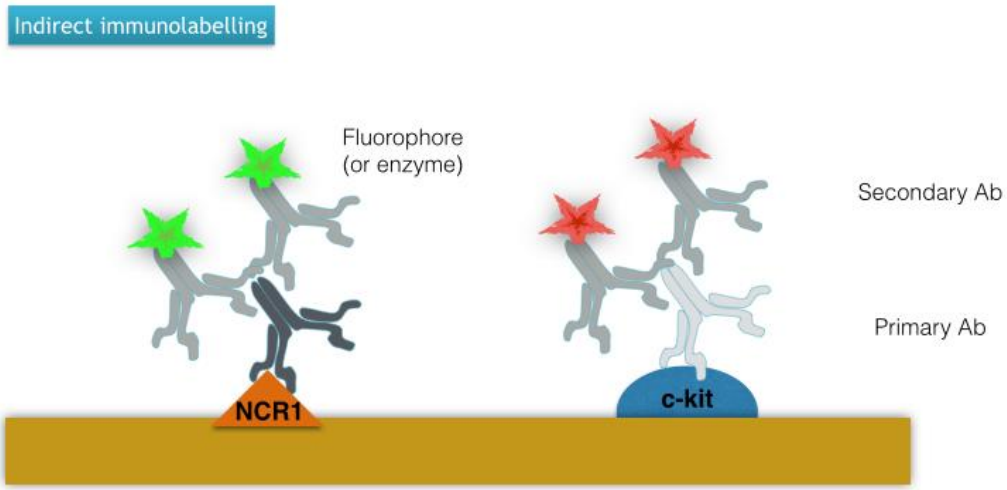

Figure 7. *Schematic illustration of indirect immune-labelling with co-localization of NCR1 and c-kit on the same lymphocyte using fluorophores. For immunohistochemistry, an enzyme that result in a brown or red colour is attached to the secondary Ab.*

Immunohistochemistry

Immuno-labelling of cells and other structures on tissue sections is called immunohistochemistry. In this work, an indirect method utilizing the enzyme horseradish peroxidase for visualization was used on FFPE sections to detect cryptosporidia (Paper III). This method is useful when the architecture of the tissue and localization of target antigen within tissues are important to visualize. The counterstaining of tissue sections helps to demonstrate the localization of the stained antigen in relation to tissue structures or cells. However, this method is not the best alternative in co-localization studies as chromogens used in these methods often produce a red or brownish colour, which may be difficult to separate from each other. When using an enzymatic method on FFPE tissue sections, the signal from the NCR1 Ab was too weak to be detected.

Fluorescent immuno-labelling

Fluorescence is natural emission of light after absorption of light with a shorter wavelength. Substances with this trait are designated fluorophores and have been used for decades after it was discovered that they could be conjugated to Abs. The use can provide endless possibilities, but it is important to know about their limitations.

> *Autofluorescence:* Tissue structures often have natural emission of light without prior labelling, generally called autofluorescence. In our procedures, autofluorescence interfere with the specific signals and should preferably be reduced to a minimum.

Factors that may influence results in fluorescent labelling include tissue preparation, fixation, labelling procedures, fluorophore type and detection methods. Unlike the use of light microscopy, the use of fluorescence will mainly allow the observation of structures that has been labelled, making it difficult to identify general tissue architecture.

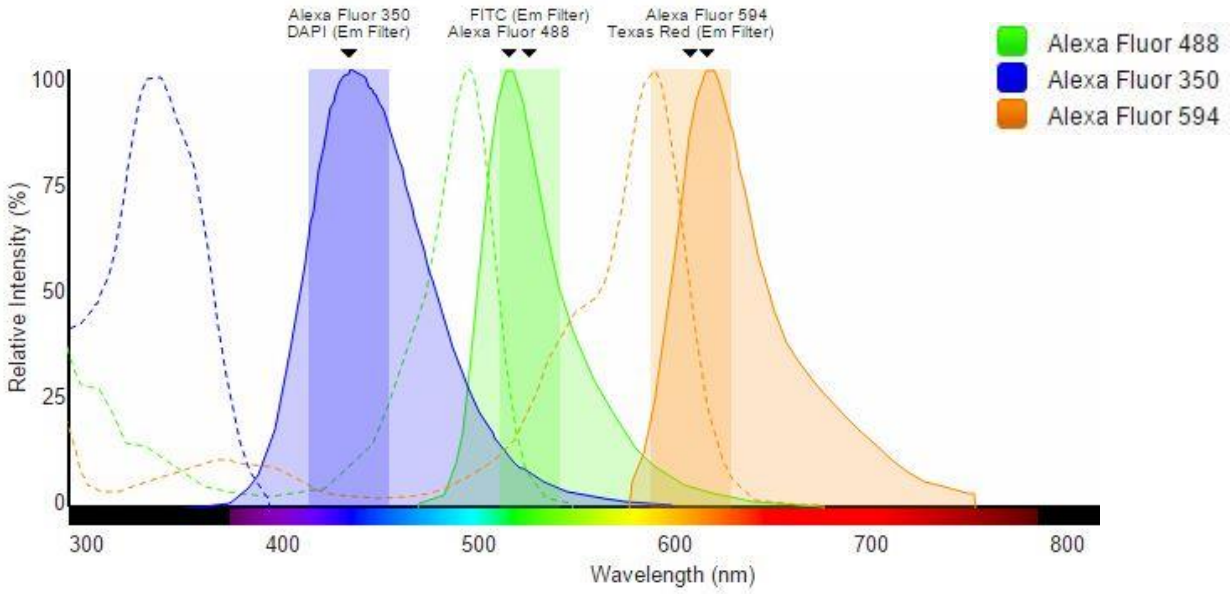

Figure 8. *The excitation (dotted empty histogram) and emission (filled histogram) spectrum of secondary antibodies used in this thesis for conventional microscopy. Emission filters (columns) DAPI, FITC and Texas Red. Constructed with Fluorescence SpectraViewer from Life Technologies.*

Each fluorophore has a characteristic peak excitation and emission wavelength (Figure 8). Depending on the detection method, several fluorophores can be applied at the same time. For this study, we had available two different types of microscopes with appropriate filters to detect the wavelength spectre of the fluorophores, confocal and conventional microscopy. In addition to morphological studies, we utilized fluorescence in flow cytometry.

Fluorescent detection by microscopy

There is a vast amount of secondary fluorescent Abs available, in addition to directly conjugated Abs with stable and strong fluorophores. The use depends on whether a direct or indirect immuno-labelling protocol is used. We used the indirect method for morphological studies that gave us freedom of choice in the desired combination of fluorophores in addition to an amplification of signal as several secondary Abs can attach to each primary Ab. The light source, excitation and emission filters available in each microscope decide what combination is applicable. The main difference between the two microscopes used in this study, is the light source. While the conventional microscope has a mercury lamp to excite the fluorophores, the confocal use lasers and thus the chance of exciting other fluorophores applied, is smaller. Figure 8 shows the combination of fluorophores linked to secondary Abs

used in this study when tissue sections were investigated under conventional microscope. This microscope detected a wider range of emitted light than the available confocal microscopy due to the different light source and filters. This was important for the sensitivity, as the Ab against NCR1 generally gave a weak fluorescent signal in the tissues, either due to low affinity or low expression of the antigen on the cell surface. To optimize this signal, cryosections and a weak fixative was ideal. By detecting emitted light of a wider spectrum from each fluorophore, like in the conventional fluorescent microscope, we were able to perceive a stronger signal in our images. Thus, the limitation of the NCR1 Ab required careful selection of combinations of fluorophores to avoid *bleed through* (crossover), which might happen when two (or more) excitation and/or emission spectra overlap, and this overlap cannot be avoided by the use of proper filters. The goal is to avoid detecting more than one fluorophore in each channel, and thus be able to study co-localization (Figure 7) of Abs attached on the same cell.

Loss of fluorescent signal caused by exposure with light on the fluorophore, also called *photo bleaching* is also something one has to combat. The confocal laser will fade the signal faster than a mercury lamp. Thus, a shortest possible exposure time in each channel when using several fluorophores, has to be balanced against the too short exposures that may result in poor image quality. To get around the undesirable autofluorescence often seen in our sections, we used a method that helped us separate specific signals from autofluorescence. By always merging images from all three available channels, even when only two Abs had been used at the same time, it was possible to easily detect triple-labelled, potentially autofluorescent structures, and in combination with morphological features decide if these signals were caused by autofluorescence or not.

Morphometric measurements

Evaluation of *in situ* immuno-labelling of tissues has several approaches. In this thesis, we have used different semi-quantitative methods. A commonly used method in pathological evaluation is the visual analogue scale (VAS). This was appropriate for the evaluation of slides stained with haematoxylin and eosin with histopathological changes due to cryptosporidiosis (Paper III). This method gave us a semi-quantitative description of all the gut segments including changes in morphology, presence of lymphocytes, epithelium and parasitic load in comparison to the uninfected control group. To evaluate the relative number

of NCR1$^+$ cells and the fraction of these cells co-expressing either c-kit, CD34 or Ki-67 (Paper I), a blinded evaluation was conducted by two researchers in parallel using micrographs from immuno-labelled sections and a scale from "-" to "+++" was used. Morphometric quantification of lymphocyte populations, as it was done for NCR1$^+$ (Paper II) and CD8$^+$ (Paper III) lymphocytes, was performed to compare the density of cells in different compartments of the same organ. The area of interest was defined based on tissue morphology and then cells labelled positive were counted manually. This provided us with interesting observations of cell density with the emphasis on the different compartments and their roles in the GALT.

Flow cytometry

Flow cytometry is a sensitive and powerful method for detection of surface markers or cell cycle molecules. It was used (Paper II and III) to study the lymphoid cell populations by investigating the proportion of a subpopulation of cells in relation to total amount and to describe their phenotypic properties. This method is advantageous because one can study the total amount of live cells in a piece of organ and with a combination of many Abs. The level of antigen expression can be estimated which is challenging in tissue sections. The requirement of fresh tissues limits this method to be applied the same day whenever animals are scheduled for euthanization/surgery, which was not possible for Paper I where the candidate did not sample the individuals. The method is also limited by its requirement for analysing cells in suspension, making information on tissue architecture and cell-to-cell interactions unavailable. In combining this and *in situ* investigation, we were able to obtain concordant and complementary results.

Quantification of NCR1$^+$ cells

To study the frequency of NCR1$^+$ lymphocytes in our tissues, we have used two different methods of investigation; histology and flow cytometry. The pros and cons of each of these methods have been discussed earlier. There was some discrepancy in the results regarding NCR1$^+$ lymphocytes in our study (Paper II and III) between quantification with *in situ* morphometric quantification and flow cytometry. This can probably be explained by the structure of the GALT, as when applying flow cytometry the vast number of B cells of the

follicle will exert a major dilution effect on the NCR1$^+$ cells. When describing the number of cells in histology, we use parameters describing density to do so.

General discussion

Before this work was initiated, there was no detailed information on NCR1$^+$ cells in sheep lymphoid tissues, and the information on these cells was scarce in other species. The newly implemented nomenclature for cells that are important effectors of innate immunity, the ILCs (Spits et al., 2013), has changed the way we perceive cells presenting the membranous NCR1. If we transfer the classification based on studies performed in mice and humans to the ruminant species, NCR1 would mark both cNK cells and ILC22s, in addition to a smaller subclass of T cells, the NKT cell. With the aid of the pan T cell marker CD3, we could prove that the great majority of NCR1$^+$ lymphocytes are not a subclass of T cells, neither in ovine foetuses (Paper I) nor in lambs (Paper II). The minute population of NCR1$^+$ cells that co-expressed CD3, observed by flow cytometry (Paper II) could, interestingly, be similar cells to the novel population of NCR1$^+$ lymphocytes expressing CD3 and TCR, which recently were isolated from the blood of cattle and suggested to be distinct from cNK, T and NKT cells (Connelley et al., 2014). These cells differed from NKT cells in having a more diverse TCR repertoire, and further studies are needed for a more precise classification of the NCR1$^+$/CD3$^+$ cells found in ovine GALT.

Previous studies have established the existence of a cNK cell population in peripheral tissues in sheep (Connelley et al., 2011; Elhmouzi-Younes et al., 2010) that exhibits the phenotype, perforin content and cytotoxicity classical for this population. With the focus on different segments of the intestine, the GALT of normal one month old lambs were analysed, and it was demonstrated that around half of the NCR1$^+$ cells indeed may be cNK cells based on the high level of CD16 that has been associated with enhanced cytotoxic properties in human (Ferlazzo et al., 2004) and bovine (Boysen et al., 2008) cNK cells (Paper II). Even the other cell fraction with low expression of CD16 could be cNK cells, as CD16$^-$ cells also can achieve high perforin levels, as shown in Paper III, and thus probably be cytotoxic. Some or all of the NCR1$^+$CD16$^-$ cells described in Paper II could alternatively belong to the ILC3 group, which are cells with little cytotoxicity (Cording, 2014). There was noted a difference in the level of CD16 expression on NCR1$^+$ cells between the two groups of unchallenged control lambs in Paper II and III. The lower proportion reported in the young control lambs in Paper III

compared to the two to four weeks older lambs in Paper II may be due to the age difference. It is likely that the lambs one week of age were not as immunologically stimulated as the one month old lambs, and therefore harbour less mature and less activated $NCR1^+$ cells. On the other hand, as noted above, the $NCR1^+$ cells were analysed with respect to perforin (Paper III), and it was found that the perforin content increased in both $CD16^+$ and $CD16^-$ subpopulations of $NCR1^+$ cells during infection. This may suggest that the $NCR1^+$ cells, low in CD16, are able to mature and effectuate cytotoxicity, especially after being immunologically challenged.

Since lack of CD16 on $NCR1^+$ cells is not a reliable criterion for classifying them as ILC22s, the results from sheep presented in this thesis are not directly comparable to murine and human studies where more precise markers are available. In the human gut, the largest fraction of $NCR1^+$ cells are classified as cNK cells, and not ILC22s (Tomasello et al., 2012). In mice, however, the number of ILC22s has been found somewhat higher than the number of cNK cells in the lamina propria. The cells were differentiated and detected by flow cytometry and *in situ* immuno-fluorescence by the use of Abs against NCR1 and RORγt (Reynders et al., 2011). The lack of sheep specific lymphoid markers is generally a challenge for detailed studies of cell subtypes in this species. The CD16 marker was only applicable in flow cytometry analyses, and not in *in situ* studies. We have without success tested important ILC markers in our analyses, including RORγt, CD127 and IL-22. However, even with the aid of such markers, the definition of ILCs would still be complicated. As an example, it was reported that a small fraction of an ILC22-like population (NCR1+/ RORγt+) in young mice were able to down regulate RORγt to acquire a phenotype similar to cNK cells (Vonarbourg et al., 2010). This proves there is still much to learn from this universe of the immune system puzzle, also in humans and mice.

In the ovine foetuses (Paper I), we found an increasing fraction of $NCR1^+$ cells to co-express c-kit late in foetal GALT development. To date, there are no known reports showing that NCR1 specific c-kit ligation renders any functional feature to the cells. Nevertheless, c-kit is expressed on NCR^+ ILCs, both ILC22s (Killig et al., 2014; Spits et al., 2013) and in some intermediate developmental stages of immature cNK cells (Freud et al., 2014). In culture, human cNK cells have shown to upregulate c-kit (Pradier et al., 2014) and high expression have been associated with decreased cell cytotoxicity, suggesting they are somewhat immature (Colucci and Di Santo, 2000; Matos et al., 1993). ILC22s are constantly expressing c-kit, and based on our findings in foetal sheep it may be speculated that this population

increases in the foetal gut in late gestation because it is unlikely that a population of immature cNK cells, expressing c-kit only a short period in their development would be prominent as noted in GALT in the late gestation. In mice the ILC22 population has been found to develop mainly postnatally (Reynders et al., 2011; Sanos et al., 2009; Sawa et al., 2010), and in line with other comments on murine-ovine developmental differences in this thesis, the presence of these cells relatively earlier in the gestation period may reflect the general notion that the murine lymphoid tissues is less developed at birth than their ovine counterparts.

The cytokine IL-22 produced by ILC22s, amongst others, are shown not to be crucial for PP structure development (Eberl, 2012). However, in adults IL-22 promotes tissue healing by inhibiting apoptosis, increasing cell cycle progression, and inducing production of mitogenic factors (Eyerich et al., 2010; Sonnenberg et al., 2011). IL-22 induces epithelial cells to produce antimicrobial peptides as well as mucin, which kill microbes and maintain the barrier between the host and bacteria (reviewed in Chen and Kasper, 2014). This indicates that a possible increase of the ILC22 population in the end of gestation prepares the gut for microbial colonization and subsequent epithelial protection. In Paper II, we showed that $NCR1^+$ cells in the gut of older lambs were mainly cNK cells. A possible shift of the ILC22 subtype towards cNK cells in the growing lamb may be due to the microbial colonization and need for innate immune surveillance in the gut mucosa.

It was beyond the scope of the study to solve the possible classifications of ILCs in sheep. The presence of $NCR1^+$ cells in the GALT of sheep have been described with different methods and markers, and in different situations (Paper I, II, and III), and if we transfer what we know about ILCs in humans and mice, we could speculate on the following:

1. NCR1 is found on cells that are cNK cells.
    a. The cNK cells developed directly from a progenitor ILC, or
    b. NCR1 is present on cNK-like cells developed from group 3 ILCs (LTi through ILC22) that has upregulated NCR1 and downregulated RORγt to share phenotypic similarities with cNK cells.
2. NCR1 label cells that are ILC22.
    a. The ILC22 developed directly from a progenitor ILC, or
    b. The ILC22s are originating from LTi cells that have upregulated NCR1 on their surface.

The characterization of NCR1$^+$ cells according to their anatomical localization may contribute to unveiling the physiological role of these unconventional lymphocytes. In this thesis, I present in three separate papers a study of the localization of NCR1$^+$ cells from foetus to lamb, and during neonatal parasitic infection. Although NCR1$^+$ cells have been identified in the gut wall and lymph node anlagen in foetuses of mice and briefly in adult sheep (Connelley et al., 2011), there has never been a description of these cells during ruminant foetal development, and in different GALT segments.

In this thesis, GALT segments from both small and large intestine were investigated. The description of the NCR1$^+$ cells distribution in primary (IPP) and secondary (JPP and CP) lymphoid organs, propose their importance in these types of tissues as both immunoregulators and innate protectors of the mucosal surfaces. This is supported by Renström et. al. (Renström et al., 1996) that also has observed traits of both primary and secondary lymphoid functions in the ileum. Whether some of the NCR1+ cells, possibly localised in certain areas, may have specific influence on the processes occurring in the primary lymphoid tissues remains to be investigated.

For tissue orientation, we have used the compartmentalization of the PPs as previously described. The Abs against T (CD3) and B (CD79) cells stained well in our tissues and outlined the anticipated compartments of the GALT. We found NCR1$^+$ cells in the gut wall long before organized structures of GALT were present in the foetus. Later, when compartmentalization was visible in the foetal gut, NCR1$^+$ cells resided mainly in areas between follicles and in the dome, already prior to any challenge by foreign antigens. Independent of the age of the foetus or lamb, NCR1$^+$ cells seemed to be stationary and stable in their localization. According to *in vitro* studies of cNK cells, it is possible that these cells have direct and indirect communication with myeloid and lymphoid cells here for immunoregulatory functions (Bajenoff et al., 2006; Ferlazzo and Munz, 2004; Walzer et al., 2007). The localization of NCR1$^+$ cells in these three papers correspond to findings in human gut (Cella et al., 2009). Overall, the studies on localization of NCR1$^+$ cells in this thesis indicate that this is a cell population that is a faithful component of the developing and fully functional lymphoid tissues.

The marked presence of NCR1$^+$ cells in the sub FAE region of the dome argued for the interpretation of these cells being cNK cells. The dome area of PPs is known to be covered by FAE containing specialized M cells. The M cells are capable of transcytosing macromolecules

(Neutra et al., 1988; Owen and Jones, 1974), and are thought to transcytose various antigens, including *Cryptosporidium parvum* (Landsverk, 1987), to the underlying immune competent areas of the dome, follicle and IFA to educate the mucosal immune system. The transportation of IgA, which occurs across the absorptive epithelium of villi, is absent in the FAE. This may cause the mucosal area of FAE to be more vulnerable to infections. This area is therefore dependent on an immediate protective reaction, and it strengthens the idea that the $NCR1^+$ cells residing in this region are cNK cells, being able to kill infected cells promptly or stimulate phagocytizing macrophages.

In the present work, the proliferation of $NCR1^+$ cells in GALT were investigated in both foetal and one month old lambs (Paper I and II) and we found a detectable proliferation only in the foetal tissue, particularly in the earliest stages of gestation (Paper I). This likely indicates that the foetal cells are more immature than in the juvenile lambs and that these immature cells can even proliferate in peripheral tissues. In mice, all subsets of common lymphoid precursors were shown to be able to divide and further differentiate in peripheral organs (Possot et al., 2011). These cells could differentiate into $ROR\gamma t^+$ cells, half of them eventually co-expressing NCR1, a phenotype consistent with ILC22s. Whether the $NCR1^+$ cells in foetal ovine GALT are ILC22s or some stage of cNK cells is an open question. In the development of cNK cells in adults, NCR seems to be expressed relative late (Freud et al., 2014) and it would not be likely that substantial cell division occur in those stages. However, this might be different in the foetus and it needs to be studied if $NCR1^+$ cells, maybe differentiating to cNK cells, proliferate in the early foetal intestine.

The cNK cells have a pivotal role in early protection through their cytolytic function and IFNγ production against bacteria, fungi, viruses, and parasitic infections. Whilst many of these infections can be eliminated in the absence of cNK cells, clearance of these organisms in most cases is more efficient and more complete in the presence of a functional cNK cell response. This is also true for orally acquired infections such as Salmonella, Toxoplasma, Citrobacter and Yersinia sp (Ivanova et al., 2014). In Paper III, evidence of both innate and acquired immune responses occurred early during the experimental ovine cryptosporidiosis. There was a slight increase in the activation status of $NCR1^+$ cells in the inoculated lambs compared to the controls. However, there was no clear evidence of their increase in number or change of localization in the gut tissue. It is shown during a *Cryptosporidium parvum* infection that $NCR1^+$ lymphocytes become activated by IL-15 (Dann et al., 2005) and release IFNγ (Barakat et al., 2009). The release of IFNγ induces enterocyte resistance against

Cryptosporidium parvum (Pollok et al., 2001) in addition to stimulation of macrophages. Also, $NCR1^+$ ILC3 plays a pivotal role in controlling early susceptibility to infection with *Citrobacter rodentium* by increasing IL-22 production and tissue repair in the intestine (Zheng et al., 2008). These reports of experimental gastrointestinal infections support the increase of activation status found in the neonatal lambs (Paper III).

Conclusions

This thesis addressed the ovine NCR1$^+$ cells, with particular focus on the GALT during foetal development, in steady state of lambs and their response to cryptosporidiosis.

- Investigations showed that the majority of ovine NCR1$^+$ cells do not express the T cell marker CD3, thus belongs to either the ILC1 or ILC3 group
- The majority of NCR1$^+$ cells in the GALT were most likely cNK cells based on their high expression of CD16, all though a subgroup could also be classified as ILC22
- NCR1$^+$ cells are present in GALT of both small and large intestine
- Investigations revealed that NCR1$^+$ cells appear early during GALT development and the majority compartmentalize in the T cell rich IFAs and domes
- During experimental cryptosporidiosis NCR1$^+$ lymphocytes did not increase conspicuously nor change localization within the GALT, but became more activated
- NCR1$^+$ cells are localized in the same compartments of the GALT of foetuses, young healthy lambs and during cryptosporidial infection

Future perspectives

Our view on the classification of innate lymphoid cells and their role in development, steady state and during disease has changed tremendously during the last years and there is no reason to believe that the final chapter on these cells has been written yet. As most studies are performed in mouse and human model systems, knowledge from other species is lagging behind. In sheep, the prototype of ILCs, the conventional Natural Killer cell, has only recently been demonstrated, so the primary aim of the work in this thesis was to further characterize this cell type. However, during the work on the ovine NK cells, a wish to study subgroups that might be analogous to the cells found in mouse and human in a more precise manner, soon came up. The discussion of this thesis has identified directions for future research and proposed topics are:

- Technical developments:
    - Create new ovine specific antibodies against relevant markers used to classify mouse and human ILCs
    - Work on protocols for the study of gene transcription, either on tissues or isolated cells using RT-PCR, or on tissue sections using *in situ* hybridisation
- Further characterize the two populations of $NCR1^+$ cells in the gut of sheep, and define their localization
- Investigate the presence and distribution of $NCR1^+$ cells in the other ovine tissues, such as bone marrow, liver and spleen
- Identify possible presence of LTi cells in foetuses of sheep including earlier stages in the gestation than in the present thesis
- Investigate the $NCR1^+$ cell population with another experimental stressor than cryptosporidiosis, such as a gastrointestinal bacterial or viral infection
- Investigate whether $NCR1^+$ cells proliferate during immunological challenge
- Address the interactions between ILCs in sheep and antigen-presenting and other immune-regulatory cells, like macrophages and dendritic cells

References

Ahn, Y.O., Blazar, B.R., Miller, J.S., Verneris, M.R., 2013. Lineage relationships of human IL-22 producing CD56+ RORγt+ innate lymphoid cells and conventional NK cells. Blood.

Aleksandersen, M., Hein, W.R., Landsverk, T., Mcclure, S., 1990. Distribution of lymphocyte subsets in the large intestinal lymphoid follicles of lambs. Immunology 70, 391-397.

Aleksandersen, M., Nicander, L., Landsverk, T., 1991. Ontogeny, distribution and structure of aggregated lymphoid follicles in the large-intestine of sheep. Developmental and Comparative Immunology 15, 413-422.

Angus, K.W., Tzipori, S., Gray, E.W., 1982. Intestinal lesions in specific-pathogen-free lambs associated with a cryptosporidium from calves with diarrhea. Vet.Pathol. 19, 67-78.

Atochina, O., Harn, D., 2005. LNFPIII/LeX-stimulated macrophages activate natural killer cells via CD40-CD40L interaction. Clin.Diagn.Lab Immunol. 12, 1041-1049.

Bajenoff, M., Breart, B., Huang, A.Y.C., Qi, H., Cazareth, J., Braud, V.M., Germain, R.N., Glaichenhaus, N., 2006. Natural killer cell behavior in lymph nodes revealed by static and real-time imaging. Journal of Experimental Medicine 203, 619-631.

Barakat, F.M., McDonald, V., Di Santo, J.P., Korbel, D.S., 2009. Roles for NK cells and an NK cell-independent source of intestinal gamma interferon for innate immunity to *Cryptosporidium parvum* infection. Infect.Immun. 77, 5044-5049.

Blum, K.S., Pabst, R., 2007. Lymphocyte numbers and subsets in the human blood: Do they mirror the situation in all organs? Immunology letters 108, 45-51.

Boyd, R.D., Haworth, C., Stacey, T.E., Ward, H.T., 1976. Permeability of the sheep placenta to unmetabolized polar non-electrolytes. The Journal of physiology 256, 617-634.

Boysen, P., Eide, D.M., Storset, A.K., 2011. Natural killer cells in free-living *Mus musculus* have a primed phenotype. Molecular Ecology 20, 5103-5110.

Boysen, P., Gunnes, G., Pende, D., Valheim, M., Storset, A.K., 2008. Natural killer cells in lymph nodes of healthy calves express CD16 and show both cytotoxic and cytokine-producing properties. Dev.Comp Immunol. 32, 773-783.

Boysen, P., Storset, A.K., 2009. Bovine natural killer cells. Veterinary Immunology and Immunopathology 130, 163-177.

Brambell, F.W.R., 1970. The transmission of passive immunity from mother to young (Frontiers of biology), Amsterdam, North Holland.

Brandtzaeg, P., 2009. Mucosal immunity: induction, dissemination, and effector functions. Scandinavian Journal of Immunology 70, 505-515.

Brandtzaeg, P., 2011. The gut as communicator between environment and host: Immunological consequences. European Journal of Pharmacology 668, S16-S32.

Brandtzaeg, P., Kiyono, H., Pabst, R., Russell, M.W., 2008. Terminology: nomenclature of mucosa-associated lymphoid tissue. Mucosal.Immunol. 1, 31-37.

Cella, M., Fuchs, A., Vermi, W., Facchetti, F., Otero, K., Lennerz, J.K., Doherty, J.M., Mills, J.C., Colonna, M., 2009. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature 457, 722-725.

Chan, A., Hong, D.L., Atzberger, A., Kollnberger, S., Filer, A.D., Buckley, C.D., McMichael, A., Enver, T., Bowness, P., 2007. CD56bright human NK cells differentiate into CD56dim cells: role of contact with peripheral fibroblasts. J Immunol 179, 89-94.

Chen, V.L., Kasper, D.L., 2014. Interactions between the intestinal microbiota and innate lymphoid cells. Gut microbes 5, 129-140.

Colucci, F., Di Santo, J.P., 2000. The receptor tyrosine kinase c-kit provides a critical signal for survival, expansion, and maturation of mouse natural killer cells. Blood 95, 984-991.

Connelley, T., Storset, A.K., Pemberton, A., MacHugh, N., Brown, J., Lund, H., Morrison, W.I., 2011. NKp46 defines ovine cells that have characteristics corresponding to NK cells. Veterinary Research 42.

Connelley, T.K., Longhi, C., Burrells, A., Degnan, K., Hope, J., Allan, A.J., Hammond, J.A., Storset, A.K., Morrison, W.I., 2014. NKp46+ CD3+ cells: a novel nonconventional T cell subset in cattle exhibiting both NK cell and T cell features. J Immunol 192, 3868-3880.

Corpa, J.M., Juste, R.A., Marin, J.F.G., Reyes, L.E., Gonzalez, J., Perez, V., 2001. Distribution of lymphocyte subsets in the small intestine lymphoid tissue of 1-month-old lambs. Anatomia Histologia Embryologia-Journal of Veterinary Medicine Series C 30, 121-127.

Dann, S.M., Wang, H.C., Gambarin, K.J., Actor, J.K., Robinson, P., Lewis, D.E., Caillat-Zucman, S., White, A.C., Jr., 2005. Interleukin-15 activates human natural killer cells to clear the intestinal protozoan cryptosporidium. J.Infect.Dis. 192, 1294-1302.

Degli-Esposti, M.A., Smyth, M.J., 2005. Close encounters of different kinds: dendritic cells and NK cells take centre stage. Nature reviews. Immunology 5, 112-124.

Eberl, G., 2012. Development and evolution of RORgammat+ cells in a microbe's world. Immunol Rev 245, 177-188.

Elhmouzi-Younes, J., Boysen, P., Pende, D., Storset, A.K., Le, V.Y., Laurent, F., Drouet, F., 2010. Ovine CD16+/CD14- blood lymphocytes present all the major characteristics of natural killer cells. Veterinary Research 41, 4.

Eyerich, S., Eyerich, K., Cavani, A., Schmidt-Weber, C., 2010. IL-17 and IL-22: siblings, not twins. Trends Immunol 31, 354-361.

Ferlazzo, G., Munz, C., 2004. NK cell compartments and their activation by dendritic cells. J.Immunol. 172, 1333-1339.

Ferlazzo, G., Pack, M., Thomas, D., Paludan, C., Schmid, D., Strowig, T., Bougras, G., Muller, W.A., Moretta, L., Munz, C., 2004. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. Proc.Natl.Acad.Sci.U.S.A 101, 16606-16611.

Freud, A.G., Yu, J., Caligiuri, M.A., 2014. Human natural killer cell development in secondary lymphoid tissues. Semin Immunol 26, 132-137.

Glick, B., Chang, T.S., Jaap, R.G., 1956. The Bursa of Fabricius and antibody production. Poultry Science 35, 224-225.

Gregoire, C., Chasson, L., Luci, C., Tomasello, E., Geissmann, F., Vivier, E., Walzer, T., 2007. The trafficking of natural killer cells. Immunological Reviews 220, 169-182.

Haller, D., Serrant, P., Granato, D., Schiffrin, E.J., Blum, S., 2002. Activation of human NK cells by staphylococci and lactobacilli requires cell contact-dependent costimulation by autologous monocytes. Clin.Diagn.Lab Immunol. 9, 649-657.

Hein, W.R., Griebel, P.J., 2003. A road less travelled: large animal models in immunological research. Nature reviews. Immunology 3, 79-84.

Hercend, T., Schmidt, R.E., 1988. Characteristics and uses of natural killer cells. Immunol Today 9, 291-293.

Horst, D., Verweij, M.C., Davison, A.J., Ressing, M.E., Wiertz, E.J., 2011. Viral evasion of T cell immunity: ancient mechanisms offering new applications. Curr Opin Immunol 23, 96-103.

Ivanova, D., Krempels, R., Ryfe, J., Weitzman, K., Stephenson, D., Gigley, J.P., 2014. NK cells in mucosal defense against infection. BioMed research international 2014, 413982.

Jeffrey, M., Gonzalez, L., Espenes, A., Press, C.M., Martin, S., Chaplin, M., Davis, L., Landsverk, T., MacAldowie, C., Eaton, S., McGovern, G., 2006. Transportation of prion protein across the intestinal mucosa of scrapie-susceptible and scrapie-resistant sheep. The Journal of pathology 209, 4-14.

Killig, M., Glatzer, T., Romagnani, C., 2014. Recognition strategies of group 3 innate lymphoid cells. Frontiers in immunology 5, 142.

Korbel, D.S., Barakat, F.M., Di Santo, J.P., McDonald, V., 2011. CD4+ T cells are not essential for control of early acute *Cryptosporidium parvum* infection in neonatal mice. Infect.Immun. 79, 1647-1653.

Landsverk, T., 1981. The epithelium covering Peyer's patches in young milk-fed calves. An ultrastructural and enzyme histochemical investigation. Acta Vet Scand 22, 198-210.

Landsverk, T., 1984. Is the ileo-caecal Peyer´s patch in ruminants a mammalian "bursa-equivalent"? Acta Pathologica Microbiologica et Immunologica Scandinavica Section A-Pathology 92, 77-79.

Landsverk, T., 1987. Cryptosporidiosis and the follicle-associated epithelium over the ileal Peyer's patch in calves. Res.Vet.Sci. 42, 299-306.

Landsverk, T., Halleraker, M., Aleksandersen, M., Mcclure, S., Hein, W., Nicander, L., 1991a. The intestinal habitat for organized lymphoid-tissues in ruminants - Comparative aspects of structure, function and development. Veterinary Immunology and Immunopathology 28, 1-16.

Landsverk, T., Halleraker, M., Aleksandersen, M., McClure, S., Hein, W., Nicander, L., 1991b. The intestinal habitat for organized lymphoid tissues in ruminants; comparative aspects of structure, function and development. Vet Immunol Immunopathol 28, 1-16.

Lantier, L., Lacroix-Lamande, S., Potiron, L., Metton, C., Drouet, F., Guesdon, W., Gnahoui-David, A., Le Vern, Y., Deriaud, E., Fenis, A., Rabot, S., Descamps, A., Werts, C., Laurent, F., 2013. Intestinal CD103+ dendritic cells are key players in the innate immune control of Cryptosporidium parvum infection in neonatal mice. PLoS pathogens 9, e1003801.

Leon, F., Roldan, E., Sanchez, L., Camarero, C., Bootello, A., Roy, G., 2003. Human small-intestinal epithelium contains functional natural killer lymphocytes. Gastroenterology 125, 345-356.

Lie, K.I., Aleksandersen, M., Landsverk, T., 2005. Lymphoid follicles of different phenotype appear in ileum during involution of the sheep ileal Peyer's patch. Developmental and Comparative Immunology 29, 539-553.

Liebler, E.M., Lemke, C., Pohlenz, J.F., 1995. Ultrastructural study of the uptake of ferritin by M cells in the follicle-associated epithelium in the small and large intestines of pigs. American journal of veterinary research 56, 725-730.

Ljunggren, H.G., Karre, K., 1990. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today 11, 237-244.

Lucas, M., Schachterle, W., Oberle, K., Aichele, P., Diefenbach, A., 2007. Dendritic cells prime natural killer cells by *trans*-presenting interleukin 15. Immunity. 26, 503-517.

Luci, C., Reynders, A., Ivanov, I.I., Cognet, C., Chiche, L., Chasson, L., Hardwigsen, J., Anguiano, E., Banchereau, J., Chaussabel, D., Dalod, M., Littman, D.R., Vivier, E., Tomasello, E., 2009. Influence of the transcription factor ROR gamma t on the development of NKp46(+) cell populations in gut and skin. Nature Immunology 10, 75-82.

Mancassola, R., Reperant, J.M., Naciri, M., Chartier, C., 1995. Chemoprophylaxis of Cryptosporidium parvum infection with paromomycin in kids and immunological study. Antimicrobial agents and chemotherapy 39, 75-78.

Mancassola, R., Richard, A., Naciri, M., 1997. Evaluation of decoquinate to treat experimental cryptosporidiosis in kids. Vet Parasitol 69, 31-37.

Martin-Fontecha, A., Thomsen, L.L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., Sallusto, F., 2004. Induced recruitment of NK cells to lymph nodes provides IFN-γ for T(H)1 priming. Nat.Immunol. 5, 1260-1265.

Martin, C., Pastoret, P.P., Brochier, B., Humblet, M.F., Saegerman, C., 2011. A survey of the transmission of infectious diseases/infections between wild and domestic ungulates in Europe. Vet.Res. 42, 70.

Matos, M.E., Schnier, G.S., Beecher, M.S., Ashman, L.K., William, D.E., Caligiuri, M.A., 1993. Expression of a functional c-kit receptor on a subset of natural killer cells. The Journal of experimental medicine 178, 1079-1084.

McDonald, V., Bancroft, G.J., 1994. Mechanisms of innate and acquired resistance to *Cryptosporidium parvum* infection in SCID mice. Parasite Immunol. 16, 315-320.

McDonald, V., Deer, R., Uni, S., Iseki, M., Bancroft, G.J., 1992. Immune responses to Cryptosporidium muris and Cryptosporidium parvum in adult immunocompetent or immunocompromised (nude and SCID) mice. Infect Immun 60, 3325-3331.

McDonald, V., Korbel, D.S., Barakat, F.M., Choudhry, N., Petry, F., 2013. Innate immune responses against *Cryptosporidium parvum* infection. Parasite Immunol. 35, 55-64.

McDonald, V., Robinson, H.A., Kelly, J.P., Bancroft, G.J., 1994. Cryptosporidium muris in adult mice: adoptive transfer of immunity and protective roles of CD4 versus CD8 cells. Infect Immun 62, 2289-2294.

Medzhitov, R., 2007. Recognition of microorganisms and activation of the immune response. Nature 449, 819-826.

Meinhardt, P.L., Casemore, D.P., Miller, K.B., 1996. Epidemiologic aspects of human cryptosporidiosis and the role of waterborne transmission. Epidemiol.Rev. 18, 118-136.

Min-Oo, G., Kamimura, Y., Hendricks, D.W., Nabekura, T., Lanier, L.L., 2013. Natural killer cells: walking three paths down memory lane. Trends Immunol 34, 251-258.

Montaldo, E., Vacca, P., Moretta, L., Mingari, M.C., 2014. Development of human natural killer cells and other innate lymphoid cells. Semin Immunol 26, 107-113.

Moreau, M.C., Corthier, G., 1988. Effect of the gastrointestinal microflora on induction and maintenance of oral tolerance to ovalbumin in C3H/HeJ mice. Infect Immun 56, 2766-2768.

Moretta, L., Bottino, C., Pende, D., Vitale, M., Mingari, M.C., Moretta, A., 2005. Human natural killer cells: Molecular mechanisms controlling NK cell activation and tumor cell lysis. Immunology letters 100, 7-13.

Naciri, M., Mancassola, R., Yvore, P., Peeters, J.E., 1993. The effect of halofuginone lactate on experimental *Cryptosporidium parvum* infections in calves. Vet.Parasitol. 45, 199-207.

Neutra, M.R., Wilson, J.M., Weltzin, R.A., Kraehenbuhl, J.P., 1988. Membrane domains and macromolecular transport in intestinal epithelial cells. The American review of respiratory disease 138, S10-16.

Nicander, L., Halleraker, M., Landsverk, T., 1991. Ontogeny of reticular cells in the ileal Peyer's patch of sheep and goats. Am.J.Anat. 191, 237-249.

Nieuwhof, G.J., Bishop, S.C., 2005. Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. Animal Science 81, 23-29.

Owen, R.L., Jones, A.L., 1974. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. Gastroenterology 66, 189-203.

Pearson, C., Uhlig, H.H., Powrie, F., 2012. Lymphoid microenvironments and innate lymphoid cells in the gut. Trends Immunol. 33, 289-296.

Peyer, J.C., 1677. Exercitatio anatomico-medica de glandulis intestinorum earumque usu et affectionibus. Impensis Onophrii a Waldkirch, typis Alexandri Riendingii, Schaffausen, Switzerland.

Pollok, R.C., Farthing, M.J., Bajaj-Elliott, M., Sanderson, I.R., McDonald, V., 2001. Interferon gamma induces enterocyte resistance against infection by the intracellular pathogen Cryptosporidium parvum. Gastroenterology 120, 99-107.

Pradier, A., Tabone-Eglinger, S., Huber, V., Bosshard, C., Rigal, E., Wehrle-Haller, B., Roosnek, E., 2014. Peripheral blood CD56(bright) NK cells respond to stem cell factor and adhere to its membrane-bound form after upregulation of c-kit. Eur J Immunol 44, 511-520.

Press, C.M., Halleraker, M., Landsverk, T., 1992. Ontogeny of leukocyte populations in the ileal Peyer´s patch of sheep. Developmental and Comparative Immunology 16, 229-241.

Press, C.M., Reynolds, J.D., McClure, S.J., Landsverk, T., 1998. Development of accessory cells in B-cell compartments is retarded in B-cell-depleted fetal sheep. Developmental Immunology 6, 223-231.

Press, C.M., Reynolds, J.D., McClure, S.J., Simpson-Morgan, M.W., Landsverk, T., 1996. Fetal lambs are depleted of IgM+ cells following a single injection of an anti-IgM antibody early in gestation. Immunology 88, 28-34.

Renström, L.H., Press, C.M., Trevella, W., Landsverk, T., 1996. Response of leucocyte populations in the illeal Peyer's patch of fetal lambs treated with ferritin *per os*. Developmental Immunology 4, 289-298.

Reynders, A., Yessaad, N., Manh, T.P.V., Dalod, M., Fenis, A., Aubry, C., Nikitas, G., Escaliere, B., Renauld, J.C., Dussurget, O., Cossart, P., Lecuit, M., Vivier, E., Tomasello, E., 2011. Identity, regulation and *in vivo* function of gut NKp46(+)RORγt(+) and NKp46(+)RORγt(-) lymphoid cells. EMBO Journal 30, 2934-2947.

Reynolds, J.D., Morris, B., 1983. The evolution and involution of Peyer's patches in fetal and postnatal sheep. European Journal of Immunology 13, 627-635.

Robertson, L.J., Chalmers, R.M., 2013. Foodborne cryptosporidiosis: is there really more in Nordic countries? Trends Parasitol. 29, 3-9.

Romagnani, C., Juelke, K., Falco, M., Morandi, B., D'Agostino, A., Costa, R., Ratto, G., Forte, G., Carrega, P., Lui, G., Conte, R., Strowig, T., Moretta, A., Munz, C., Thiel, A., Moretta, L., Ferlazzo, G., 2007. CD56brightCD16- killer Ig-like receptor-NK cells display longer telomers and acquire features of CD56dim NK cells upon activation. J.Immunol. 178, 4947-4955.

Sanos, S.L., Bui, V.L., Mortha, A., Oberle, K., Heners, C., Johner, C., Diefenbach, A., 2009. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. Nat.Immunol. 10, 83-91.

Sawa, S., Cherrier, M., Lochner, M., Satoh-Takayama, N., Fehling, H.J., Langa, F., Di Santo, J.P., Eberl, G., 2010. Lineage relationship analysis of RORgammat+ innate lymphoid cells. Science 330, 665-669.

Snodgrass, D.R., Angus, K.W., Gray, E.W., 1984. Experimental cryptosporidiosis in germfree lambs. J.Comp Pathol. 94, 141-152.

Sonnenberg, G.F., Fouser, L.A., Artis, D., 2011. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. Nat Immunol 12, 383-390.

Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J.P., Eberl, G., Koyasu, S., Locksley, R.M., McKenzie, A.N., Mebius, R.E., Powrie, F., Vivier, E., 2013. Innate lymphoid cells--a proposal for uniform nomenclature. Nat.Rev.Immunol. 13, 145-149.

Spits, H., Cupedo, T., 2012. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. Annu.Rev.Immunol. 30, 647-675.

Spits, H., Di Santo, J.P., 2011. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. Nature Immunology 12, 21-27.

Steel, A.W., Mela, C.M., Lindsay, J.O., Gazzard, B.G., Goodier, M.R., 2011. Increased proportion of CD16(+) NK cells in the colonic lamina propria of inflammatory bowel disease patients, but not after azathioprine treatment. Alimentary Pharmacology & Therapeutics 33, 115-126.

Strowig, T., Brilot, F., Munz, C., 2008. Noncytotoxic functions of NK cells: direct pathogen restriction and assistance to adaptive immunity. J Immunol 180, 7785-7791.

Sweeny, J.P., Ryan, U.M., Robertson, I.D., Jacobson, C., 2011. *Cryptosporidium* and *Giardia* associated with reduced lamb carcase productivity. Vet.Parasitol. 182, 127-139.

Tagliabue, A., Befus, A.D., Clark, D.A., Bienenstock, J., 1982. Characteristics of natural killer cells in the murine intestinal epithelium and lamina propria. The Journal of experimental medicine 155, 1785-1796.

Tizard, I., 2013. Organs of the immune system, Veterinary Immunology, 9 ed. Elsevier, pp. 113-126.

Tomasello, E., Yessaad, N., Gregoire, E., Hudspeth, K., Luci, C., Mavilio, D., Hardwigsen, J., Vivier, E., 2012. Mapping of NKp46(+) cells in healthy human lymphoid and non-lymphoid tissues. Front Immunol. 3, 344.

Trinchieri, G., 1989. Biology of natural killer cells. Adv.Immunol. 47, 187-376.
Tzipori, S., Angus, K.W., Campbell, I., Clerihew, L.W., 1981. Diarrhea due to *Cryptosporidium* infection in artificially reared lambs. J.Clin.Microbiol. 14, 100-105.

Ungar, B.L., Kao, T.C., Burris, J.A., Finkelman, F.D., 1991. *Cryptosporidium* infection in an adult mouse model. Independent roles for IFN-γ and CD4+ T lymphocytes in protective immunity. J.Immunol. 147, 1014-1022.

Veiga-Fernandes, H., Coles, M.C., Foster, K.E., Patel, A., Williams, A., Natarajan, D., Barlow, A., Pachnis, V., Kioussis, D., 2007. Tyrosine kinase receptor RET is a key regulator of Peyer's patch organogenesis. Nature 446, 547-551.

Vonarbourg, C., Mortha, A., Bui, V.L., Hernandez, P.P., Kiss, E.A., Hoyler, T., Flach, M., Bengsch, B., Thimme, R., Holscher, C., Honig, M., Pannicke, U., Schwarz, K., Ware, C.F., Finke, D., Diefenbach, A., 2010. Regulated expression of nuclear receptor RORgammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes. Immunity 33, 736-751.

Walzer, T., Blery, M., Chaix, J., Fuseri, N., Chasson, L., Robbins, S.H., Jaeger, S., Andre, P., Gauthier, L., Daniel, L., Chemin, K., Morel, Y., Dalod, M., Imbert, J., Pierres, M., Moretta, A., Romagne, F., Vivier, E., 2007. Identification, activation, and selective *in vivo* ablation of mouse NK cells via NKp46. Proceedings of the National Academy of Sciences of the United States of America 104, 3384-3389.

Wyatt, C.R., Brackett, E.J., Perryman, L.E., Rice-Ficht, A.C., Brown, W.C., O'Rourke, K.I., 1997. Activation of intestinal intraepithelial T lymphocytes in calves infected with *Cryptosporidium parvum*. Infect.Immun. 65, 185-190.

Wyatt, C.R., Brackett, E.J., Savidge, J., 2001. Evidence for the emergence of a type-1-like immune response in intestinal mucosa of calves recovering from cryptosporidiosis. J.Parasitol. 87, 90-95.

Xiao, L., Fayer, R., Ryan, U., Upton, S.J., 2004. Cryptosporidium taxonomy: recent advances and implications for public health. Clinical microbiology reviews 17, 72-97.

Yasuda, M., Fujino, M., Nasu, T., Murakami, T., 2004. Histological studies on the ontogeny of bovine gut-associated lymphoid tissue: appearance of T cells and development of IgG(+) and IgA(+) cells in lymphoid follicles. Developmental and Comparative Immunology 28, 357-369.

Yasuda, M., Jenne, C.N., Kennedy, L.J., Reynolds, J.D., 2006. The sheep and cattle Peyer's patch as a site of B-cell development. Veterinary Research 37, 401-415.

Zheng, Y., Valdez, P.A., Danilenko, D.M., Hu, Y., Sa, S.M., Gong, Q., Abbas, A.R., Modrusan, Z., Ghilardi, N., de Sauvage, F.J., Ouyang, W., 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nature medicine 14, 282-289.

Åkesson, C.P., Press, C.M., Tranulis, M.A., Jeffrey, M., Aleksandersen, M., Landsverk, T., Espenes, A., 2012. Phenotypic characterization of cells participating in transport of prion protein aggregates across the intestinal mucosa of sheep. Prion 6, 261-275.

Paper I

NCR1+ cells appear early and accumulate c-kit+ phenotype during foetal GALT development

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Abstract

As NCR+ ILCs are found important in GALT development we aimed to describe the amount, distribution and phenotypic characteristics of ovine NCR1+ cells during the development of GALT from day 70 of gestation. Using immunofluorescence on cryosections, antibodies against CD3 and CD79 were used to identify the compartments described for GALT, and the localization of NCR1+ cells were correlated with these structures. The progenitor markers CD34 and c-kit, in addition to the proliferation marker Ki67, was used to investigate possible origin and the stage of development of the NCR1+ cells. NCR1+ cells were present as early as 70 days of gestation, and that they appeared at large in the CD3+ T cell rich IFAs and in the sub FAE domes synchronized with the development of these compartments. There was a gradual increase of NCR1+/c-kit+ cells, particularly towards the end of gestation. NCR1+ cells were found to proliferate, more pronounced at day 70-104 than later. In conclusion, NCR1+ appeared early in T cell rich areas of the gut of ovine foetuses and the cells displayed a phenotype consistent with intermediate stages of developing cNK cells and/or a subpopulation of ILC22.

Keywords: NK cells, foetus, GALT, innate lymphoid cells, NCR1, ovine

1. Introduction

The Peyer's patches (PPs) of the small intestine and colon patches (CPs) are organized lymphoid tissues of the gut and constitute, together with isolated lymphoid follicles, the gut-associated lymphoid tissue (GALT). These structures are important for the balance between tolerance to food antigens and normal microbiota and responses against harmful agents. The fully developed PPs and CPs can be divided into compartments based on morphology, cellular composition, and function (Aleksandersen et al., 1991; Landsverk et al., 1991; Reynolds and Morris, 1983). The tissue compartments include the follicle-associated epithelium (FAE), the underlying dome, the submucosal B cell follicles, and the T cell rich inter-follicular areas (IFAs). These compartments are all considered mainly to be inductive sites. The lamina propria of the intestinal villi that is covered with the absorptive epithelium is considered to be an effector site and found alongside the whole length of the gut (Brandtzaeg, 2009).

Lymphoid tissue development has been described to take place in three stages (Coles et al., 2006; Constantinides et al., 2014); initially in the gut, precursors of hematopoietic innate lymphoid cells (ILCs) migrate into the intestinal wall and are evenly distributed where future lymphoid tissue will form (Bando et al., 2015). The precursor ILCs then bind to and interact with stromal cells to secrete cytokines and chemokines to further promote the maturation of the lymphoid tissue anlage and the clustering of cells. In the third and final stage, mature lymphocytes migrate into these fetal structures to generate adult lymphoid tissues with B and T cell compartments. A substantial number of lymphocytes in these structures are recognized as ILCs, which also include conventional NK (cNK) cells, a recently characterized cell group which are central in organogenesis as well as mucosal homeostasis and immunity in post-natal life (Spits, 2013). Several ILC subgroups express the natural cytotoxicity receptor 1 (NCR1), also denoted NKp46, but the expression pattern vary between

species. In humans NCR2 (NKp44) thought to be analogous to murine NCR1+ ILCs, appeared in the small intestine early in the second trimester in foetuses (Hoorweg et al., 2012), while in mice NCR1+ ILCs first appeared after birth (Reynders et al., 2011; Sawa et al., 2010). Apart from humans and mice, little is known about ILCs in fetal tissues.

Sheep have been used as a model for GALT development in ruminants, and many studies have addressed the difference in function between the jejunal and ileal PPs as secondary and primary lymphoid organs, respectively has been an important focus (Griebel and Hein, 1996; Landsverk, 1984). The gestation period for sheep is 150 days, and the development of PPs and CPs in lamb foetuses starts in the second trimester. The PP development is identified initially with the formation of a dome that is characterized by high folds of primordial epithelium. Subsequently, aggregates of lymphoid cells form beneath the epithelium, first appearing in the colon and jejunum at 70-75 days of gestation (Aleksandersen et al., 1991; Reynolds and Morris, 1983), and in the ileum at around 97 days of gestation (Nicander et al., 1991). The further expansion of the follicles filled with B cells (Aleksandersen et al., 1991) is accompanied by an interfollicular accumulation of heterogenic leukocyte populations, including various T cells and dendritic cells (Press et al., 1992).

Connelley and coworkers (2011) have recently described NCR1+ cells in sheep. Investigation of NCR1+ cells in the GALT of one month old lambs has found at least two subpopulations. More than half of the NCR1+ cells in lambs showed high expression of CD16, a receptor associated with cytotoxicity whereas the other fraction was associated with other ILCs (Olsen et al., 2013). Analysis of NCR1+ cells in lamb foetuses can yield information of early events in the formation of PPs in larger mammals. As far as the authors are aware, there are no reports on NCR1+ cells in ruminant foetuses.

In the present study we detected NCR1+ cells during GALT development in intestinal tissues from ovine foetuses by immunofluorescent techniques on cryosections. The aim of this study was to describe the presence, distribution and phenotype of NCR1+ cells, and their relation to other immune cells from the second trimester until birth. The T and B lymphocyte markers CD3 and CD79, respectively, were used to identify early lymphoid compartments of GALT, and the localization of NCR1+ cells was correlated with these lymphocyte populations. Further, whether the NCR1+ cells in the foetal gut carried the progenitor markers and/or proliferation markers was investigated.

2. Materials and methods

2.1. Animals and tissue sampling procedures

Sheep foetuses were Norwegian Dala and Spæl (Research Station, Heggedal, Norway), Australian Merino (Research Station, Canberra, Australia) and Swiss White Alpine x Black Jura (Research Station, Basel Institute for Immunology, Switzerland). Developmental details and groups of the foetuses are presented in Table 1. Samples of jejunum, jejunal Peyer's patches (JPPs), ileal Peyer's patch (IPP), and colon patches (CPs) were used for this study (Additional Table 2.). The foetuses used in this study have been described in detail elsewhere (Press et al., 1998; Press et al., 1996; Renström et al., 1996). Only the control foetuses from the study were used here.

2.2. Antibodies

The antibodies (Abs) used in this study were against: ovine NCR1/NKp46 (EC1.1; IgG1 kindly donated by Dr. Timothy Connelley, human c-kit (CD117) (A4502; pAb) from Dako, CD34 (EP373Y; IgG) from Abcam, human CD3ε (A0452; pAb) from Dako, human CD79αcγ (HM57; IgG1/APC) from Dako, human Ki67 (ab15580; pAb) from Abcam.

2.3. Immunofluorescence

Indirect immunolabelling was performed according to a protocol described earlier (Olsen et al., 2013; Åkesson et al., 2008). In brief, 7 µm thick cryosections on poly-lysine-coated slides were fixed in acetone before blocking of non-specific binding with 10% normal goat serum in phosphate-buffered saline (PBS)/0.5% Tween®80 (Sigma-Aldrich, MO, USA). After application of a mixture of two Abs that were incubated for 1 hour at room temperature, the mixture of isotype-specific Alexa Fluor® secondary Abs (Molecular Probes, Inc., OR, USA) was incubated in the dark. The slides were mounted in polyvinyl alcohol at pH 8. Control sections included slides with dilution solution replacing primary Abs, secondary Abs, or sections replaced with dilution solution in both incubation periods.

2.4. Microscopy

Immunolabelled sections were examined under a Zeiss Axiovert 100 inverted microscope, equipped with an LSM 710 laser confocal unit and the Zeiss ZEN 2012 Software (Carl Zeiss, Germany). In addition, a Zeiss Axio Imager 2 equipped with an AxioCam 506 mono, using FL Filter Set 20 DAPI, 38 Rhodamine, and 49 Endow GFP for Alexa Fluor Secondary Abs 350, 594 and 488, respectively, was used. For semi-quantitative analysis of cells labelled for NCR1, c-kit, CD34 and Ki67, 3 – 7 images of each intestinal segment were collected. The average number of cells in comparable areas were denoted as -: <1 cells (none), +: 1-3 cells (few), ++: >3-10 cells (moderate) and +++: >10 cells (many/numerous).

3. Results

3.1. Colonization of the developing GALT structures by lymphocytes

With the aid of Abs against the pan-T cell marker CD3 and the B cell marker CD79, we identified structures associated with the development of GALT (Figure 1). The distribution

and time of appearance of T and B lymphocytes in the gut were consistent with previous studies using other antibodies against these lymphocyte populations (Aleksandersen et al., 1991; Press et al., 1992). In the foetuses of group 1 aged between 70-83 days of gestation, the epithelium formed small folds. A small number of B cells were observed dispersed in lamina propria under the epithelium. In a few sections of the jejunum at this gestation period, small B cell clusters had started to take form under some epithelial folds, possibly representing early PP follicle anlagen. The moderate presence of T cells was dispersed evenly in the subepithelial tissues in all segments of the gut. Compartments typical of GALT structure, with a clear division between T and B cell areas were absent at this stage of gestation. In group 2 (95-104 days of gestation), the mucosal folds were observed to be increased in height to have formed villi. Recognizable domes were seen above B cell follicles. A moderate to large numbers of T cells were present in the lamina propria of jejunum and ileum, while only small numbers were observed in the colon. The T cells were distributed evenly in the lamina propria, both in the villi and deeper mucosa, without showing any tendency to compartmentalisation. In groups 3 (114-129 days of gestation) and 4 (141 days of gestation), the B cell follicles resembled fully developed PPs of new born lambs. At the same time clustering of T cells in the IFAs was first observed in the jejunum and colon in group 3, and later also in the ileum in group 4. The CD3+ T cells were also present in the dome regions, and in the lamina propria in the final gestation period investigated (Table 1; Fig. 1).

3.2. Distribution of NCR1+ cells in developing GALT compartments

NCR1+ cells were observed in all gut segments of foetuses already in group 1, and the numbers in all gut segments increased throughout gestation (Table 2 and 3). In foetuses of group 1, NCR1+ cells appeared as single cells in the subepithelial lamina propria where CD3+ T cells and the scarce CD79+ B cells resided (Figure 1). In group 2, more NCR1+ cells

were dispersed among T cells of the submucosa in the ileal segment compared with the distribution of NCR1+ cells in the jejunum and colon. The lamina propria in group 2 had few NCR1+ cells in all segments. In all intestinal segments of groups 3 and 4, the largest presence of NCR1+ cells was observed in the domes, IFAs and to a lesser degree in the lamina propria of the villi. When identified, follicles did not contain NCR1+ cells in any of the groups. None of the T or B cells co-labelled the NCR1 Ab.

3.3. Phenotypic properties of NCR1+ cells.

Antibodies against the progenitor marker and cytokine receptor c-kit labelled cells in gut tissues from all tissue sections. Double labelled cells (NCR1+/c-kit+) were first observed in the jejunum and ileum of foetuses in group 2 (Figure 2A,B; Table 2). The fraction (0-6%) of NCR1+ cells labelling for c-kit remained stable between groups 2 and 3. This fraction increased in the oldest foetuses, particularly in the jejunum and ileum, where more than 30% of the NCR1+ cells co-labelled for c-kit in the IFA and dome. Ki67+ cells were found in all segments at all gestational stages (Figure 2C; Table 3). Proliferating cells were most frequently observed in the follicles, when present, and in the crypt epithelium. Some NCR1+ cells also labelled with Ki67. Double labelled Ki67+NCR1+ cells were mainly seen in the submucosal area, where the fraction could reach up to 20% of NCR1+ cells in group 1. This fraction decreased to only 0-3% during gestation. In all gut segments examined and at all stages of development, NCR1+ cells did not label for the progenitor marker CD34, consistent with findings in human studies where CD34 expression does not persist into NCR+ expressing intermediate or mature NK cell stages (Freud 2006). CD34 staining was found mainly in cells resembling endothelial tissue in the subepithelial space and the interstitial cells of Cajal in the lamina muscularis (Figure 2D).

4. Discussion

To our knowledge, this is the first *in situ* study of NCR1+ cells in relation to the foetal development of the GALT in ruminants. We have shown that cells bearing NCR1 are present in the foetal gut before compartmentalization of the GALT and that NCR1+ cells expands during PP development. The present study further shows that NCR1+ cells segregate to the T cell rich IFAs and sub FAE areas of the dome during PP development.

In general, the GALT of mice is considered more immature at birth and needs stimulation from the gut flora in post-natal life to mature whereas in sheep the GALT structures are fully developed at birth (Griebel, 1996; Pearson, 2012). We observed NCR1+ cells to be present as early as day 70 of gestation (total gestation 150 days) in foetal gut segments. In the mouse foetus, NK1.1+NCR1+ cells with functions consistent with mature NK cells have been found in the third trimester, gestation day 16.5 in the liver and day 17.5 in the spleen (gestation period 19-21) (Tang et al., 2012). The NCR1+ cells in foetal ovine GALT of this present study appeared in the second trimester, thus relatively early compared with the appearance of these cells in mice foetuses although the data are not directly comparable since GALT was not included in the mouse study. Veiga-Fernandes et al. (2007) studied the development of intestinal lymphoid tissue, and found a NK1.1+ population at around gestation day 16 that was speculated to be important in PP organogenesis. It is likely that the NCR1+ cells appear at an earlier gestation stage in the ovine than in murine GALT and this could reflect species differences in the maturation stages of the lymphoid tissues, since in mice the first B and T cells enter and are evenly distributed in the PP anlage late in gestation at day 18.5, before start of compartmentalization which is completed after birth (Hashi et al., 2001).

In our study, the NCR1+ cells were present before T and B cells showed signs of compartmentalization, and later segregated into PP compartments together with T cells. To

our knowledge, this is the first time antibodies against CD3 and CD79 have been used on ovine foetal intestinal tissues. The distribution of T and B cells was visualised in relation to the different compartments of the GALT, which was in agreement with the findings using Abs against other T and B cell antigens in calves and lambs (Aleksandersen et al., 1991; Press et al., 1992). The identification of PP structures enabled us to detect a moderate to large presence of NCR1+ cells in T cell rich areas, namely the IFAs and domes, whereas the B cell rich areas were devoid of NCR1+ cells. The presence of NCR1+ cells in our study suggests that these cells play a part in mobilization of the innate immune system and the organogenesis of the developing GALT in a foetus shielded from external antigens.

We were not able to identify NCR1+ cells co-labelling CD3 in this material, indicating that they are ILCs and not the NKT subset of T cells. ILCs are able to respond promptly to insults and are important for the initiation and organization of lymphoid tissue genesis in foetuses. ILCs are subdivided into three groups, of interest are group 1 and 3. Group 1 ILCs include cytotoxic cNK cells that carry NCR1 and produce IFNγ. Group 3 ILCs include NCR1+ cells producing IL-22 (ILC22), which lack perforin and granzymes, and do not induce cell lysis. In adult mice and man, ILC22 have been recognized to have an essential role in mucosal homeostasis and immunity after insult (Montaldo et al., 2014; Spits and Cupedo, 2012; Spits and Di Santo, 2011), but have not been assigned any function in the foetal development.

Interestingly, we found that the fraction of the NCR1+ cell population expressing c-kit increased during GALT development. In both mouse and humans, c-kit is expressed on NCR+ ILCs, including ILC22 cells (Killig et al., 2014; Spits et al., 2013) and in some intermediate developmental stages of cNK cells, but not in mature cNK cells (Freud et al., 2014). Stimulation of c-kit contributes to regulation of cellular activities, such as apoptosis, cell differentiation, proliferation, and cell adhesion (Lasota and Miettinen, 2008). The

increase of NCR1+/c-kit+ cells during GALT development observed in the present study could either be a result of proliferation and/or immigration of immature cNK cells or of the ILC22 subtype in the gut. Both cell types exert important functions in the GALT environment of neonatal lambs (Olsen et al., 2015; Olsen et al., 2013). Studies on cNK cell development in man have associated the expression of c-kit on cNK cells with immature stages of development (Freud and Caligiuri, 2006). If the NCR1+ cells found in foetal sheep are cNK cells, the increased presence of c-kit+ NCR1+ cells in ovine GALT at the end of the gestation would indicate the accumulation of immature cNK cells in organized lymphoid tissue as parturition approaches.

The presence of NCR1+ cells increased concomitantly with the development of the gut and GALT in the growing foetus. A substantial part of the NCR1+ cells co-labelled the proliferation marker Ki67. This finding indicates that there is a continuous proliferation of NCR1+ cells in GALT before birth, which is in contrast to the observations in young lambs, where the detected NCR1+ cells were not proliferating (Olsen et al., 2013). Proliferation of common lymphoid progenitors outside the bone marrow has been shown to occur in peripheral tissues (Possot et al., 2011). This report has been supported by Vitale et. al. (2004) in an early study of human NK cells cultured *in vitro*. These investigators found that the NK cells that were completing maturation ($CD56^{bright}CD16^{-}$ subset) were the only NK cell phenotype proliferating when in contact with dendritic cells (Vitale et al., 2004). This observation invites speculation that in the foetal lamb many cNK cells may be trafficked to the gut during their development, where they proliferate and complete their maturation. Whether ILC22 are able to proliferate or not after homing to the gut has to our knowledge not been described.

5. Conclusion

In ovine foetal intestine, NCR1+ cells were found *in situ* where lymphoid tissues develop, thus being potentially important in early stages of GALT organogenesis. There were two distinct phenotypes of NCR1+ cells; the NCR1+/CD34-/CD3-/c-kit+ cell population may be either cNK cells or ILC22, while NCR1+/CD34-/CD3-/c-kit- cells are most likely cNK cells. For further studies, markers against early stages of cNK or ILC22 cells that are NCR1- which are presently not available should be included.

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References

Aleksandersen, M., Nicander, L., Landsverk, T., 1991. Ontogeny, distribution and structure of aggregated lymphoid follicles in the large-intestine of sheep. Developmental and Comparative Immunology 15, 413-422.

Bando, J.K., Liang, H.E., Locksley, R.M., 2015. Identification and distribution of developing innate lymphoid cells in the fetal mouse intestine. Nat Immunol 16, 153-160.

Brandtzaeg, P., 2009. Mucosal immunity: induction, dissemination, and effector functions. Scandinavian Journal of Immunology 70, 505-515.

Coles, M.C., Veiga-Fernandes, H., Foster, K.E., Norton, T., Pagakis, S.N., Seddon, B., Kioussis, D., 2006. Role of T and NK cells and IL7/IL7r interactions during neonatal maturation of lymph nodes. Proc Natl Acad Sci U S A 103, 13457-13462.

Connelley, T., Storset, A.K., Pemberton, A., MacHugh, N., Brown, J., Lund, H., Morrison, W.I., 2011. NKp46 defines ovine cells that have characteristics corresponding to NK cells. Veterinary Research 42.

Constantinides, M.G., McDonald, B.D., Verhoef, P.A., Bendelac, A., 2014. A committed precursor to innate lymphoid cells. Nature 508, 397-401.

Freud, A.G., Caligiuri, M.A., 2006. Human natural killer cell development. Immunol.Rev. 214, 56-72.

Freud, A.G., Yu, J., Caligiuri, M.A., 2014. Human natural killer cell development in secondary lymphoid tissues. Semin Immunol 26, 132-137.

Griebel, P.J., Hein, W.R., 1996. Expanding the role of Peyer's patches in B-cell ontogeny. Immunol.Today 17, 30-39.

Hashi, H., Yoshida, H., Honda, K., Fraser, S., Kubo, H., Awane, M., Takabayashi, A., Nakano, H., Yamaoka, Y., Nishikawa, S., 2001. Compartmentalization of Peyer's patch anlagen before lymphocyte entry. J Immunol 166, 3702-3709.

Hoorweg, K., Peters, C.P., Cornelissen, F., Aparicio-Domingo, P., Papazian, N., Kazemier, G., Mjosberg, J.M., Spits, H., Cupedo, T., 2012. Functional Differences between Human NKp44(-) and NKp44(+) RORC(+) Innate Lymphoid Cells. Frontiers in immunology 3, 72.

Killig, M., Glatzer, T., Romagnani, C., 2014. Recognition strategies of group 3 innate lymphoid cells. Frontiers in immunology 5, 142.

Landsverk, T., 1984. Is the ileo-caecal Peyer´s patch in ruminants a mammalian "bursa-equivalent"? Acta Pathologica Microbiologica et Immunologica Scandinavica Section A-Pathology 92, 77-79.

Landsverk, T., Halleraker, M., Aleksandersen, M., McClure, S., Hein, W., Nicander, L., 1991. The intestinal habitat for organized lymphoid tissues in ruminants; comparative aspects of structure, function and development. Vet Immunol Immunopathol 28, 1-16.

Lasota, J., Miettinen, M., 2008. Clinical significance of oncogenic KIT and PDGFRA mutations in gastrointestinal stromal tumours. Histopathology 53, 245-266.

Montaldo, E., Vacca, P., Moretta, L., Mingari, M.C., 2014. Development of human natural killer cells and other innate lymphoid cells. Semin Immunol 26, 107-113.

Nicander, L., Halleraker, M., Landsverk, T., 1991. Ontogeny of reticular cells in the ileal Peyer's patch of sheep and goats. Am.J.Anat. 191, 237-249.

Olsen, L., Akesson, C.P., Storset, A.K., Lacroix-Lamande, S., Boysen, P., Metton, C.,

342 Connelley, T., Espenes, A., Laurent, F., Drouet, F., 2015. The early intestinal immune
343 response in experimental neonatal ovine cryptosporidiosis is characterized by an increased
344 frequency of perforin expressing NCR1(+) NK cells and by NCR1(-) CD8(+) cell
345 recruitment. Vet Res 46, 28.
346
347 Olsen, L., Boysen, P., Åkesson, C.P., Gunnes, G., Connelley, T., Storset, A.K., Espenes, A.,
348 2013. Characterization of NCR1+ cells residing in lymphoid tissues in the gut of lambs
349 indicates that the majority are NK cells. Veterinary Research 44.
350
351 Possot, C., Schmutz, S., Chea, S., Boucontet, L., Louise, A., Cumano, A., Golub, R., 2011.
352 Notch signaling is necessary for adult, but not fetal, development of RORgammat(+) innate
353 lymphoid cells. Nat Immunol 12, 949-958.
354
355 Press, C.M., Halleraker, M., Landsverk, T., 1992. Ontogeny of leukocyte populations in the
356 ileal Peyer´s patch of sheep. Developmental and Comparative Immunology 16, 229-241.
357
358 Press, C.M., Reynolds, J.D., McClure, S.J., Landsverk, T., 1998. Development of accessory
359 cells in B-cell compartments is retarded in B-cell-depleted fetal sheep. Developmental
360 Immunology 6, 223-231.
361
362 Press, C.M., Reynolds, J.D., McClure, S.J., Simpson-Morgan, M.W., Landsverk, T., 1996.
363 Fetal lambs are depleted of IgM+ cells following a single injection of an anti-IgM antibody
364 early in gestation. Immunology 88, 28-34.
365
366 Renström, L.H., Press, C.M., Trevella, W., Landsverk, T., 1996. Response of leucocyte
367 populations in the illeal Peyer's patch of fetal lambs treated with ferritin *per os*.
368 Developmental Immunology 4, 289-298.
369
370 Reynders, A., Yessaad, N., Manh, T.P.V., Dalod, M., Fenis, A., Aubry, C., Nikitas, G.,
371 Escaliere, B., Renauld, J.C., Dussurget, O., Cossart, P., Lecuit, M., Vivier, E., Tomasello, E.,
372 2011. Identity, regulation and *in vivo* function of gut NKp46(+)RORγt(+) and
373 NKp46(+)RORγt(-) lymphoid cells. EMBO Journal 30, 2934-2947.
374
375 Reynolds, J.D., Morris, B., 1983. The evolution and involution of Peyer's patches in fetal and
376 postnatal sheep. European Journal of Immunology 13, 627-635.
377
378 Sawa, S., Cherrier, M., Lochner, M., Satoh-Takayama, N., Fehling, H.J., Langa, F., Di Santo,
379 J.P., Eberl, G., 2010. Lineage relationship analysis of RORgammat+ innate lymphoid cells.
380 Science 330, 665-669.
381
382 Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J.P., Eberl, G., Koyasu, S.,
383 Locksley, R.M., McKenzie, A.N., Mebius, R.E., Powrie, F., Vivier, E., 2013. Innate lymphoid
384 cells--a proposal for uniform nomenclature. Nat.Rev.Immunol. 13, 145-149.
385
386 Spits, H., Cupedo, T., 2012. Innate lymphoid cells: emerging insights in development, lineage
387 relationships, and function. Annu.Rev.Immunol. 30, 647-675.
388
389 Spits, H., Di Santo, J.P., 2011. The expanding family of innate lymphoid cells: regulators and
390 effectors of immunity and tissue remodeling. Nature Immunology 12, 21-27.
391

392 Tang, Y., Peitzsch, C., Charoudeh, H.N., Cheng, M., Chaves, P., Jacobsen, S.E., Sitnicka, E.,
393 2012. Emergence of NK-cell progenitors and functionally competent NK-cell lineage subsets
394 in the early mouse embryo. Blood 120, 63-75.
395
396 Veiga-Fernandes, H., Coles, M.C., Foster, K.E., Patel, A., Williams, A., Natarajan, D.,
397 Barlow, A., Pachnis, V., Kioussis, D., 2007. Tyrosine kinase receptor RET is a key regulator
398 of Peyer's patch organogenesis. Nature 446, 547-551.
399
400 Vitale, M., Della Chiesa, M., Carlomagno, S., Romagnani, C., Thiel, A., Moretta, L., Moretta,
401 A., 2004. The small subset of CD56brightCD16- natural killer cells is selectively responsible
402 for both cell proliferation and interferon-gamma production upon interaction with dendritic
403 cells. Eur J Immunol 34, 1715-1722.
404
405 Åkesson, C.P., Press, C.M., Espenes, A., Aleksandersen, M., 2008. Phenotypic
406 characterisation of intestinal dendritic cells in sheep. Developmental and Comparative
407 Immunology 32, 837-849.
408

Figure legends

Figure 1. Micrographs of triple fluorescent labelling with Abs against CD3 (red), CD79 (blue) and NCR1 (green). Representative sections from jejunum, ileum and colon from the four groups of ovine foetuses. Note the tissue expansion during gestation, with higher mucosal folds and larger lymphoid accumulations. The first B cell cluster is seen in the jejunum in group 1. Scattered NCR1+ cells (arrows) in the first group examined, which is later found in T cell areas as the interfollicular areas (i). Unspecific (auto)fluorescence in epithelium, lamina muscularis and in mucosa is identified morphologically. Villi (**v**); follicle (**f**); lumen (**l**); dome (**d**). Scale bars 100 µm.

Figure 2. Double immunolabelling of c-kit (red) and NCR1 (green) in an ileal segment from a foetus at days 80 (**A**) and 141 (**B**). Note the increased number of double labelled cells (yellow, arrows) in the older foetus. Double labelling (**C**) of Ki67 (red) and NCR1 (green) of a foetus aged 89 days of gestation. Some NCR1+ cells exhibited signs of ongoing mitoses with a red stained Ki67+ nucleus (inset). Merged image (**D**) of a double immunofluorescent labelling of CD34 (red), NCR1 (green). Tissue from the ileal PP from a foetus at day 125 of gestation. No double labelling of NCR1+ and CD34+ cells could be observed. Unspecific autofluorescence was identified with an empty channel image (blue) and morphologically in all images. Scale bars 50 µm.

Table 1. Number of foetuses used in the study with the material variation given as mean, median and range, of gestational ages. Full term for ovine spp. is 142-152 days of gestation.

Additional table 1. Detailed information of the material used in this study with the age of gestation and organs studied for each animal that are identified with a unique number.

Table 2. Number of NCR1+ cells in cryosections (average per image) in different gut segments of foetuses from four age groups. Based on structural assessment, cells were counted in lamina propria (**LP**), submucosa with the interfollicular (when present) T-cell rich area (**IFA**) and the dome. The number of labelled cells were subjectively assessed and graded as none (-), few (+), moderate (++) and many/numerous (+++). In bracket the percentage of NCR1+ cells that were also c-kit+. Jejunum (**Je**); Ileal Peyer`s patch; (**IPP**); Jejunal Peyer`s patch (**JPP**); Colonic patch (**CP**).

Table 3. Number of NCR1+ cells in cryosections (average per image) in different gut segments of foetuses from four age groups. Based on structural assessment, cells were counted in lamina propria (**LP**), submucosa with the interfollicular (when present) T-cell rich area (**IFA**) and the dome. The number of labelled cells were subjectively assessed and graded as none (-), few (+), moderate (++) and many/numerous (+++). In brackets the percentage of NCR1+ cells that were also Ki67+. Jejunum (**Je**); Ileal Peyer`s patch; (**IPP**); Jejunal Peyer`s patch (**JPP**); Colonic patch (**CP**).

Tables

Table 1. Age characteristics of the 4 groups of foetuses used in this study

Group nr.	1	2	3	4
n	4	4	7	7
Age mean	78,25	100,75	122,14	141
Age median	80	100	125	141
Age range	70-83	95-104	114-129	141

Additional Table 1. Individual details of the foetuses investigated in this study

Gr.1	**101**	**102**	**103**	**104**	**-**	**-**	**-**
Age (days)	70	80	80	83			
Organs	IPP Colon	IPP Cecum	IPP Cecum	IPP Jejunum			

Gr.2	**201**	**202**	**203**	**204**	**-**	**-**	**-**
Age (days)	95	100	104	104			
Organs	JPPs IPP CP	JPPs IPP CP	JPPs IPP -	JPPs IPP -			

Gr.3	**301**	**302**	**303**	**304**	**305**	**306**	**307**
Age (days)	114	116	117	125	126	128	129
Organs	JPPs IPP	JPPs IPP CP	JPPs IPP	JPPs IPP CP	JPPs IPP	JPPs IPP CP	IPP CP

Gr.4	**401**	**402**	**403**	**404**	**405**	**406**	**407**
Age (days)	141	141	141	141	141	141	141
Organs	IPP JPP CP	IPP JPP CP	IPP JPP CP	IPP JPP CP	IPP JPP CP	IPP JPP CP	IPP JPP CP

Table 2. NCR1+ cells and their double labelling with c-kit

Group (age)	Gut segment	LP	Submucosa* / IFA**	Dome
1	Je	+ (0)	+ (0)	
(70-83)	IPP	+ (0)	+ (0)	
	CP	-	+ (0)	
2	Je	+ (0)	+ (5)	
(95-104)	IPP	+ (0)	++ (1)	++ (6)
	CP	-	+ (0)	
3	JPP	+ (0)	++ (5)	+++ (4)
(114-129)	IPP	+ (0)	+++ (5)	++ (0)
	CP	++ (0)	++ (0)	+++ (0)
4	JPP	+ (11)	++ (25)	+++ (31)
(141)	IPP	+ (9)	+++ (25)	+++ (33)
	CP	++ (7)	+++ (12)	+++ (15)

* Group 1 and 2.
** Group 3 and 4.

461 **Table 3. NCR1+ cells and their double labelling with Ki67**

Group (age)	Gut segment	LP	Submucosa* / IFA**	Dome
1	Je	+ (0)	+ (20)	
(70-83)	IPP	+ (9)	+ (12)	
	CP	-	+ (14)	
2	Je	+ (10)	+ (9)	
(95-104)	IPP	+ (2)	++ (10)	++ (13)
	CP	-	+ (7)	
3	JPP	+ (4)	++ (4)	+++ (1)
(114-129)	IPP	+ (0)	+++ (3)	++ (2)
	CP	++ (11)	++ (4)	+++ (5)
4	JPP	+ (2)	++ (3)	+++ (2)
(141)	IPP	+ (0)	+++ (3)	+++ (1)
	CP	++ (2)	+++ (3)	+++ (2)

462 * Group 1 and 2.
463 ** Group 3 and 4.
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Figure 1

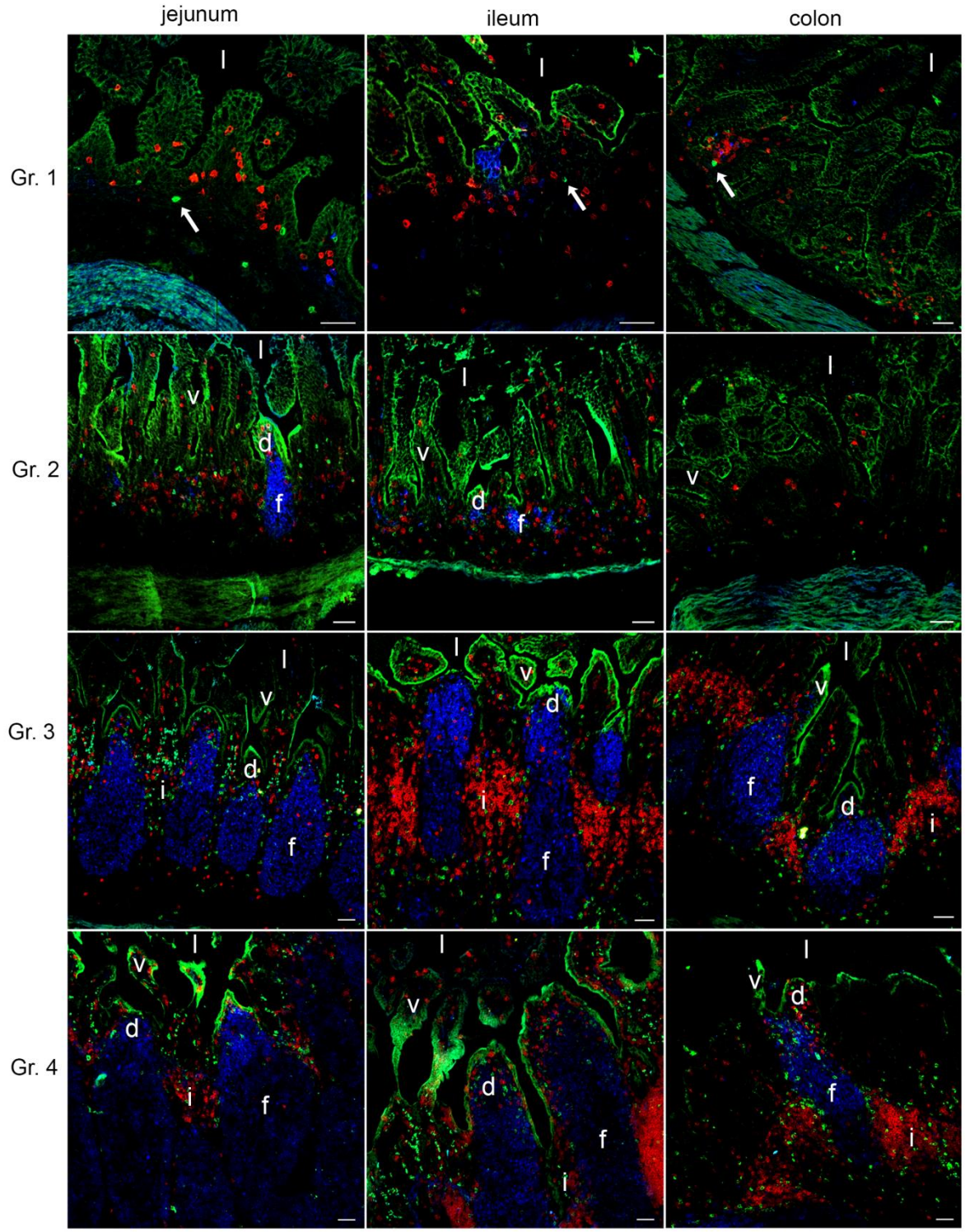
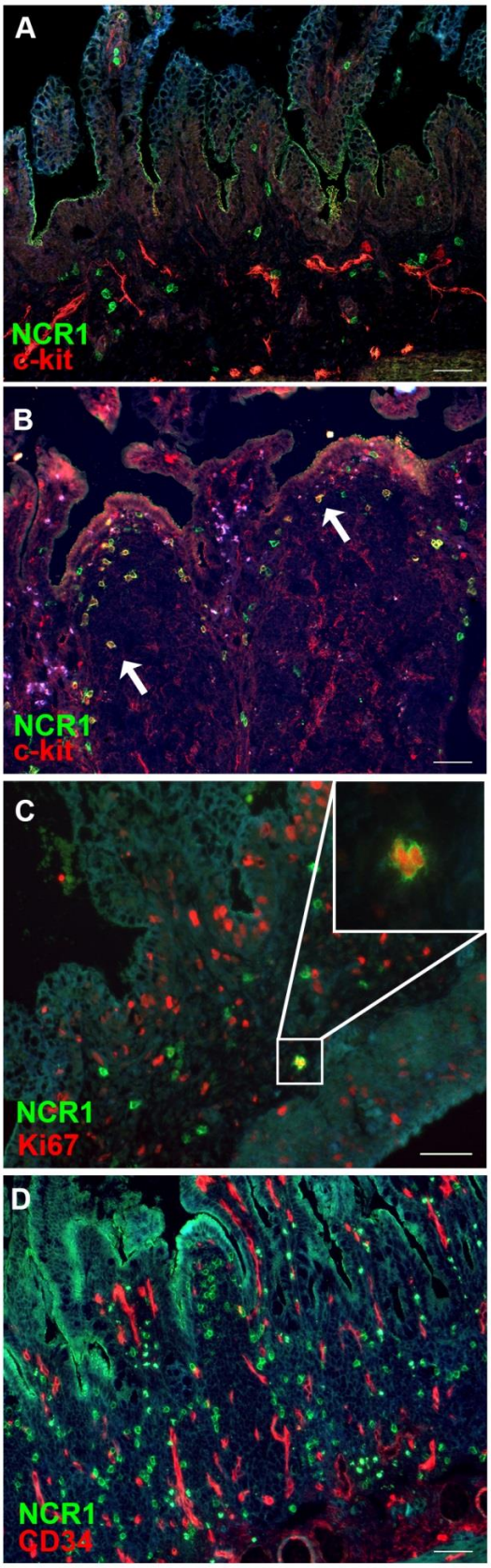


Figure 2



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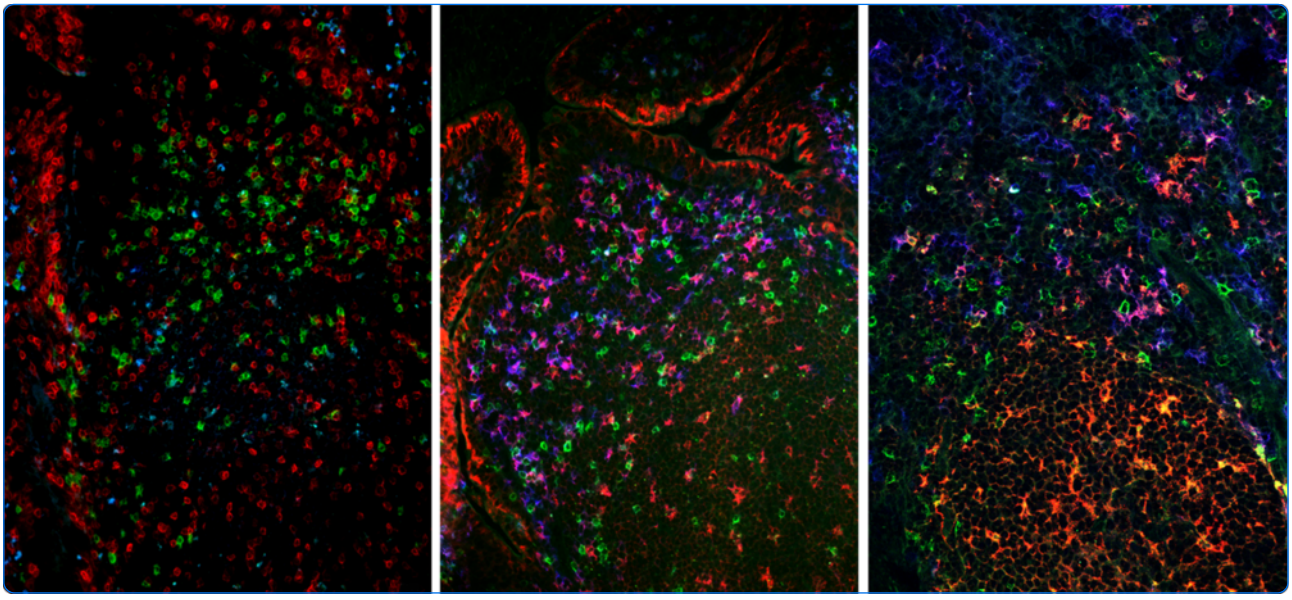

Characterization of NCR1+ cells residing in lymphoid tissues in the gut of lambs indicates that the majority are NK cells

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VETERINARY RESEARCH

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Characterization of NCR1+ cells residing in lymphoid tissues in the gut of lambs indicates that the majority are NK cells

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Abstract

Natural killer (NK) cells are important for immune protection of the gut mucosa. Previous studies have shown that under pathologic conditions NK cells, T cells and dendritic cells are found co-localised in secondary lymphoid organs where their interaction coordinates immune responses. However, in the gut-associated lymphoid tissues (GALTs), there are few detailed reports on the distribution of NK cells. Sheep harbour several types of organised lymphoid tissues in the gut that have different functions. The ileal Peyer's patch (IPP) functions as a primary lymphoid tissue for B cell generation, while the jejunal Peyer's patches (JPPs) and colon patches (CPs) are considered secondary lymphoid tissues. In the present study, we analysed tissues from healthy lambs by flow cytometry and *in situ* multicolour immunofluorescence, using recently described NCR1 antibodies to identify ovine NK cells. Most NCR1+ cells isolated from all tissues were negative for the pan T cell marker CD3, and thus comply with the general definition of NK cells. The majority of NCR1+ cells in blood as well as secondary lymphoid organs expressed CD16, but in the GALT around half of the NCR1+ cells were negative for CD16. A semi-quantitative morphometric study on tissue sections was used to compare the density of NK cells in four compartments of the IPPs, JPP and CPs. NCR1+ cells were found in all gut segments. Statistical analysis revealed significant differences between compartments of the primary lymphoid organ IPP and the secondary lymphoid organs of the JPPs and CP. NK cells co-localised and made close contact with T cells, dendritic cells and other NK cells, but did not show signs of proliferation. We conclude that NK cells are present in all investigated segments of the sheep gut, but that presence of other innate lymphoid cells expressing NCR1 cannot be excluded.

Introduction

Natural killer (NK) cells are lymphocytes of the innate immune system traditionally known for their immediate cytotoxic activity against stressed, transformed or infected cells [1]. More recently, they have been shown to be present in lymphoid tissues, mucosal tissues and several other organ systems, where they exhibit direct effector functions as well as immunoregulatory actions on other cells through cytokine production [2-5]. By direct interactions with macrophages [6,7] or dendritic cells (DCs) [8], NK cells provide an early source of interferon-γ (IFNγ), which is necessary for T_H1 polarization in the lymph nodes [9]. NK cells are known to be present in the intestinal mucosa of humans and mice, but their precise tissue compartmentalization and function have been a matter of debate, as other distinct lymphoid cell populations also express NK cell markers [10,11]. The distribution and phenotype of NK cells in the gut-associated lymphoid tissues (GALTs) of sheep have not yet been described.

The gut mucosa is constantly challenged with dietary and other exogenous antigens, and the immune system needs to react appropriately to both harmless and dangerous antigens. The organised lymphoid tissue of small intestinal Peyer's patches (PPs) and the lymphoid patches of the colon (CPs), as well as the solitary lymphoid follicles present along the gastrointestinal tract, are the main inductive sites of the gut immune system. The lamina propria, which is found subepithelially throughout the gut, is regarded mainly as an effector site [12]. In lambs and calves, the continuous ileal PP (IPP) is responsible for the

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generation of B cells, and is considered a primary lymphoid tissue, unlike the jejunal PPs (JPPs) and CPs, which are recognised as secondary lymphoid tissues [13-16]. The PPs and CPs of sheep can be divided into immunologically relevant tissue compartments based on morphology, cellular composition, and function [13,17,18]. Each B cell containing follicle in the submucosa is surrounded by a capsule except on the luminal side where the follicle extends into the mucosa and blends with the dome. The dome contains myeloid and lymphoid cells and is covered by a specialized follicle-associated epithelium (FAE). Between the follicles and beneath the lamina muscularis mucosae is an area rich in T cells; the interfollicular area (IFA). A further compartment is the lamina propria, which is found along the whole length of the gut, and is present both within and beyond the borders of PPs. T- and B cells predominate in the IFA and follicle, respectively, of the sheep PPs [19-21]. In the lamb and sheep gut, DCs are mostly found in the dome, IFA and lamina propria and have been shown to express CD11c, CD205, and MHCII [22].

CD16+/CD14- lymphocytes in the blood of sheep have been identified as NK cells [23], and NK cells were later found to be more precisely defined by the expression of NCR1 (CD335, NKp46), a natural cytotoxicity receptor (NCR) [24]. The NCR1+/CD3- phenotype of lymphocytes has proven to be a reliable definition of NK cells in many species [25], but a flow cytometric technique has not been available for antibodies that label CD3 in sheep. In mice and humans, NK cells in tissues display aberrant phenotypes compared to NK cells in blood [2]. The presence of NK cells in lymphoid and mucosal tissues of sheep has not been previously described in detail.

The aim of this study was to characterize NCR1+ cells present in the intestine with respect to CD3 (pan-T cell marker) and CD16 (Fcγ receptor IIIa), and to compare the relative number of NCR1+ cells in different lymphoid tissues, with focus on GALT. The localisation of NCR1+ cells in the various lymphoid tissues of healthy sheep was investigated using multicolour immunofluorescence. Morphometry was further used to compare the density of NCR1+ cells in different compartments of JPPs, IPP and CPs. Finally, we wanted to investigate the spatial relation of NCR1+ cells to T cells and DCs.

Materials and methods

Animals and collection of tissues

Five ($n = 5$) clinically healthy young lambs, aged 31–43 days, were used in the present study. Three lambs belonged to the Norwegian white sheep breed, while 2 lambs were a cross between the Norwegian white sheep and the Texel breed. A second cohort was collected from an additional 5 older lambs at an abattoir. These animals were approximately 6 months of age and tissues were collected to perform multi-parameter flow cytometric labelling with an antibody against the cytoplasmic region of the human CD3 epsilon chain (cytCD3), reported to be cross-reactive to multiple species by the manufacturer. All tissues were collected immediately after the lambs were euthanized in a way that complied with the Norwegian Animal Welfare Act of 28 December 2009. An overview of the tissues and method of analysis are shown for each of the two age groups in Additional file 1.

Antibodies

Primary antibodies used in the present study are shown in Table 1.

Flow cytometry

Peripheral blood mononuclear cells (MNC) were isolated using Lymphoprep (Axis-Shield, Dundee, UK) gradient, as previously described [26]. Tissues were prepared as previously described [30]; briefly, lymph nodes were sectioned through the cortex and medulla, PPs and CPs had the mucosal layer gently scraped off, and representative pieces of spleen and tonsils were sliced. These samples were disaggregated in a Medimachine (BD Biosciences, NJ, USA) and filtered. In tissues from the 5 older lambs collected from the abattoir, cells were incubated with LIVE/DEAD Fixable Far Red Dead Cell Stain Kit (Life Technologies, Ltd., Paisley, UK), according to the manufacturer's instructions. Cells were surface stained with primary monoclonal antibodies (mAbs) against NCR1, then incubated briefly with normal goat serum for Fc receptor blocking, followed by PE-conjugated goat anti-mouse secondary antibodies (Southern Biotechnologies, AL, USA). For labeling of cytCD3, and following surface labeling, cells were permeabilized using Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions, and finally incubated with an anti-CD3 antibody conjugated with Pacific blue. Labeled cells were resuspended in FACS Lysing Solution (BD Biosciences) and analyzed in a FACS Calibur (BD Biosciences) for the analysis done in 2010, or in a Gallios (Beckman Coulter, Inc., CA, USA) flow cytometer acquired in 2012. Data were processed using Kaluza 1.2 software (Beckman Coulter, Inc.).

In situ immunofluorescence

The intestinal tissue samples for cryostat sectioning were placed with their mucosal side onto thin slices of liver to protect the mucosa against mechanical damage and to facilitate cryostat sectioning. All tissues were snap frozen in chlorodifluoromethane (Isceon^{TM}) chilled with liquid nitrogen. The tissues were stored at −70 °C until further preparation. 7 μm thick cryosections were mounted onto poly-lysine-coated slides and stored at −70 °C before use. The sections were air dried at room temperature for one hour, fixed in acetone for ten minutes and then air dried for another ten minutes. The sections were rinsed

Table 1 Primary antibodies used in this study

Antibody	Clone	Isotype	Specificity	Cellular expression	Source	Used in	Cross reactivity[a]	References
Mouse anti-bovine NCR1	AKS4	IgG1	NCR1	Natural killer cells		Flow	Sheep, goat	[26-28]
	AKS6	IgG2b						
Rat anti-human CD3: Pacific blue (MCA1477)	CD3-12	IgG1	CD3ε	All T cells	AbD Serotec, Ltd., Oxford, UK	Flow	Cattle, pig, horse, dog, cat and other mammals	
Mouse anti-human CD16	KD1	IgG2a	CD16	NK cells, macrophages, some T cells		Flow	Sheep, cattle	[5,23]
Mouse anti-ovine NCR1	EC1.1	IgG1	NCR1	Natural killer cells		IF		[24]
Rabbit anti-human CD3 (A0452)		Poly-clonal	CD3ε	All T cells	Dako, Glostrup, Denmark	IF	Sheep, goat, cattle and others	[29]
Mouse anti-bovine CD205 (MCA1651)	CC98	IgG2b	CD205	Dendritic cells, some T cells, some B cells, some epithelial cells	AbD Serotec, Ltd., Oxford, UK	IF	Sheep	
Mouse anti-bovine CD11c (BAQ153A)	BAQ153A	IgM	CD11c	Dendritic cells, some macrophages	VMRD, Inc., WA, USA	IF	Sheep, goat, cattle and others	
Rabbit anti-Ki67 (ab15580)		Poly-clonal	Ki67	Proliferating cells	Abcam, Cambridge, UK	IF	Cattle, horse, dog, mouse, rat and other mammals	

[a]For antibodies not raised against sheep antigens.
IF, *in situ* immunofluorescence; Flow, flow cytometry.

and rehydrated before they were blocked for non-specific binding with 10% normal goat or sheep serum in phosphate-buffered saline (PBS)/0.5% Tween®80 (Sigma-Aldrich, MO, USA) for 30 min at room temperature. The blocking solution was carefully tapped off and a mixture composed of either two or three primary antibodies was applied and left at 4 °C overnight. Following a washing step, slides were incubated with isotype-specific Alexa Fluor® secondary antibodies (Molecular Probes, Inc., OR, USA) for one hour at room temperature. After a final washing, slides were mounted in polyvinyl alcohol at pH 8. All incubations were performed in a slowly rotating humid incubation chamber, and washing between each step was done in PBS/0.5% Tween®80 for five minutes. The primary and the secondary antibodies were diluted in the blocking solution. Control sections included (a) replacement of both primary and secondary antibodies by blocking solution, (b) replacement of primary antibodies by blocking solution and (c) replacement of primary antibody with an irrelevant antibody of the same isotype as the primary antibody.

Microscopy

For quantitative analysis, sections labelled by immunofluorescence were stored in darkness at 4 °C until examination with a Leica DM RXA fluorescence microscope (Leica Microsystems, Wetzlar, Germany), and images were captured using a SPOT RT Slider™ camera (Diagnostic Instruments, MI, USA) with SPOT 5.0 Advanced Software (Diagnostic Instruments). The filter cubes (Leica Microsystems, Wetzlar, Germany) A4 with excitation BP 360/40, L5 with excitation BP 480/40 and TX2 with excitation BP 560/40 were used for Alexa Fluor® 350, Alexa Fluor® 488 and Alexa Fluor® 594, respectively. In addition, images were captured using a Zeiss Axiovert 100 inverted microscope, equipped with an LSM 510 laser confocal unit with the Zeiss ZEN 2009 Software, Release Version 5.5 SP1 (Carl Zeiss, Jena, Germany). A combination of filters corresponding to the secondary antibodies Alexa Fluor® 488, Alexa Fluor® 546 and Alexa Fluor® 633 were used when capturing the confocal images.

Evaluation of the immuno labelled slides

To compare the density of NK cells in the different compartments of JPPs, IPP and CPs, images were obtained at original magnification of 200× from at least 4 individual areas for each compartment of each of the gut segments. The compartments studied were lymphoid follicles, IFA, domes and lamina propria of comparable pixel areas. The semi-quantative analysis was performed in Image-Pro® Plus software version 5.1 (Media Cybernetics, Inc., MD, USA) on JPEG files with a resolution of 1520 × 1080 pixels. An Image-Pro® Plus macro was written to reduce workload and minimise user bias. The macro loaded specified images, added pseudocolours and allowed the user to interactively define the area of interest and perform manual cell counts (Figure 1). Due to autofluorescence in the gut lamina propria, NCR1+ cells were identified based on the morphology of the circular membrane bound signal on medium sized cells and further by revealing the unwanted autofluorescent signals by merging at least two images taken with different colour filters. This method facilitated the differentiation between the autofluorescence (Figure 1, arrows) and the specific fluorescence from the secondary antibodies (Figure 1, circles).

Statistical analysis

The flow cytometric data were assessed by nonparametric Wilcoxon rank-sum test for each pair, using the JMP 10.0 statistical software (SAS Institute, NC, USA). For the morphometric analysis of the density of NK cells, it was necessary to compensate for natural variability between individuals; therefore the non-parametric

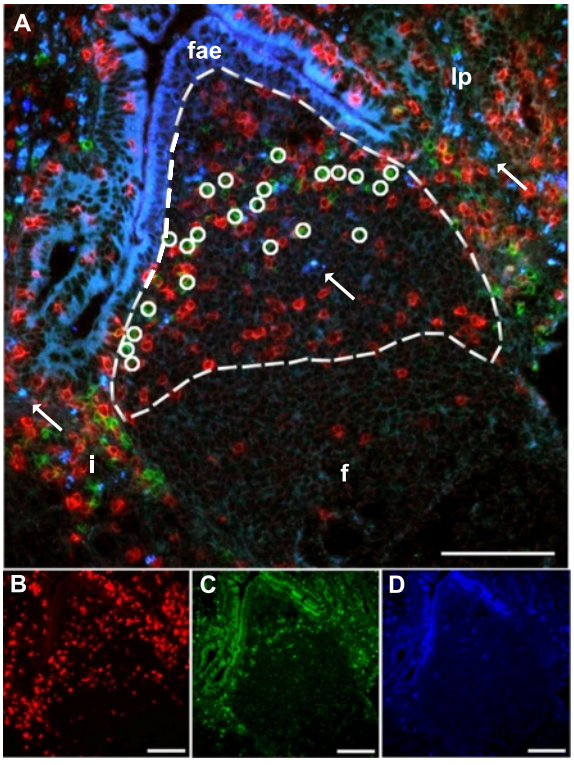

Figure 1 Morphometric quantification of NCR1+ cells of the compartments in JPPs, IPP and CPs. A dome of jejunal Peyer's patch (delineated) is shown to illustrate the method of quantification. The area of interest was interactively defined based on tissue morphology; here shown with a merged image **(A)** of CD3 in red **(B)**, NCR1 in green **(C)** and unlabeled filter in blue **(D)**. The cells were counted manually (circles) and were distinct from the autofluorescence (arrows) particularly present in the lamina propria and dome. Follicle **(f)**, follicle-associated epithelium **(fae)**, interfollicular area **(i)**, lamina propria **(lp)**. Bars 100 μm.

Wilcoxon-van Elteren [31] test was used to calculate the significance of differences between gut segments. Two-tailed tests were performed and differences were considered significant for p-values < 0.05.

Results

Quantification and characterization of NCR1+ cells by flow cytometry

The quantity of NCR1+ cells was given as the number of positive cells in relation to the total number of MNC gated in a FSC/SSC scatter plot. Peripheral blood contained 1-3% NCR1+ cells and the spleen 3-6% (Figure 2). Lymph nodes, draining mucosal or peripheral tissues, as well as tonsils and CPs, contained NCR1+ cells at proportions comparable to blood, mostly around 1-2%. The gut PPs and particularly IPP contained less than 1% NCR1+ cells, which was markedly lower than blood. To assess whether NCR1 could be present on a subclass of T cells, we labelled cells isolated from various tissues collected from 5 older abattoir lambs using an antibody against the cytCD3. This was done because mAbs against extracellular regions of ovine CD3 were not available. This additional analysis showed that almost all NCR1+ cells separated from these tissues was cytCD3- (Figure 3A). A minimal population of NCR1+ cells expressing CD3 (< 1% of MNC) was observed in JPP (0.4% of MNC), distal jejunal lymph node and blood (both 0.2% of MNC), but not in retropharyngeal lymph node (< 0.05% of MNC) (Figure 3A and not shown); however, such double expression was not observed by *in situ* immunofluorescence analysis (presented below and in Figure 3B-G).

The literature reports considerable variability in the presence of CD16 on NK cells between organs and between species, so we went on to measure the expression of this marker. The great majority of NCR1+ cells in blood, spleen and lymph nodes expressed CD16, whereas in tonsils, JPPs and CPs only approximately half of the NCR1+ cells were CD16+ (Figure 4). The low number of NCR1+ cells obtained from IPP made subset characterization unreliable.

In situ study of NCR1+ cells in GALT, tonsils and lymph nodes by immunofluorescence

NCR1+ cells were present in all organs studied, and these cells generally did not co-label with antibodies against CD3 (Figure 3B-G). NCR1+/CD3- cells were present in all investigated segments of the gut. The lamina propria contained moderate numbers of NCR1+/CD3- cells that were scattered throughout the stroma. A low number of these cells was located within the absorptive epithelium, but never observed in the lacteals. In the IFA, numerous NCR1+/CD3- cells were evenly distributed. Sometimes these cells were found within the fibres of the lamina muscularis mucosae, but none was observed in the submucosal lymphatics. A high number of NCR1+/CD3- cells were evenly distributed in the dome and a few cells

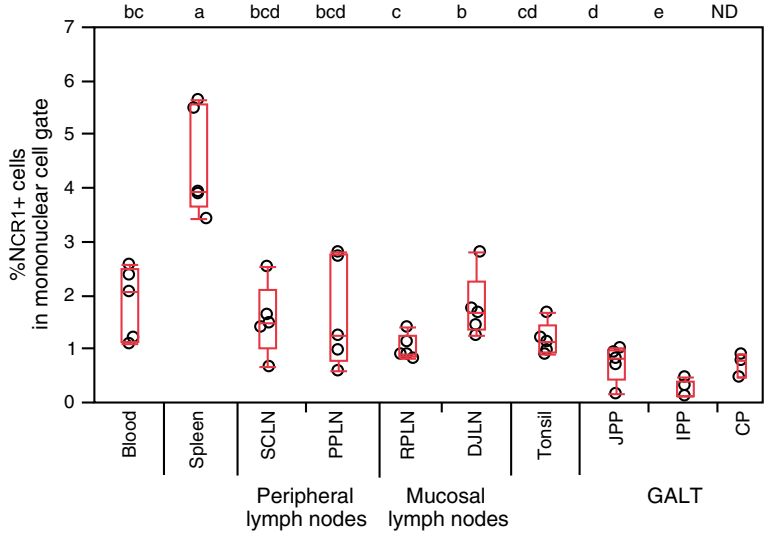

Figure 2 Relative NCR1+ cell numbers in tissues of young lambs. Mononuclear cells from peripheral blood or disaggregated tissues were analysed in flow cytometry. NCR1+ cells are stated as % of cells in the mononuclear gate. Dots represent individual animals ($n = 5$ and for colon patch $n = 3$), outlier boxes indicate median and quartiles, and whiskers indicate data points within 1.5× (interquartile range). Letters shared between groups indicate no significant difference according to the Wilcoxon Each Pair non-parametric rank sum test ($P < 0.05$). Not determined **(ND)**; superficial cervical lymph node **(SCLN)**; popliteal lymph node **(PPLN)**; retropharyngeal lymph node **(RPLN)**; distal jejunal lymph node **(DJLN)**; jejunal Peyer's patch **(JPP)**; ileal Peyer's patch **(IPP)**; colon patch **(CP)**; gut-associated lymphoid tissue **(GALT)**.

were occasionally observed within the FAE (Figure 3B, arrow). A few NCR1+/CD3- cells were found in the centre of the follicles in JPPs and CPs, while the follicles of IPP were chiefly devoid of this cell population. In the lymph nodes and tonsils, NCR1+/CD3- cells were present in abundance in the diffuse extra-follicular areas, particularly in regions close to the follicles, while few or none were found in the follicles (Figure 3F-G). In the lymph

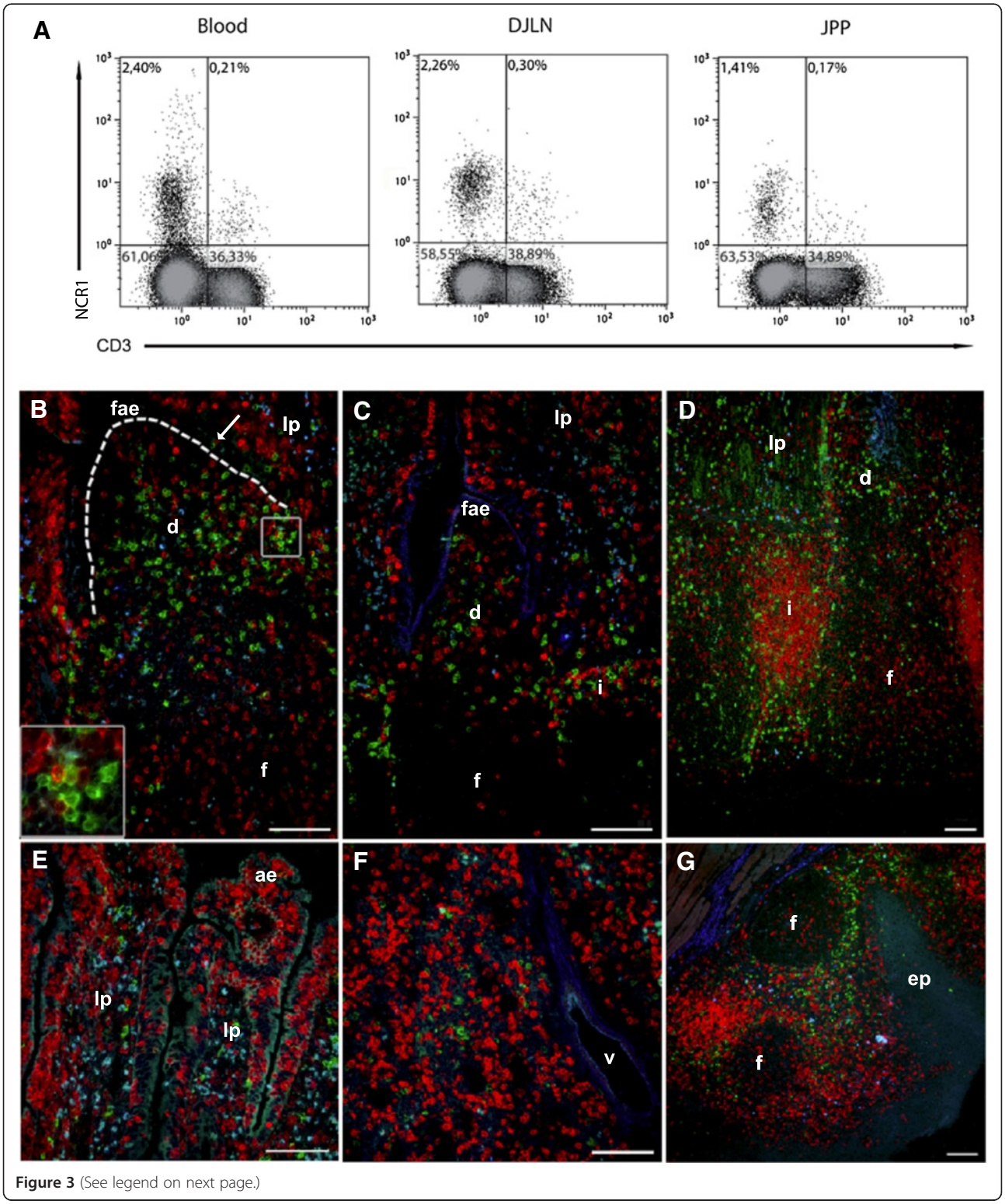

Figure 3 (See legend on next page.)

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Figure 3 NCR1+ cells in relation to CD3. (A) Flow cytometric plots of viable mononuclear cells from blood, distal jejunal lymph node **(DJLN)** and jejunal Peyer's patch **(JPP)** of lambs. Cells were labeled with fixable Live/Dead (aqua) dye, followed by labeling for NCR1, then permeabilization and labeling for a conserved intracellular CD3 epitope (CytCD3). Plots are representative of five animals, at approx. 6 months of age. **(B-G)** Representative figures of 5 one month old lambs, showing the *in situ* distribution of NCR1+/CD3- cells and T cells in jejunal Peyer's patch **(B)**, ileal Peyer's patch **(C)**, colon patch **(D)**, jejunum **(E)**, superficial cervical lymph node **(F)** and tonsil **(G)**. A two-colour fluorescent labeling with the NK cell antibody NCR1 (green) and the pan T cell marker CD3 (red) was used. The NCR1+/CD3- cells were present at a moderate to large amount in the compartments of dome **(d)**, interfollicular area **(i)**, at a moderate amount in lamina propria **(lp)**, while very few NCR1+ cells were found in the follicle **(f)**. In the follicle-associated epithelium **(fae)**, NCR1+/CD3- cells were observed intermittently (arrow). NCR1+/CD3+ cells were not observed, but NK cells and T cells were localised in the same areas, and were often seen in close contact with each other (inset; merged membranes appear yellow). To illustrate autofluorescence, photos were taken with a blue filter which did not detect signals from the secondary antibodies used in the study. The basal lamina in dome is delineated **(B)**. Absorptive epithelium **(ae)**; epithelium **(ep)**; vessel **(v)**. Bars 50 μm **(B-G)**.

nodes, the density of NCR1+/CD3- cells gradually decreased from the cortex towards the medulla (Figure 3F). Attempts to analyse ovine tissues with the CD16 mAb were unsuccessful by the *in situ* immunofluorescent technique used as no NCR1+ cells did co-label with the CD16 mAb.

Morphometric and statistical analysis of the density of NCR1+/CD3- cells in compartments of JPPs, IPP and CPs

Statistical analysis of the morphometric observations in GALT revealed significant differences between gut segments with respect to the density of NCR1+/CD3- cells

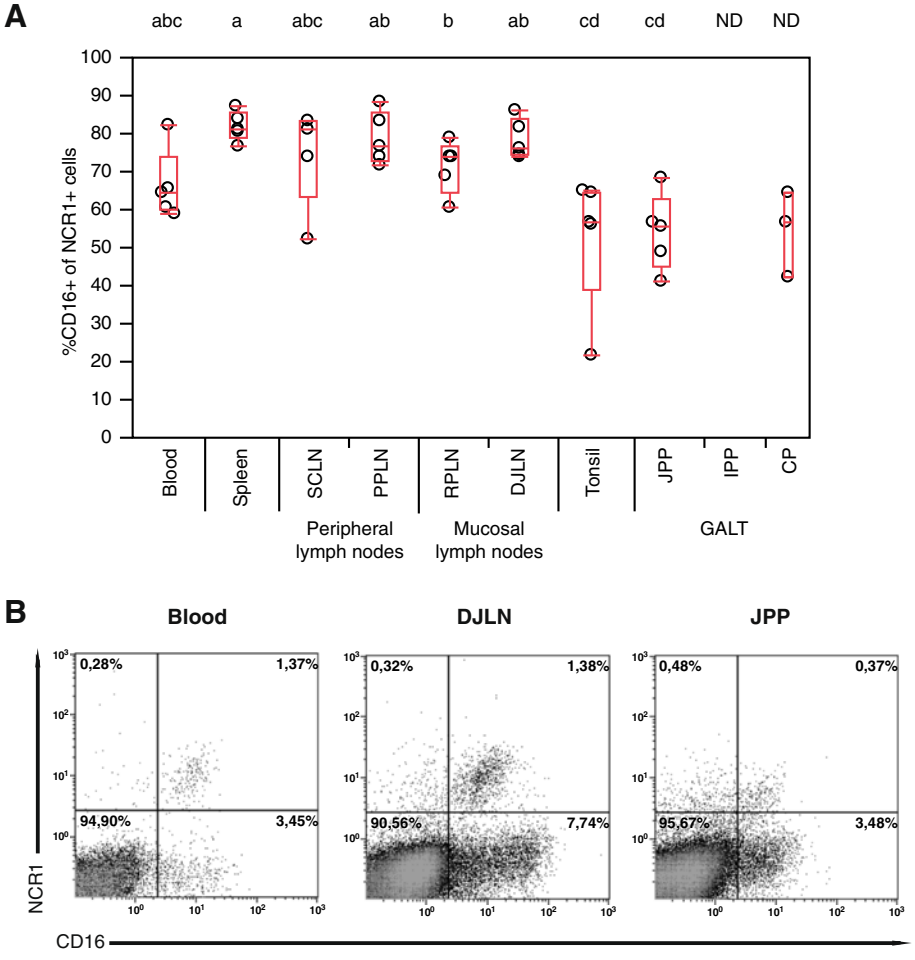

Figure 4 CD16 expression on NCR1+ cells. (A) Proportions of NCR1+ cells that express CD16. Plot elements and statistics as in Figure 2. Not determined **(ND)**. **(B)** Corresponding flow cytometric plots, showing CD16 in relation to NCR1 expression in mononuclear cells, representing one out of five lambs. Not determined **(ND)**; superficial cervical lymph node **(SCLN)**; popliteal lymph node **(PPLN)**; retropharyngeal lymph node **(RPLN)**; distal jejunal lymph node **(DJLN)**; jejunal Peyer's patch **(JPP)**; ileal Peyer's patch **(IPP)**; colon patch **(CP)**; gut-associated lymphoid tissue **(GALT)**.

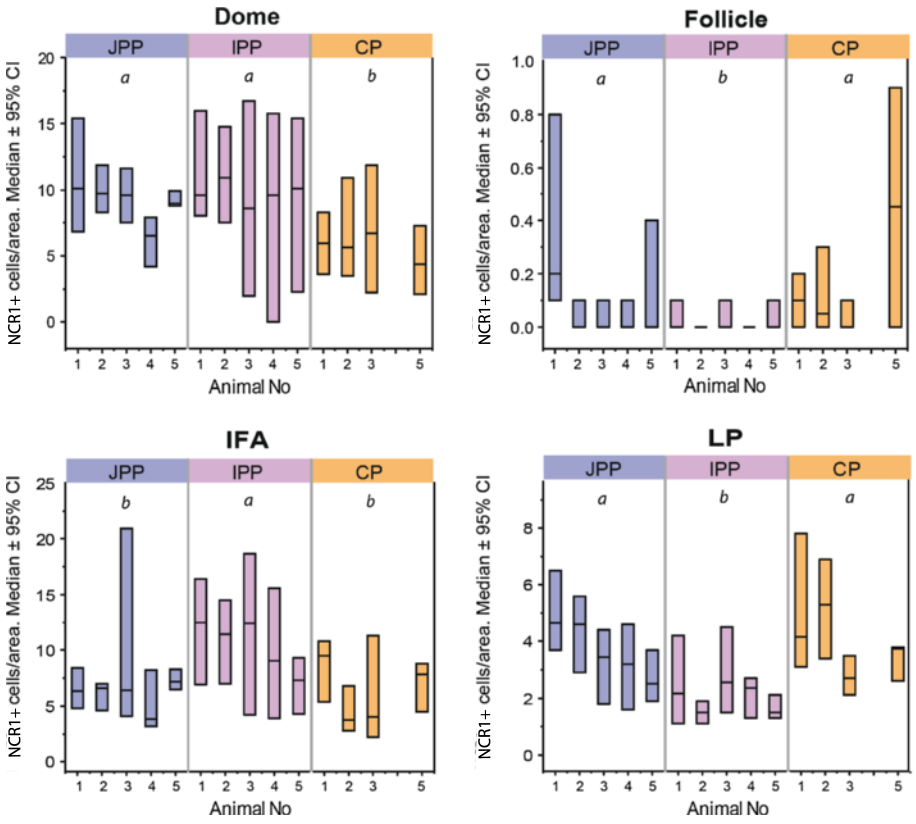

Figure 5 Statistical analysis of the density of NCR1+ cells in JPPs, IPP and CPs. The results are expressed as median with 95% confidence interval constructed using the Bernoulli-Wilcoxon procedure. Note that animal nr. 4 has missing data for colon patch due to the macroscopic inability to localise it in this animal. Within each compartment, letters shared between gut segments indicate no significant difference ($P < 0.05$). Jejunal Peyer's patch **(JPP)**; ileal Peyer's patch **(IPP)**; colon patch **(CP)**; interfollicular area **(IFA)**; lamina propria **(LP)**.

in the four compartments (Figure 5). The density of NCR1+/CD3- cells in IFA of IPP was higher compared with this tissue compartment in JPPs and CPs, while the lamina propria and follicles had a lower density in IPP compared with these tissue compartments in JPPs and CPs. The NCR1+/CD3- cell density of the dome in IPP and JPPs was similar, and the domes in both these gut segments had a higher density than the domes of CPs.

Localisation of NCR1+/CD3- cells in relation to T cells and DCs

Generally, NCR1+/CD3- cells were found in areas where T cells were abundant (Figure 3B-G). However, the number of NCR1+/CD3- cells did not always seem to correspond to the number of T cells as some areas had a relatively high number of T cells and a low number of NCR1+/CD3- cells, and vice versa. In all lymphoid tissues, including GALT, lymph nodes and tonsils, the NCR1+/CD3- cells had a similar tissue compartment distribution as the CD11c+/CD205+ DCs (Figure 6). Often, distinct cell-to-cell contact between NCR1+/CD3- cells and T cells (Figure 3B, inset) or DCs (Figure 6, long arrow) was observed. In addition, there were many cell-to-cell contacts between the NCR1+/CD3- cells (Figures 3B-G and Figure 6, arrowhead). To study whether this cell contact was associated with cell division, a double immunofluorescent labelling with NCR1 and Ki67 was performed. No NCR1+ cells, including those in close contact, were found to co-label with Ki67, indicating that NK cells are not undergoing cell division in these tissues (not shown).

Discussion

The NCR molecule NCR1 is a conserved cell membrane marker identifying NK cells in many mammalian species [25,26,32,33]. The present study utilized immunofluorescence with combinations of antibodies against NCR1 and other relevant immune cell markers, to characterize ovine NK cells, their *in situ* distribution and relation to other immune cells in various lymphoid tissues with focus on GALT. Basic information on a possible cellular overlap between NCR1 and the pan-T cell marker CD3 was obtained by flow cytometry and *in situ* immunofluorescence. As found in human intestine and in lymphoid tissues of mice [25,32], only a minor population of the ovine NCR1+ cells co-labelled for CD3. This finding was consistent in blood and all lymphoid tissues including GALT

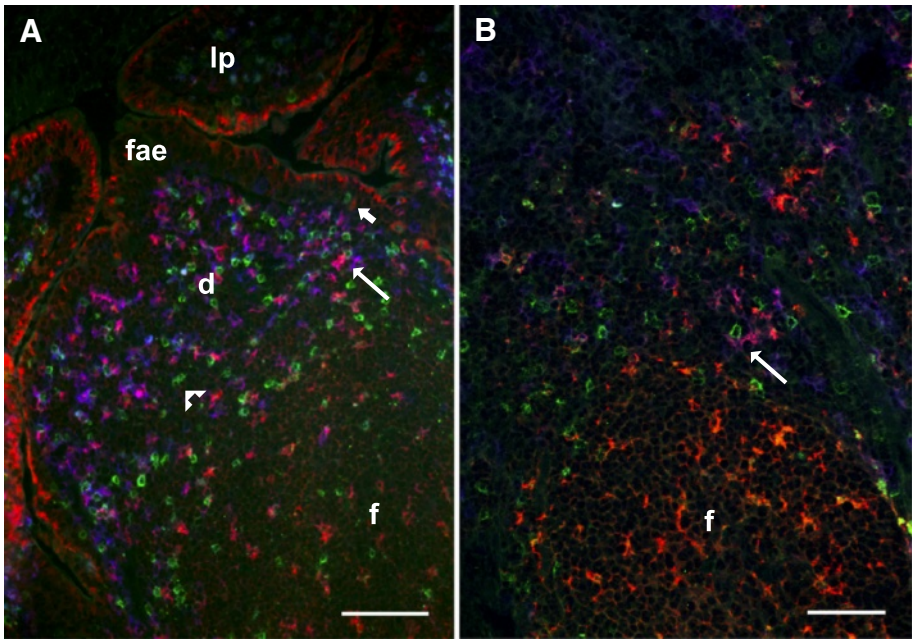

Figure 6 NCR1+ cells in relation to DCs. *In situ* co-localisation studies of NCR1+ cells and dendritic cells, represented here in the jejunal Peyer's patch **(A)** and superficial cervical lymph node **(B)**. NCR1+ (green) cells were found in the same compartments as CD205+ (red)/CD11c+ (blue) dendritic cells (pink-purple) and were sometimes observed in close contact with each other (long arrow). Note that occasionally, the NK cells were found intraepithelially, including in the follicle-associated epithelium **(fae)** (short arrow) and were often observed to be in cell-to-cell contact with each other (arrowhead). Lamina propria **(lp)**; dome **(d)**; follicle **(f)**. Bars 100 μm.

and it is the first time NCR1+ cells in ovine tissues have been shown to be CD3 negative. Thus, the present study shows that NCR1+ cells are mainly NK cells and not a subpopulation of T cells, which is a finding that adds support to previous studies characterizing ovine NCR1+ cells as typical NK cells [24].

CD16 is an activation receptor present on classical NK cells mediating recognition of antibody-opsonized targets. Flow cytometric analysis of NCR1+/CD3- cells showed that most NCR1+ cells in blood, spleen and lymph nodes were CD16+. This observation is in accordance with previous studies of NK cells in the blood of sheep [23,24], as well as in blood, lymph nodes and spleen of cattle and cats [5,33]. Compared with NK cells in blood, lymph nodes and spleen, the JPPs and CPs showed lower, but still relatively high expression of CD16. We were unable to support these findings *in situ* as the CD16 mAb was not compatible with the immunofluorescence technique used. In humans, a CD16+ NK cell subset dominates in blood, spleen and liver, while a CD16 low/- population dominates in lymph nodes, uterus, skin, and in the mucosal tissues such as tonsils and intestinal wall [2,3,34-37]. The latter "regulatory" NK cell population has strong IFNγ producing capabilities and has a poor cytotoxic potential, but the absence of the CD16 molecule does not seem to be constant and expression of CD16 can be up-regulated during inflammatory bowel disease in humans [38]. A previous report in sheep showed that cultured ovine NK cells expressed CD16 in the majority of cells but at a slightly lower level [24], in contrast to cattle where up-regulation was seen [5]. In mice, a CD16 antibody has only recently become available, resulting in conflicting reports whether or not CD16 is present on NK cells in mice at all [39,40]. Thus with respect to CD16 expression, plasticity and species differences are apparent, owing to genetic as well as environmental factors.

Recently, NK cells in the gut mucosa have been grouped under innate lymphoid cells (ILCs), a category of cells that plays a significant role in gut immunity as well as tissue development and remodelling [10]. The ILCs have been organized into three main groups named ILC1, ILC2 and ILC3, based on their cytokine producing profile [11]. Classical NK cells belong to the ILC1 group. ILC2s are NCR negative and important for helminth expulsion. ILC3s are important for lymphoid tissue organogenesis and the protective function of epithelial cells during infection [10]. The expression of NCRs has been proposed as a signature of most ILC3s across species. The ILC3 cells may be particularly important for immunity against pathogenic bacteria and may also play a role in inflammatory bowel disease [11]. In human intestine, the low expression of CD16 under non-inflammatory conditions appears to be common for all ILCs, including classical NK cells [35,37]. Although the definition of ILC groups in sheep is

lacking, it is likely that the NCR1+/CD16+ population represents classical (ILC1) cells, while NCR1+/CD16- cell population in sheep could be associated with any of the ILC groups. Unfortunately, a scarcity of antibodies against ILC markers (including RORγt, CD117, CD127 and IL-22) hampers the detailed analysis of such cells in sheep.

The relative numbers of NK cells quantified by flow cytometry in blood, lymph nodes and spleen of sheep were largely in accordance with previous studies [23,24]. Mucosal and non-mucosal lymph nodes appeared to have similar levels of NK cells, unlike observations in cattle, where a higher level was found in the non-mucosal lymph nodes [5]. A distinct population of NK cells was observed by flow cytometry in JPPs, CPs and tonsils. In IPP, a very low proportion of mononuclear cells were found to be NCR1+, although in tissue sections a substantial number of such cells was detected. This discrepancy can probably be explained by the structure of this organ, as when applying flow cytometry, the vast number of B cells of the follicles will exert a major dilution effect on the NK cells (Figure 7).

A general description of the presence of NK cells in the ovine gastrointestinal tract has previously been published [24]. However, the different segments of GALT in sheep do not only differ in structure and cell composition, but also in function and development [13,17,41,42]. Therefore, a more detailed investigation of the distribution and density of NK cells were undertaken and these results were correlated to the immune function of the different GALT segments. The lymphoid follicles of the JPPs and CPs had a higher density of NK cells than IPP. The JPPs and CPs are secondary lymphoid tissues [16,17,43-46], and it is plausible that NK cells in the follicles may provide priming signals to T_H1 CD4+ T cells found here [15,41,42,47,48], as reported in lymph nodes [9]. In contrast, the lower density of NK cells in the follicles of IPP could be related to the function as a primary lymphoid organ where the follicles are generation sites for circulating B lymphocytes before involution at puberty [16]. Similarly, the lamina propria of the ovine IPP was shown to have less density of NK cells compared to the JPPs and CPs, and this could indicate that the lamina propria of IPP is a less efficient effector site compared with lamina propria of the secondary lymphoid tissues. The finding could be consistent with the presence of reduced local cellular and humoral immune responses in IPP [49], and possibly lowered innate immune function such as reduced NK cell density found in the present study. The relatively high, but variable density of NCR1+ cells in the dome and IFA of IPP, JPPs and CPs could, in addition to immune responses, also be related to organ

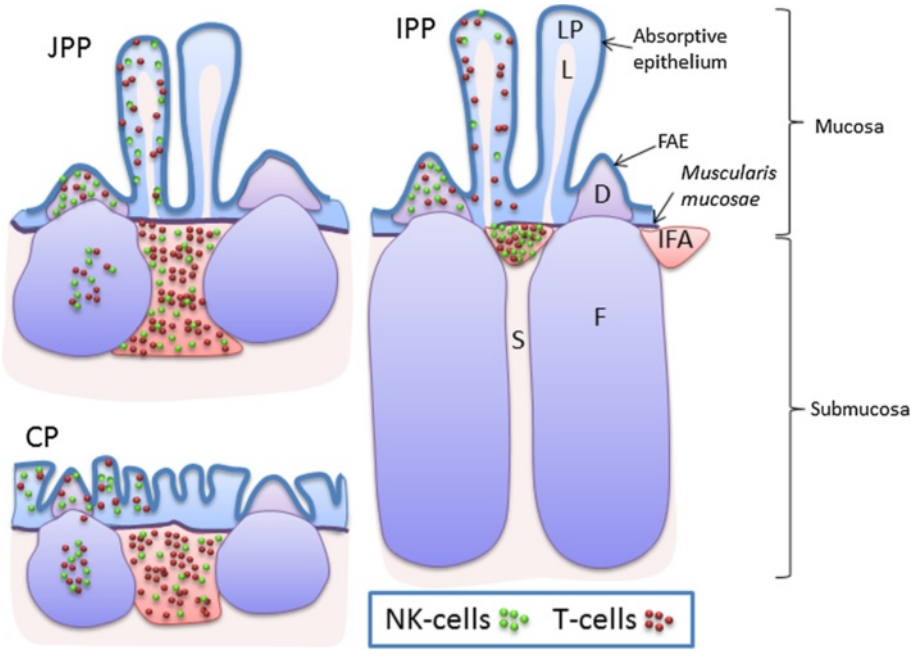

Figure 7 Illustration of the density of NK cells in ovine JPPs, IPP and CP. In the B cell containing follicles located in the submucosa, a higher density was found in jejunal Peyer's patches **(JPPs)** and colon patch **(CP)** compared to the almost NK cell empty follicles of the ileal Peyer's patch **(IPP)**. In the T cells rich area between the follicles and beneath the lamina muscularis mucosae; the interfollicular area (IFA), the density of NK cells was higher in IPP than in JPPs and CP, while in lamina propria of the villi had a lower density in IPP compared to JPPs and CP. In the dome area, covering the follicles, the density was lower in CP compared to IPP and JPPs. Dome **(D)**; follicle **(F)**; follicle-associated epithelium **(FAE)**; interfollicular area **(IFA)**; lacteal in villus **(L)**; lamina propria **(LP)**; submucosa **(S)**.

development, as previously reported for ILC3s in thymus development [50]. Thus, further sub-typing of NCR1+ cells in different GALT segments and compartments in these organs is needed to distinguish conventional NK cells from other ILCs in sheep.

Similar to the co-localisation of NK cells, DCs and T cells in lymphoid organs of humans and mice [4,25,51], we observed DCs and NK cells in close proximity in the T cell rich areas, like the dome, lamina propria and IFA of the gut, in addition to the diffuse extra-follicular areas of lymph nodes and tonsils. The reciprocal crosstalk between NK cells and DCs plays a pivotal role in tolerogenic regulation under normal conditions [52] and innate cytolytic immune responses against infections [53] and cancer [4,54,55]. Distinct NK cell immunological synapses have been described for the different lytic, inhibitory or regulatory functions of these cells [56,57]. We propose the possibility that the NCR1+/CD3- cells detected in this study execute a similar DC-NK cell communication demonstrated in mice and humans, as these two cell populations reside in the same compartments and were demonstrated to be in close contact, both in gut, lymph nodes and tonsils.

In conclusion we have found ovine NCR1+ cells to be chiefly CD3-/CD16+ in blood, spleen and lymph nodes, and to a lesser extent also in GALT organs. Accordingly, we postulate that most of these NCR1+ cells are classical NK cells of the ILC1 group. NK cells resided in compartments rich in, and in close contact to both T cells and DCs, consistent with their need for cell-to-cell contact for important functions in immunoregulatory activities. Differences found in the density of NK cells between the primary and secondary lymphoid tissues of the GALTs indicate dissimilar functions, and since about half of the NCR1+ cells in JPPs, CPs and tonsils were CD16 negative, it is necessary to determine if this cell phenotype will be associated with other ILC groups. Moreover, it remains to be elucidated whether all or some of the NK cells under steady state conditions in lambs are cytolytic, inhibitory or regulatory in function and whether these NK cells will change expression patterns upon infection.

Additional file

Additional file 1: Tissues obtained and the method of analysis used in this study. The table gives a list of all tissues obtained from the animals euthanized at the two time-points and their respective method of analysis.

Abbreviations

NK: Natural killer; DC: Dendritic cell; IFNγ: Interferon gamma; GALT: Gut-associated lymphoid tissue; PP: Peyer's patch; CP: Colon patch; IPP: Ileal Peyer's patch; JPP: Jejunal Peyer's patch; FAE: Follicle-associated epithelium; IFA: Interfollicular area; NCR: Natural cytotoxicity receptor; cytCD3: Cytoplasmic region of CD3; MNC: Mononuclear cell; mAb: Monoclonal antibody; PBS: Phosphate-buffered saline; ILC: Innate lymphoid cell.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

LO, PB, CPÅ, GG, AKS, and AE designed research, performed research, analyzed data, and contributed to the manuscript. LO wrote the manuscript. TC made contribution to conception and design, and helped to draft the manuscript. All authors read and approved the final manuscript.

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References

1. Trinchieri G: **Biology of natural killer cells.** *Adv Immunol* 1989, **47:**187–376.
2. Shi FD, Ljunggren HG, La CA, Van KL: **Organ-specific features of natural killer cells.** *Nat Rev Immunol* 2011, **11:**658–671.
3. Fehniger TA, Cooper MA, Nuovo GJ, Cella M, Facchetti F, Colonna M, Caligiuri MA: **CD56(bright) natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity.** *Blood* 2003, **101:**3052–3057.
4. Ferlazzo G, Munz C: **NK cell compartments and their activation by dendritic cells.** *J Immunol* 2004, **172:**1333–1339.
5. Boysen P, Gunnes G, Pende D, Valheim M, Storset AK: **Natural killer cells in lymph nodes of healthy calves express CD16 and show both cytotoxic and cytokine-producing properties.** *Dev Comp Immunol* 2008, **32:**773–783.
6. Haller D, Serrant P, Granato D, Schiffrin EJ, Blum S: **Activation of human NK cells by staphylococci and lactobacilli requires cell contact-dependent costimulation by autologous monocytes.** *Clin Diagn Lab Immunol* 2002, **9:**649–657.
7. Atochina O, Harn D: **LNFPIII/LeX-stimulated macrophages activate natural killer cells via CD40-CD40L interaction.** *Clin Diagn Lab Immunol* 2005, **12:**1041–1049.
8. Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A: **Dendritic cells prime natural killer cells by *trans*-presenting interleukin 15.** *Immunity* 2007, **26:**503–517.
9. Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, Sallusto F: **Induced recruitment of NK cells to lymph nodes provides IFN-γ for T(H)1 priming.** *Nat Immunol* 2004, **5:**1260–1265.
10. Rankin L, Groom J, Mielke LA, Seillet C, Belz GT: **Diversity, function, and transcriptional regulation of gut innate lymphocytes.** *Front Immunol* 2013, **4:**22.
11. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, Koyasu S, Locksley RM, McKenzie AN, Mebius RE, Powrie F, Vivier E: **Innate lymphoid cells–a proposal for uniform nomenclature.** *Nat Rev Immunol* 2013, **13:**145–149.
12. Brandtzaeg P: **The gut as communicator between environment and host: immunological consequences.** *Eur J Pharmacol* 2011, **668:**S16–S32.
13. Reynolds JD, Morris B: **The evolution and involution of Peyer's patches in fetal and postnatal sheep.** *Eur J Immunol* 1983, **13:**627–635.
14. Landsverk T: **Is the ileo-caecal Peyer's patch in ruminants a mammalian "bursa-equivalent"?** *Acta Pathol Microbiol Immunol Scand A* 1984, **92:**77–79.
15. Aleksandersen M, Hein WR, Landsverk T, Mcclure S: **Distribution of lymphocyte subsets in the large intestinal lymphoid follicles of lambs.** *Immunology* 1990, **70:**391–397.
16. Yasuda M, Jenne CN, Kennedy LJ, Reynolds JD: **The sheep and cattle Peyer's patch as a site of B-cell development.** *Vet Res* 2006, **37:**401–415.
17. Landsverk T, Halleraker M, Aleksandersen M, Mcclure S, Hein W, Nicander L: **The intestinal habitat for organized lymphoid-tissues in ruminants; comparative aspects of structure, function and development.** *Vet Immunol Immunopathol* 1991, **28:**1–16.

18. Aleksandersen M, Nicander L, Landsverk T: **Ontogeny, distribution and structure of aggregated lymphoid follicles in the large-intestine of sheep.** *Dev Comp Immunol* 1991, **15:**413–422.
19. Press CM, Halleraker M, Landsverk T: **Ontogeny of leukocyte populations in the ileal Peyer's patch of sheep.** *Dev Comp Immunol* 1992, **16:**229–241.
20. Renström LH, Press CM, Trevella W, Landsverk T: **Response of leucocyte populations in the illeal Peyer's patch of fetal lambs treated with ferritin *per os*.** *Dev Immunol* 1996, **4:**289–298.
21. Corpa JM, Juste RA, Marin JFG, Reyes LE, Gonzalez J, Perez V: **Distribution of lymphocyte subsets in the small intestine lymphoid tissue of 1-month-old lambs.** *Anat Histol Embryol* 2001, **30:**121–127.
22. Åkesson CP, Press CM, Espenes A, Aleksandersen M: **Phenotypic characterisation of intestinal dendritic cells in sheep.** *Dev Comp Immunol* 2008, **32:**837–849.
23. Elhmouzi-Younes J, Boysen P, Pende D, Storset AK, Le VY, Laurent F, Drouet F: **Ovine CD16+/CD14- blood lymphocytes present all the major characteristics of natural killer cells.** *Vet Res* 2010, **41:**4.
24. Connelley T, Storset AK, Pemberton A, MacHugh N, Brown J, Lund H, Morrison WI: **NKp46 defines ovine cells that have characteristics corresponding to NK cells.** *Vet Res* 2011, **42:**37.
25. Walzer T, Blery M, Chaix J, Fuseri N, Chasson L, Robbins SH, Jaeger S, Andre P, Gauthier L, Daniel L, Chemin K, Morel Y, Dalod M, Imbert J, Pierres M, Moretta A, Romagné F, Vivier E: **Identification, activation, and selective *in vivo* ablation of mouse NK cells via NKp46.** *Proc Natl Acad Sci USA* 2007, **104:**3384–3389.
26. Storset AK, Kulberg S, Berg I, Boysen P, Hope JC, Dissen E: **NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics.** *Eur J Immunol* 2004, **34:**669–676.
27. Lund H, Boysen P, Dean GA, Davis WC, Park KT, Storset AK: **Interleukin-15 activated bovine natural killer cells express CD69 and produce interferon-γ.** *Vet Immunol Immunopathol* 2012, **150:**79–89.
28. Crozat K, Guiton R, Contreras V, Feuillet V, Dutertre CA, Ventre E, Manh TPV, Baranek T, Storset AK, Marvel J, Boudinot P, Hosmalin A, Schwartz-Cornil I, Dalod M: **The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8α + dendritic cells.** *J Exp Med* 2010, **207:**1283–1292.
29. Jones M, Cordell JL, Beyers AD, Tse AG, Mason DY: **Detection of T and B cells in many animal species using cross-reactive anti-peptide antibodies.** *J Immunol* 1993, **150:**5429–5435.
30. Gunnes G, Valheim M, Press CM, Tverdal A, Storset A: **Comparison of flow cytometry and image morphometry in the quantitative analysis of cell population markers in the lymph node of sheep.** *Vet Immunol Immunopathol* 2003, **94:**177–183.
31. van Elteren PH: **On the combination of independent two-sample tests of Wilcoxon.** *Bull Int Stat Inst* 1960, **37:**351–361.
32. Tomasello E, Yessaad N, Gregoire E, Hudspeth K, Luci C, Mavilio D, Hardwigsen J, Vivier E: **Mapping of NKp46(+) cells in healthy human lymphoid and non-lymphoid tissues.** *Front Immunol* 2012, **3:**344.
33. Vermeulen BL, Devriendt B, Olyslaegers DA, Dedeurwaerder A, Desmarets LM, Grauwet KL, Favoreel HW, Dewerchin HL, Nauwynck HJ: **Natural killer cells: frequency, phenotype and function in healthy cats.** *Vet Immunol Immunopathol* 2012, **150:**69–78.
34. Leon F, Roldan E, Sanchez L, Camarero C, Bootello A, Roy G: **Human small-intestinal epithelium contains functional natural killer lymphocytes.** *Gastroenterology* 2003, **125:**345–356.
35. Ferlazzo G, Thomas D, Lin SL, Goodman K, Morandi B, Muller WA, Moretta A, Munz C: **The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic.** *J Immunol* 2004, **172:**1455–1462.
36. Ebert LM, Meuter S, Moser B: **Homing and function of human skin γδ T cells and NK cells: relevance for tumor surveillance.** *J Immunol* 2006, **176:**4331–4336.
37. Chinen H, Matsuoka K, Sato T, Kamada N, Okamoto S, Hisamatsu T, Kobayashi T, Hasegawa H, Sugita A, Kinjo F, Fujita J, Hibi T: **Lamina propria c-kit + immune precursors reside in human adult intestine and differentiate into natural killer cells.** *Gastroenterology* 2007, **133:**559–573.
38. Steel AW, Mela CM, Lindsay JO, Gazzard BG, Goodier MR: **Increased proportion of CD16(+) NK cells in the colonic lamina propria of inflammatory bowel disease patients, but not after azathioprine treatment.** *Aliment Pharmacol Ther* 2011, **33:**115–126.
39. Narni-Mancinelli E, Chaix J, Fenis A, Kerdiles YM, Yessaad N, Reynders A, Gregoire C, Luche H, Ugolini S, Tomasello E, Walzer T, Vivier E: **Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor.** *Proc Natl Acad Sci USA* 2011, **108:**18324–18329.
40. Biburger M, Nimmerjahn F: **Low level of FcγRIII expression on murine natural killer cells.** *Immunol Lett* 2012, **143:**53–59.
41. Larsen HJ, Landsverk T: **Distribution of T and B lymphocytes in jejunal and ileocaecal Peyer's patches of lambs.** *Res Vet Sci* 1986, **40:**105–111.
42. Hein WR, Dudler L, Mackay CR: **Surface expression of differentiation antigens on lymphocytes in the ileal and jejunal Peyer's patches of lambs.** *Immunology* 1989, **68:**365–370.
43. Craig SW, Cebra JJ: **Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit.** *J Exp Med* 1971, **134:**188–200.
44. Husband AJ, Gowans JL: **The origin and antigen-dependent distribution of IgA-containing cells in the intestine.** *J Exp Med* 1978, **148:**1146–1160.
45. Kroese FG, Timens W, Nieuwenhuis P: **Germinal center reaction and B lymphocytes: morphology and function.** *Curr Top Pathol* 1990, **84:**103–148.
46. Weinstein PD, Schweitzer PA, Cebra-Thomas JA, Cebra JJ: **Molecular genetic features reflecting the preference for isotype switching to IgA expression by Peyer's patch germinal center B cells.** *Int Immunol* 1991, **3:**1253–1263.
47. Griebel PJ, Hein WR: **Expanding the role of Peyer's patches in B-cell ontogeny.** *Immunol Today* 1996, **17:**30–39.
48. Kozuka Y, Nasu T, Murakami T, Yasuda M: **Comparative studies on the secondary lymphoid tissue areas in the chicken bursa of Fabricius and calf ileal Peyer's patch.** *Vet Immunol Immunopathol* 2010, **133:**190–197.
49. Mutwiri G, Watts T, Lew L, Beskorwayne T, Papp Z, Baca-Estrada ME, Griebel P: **Ileal and jejunal Peyer's patches play distinct roles in mucosal immunity of sheep.** *Immunology* 1999, **97:**455–461.
50. Rossi SW, Kim MY, Leibbrandt A, Parnell SM, Jenkinson WE, Glanville SH, McConnell FM, Scott HS, Penninger JM, Jenkinson EJ, Lane PJ, Anderson G: **RANK signals from CD4(+)3(−) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla.** *J Exp Med* 2007, **204:**1267–1272.
51. Bajenoff M, Breart B, Huang AYC, Qi H, Cazareth J, Braud VM, Germain RN, Glaichenhaus N: **Natural killer cell behavior in lymph nodes revealed by static and real-time imaging.** *J Exp Med* 2006, **203:**619–631.
52. Guimond M, Freud AG, Mao HC, Yu J, Blaser BW, Leong JW, Vandeusen JB, Dorrance A, Zhang J, Mackall CL, Caligiuri MA: **In vivo role of Flt3 ligand and dendritic cells in NK cell homeostasis.** *J Immunol* 2010, **184:**2769–2775.
53. Moretta A, Marcenaro E, Parolini S, Ferlazzo G, Moretta L: **NK cells at the interface between innate and adaptive immunity.** *Cell Death Differ* 2008, **15:**226–233.
54. Waldhauer I, Steinle A: **NK cells and cancer immunosurveillance.** *Oncogene* 2008, **27:**5932–5943.
55. Munz C, da Barreira Silva R: **Natural killer cell activation by dendritic cells: balancing inhibitory and activating signals.** *Cell Mol Life Sci* 2011, **68:**3505–3518.
56. Munz C, Graf C, da Barreira Silva R: **Cytoskeletal stabilization of inhibitory interactions in immunologic synapses of mature human dendritic cells with natural killer cells.** *Blood* 2011, **118:**6487–6498.
57. Mace EM, Orange JS: **Multiple distinct NK-cell synapses.** *Blood* 2011, **118:**6475–6476.

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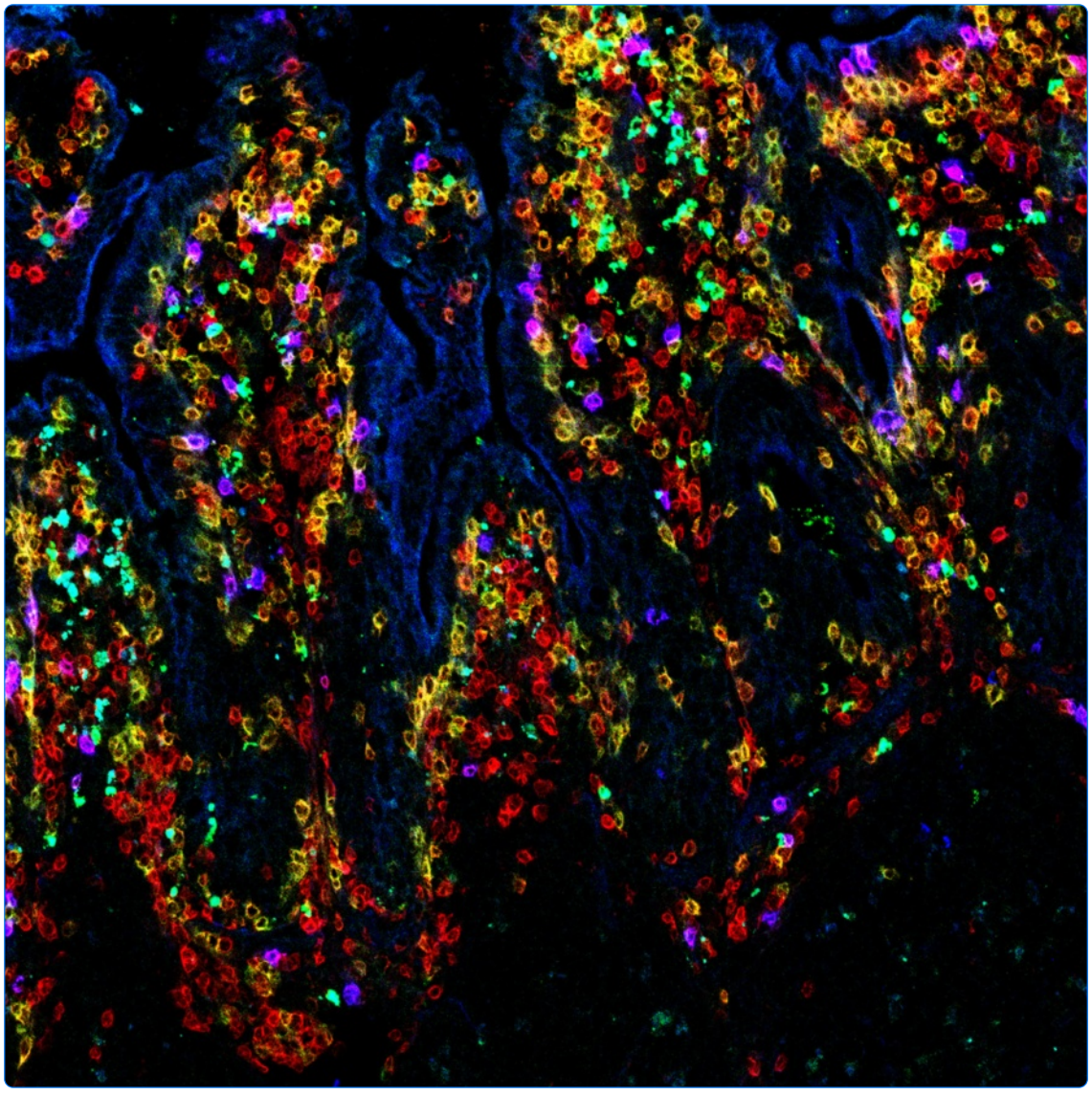
Paper III

VETERINARY RESEARCH



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VETERINARY RESEARCH

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The early intestinal immune response in experimental neonatal ovine cryptosporidiosis is characterized by an increased frequency of perforin expressing NCR1$^+$ NK cells and by NCR1$^-$ CD8$^+$ cell recruitment

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Abstract

Cryptosporidium parvum, a zoonotic protozoan parasite, causes important losses in neonatal ruminants. Innate immunity plays a key role in controlling the acute phase of this infection. The participation of NCR1+ Natural Killer (NK) cells in the early intestinal innate immune response to the parasite was investigated in neonatal lambs inoculated at birth. The observed increase in the lymphocyte infiltration was further studied by immunohistology and flow cytometry with focus on distribution, density, cellular phenotype related to cytotoxic function and activation status. The frequency of NCR1+ cells did not change with infection, while their absolute number slightly increased in the jejunum and the CD8+/NCR1- T cell density increased markedly. The frequency of perforin+ cells increased significantly with infection in the NCR1+ population (in both NCR1+/CD16+ and NCR1+/CD16- populations) but not in the NCR1-/CD8+ population. The proportion of NCR1+ cells co-expressing CD16+ also increased. The fraction of cells expressing IL2 receptor (CD25), higher in the NCR1+/CD8+ population than among the CD8+/NCR1- cells in jejunal Peyer's patches, remained unchanged during infection. However, contrary to CD8+/NCR1- lymphocytes, the intensity of CD25 expressed by NCR1+ lymphocytes increased in infected lambs. Altogether, the data demonstrating that NK cells are highly activated and possess a high cytotoxic potential very early during infection, concomitant with an up-regulation of the interferon gamma gene in the gut segments, support the hypothesis that they are involved in the innate immune response against *C. parvum*. The early significant recruitment of CD8+/NCR1- T cells in the small intestine suggests that they could rapidly drive the establishment of the acquired immune response.

Introduction

As with all neonatal mammals, the new-born ruminant is challenged by infections at vulnerable mucosal sites like the gut mucosa, frequently leading to enteritis. *Cryptosporidium parvum* (*C. parvum*), a protozoan parasite highly prevalent in cattle and small ruminant flocks throughout the world is a zoonotic agent. In sheep, *C. parvum* causes moderate to severe, but usually self-limiting enteric neonatal disease [1,2] with low mortality. However, in very young ruminants, this parasite may cause profuse diarrhoea and can lead to death by dehydration if combined with co-infections or deficiencies in nutrition and husbandry [3]. The parasite cycle ends with either thin-walled oocysts that auto-infect the host or thick-walled oocysts that are released in the environment [4]. Both animal health and welfare, economic impact and the zoonotic aspect make cryptosporidiosis one of the most important gastro-intestinal diseases in ruminant production. To date there is no vaccine available, and halofuginone lactate is the only drug with marketing

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authorization for preventive treatment of cryptosporidiosis [5,6]. To develop an adequate immunoprophylaxis strategy, it is therefore important to clarify the early immune events leading to a protective response against this parasite as neonates frequently become infected within the few hours following birth.

Only limited information is available on the neonatal ruminant intestinal immune response to *C. parvum* during the early stages of the infection. Pathogenicity and brief pathology of ovine cryptosporidiosis were described in lambs for the first time [1,2,7] more than three decades ago and more recent data were obtained in calves describing the intestinal response to the parasite with an increase of T cell subsets [8-12]. Nevertheless, our understanding of the immuno-pathological response to *C. parvum* remains poor in these species.

Recovery and protection from reinfection have been associated with a CD4+ T cell response starting from the second week post inoculation [13-15]. In cattle, this response has been associated with a production of gamma interferon (IFNγ) [11,12]. SCID mice lacking B and T cells develop chronic inflammation upon *C. parvum* infection, which progressively becomes fatal [13,15,16]. More recent experiments performed with mice tend to demonstrate that the innate immune system could be sufficient to resolve the infection [17] and we recently showed in neonatal mice that innate immunity can control the acute phase of the disease [18]. As Natural Killer (NK) cells are key players in innate immune responses they might play a role in the early host immune response against this parasite in young lambs. NK cells have been suggested to be important participants in the immune response against *C. parvum* infection; Barakat et al. [19] found that NK cells had an important role for the innate control of *C. parvum* infection in mice and Dann et al. [20] showed that NK cells lead to clearance of cryptosporidia from the intestine of humans.

Most of the studies on the role of NK cells in *C. parvum* infections have been performed with adult murine models which are not the most suitable species for studying *C. parvum* pathogenesis; indeed they are not naturally susceptible, rarely develop diarrhoea and do not develop the same mucosal pathology as observed in larger animals and humans [21,22].

The jejunum and ileum contain Peyer's patches (PPs) that are considered as immune sensors of the intestine and are important for immune protection at mucosal surfaces and the induction of mucosal immune responses in the intestine [23,24]. Whereas the PPs of the jejunum (JPPs) are recognized as secondary lymphoid organs of the intestinal wall, the continuous ileal PP (IPP) is also responsible for the generation of B cells and is thus considered as a primary lymphoid tissue [25-28]. The specialized follicle associated epithelium (FAE) that overlies PPs is capable of transporting luminal antigens [29] to the underlying immune cells to promote a tolerogenic or an inflammatory response, which will be set in action in the lamina propria. Our aim was to get an insight into the early local immune response in the different sections of the small intestine and associated lymphoid tissues of lambs during the neonatal period with a particular focus on NK cells, which we have shown to be active in neonatal calves [30], and CD8 T lymphocytes, that have been shown to be important in controlling *C. parvum* infection in humans [31].

In lambs inoculated soon after birth, we observed an activation of the NCR1+ NK population in the gut with increased expression of perforin, CD16 and CD25. In contrast, the expression of perforin and CD25 by CD8+/NCR1- T lymphocytes did not increase in infected lambs although the density and percentages of this population increased from day 3 post-inoculation (pi) in both the inductive and effector sites of the small intestine.

Materials and methods

Animals and experimental design

The lambs used for this study were born from Préalpes ewes maintained in protected facilities with a conventional status (PFIE-INRA-37380 Nouzilly). At birth the lambs were allowed to suckle the colostrum and then received artificial milk *ad libitum* until euthanasia. Within 24 h, age-matched "pairs of lambs" (occasionally triplets), i.e. lambs born within a 12 h interval, were relocated to two identical rooms, one for the inoculated lambs and one for the controls. The day following birth, the animals were inoculated *per os* with 2×10^6 oocysts of *C. parvum* (day 0 pi). During the experiment, symptoms were registered and pathological signs briefly recorded at the time of slaughter. Animals were slaughtered at various days pi (dpi), i.e. 0, 1, 2, 3, 6 and 11 dpi by electric stunning and bleeding according to the AMVA guideline on euthanasia; matched pairs of lambs were slaughtered the same day and their organs processed simultaneously. All experimental protocols were conducted in compliance with French legislation (Décret: 2001–464 29/05/01) and EEC regulations (86/609/CEE) governing the care and use of laboratory animals, after validation by the local ethics committee for animal experimentation (CEEA VdL: 2011-05-2).

Parasite and infection

Collection of oocysts

C. parvum oocysts were isolated from the faeces of neonatal calves infected with oocysts initially obtained from an infected child and maintained by repeated passage in calves. Oocysts were purified as previously described [32].

Parasitic load detection

In a set of animals, faeces were collected daily to assess the oocyst excretion pattern (Figure 1A). The first oocysts pass in the faeces at day 3 or 4 pi. Therefore, to assess the parasitized status of the inoculated lambs slaughtered early after inoculation (before parasite excretion) and the uninfected status of their controls, the presence of *C. parvum* in the mucosa was tested on fragments of intestine by assessing the expression of a cryptosporidium-specific gene by real time RT-PCR as previously described [32]. From day 4 pi the level of infection was also assessed by counting oocysts in the faeces as described by Naciri et al. [5].

Collection of tissue specimens

Samples of jejunum, JPPs, IPP, spleen and small intestinal mesenteric lymph nodes (MLN) were taken. Tissue sampling for cryostat sectioning was performed as previously described [33]. In short, tissues were chilled in isopentane before freezing in liquid nitrogen and storage at −70 °C. In addition, tissues were fixed in formalin and embedded in paraffin wax (FFPE). Some tissues were snap frozen in liquid nitrogen for RNA analyses. In pairs of age matched control and inoculated lambs, fresh tissues were collected in ice-cold RPMI medium supplemented with 5% foetal calf serum (FCS) and 1% penicillin streptomycin (P/S) for extraction of the cells.

Antibodies used for labelling

The antibodies (Abs) used in this study were against: ovine NCR1/NKp46 (EC1.1; IgG1 [34]), bovine NCR1/NKp46 (AKS6; IgG2b [35]), bovine TcR1-N7 (86D; IgG1) that labels γδ-T lymphocytes, bovine CD25 (CACT116A; IgG1), from VMRD/WSU (Pullman, USA), human CD16 (KD1; IgG2a, [36,37]), human CD3 (A0452; pAb) from Dako (Trappes, France), ovine CD8 (38.65; IgG2a) from Serotec (UK) and Ki67 (ab15580; pAb and NCL-L-Ki67-MM1; IgG1) from Abcam (Cambridge, UK) and Novocastra Laboratories-Leica (UK) respectively. The anti-human perforin-FITC kit (δG9; IgG2b) was from BD Pharmingen (France). IgG1, IgG2a, IgM mouse isotype controls for flow cytometry were from Dako and IgG2b from Caltag- Invitrogen (France). Isotype controls for IHC against IgG1, IgG2a and IgG2b were from BD Biosciences (USA). Subtype-specific secondary Abs conjugated with Tricolor (TC) or R-Phycoerythrin (PE) were from Caltag. Goat anti-mouse IgG Fab'2 secondary Abs conjugated with Fluo Probe (FP) 488 were from Fluo Probes- Interchim (France). Alexa Fluor-conjugated secondary Abs AF 350, 488, 546, 594 and 633 for indirect immunofluorescence were from Molecular Probes-Invitrogen.

Histology techniques

All FFPE tissues were stained and examined with haematoxylin and eosin (H&E) according to standard histological techniques [38] for routine histological examination. Samples of intestine for electron microscopy (EM) were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, then processed as previously described for transmission EM [39] and scanning EM [40]. The samples were examined with a Jeol 1010 transmission electron microscope (Jeol, Croissy-sur-Seine, France) and a FEG Gemini 982 scanning electron microscope (Carl Zeiss, Jena, Germany). For in situ immunolabelling, standard indirect methods with avidin-biotin complex peroxidase (Vectastain® ABC Kit, Vector Laboratories, USA) were used against *C. parvum*. Prior to immunolabelling of the FFPE sections, 4 μm thick sections were placed on positively charged slides and dried at 59 °C. After a standard dewaxing procedure, sections were treated for antigen retrieval in citrate buffer (0.01 M citric acid monohydrate, pH 6.0) in a microwave. Endogenous peroxidase was inhibited by treatment with 3% H_2O_2 in methanol for 10 min. Further blocking of unspecific binding and incubation with antibodies were performed as described by the manufacturer. The specific binding of the antibodies was visualized by using ImmPACT™ AEC after counter staining with Mayer's haematoxylin. Indirect immunolabelling was performed on cryosections, according to a protocol described earlier [33,41]. Fluorescent sections were examined under a Leica DM RXA fluorescence microscope (Germany), and images were captured using a SPOT RT Slider™ camera (Diagnostic Instruments, USA) with SPOT 5.0 Advanced Software (Diagnostic Instruments). In addition, images were captured using a Zeiss Axiovert 100 inverted microscope, equipped with an LSM 510 laser confocal unit with the Zeiss ZEN 2009 Software (Carl Zeiss).

Microscopic evaluation of immuno-labelled slides

To ascertain whether morphological features observed on H&E sections were related to *C. parvum* infection in the gut, the sections were blind coded. Features present in each section were listed and each feature was subjectively recorded in a visual analogue scale (VAS) ranging from 0 to 100. For immunofluorescent qualitative analysis of NCR1+ cells, single-blinded analysis was done. For quantitative analysis of CD8+ cells in the IPP, images were taken and processed as previously described [33]. Briefly, images of 400× from at least 5 individual villi and domes from the ileal segment were taken. A pixel-to-millimetre calibration was performed and the areas were defined in mm^2.

Extraction and purification of mononuclear cells from the organs

Spleen and MLN tissues were processed as previously described [42]. The whole organs were treated to assess the absolute number of mononuclear cells (MNC). All mediums and chemicals were from Sigma-Aldrich (Lyon, France) unless otherwise stated. Briefly, the tissues were

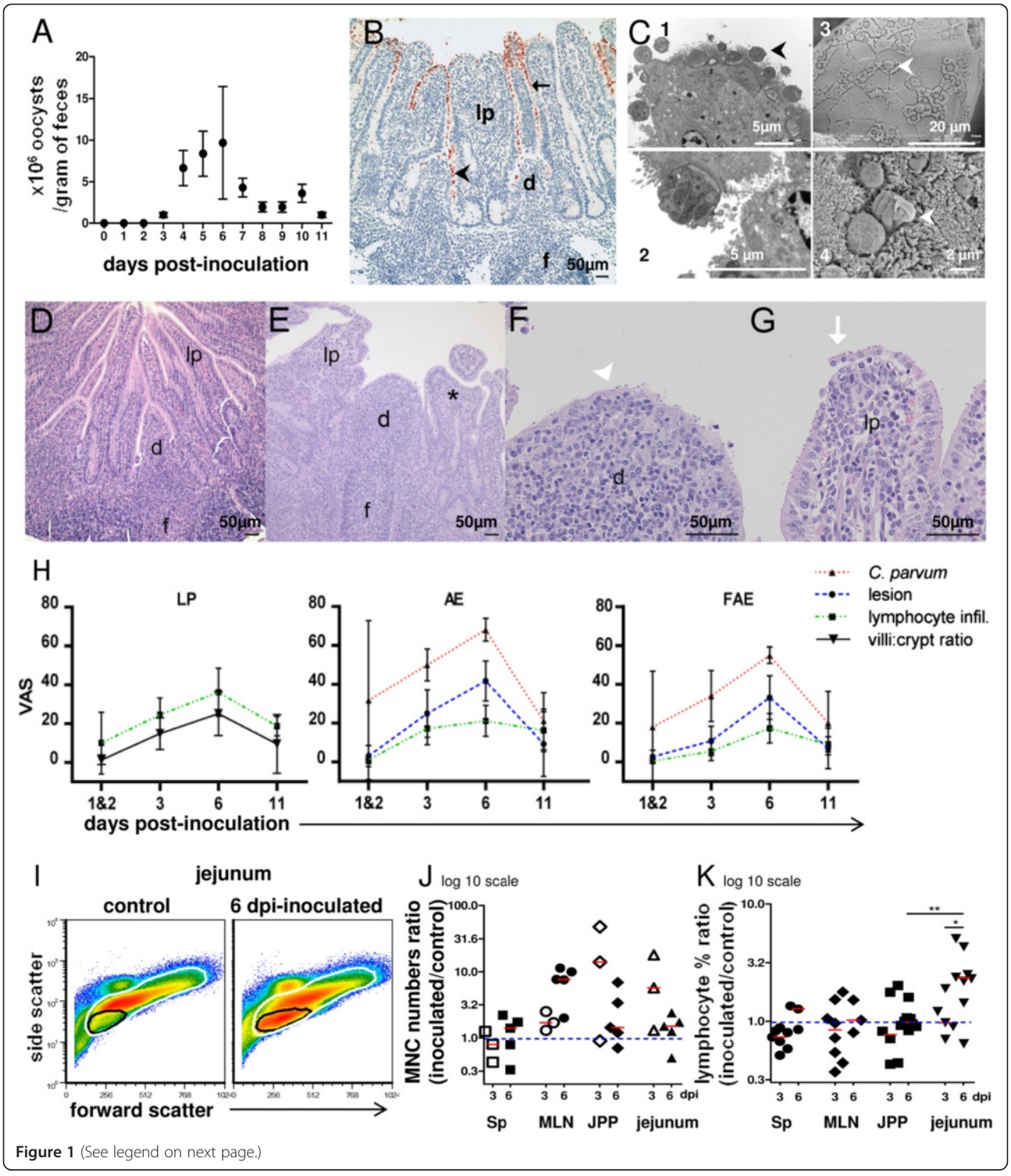

Figure 1 (See legend on next page.)

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Figure 1 *C. parvum* oocyst excretion, lesions and lymphocyte infiltration during infection. (A) *C. parvum* oocyst excretion: mean/gram of faeces ± standard error (10 to 15 animals). **(B)** *C. parvum* parasitic stages immunolabelled in brown in a 3 dpi ileal Peyer's patch (IPP): on the brush border of absorptive epithelium (AE) (arrow) and follicle-associated epithelium (fae/FAE) (arrowhead) covering the lamina propria (lp/LP) and dome (d). Lymphoid follicle (f). **(C)** Trophozoites and meronts at 6 dpi, in the JPP AE (arrowhead) observed by transmission EM (C1, C2) and scanning EM of meronts in infected FAE (C3; arrowhead) and merozoite leaving a meront (C4; arrowhead). **(D-G)** PPs representative changes during infection (HE staining). JPPs of control **(D)** and 6 dpi inoculated lambs **(E)** with villous atrophy and fusion (*) and lymphocyte infiltration in lp. **(F-G)** Attenuation, lymphocyte infiltration, detachment of fae (arrowhead) and absorptive epithelium (AE) (arrow) in an infected IPP. **(H)** Semi quantitative scoring of histopathological changes in IPP during infection. Sections from control lambs were used as a baseline and each change was rated according to severity on a 0 to 100 visual analogue scale (VAS). Values represent mean with 95% confidence intervals for 4 to 8 animals. **(I-K)** Cells extracted of spleen (Sp), mesenteric lymph nodes (MLN), JPPs and jejunum from matched pairs of lambs were purified on density gradients and mononuclear cell (MNC) and lymphocyte percentages were determined by flow cytometry on morphology parameters. **(I)** Plots show the gating of lymphocytes (black gate) and MNC (white gate) in the jejunum. In this example, lymphocytes represented 11 and 38% of MNC (88 and 84% of the cells analyzed) in control versus matched inoculated lamb, respectively. **(J)** The MNC absolute number ratio and **(K)** the lymphocyte percentage ratio were determined for each pair of lambs at 3 or 6 dpi. The red bars indicate the median values. Mann–Whitney statistic test: significance * $p < 0.05$, ** for $p < 0.01$.

disrupted in Hanks medium (HBSS) containing 2% FCS and 1% P/S by crushing on a 200 μm nylon gauze with a syringe piston. Splenic red blood cells were lysed with ammonium chloride solution (0.155 M NH4Cl, pH 7.4) then resuspended in HBSS medium. MNC were then purified on Histopaque^{TM} d = 1.077, washed and stored in ice cold RPMI medium supplemented with 10% FCS and 1% P/S until labelling.

Gut tissues were processed with a technique adapted from Renaux et al. [43] and Pérez-Cano et al. [44] to recover lamina propria MNC. Briefly, the gut tract was emptied of faecal content and rinsed with Phosphate Buffer Saline (PBS) buffer. The JPPs were dissected carefully, pooled then processed as the jejunal tissue. The whole jejunum and the JPPs were weighed separately before 30-gram samples of jejunum were taken for extraction of cells. The gut was opened and cut into 1 cm^2 fragments. The epithelial cells and intra-epithelial lymphocytes were extracted by incubation for 20 min at 37 °C in HBSS without Ca and Mg containing 3 mM ethylene diamine tetraacetic acid disodium salt (EDTA), 2 mM dithioerythritol, 10% FCS and 1% P/S under magnetic stirring and discarded. Then the lamina propria lymphocytes were extracted. The EDTA treated intestinal pieces were washed with HBSS, then incubated at 37 °C for 45 mn under magnetic stirring in RPMI medium containing 9.25U/mL type I collagenase, 30U/mL dispase II (Roche, Rosny sous Bois, France) and 2500U/mL bovine pancreas DNase I (Calbiochem, USA). The cell suspension was filtered on a 500 μM nylon mesh and the cells were washed with RPMI-10% SVF. The MNC were purified on a 75%/40% Percoll (GE Healthcare - Bio-Sciences, Sweden) gradient, washed and stored in ice cold RPMI-10% FCS until labelling. The living cells were counted with Thoma chambers and the absolute number in the organ (spleen, MLNs or JPPs) was calculated as follows: number of cells per mL multiplied by number of mL of cell suspension for the whole organ. For the jejunum, as the cells were extracted from 30 grams of tissue, the latter result was multiplied by the ratio: jejunum total weight (in grams)/30. For all the analyses and comparisons reported, the organs from age-matched control and inoculated pairs of lambs were processed simultaneously to minimize technical induced variations.

Cell labelling and flow cytometry

Single or multiple indirect labelling of surface receptors was performed on purified ovine cells using Abs against the molecules NCR1, CD8, TCR1, CD16 and CD25 revealed by subtype-specific secondary Abs. Direct intracellular perforin labelling was performed with the perforin-FITC kit and the Cytofix/Cytoperm and Permwash solutions (BD Pharmingen). The samples were analysed on a FACS CALIBUR flow cytometer (Becton Dickinson), equipped with Cell-Quest Pro software. At least 2×10^5 viable cells were analysed for spleen, MLN and PPs and at least 10^6 cells for the jejunum. The analyses of labelling were performed with the FCS express software; percentages of perforin+ and CD25+ cells and median fluorescence intensity (MFI) were determined using the histogram subtraction and statistics functions of the software. Relative numbers of cell subsets determined by flow cytometry (subset %) were converted into absolute numbers per organ according to the formula: (subset% × MNC%)/100 × total number of cells in organ (as calculated above).

RNA isolation and real-time RT-PCR

RNA was extracted from tissues with TRIZOL solution (Invitrogen), according to the manufacturer's instructions. Purified RNA was reverse-transcribed using oligo (dT) primers and M-MLV reverse transcriptase (Promega, France). For PCR experiments, primer pairs were designed using Primer 3 software (Additional file 1). Each primer was designed on different exons to span the intervening intron and thus avoid amplification from contaminating genomic DNA. Q-RT-PCR assays were carried out by

combining cDNA with primers and IQ SYBRGreen Supermix (Bio-Rad, USA) and were run on a Chromo4 (Bio-Rad). Samples were normalized internally using the average cycle quantification (Cq) of three reference genes Hypoxanthine phosphoribosyltransferase (HPRT), b-Actin and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Gene expression values are expressed as relative values after Genex macro analysis (Bio-Rad).

Statistical analyses

For the morphometric analysis of the density of CD8+ cells, it was necessary to compensate for natural variability between individuals; therefore the non-parametric Wilcoxon-van Elteren [45] test was used to calculate the significance of differences between the two groups. Two-tailed tests were performed and differences considered significant for p-values < 0.05. Flow cytometry data were analysed with GraphPad software: the nonparametric Mann–Whitney test was used to test the significance of differences between means from inoculated lambs and matched controls, the Wilcoxon test to compare different subsets of cells from the same animals and the paired t test to compare groups of paired lambs.

Results

C. parvum induces typical enteritis lesions with immune cell recruitment in the segments of the neonatal ovine small intestine

In neonatal lambs infected on the day of birth, the kinetics of *C. parvum* oocyst excretion was evaluated daily (Figure 1A). The *C. parvum* infected or uninfected status was verified by measuring the expression of a cryptosporidium specific gene in gut tissues by RT-PCR (data not shown). The excretion of oocysts started from day 3 pi coinciding with the onset of watery diarrhoea and mild dehydration and reached a peak at 6 dpi (Figure 1A). At necropsy, there was a mild to moderate enlargement of mesenteric lymph nodes (MLN) in the inoculated lambs from day 2 pi (1.5-3 fold weight increase on average compared to controls) while no visible change of the spleen was observed. Histopathological observations confirmed the macroscopical findings demonstrating a mild to moderate, diffuse and catarrhal enteritis. Parasites were observed in the brush border throughout the whole gut, with increased density in distal jejunum and ileum. In all PPs, the FAE was also infected (Figures 1B, 1C-F) but, as previously observed in the mouse model (unpublished data), we could not observe developing parasites in M cells. The lesions of superficial, lymphocytic and granulocytic diffuse enteritis, with shortening and bridging of the villi (Figures 1D-G) affected not only the absorptive epithelium, but also the FAE of the dome in which attenuation and sloughing and, to a lesser degree necrosis, could be observed. The pathological changes and the amount of *C. parvum* found on the epithelium increased gradually, reaching a peak at 6 dpi, and were partly resolved by day 11 pi (Figure 1H). In gut sections from JPPs and IPP, the mononuclear cell (MNC) infiltration (identified mainly as lymphocytes with small round and dense nuclei surrounded by narrow eosinophilic cytoplasm) was observed from day 3 pi in both villi and dome and their respective epitheliums (Figures 1E-H). Using flow cytometry, we analysed the recruitment of lymphoid cells in MLNs, the jejunum and JPPs, and spleen as a reference of a systemic organ (Figures 1I-J). There was a tendency towards an increase in the absolute number of MNCs in the gut and MLNs at 3–6 dpi: the ratio between the absolute numbers of MNCs in inoculated lambs and their matched controls was superior to one in most pairs i.e. 6 of 8 in JPPs, 7 of 8 in the jejunum and 8 of 8 in the MLNs (Figure 1J). This was consistent with the necropsy and histology findings, and suggested that the immune response to this infection occurs locally and within the draining lymph nodes. Considering the percentage of total lymphocytes in the different tissues, we found that in most (10 of 13) pairs of lambs at 3 and 6 dpi, there was an increase in the lymphocyte proportion in the jejunum of the inoculated lamb (ratio significantly superior to 1, $p < 0.01$) (Figure 1K) and this local increase could already be observed as early as 1 and 2 dpi (data not included in Figures 1J and K).

We and others have shown that interferon gamma (IFNγ) is a key cytokine for controlling infected enterocytes in the mouse model [32,46]. In contrast, interleukin 22 (IL22), that is now considered to play an important role in intestinal tissue repair [47,48], has not been investigated in response to *C. parvum* infection. Infection of lambs was associated with an increase in the expression of the IFNγ and IL22 genes which was evident from as early as 1 dpi with a further increase observed at 3 and 6 dpi (Figure 2). The up-regulation of IFNγ and IL22 during the infection may participate in the clearance of the infection and the recovery of the epithelial integrity, respectively.

The proportion of NCR1+ lymphocytes did not change with infection although a slight increase in their absolute numbers was observed in the small intestine of infected lambs

NCR1+ lymphocytes are known to be IFNγ producing cells and important players of the innate response. As an increase of lymphocytes in the gut segments was observed early in the infection at 3 and 6 dpi (Figures 1E-H, K) and even earlier at 1–2 dpi (data not included in Figure 1K), we analysed the local changes of NCR1+ lymphocytes in the GALTs at different time-points of infection. Preliminary studies revealed that the proportion of NCR1+ cells was rather low in the ileum compared to jejunum and JPPs; consequently immunohistology was

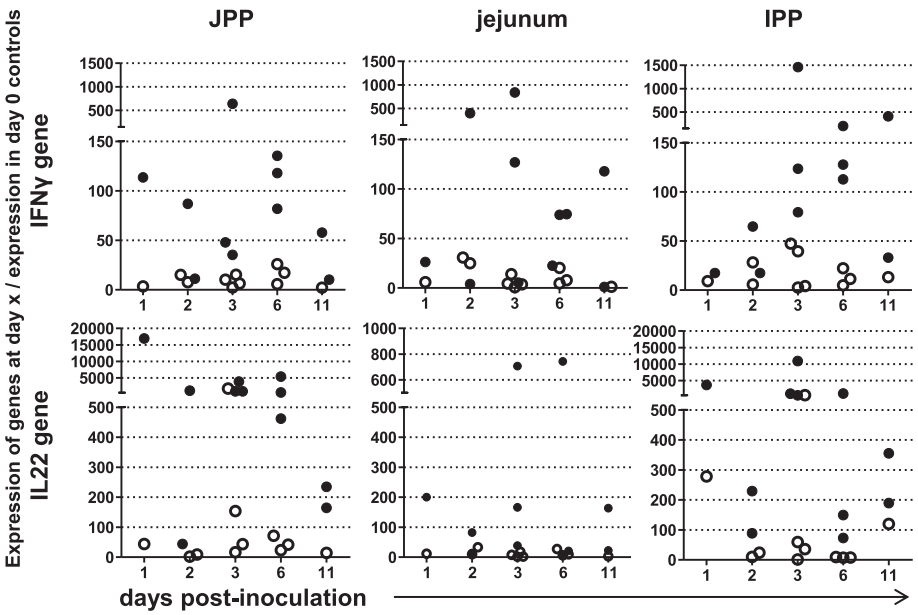

Figure 2 IFNγ and IL22 gene expression in samples from intestinal tissues. At slaughter, fragments of the small intestine were frozen in nitrogen and processed for quantification of IFNγ and IL22 gene expression by qRT-PCR. Each point represents the ratio of the number of gene copies in one individual (Ct) to the reference corresponding to the mean of the number of copies in two control animals slaughtered at birth (Ct0). The open and filled circles correspond to control and inoculated lambs respectively. JPP; jejunal Peyer´s patch, IPP; ileal Peyer´s patch.

preferred to flow cytometry to assess the cell recruitment in the IPP.

As previously found in the GALTs of other healthy 1–2 month-old lambs [33,34,49], NCR1 labelling of IPP sections (Figure 3A) revealed the presence of NCR1+ cells in the interfollicular T cell areas and domes, and to a lesser degree in the lamina propria and intraepithelial compartment, while few or none were observed in the follicles. There was neither a difference in the density (number of labelled cells per area) nor a change in localization of NCR1+ cells in the infected lambs compared to the controls at any time during the infection (Figure 3A). The NCR1+ lymphocyte proportions were also examined by flow cytometry in gut segments, MLN and spleen (Figures 3B-D). No difference in the proportion of NCR1+ lymphocytes (Figure 3C) or in the percentages ratio (inoculated/matched control) (Figure 3D) could be detected in any organ at any time-point. Also, no difference was detected in the level of expression of the NCR1 gene in the different gut segments (Additional file 2). However, both the absolute number of mononuclear cells and the lymphocyte percentage were higher in the jejunum of inoculated lambs (Figures 1H, J, K). In addition, when specifically examining the absolute numbers of NCR1+ lymphocytes in gut segments of paired lambs, a statistics test could only be applied to data from JPPs at day 6 pi ($p < 0.05$), but a similar tendency to an increase in infected lambs was seen at other time points and in the jejunum (Additional file 3). We conclude that in small intestinal segments, NCR1+ lymphocytes do not increase in relative numbers, although they most likely increase in absolute numbers during the early stages of infection.

The frequency of NCR1+ lymphocytes expressing perforin increases with infection and is concurrent with a preferential increase of the NCR1+/CD16+ subpopulation in JPPs and jejunum

Of the two subsets of NK cells described in humans, the $CD56^{dim}CD16+$ population is considered to have strong cytotoxic properties. Recently, the presence of two populations of NK cells with different CD16 expression were described in the sheep intestine [33,50]. Since the expression of CD16 might reflect the cytotoxic potential of NK cells, we analysed the balance between the NCR1+/CD16+ and NCR1+/CD16- subpopulations during the course of infection (Figures 4A-C). In control animals more than 55-60% NCR1+ lymphocytes expressed the CD16 receptor in spleen and MLN (Figure 4B) while, in JPPs and jejunum, they represented only 20-40% of NCR1+ lymphocytes (Figure 4B). In inoculated animals an increase in the NCR1+/CD16+ subpopulation (2 fold on average) was observed at 3–6 dpi in the cells extracted from JPPs and jejunum ($p < 0.01$) (Figure 4C). In addition, in around half of the inoculated lambs an increase in the level of expression of the CD16 receptor was observed (MFI), (data not shown).

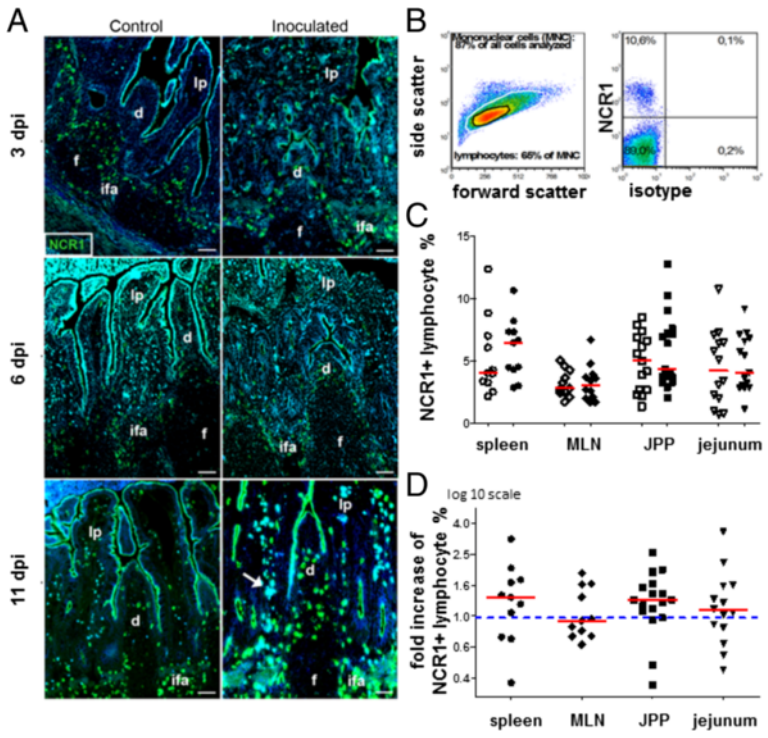

Figure 3 Proportions of NCR1+ lymphocytes in different tissues during *C. parvum* infection. (A) Sections from ileal Peyer's patches of lambs 3, 6 and 11 dpi and their age-matched controls were labelled with NCR1 (green) mAb. NCR1+ cells changed neither in density nor location during infection. Diffuse light blue spots were identified as autofluorescence using the blue filter, especially in the lamina propria (lp). Dome (d), interfollicular area (ifa), follicle (f). Bar 50 μm. **(B-D)** The NCR1+ lymphocyte percentage within the mononuclear cell population (MNC) was determined by flow cytometry. **(B)** The plots show the example of the NCR1 labelling in cells purified from jejunal Peyers'patches (JPP) at 6 dpi and gated as indicated (black gate); the right plot shows the labelling with both the anti-ovine NCR1 mAb and the isotype control of the CD8 mAb. **(C)** The NCR1+ lymphocyte percentage is shown in the different organs of lambs from 1 to 6 or 7 days of age: each dot represents one animal and the bars indicate the mean values. Control lambs (open symbols) inoculated lambs (filled symbols). Mesenteric lymph nodes (MLN). **(D)** The points represent the fold increase of NCR1+ lymphocytes (i.e. ratio inoculated/control NCR1+ lymphocyte percentage) for each pair of age-matched lambs shown in graph C, and the red bars show the medians. The mean ratio was significantly superior to one with a confidence interval of 1.21-1.57 for spleen, 1.05-1.15 for MLN, 1.17-1.55 for JPP and 1.28-1.34 for jejunum ($p < 0.01$).

The perforin content, which directly reflects the cytotoxic potential of the NCR1+ lymphocytes, was examined in the same two subpopulations at 6 dpi (Figure 4D). In both subpopulations, the percentage of perforin+ cells (Figure 4D) and their mean perforin content (MFI shown in the representative example (Figure 4E)) were increased in infected lambs.

C. parvum infection induces a strong increase of the CD8 + NCR1- cell population in the intestine

Circulating NCR1 lymphocytes from ruminants are known to express the CD8 marker, also expressed by subsets of both αβ and γδ T lymphocytes [30,34,51]. We therefore examined the expression of this marker in JPPs and jejunum and found that the vast majority of NCR1 lymphocytes expressed CD8 (Figure 5A). In addition, a large population of CD8+ NCR1- lymphocytes was also observed (Figure 5A). Three populations of CD8+ lymphocytes could be distinguished: NCR1+/CD8+ NK cells, $CD8^{tot}$/NCR1-, with a $CD8^{hi}$/NCR1- cell population included in the latter. The proportion of NCR1+/CD8+ NK cells was similar in JPPs and jejunum and did not change during the infection (Figure 5B), which was in agreement with the results shown in Figures 3C and D. However, the $CD8^{tot}$/NCR1- cells that already predominated in JPPs and particularly in the jejunum at homeostasis increased significantly during infection (3–6 dpi); this increase was not due to a specific increase of the included $CD8^{hi}$/NCR1- sub population (shown in Figure 5A). We sought to elucidate if this global increase of the $CD8^{tot}$/NCR1- population was caused by a recruitment or local proliferation of CD8+ lymphocytes. Double immunofluorescent labelling against CD8 and Ki-67 on gut sections (Figures 5C and D) showed that although the density of CD8+ cells increased in the inoculated lambs, the proportion of CD8+/Ki-67+ proliferating cells remained the same as in the controls, indicating that only a minor fraction of the local CD8+ cell population was proliferating in situ and that the increase in the CD8+ cells most probably was caused by cells recruited from blood. As several cell types

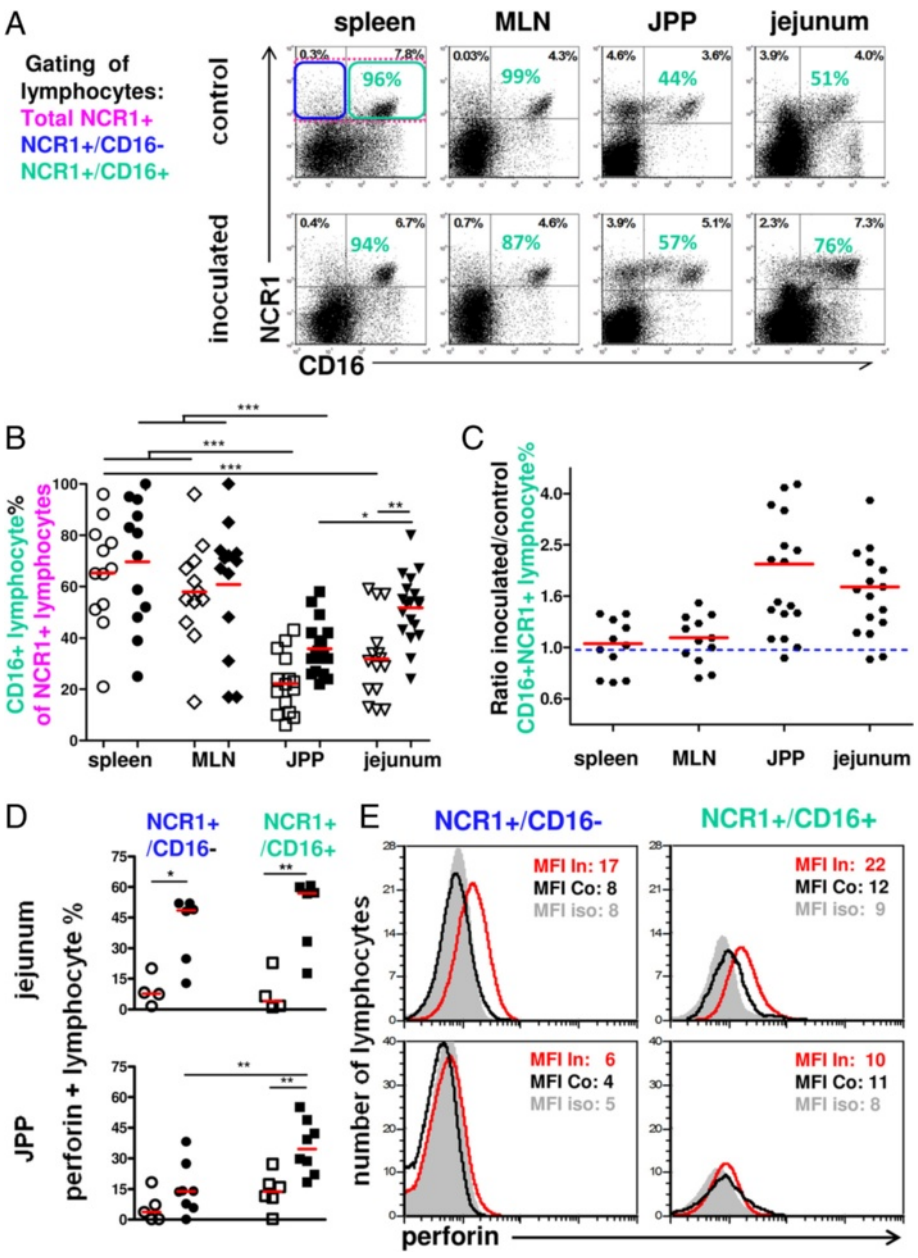

Figure 4 Increase in NCR1+/CD16+ lymphocyte percentage and perforin+ cell percentage in the jejunum during infection. (A) Cells labelled with anti NCR1 and CD16 mAbs and gated on the lymphocyte gate (shown in Figure 1I) were analyzed by flow cytometry in spleen, mesenteric lymph nodes (MLN), jejunal Peyer's patches (JPP) and jejunum. The quadrants were set according to the labelling obtained with the isotype controls for the same number of cells analyzed. Percentages indicated in black represent the CD16-/NCR1+ and CD16+/NCR1+ lymphocyte percentages of which the non-specific labelling was subtracted. Percentages of CD16+ cells among NCR1+ lymphocytes are indicated in green. Data are from an inoculated lamb at 6 dpi and its control. **(B)** Individual CD16+ percentages of NCR1+ lymphocytes are indicated for animals 2–6 dpi, control lambs (open symbols), inoculated lambs (filled symbols). The red bars indicate the mean values. **(C)** The points represent the inoculated/control ratio of NCR1+/CD16+ lymphocytes for each pair of age-matched lambs. **(D-E)** NCR1 and CD16 double labelled cells were permeabilized for intracellular labelling with an anti-human perforin mAb or isotype control to determine the perforin+ cell percentage in both NCR1+ subpopulations by histogram subtraction at 6 dpi. Control lambs (open symbols), inoculated lambs (filled symbols). **(E)** The data shown are representative of the lambs whose individual percentages of perforin+ cells are shown in Figure 4D. The red and black line limited histograms correspond to the inoculated lamb (In) and its matched control (Co) respectively, the gray filled histogram to the isotype control of the perforin mAb (iso); the mean fluorescence intensity (MFI) is indicated for each histogram. The comparisons were made with the Mann–Whitney (control/inoculated) and Wilcoxon tests (paired data). Significant difference probability: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The inoculated/control CD16+/NCR1+ lymphocyte ratio was calculated for each pair of lambs **(C)**; the mean was significantly superior to 1 with confidence interval of 1.4-2.82 for JPPs and 1.48-2.32 for jejunum ($p < 0.01$).

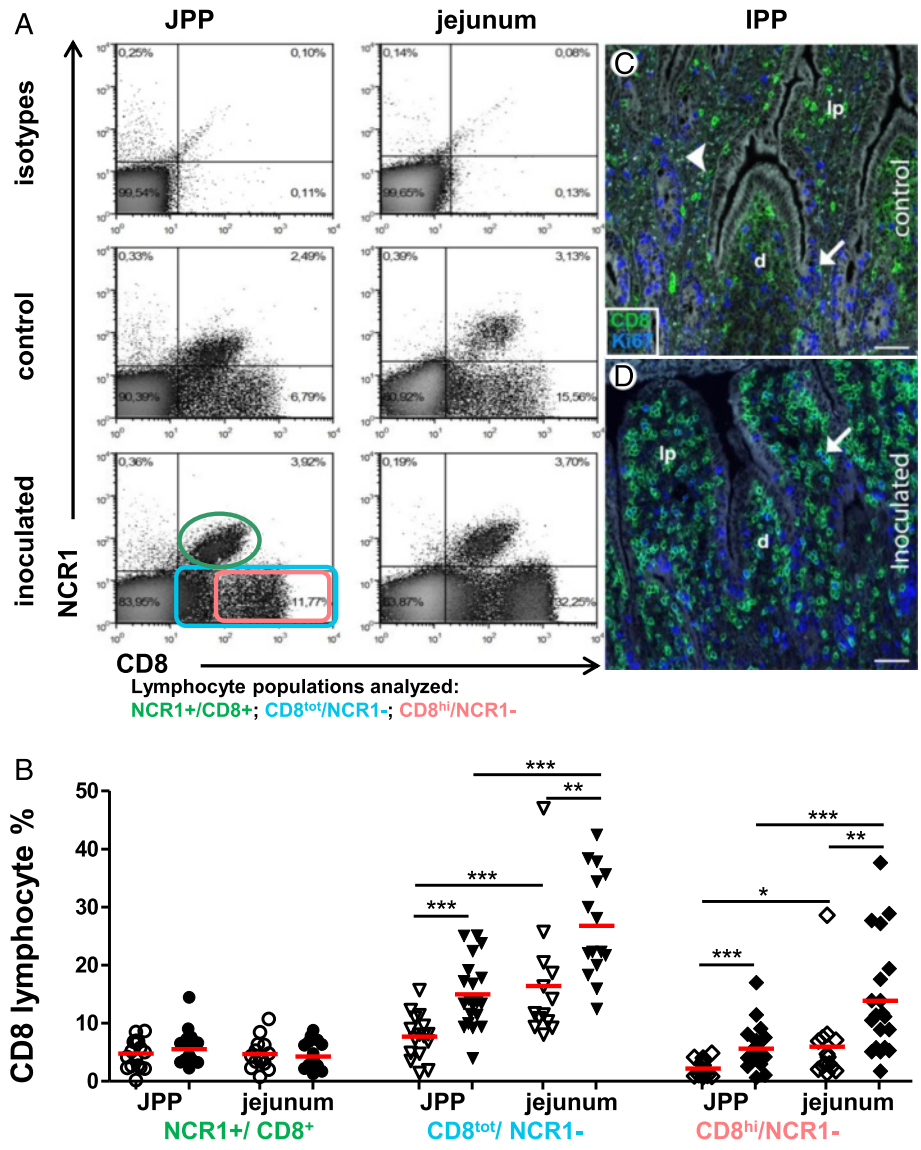

Figure 5 CD8+ lymphocyte increase in the jejunum and jejunal Peyer's patches. (A) Cells isolated from jejunal Peyer's patches (JPP) and jejunum were double labelled with anti CD8 and NCR1 mAbs and analyzed by flow cytometry. The plots show data from a 6 dpi inoculated lamb and its 7 day-old matched control and are representative of 8 matched pairs of 7 day-old lambs. The gating used for the analyses shown in B is indicated with the corresponding colours. **(B)** The percentages of the 3 different populations of CD8+ lymphocytes corresponding to the 3 gates shown in **(A)** were analyzed in jejunum and JPP from lambs 2–6 dpi. Control lambs (open symbols), inoculated lambs (filled symbols). The means are indicated in red. The comparisons were made with the Mann–Whitney (control/inoculated) and Wilcoxon tests (paired data). Statistically significant differences are indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(C-D)** Section of an ileal Peyer's patch (IPP) from a 7 day old control **(C)** and a 6 dpi lamb **(D)** were double labelled with anti CD8 (green) and Ki-67 (blue) antibodies. The images shown are obtained by merging these images with the white light image. The double positive cells (arrow) display CD8 cytoplasmic staining and Ki67 nuclear staining. Pinpoint sized spots (arrowhead) are identified as autofluorescence. Dome (d), lamina propria (lp). Bar 50 μm.

may express CD8, we sought to clarify the identity of the CD8+ cells in sections. In gut sections, the vast majority of CD8+ cells were also CD3+ (Figures 6A and B) and thus represent CD8 T lymphocytes. As the $CD8^{low}$ labelling of NCR1+ cells was not perceptible in immunohistology (not shown), we conclude that we only could visualize the $CD8^{high}$ population of the NCR1- cells with this technique. Their density increased in villi, dome and their respective epitheliums in both JPPs and IPP. As the changes were observed to be similar in the jejunal and ileal segments, IPP was chosen for quantification to verify the observations in the sections (Figure 6C). CD8+ cell density increased significantly at days 3 and 6 pi, in the lamina propria and absorptive epithelium of the villi and at 6 dpi in the dome including FAE which supported the flow cytometry data (Figure 5B). To discriminate between αβ and γδ T cells

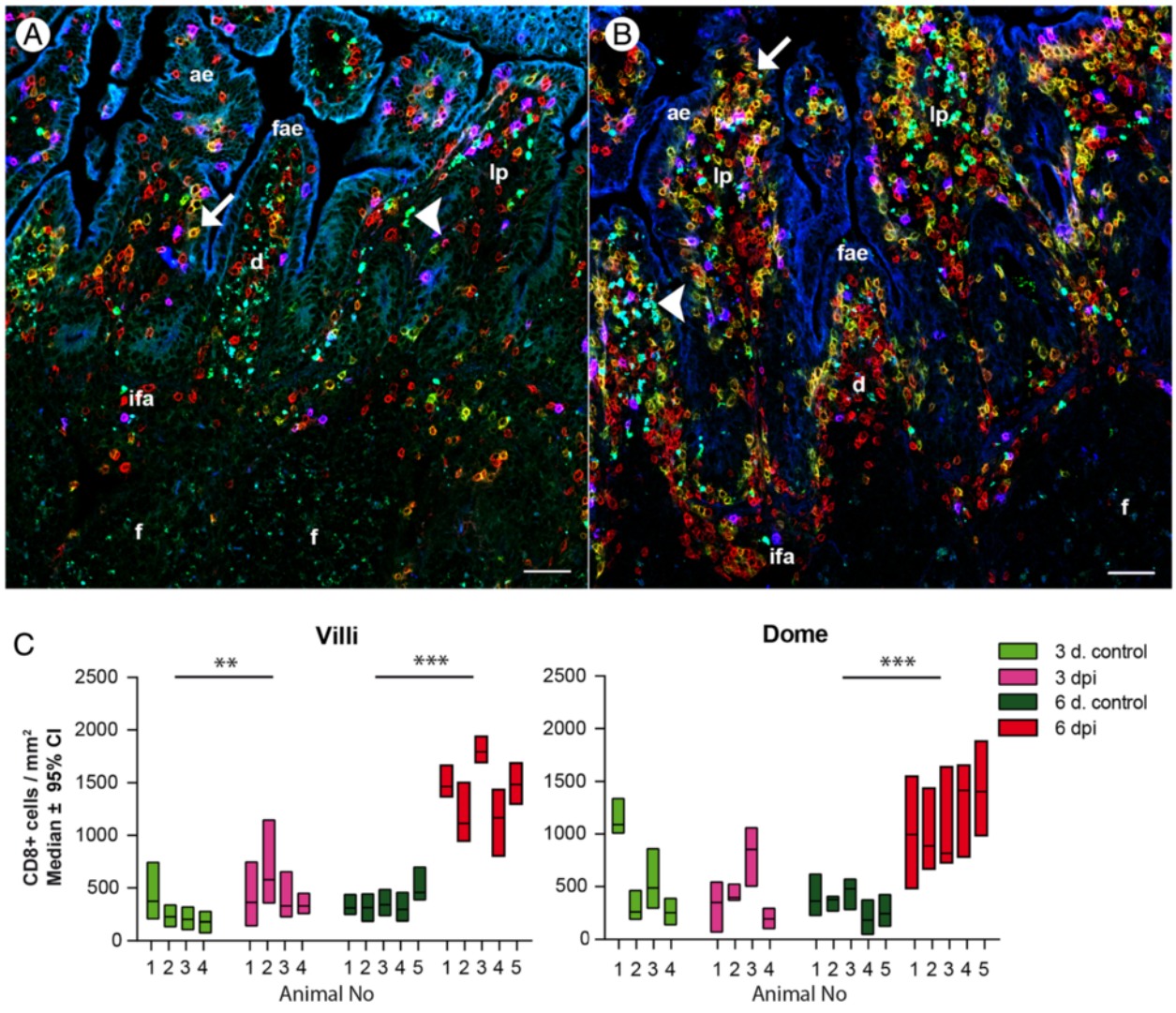

Figure 6 Significant increase of CD8+ cells in the ileum of infected lambs. Ileal Peyer's patches sections from a pair of 6 day-old matched lambs, control **(A)** and inoculated **(B)**, were labelled with Ab against CD3 (red), CD8 (green) and γδTCR (blue). Increased numbers of yellow CD3+/CD8+ cells (arrow) in the lamina propria (lp) and dome (d) were seen in the inoculated animals compared with the controls. Few or no CD3+/CD8+/γδTCR+ cells were observed. Light blue dots (arrowhead) were identified as autofluorescence and thus differentiated from the specific labelling which was localised in the cell membrane. Absorptive epithelium (ae), follicle-associated epithelium (fae), follicle (f), interfollicular area (ifa). Bar 50 μm. **(C)** The increase of CD3+/CD8+ cells in the ileal Peyer's patch was demonstrated by quantitative analysis of positive cells per mm^2 in villi and dome, including the covering epithelium. The graphs show the median with 95% confidence interval constructed using the Bernoulli-Wilcoxon procedure. Statistically significant differences are indicated as ** $p < 0.01$ and *** $p < 0.001$.

and to better characterize the CD8+/NCR1- population, we analyzed the expression of TCR1, the γδ T cell receptor, on CD8+ cells. TCR1+ T cells were scarce in gut sections of JPPs and IPP (Figures 6A and B), and no obvious increase of density was observed in infected animals compared to controls. Similar results were found with flow cytometry (Additional file 4) although this method indicated a 2–3 fold increase of γδ T cell percentage in jejunum. Only 3 to 12% of CD8+ cells co-expressed TCR1. Thus the vast majority of CD8+/NCR1- lymphocytes recruited during the infection are most probably conventional αβ CD8+ T cells.

The proportion of perforin+ cells within the intestinal NCR1+ population and their activation (CD25 MFI) increase with infection while they remain stable in the CD8+/NCR1- population

To determine the functional potential of the CD8+ subpopulations, the perforin content (Figure 7) and the activation status (Figure 8) were analyzed by flow cytometry. The

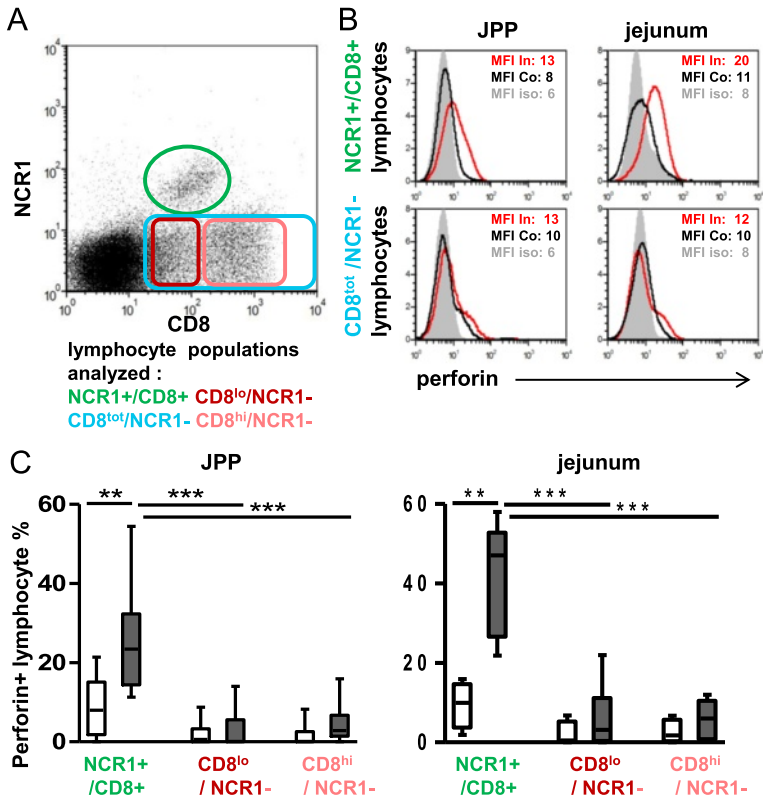

Figure 7 Perforin+ lymphocytes among CD8+ lymphocytes in the gut. (A) Cells extracted from gut tissues were double labelled with anti NCR1 and CD8 mAbs then fixed and permeabilized and labelled with an anti perforin mAb. The plot shows (example of jejunum at 6 dpi) the gating of the lymphocyte populations analyzed. **(B)** Lymphocytes from an inoculated lamb (3 dpi) and its control were gated either on the CD8+/NCR1+ or the $CD8^{tot}$/NCR1- populations then analyzed for the perforin content. The red line limited histogram corresponds to the inoculated lamb (In), the black line limited histogram to its matched control (Co) and the gray filled histogram to the isotype control of the perforin mAb (iso) and the mean fluorescence intensity (MFI) is indicated for each histogram. **(C)** The analysis of the perforin expression by CD8+ lymphocyte sub-populations (gates shown on Figure 7A) was performed in 3 control and 4 age-matched 6 dpi inoculated lambs. The comparisons were made with the Mann–Whitney (control/inoculated) and Wilcoxon tests (paired data). Statistically significant differences are indicated with ** $p < 0.01$ and *** $p < 0.001$.

percentage of perforin+ cells among the NCR1+/CD8+ population was already noticeably increased in both JPPs and jejunum while it was low in the CD8+/NCR1- population in the age-matched pair tested at 3 dpi (Figures 7A and B). At 6 dpi, this feature was confirmed (Figure 7C). The activation status of the same populations was also assessed through the expression of the IL2 receptor (CD25) (Figure 8). In JPPs of control animals, around 70% the NCR1+ lymphocytes expressed CD25 compared with 40% of the $CD8^{lo}$ and less than 30% of the $CD8^{hi}$ population. This latter population was also the less activated in the jejunum. Surprisingly, the proportion of CD25+ cells did not increase during the infection in any of the populations analysed. However, considering the higher level of expression of the CD25 marker (MFI) on the NCR1+ lymphocytes of JPPs from the inoculated lambs at day 6 pi, these cells were significantly more activated than in the controls (Additional file 5).

Discussion

The early immune response of neonatal ruminants to *C. parvum* infection is still largely unknown although some insight has been gained on the recruitment of CD8 lymphocytes by Wyatt et al. [10,52] and of mast cells by Li et al. [53] in the gut of infected calves. Natural Killer (NK) cells have been known for a long time to be important actors of the primary innate immune response through cytotoxicity and IFNγ production and also through a regulatory role in the immune response via their interaction with other cells and their ability to produce various cytokines once activated. The objective of this work was to investigate the participation of NK cells in the first steps of the innate immune response to *C. parvum* in a natural host, the neonatal lamb, in response to a controlled experimental infection. NK cells express several receptors, including CD8, that are common to several lymphocyte types. Over the last decade, the activating receptor NKp46, renamed Natural Cytotoxicity Receptor

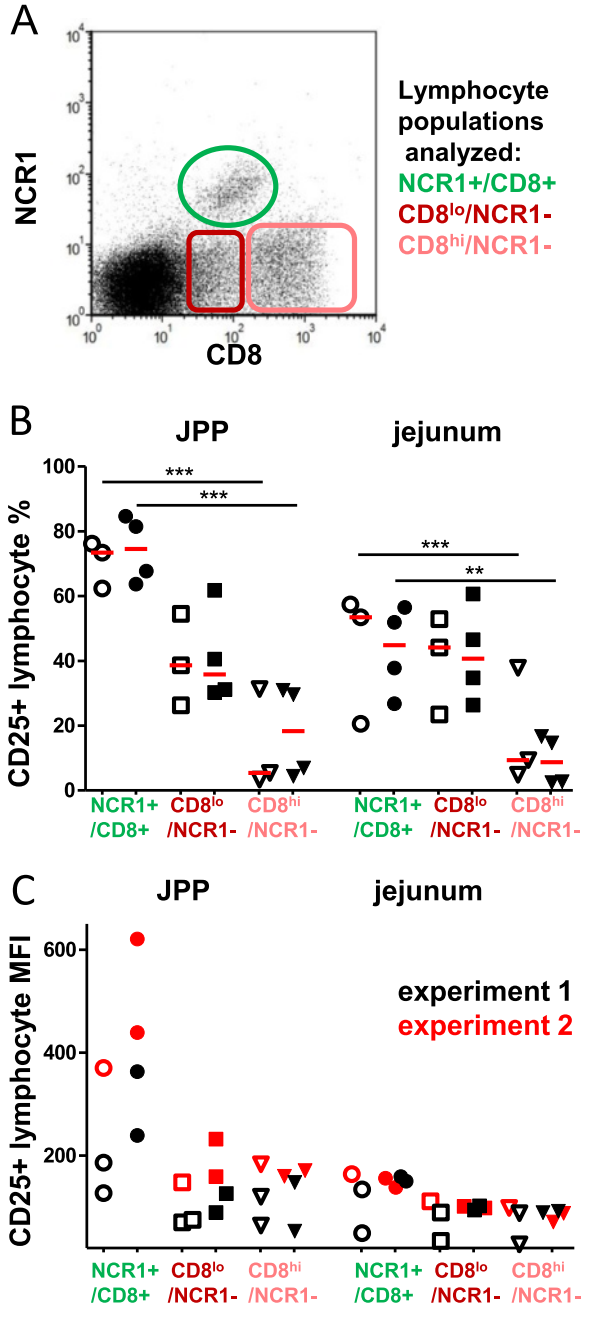

Figure 8 Expression of CD25 on small intestinal NCR1+ and CD8+ lymphocytes. (A) The expression of the activation marker CD25 was analyzed at 6 dpi in the populations gated as indicated. The individual percentages **(B)** and the mean fluorescence intensity (MFI) of CD25+ cells **(C)** are shown for the 3 populations. Medians are shown with red bars. Control lambs (open symbols), inoculated lambs (filled symbols). In C, the results of the two experiments are shown with red or black symbols. The comparisons were made with the Mann–Whitney (control/inoculated) and Wilcoxon tests (paired data). Statistically significant differences are indicated with ** $p < 0.01$ and *** $p < 0.001$.

1 (NCR1) in the new nomenclature, had come to be considered as the prototypal marker to define NK cells in most species [35,54,55]. However, various recent studies on innate immune cells in mice and humans led to the discovery that NCR1+ cells not only include conventional NK cells (cNK) but also innate lymphoid cells (ILC) of groups 1 and 3 (including NK22/ILC22) [56]. In mouse and human, ILCs participate in the intestinal defence and homeostasis but, to date, these cells have not been characterized in ruminants and their presence in this species is therefore still speculative. We examined the changes in the NCR1+ lymphocytes, in two inductive sites of the small intestine, the ileal (IPP) and jejunal Peyer's patches (JPPs), both known for playing distinct roles in the mucosal immunity of sheep, and the jejunum which is considered a major effector site with a special focus on days 3 and 6 pi [28,57,58]. The two techniques used for this study (immunohistology and flow cytometry) brought concordant and complementary results. The histopathological findings revealed a marked lymphocyte infiltration and proliferation in the intestinal mucosa already by day 3 pi (end of the parasite prepatent period) and at day 6 pi (around the peak of infection). Some Ki-67+ proliferating cells were CD8+ cells suggesting a proliferation in situ beside recruitment from blood and secondary lymphoid organs. CD8 is expressed both by T cells and most mucosal NK cells in ruminants, and both of these lymphocytes are known to be recruited in inflammatory conditions and several infections in human and mice [59,60]. NCR1+/CD8+ lymphocytes representing NK cells were part of the early lymphocyte infiltration observed in MLN and all the small intestine and the CD3+/CD8+/NCR1- population (corresponding to CD8+ T lymphocytes) also presented a marked increase in the lamina propria and absorptive epithelium of the villi and, with a short delay, within the dome and FAE of inoculated lambs during this period as previously observed by Wyatt et al. in calves [52]. Flow cytometry revealed that only a scarce proportion of the CD8+/NCR1- lymphocytes expressed the γδ TCR1 suggesting that the vast majority of these cells were conventional CD8 T lymphocytes. The increase of CD8+ T cells at the onset of oocyst shedding may indicate that the adaptive immune response was initiated in the gut tissue at that time.

As the NCR1+ cell proportion within the lymphocyte population is rather low (5% on average) and the individual variability is high in very young animals, the variations due to infection were less conspicuous for NK cells than for CD8 T lymphocytes.

However, exploring the functional potential of NK cells we found that they are likely participating in the early response to this infection. The cytotoxic potential of NCR1+ lymphocytes was explored through their perforin content. Importantly, the proportion of perforin+ cells

within the total NCR1+/CD8+ NK cell population, already higher than in the CD8+/NCR1- T cell population at homeostasis, increased from day 3 of infection. The higher cytotoxicity of purified jejunal and JPP NCR1+ cells from a 6 dpi-infected lamb compared to its control (Drouet unpublished data, Additional file 6) is in agreement with these data. Moreover, NCR1+ cells expressed high levels of the IL2 receptor (CD25 MFI) especially in infected lambs and their percentage was particularly high in JPPs, indicating that NCR1+ NK cells are activated in all the gut and especially in this inductive lymphoid tissue. In contrast, the proportion of perforin+ cells remained stable within the CD8+/NCR1- T cell population at 6 dpi. Analysing the cytotoxicity of CD8 T lymphocytes derived from healthy cryptosporidium seropositive and negative donors sensitized in vitro with a cryptosporidium antigen, on an intestinal cell line (CaCo2) infected with the parasite, Pantenbourg et al. [31] found that those from the seropositive donor were more cytotoxic. Comparing our in vivo results with those of Pantenbourg et al. we may hypothesize that in the present study, at 6 dpi, the CD8 T lymphocytes are likely still in the situation of a primary response in which they have a poor cytolytic potential because they are not yet fully sensitized to the parasitic antigens. Supporting this hypothesis, the CD8+ T cells were also globally less activated (low CD25 MFI) than the NCR1+ cells and surprisingly the level of expression of CD8 is not correlated with a higher activation status since $CD8^{hi}$ T cells expressed less CD25 than the whole CD8+ population. $CD8^{hi}$/NCR1- cells may represent cells at a different stage of differentiation.

The expression of the CD16 marker has long been associated with enhanced cytotoxic properties in human NK cells. Concerning the expression of the CD16 marker on lamb NCR1+ NK cells, we confirmed in week-old lambs the presence of CD16- and CD16+ subpopulations similar to those we have previously described in the gut of older lambs [33,49] and adults [34] and in which there was a tendency to a higher representation of the CD16+/NCR1+ NK subpopulation in older lambs [49]. The *C. parvum* infection in this study was associated with an increase in the frequency of the NCR1+/CD16+ subpopulation among total NK cells, which could indicate a higher cytotoxicity of intestinal NK cells in the infected lambs. Therefore we sought to find out if the CD16+ subpopulation displayed a higher perforin content, reflecting a higher cytotoxic potential, but the proportion of perforin+/NCR1+ lymphocytes increased in both CD16- and CD16+ subpopulations (up to 60%) with infection, especially in the jejunum. Interestingly, a cytotoxicity assay probe trial performed with CD16-/NCR1+ and CD16+/NCR1+ FACS-sorted subpopulations, failed to show any difference between the two subpopulations (Drouet unpublished data). These data suggest that the expression of CD16 on sheep NK cells might be associated with a function different from that of their human homologs, perhaps more linked with antibody dependent cell cytotoxicity than direct cytotoxicity.

Finally, considering the cytokine profile during the first days of infection, the up-regulation of IFNγ gene expression observed in inoculated lambs, is in agreement with our data obtained from rodent neonates where an early production of IFNγ is known to be a determinant for the resolution of the infection in neonatal mice [32]. Both cNK cells and T lymphocytes are known to be significant producers of IFNγ and it will be of interest to ascertain in future studies their relative contributions to its production during the course of *C. parvum* infection and resolution in sheep. The increased expression of the IL22 gene observed in all segments of the small intestine of the inoculated lambs from the very first days of infection, could come from NK22/ILC22 cells (ILC3) and/or lymphoid tissue inducers (LTi) known to produce this cytokine in mice and humans [48]. However, a recent publication [61] shows that cNK cell depletion in mouse leads to a decreased production of IL22 in the lung during *Klebsiella pneumoniae* infection, suggesting that mucosal cNK may also produce IL22. As in mouse, ovine NCR1+ cells isolated from jejunum (magnetic sorting) were able to up-regulate the expression of the IL22 gene upon in vitro stimulation by rh IL23 [50]. When more antibodies cross reactive with sheep become available, it will be of great interest to further analyse the NCR1+ together with CD8+ NCR1- lymphocytes, to study their crosstalk and characterize their subsets and respective participation in the cytokine production and resolution of cryptosporidiosis.

In the mouse model the involvement of NK cells is debated; whereas works from the team of MacDonald [19,62] support an involvement of NK1.1+ NK cells in the protective response, we recently failed [18] to demonstrate a potent role for NCR1+ NK cells in the immune response of neonatal mice to the infection with *C. parvum.* The use of different markers to analyse NK cells makes it difficult to compare results from different studies but, more importantly, the distribution of the different lymphoid cell types within the gut mucosa of neonates differs notably between ruminants and mice. The dramatic difference in their developmental status at birth is illustrated by the presence of very scarce NK cells in the gut of mice [18] and rat [44,63], while we have shown here that they are already well represented in the gut of neonatal lambs.

Altogether, the experimental data presented here demonstrate that activated NK cells that possess a high cytotoxic potential are present very early in the small intestine and likely involved in the innate response to this infection. We recently demonstrated in neonatal mice [18] that dendritic cells play a key role in the resolution of *C. parvum* infection. Dendritic cells are known

to produce both IL23 and IL12 that contribute to the production of IL22 by ILC22, and IFNγ by activated cNK cells, respectively. The crosstalk between NK cells and dendritic cells warrants further study since cooperation between these two cell types, as described for other infections in human and murine species, is a key mechanism governing innate immunity against intracellular parasites. The adaptive immune response is often required for complete control of infection [18,64]. The early increase in the CD8 + T cell population that we observed in infected lambs probably coincides with the onset of the adaptive immune response and further studies are needed for better comprehension of cell interactions during this important stage of the immune response. As a specific acquired immune response requires processing and presentation of antigens to effector cells, it would be interesting to investigate the sampling of cryptosporidium antigens from the gut lumen by FAE and dendritic cells; indeed, in this study, we did not observe any cryptosporidium labelling below the epithelium, contrary to what Landsverk [29] and Åkesson et al. [65] observed with other pathogens. More specific studies on presentation of the parasite antigens to lymphocytes in the GALT would therefore be useful.

Additional files

Additional file 1: Primer sequences. For PCR experiments, primer pairs were designed using Primer 3 software. Crypto = *Cryptosporidium parvum*.

Additional file 2: Expression of the NCR1 gene in the gut during infection. At slaughter, fragments of the small intestine were frozen in nitrogen and processed for quantification of the expression of the NCR1 gene by qRT-PCR. The individual data represent the ratio of the number of gene copies to the mean number of copies of two control animals slaughtered at birth. Control lambs (open symbols), inoculated lambs (filled symbols).

Additional file 3: Absolute numbers of NCR1+ lymphocytes in the small intestine. Cells extracted from jejunal Peyer's patches (JPP) and jejunum from matched pairs of lambs were purified on density gradients and the numbers of MNC and lymphocyte percentages were determined by flow cytometry on morphology parameters as shown in Figure 1I. The MNC absolute number, the lymphocyte percentage and the NCR1+ cell percentage of lymphocytes were determined for each pair of lambs at 3 or 6 dpi to calculate the absolute number of NCR1+ lymphocytes (log 10 transformed) in the tissues of each animal. The matched colour symbols correspond to paired lambs; control lambs (open symbols), inoculated lambs (filled symbols). The paired *t* test performed on log 10 transformed data from JPP at day 6 pi was significant (** $p < 0.01$).

Additional file 4: Gamma delta lymphocytes in the gut lamina propria. Cells isolated from jejunal Peyer's patches (JPP) and jejunum, were double labelled with anti TCR1 and CD8 mAbs. Cells with lymphocyte morphology were gated. The plots show data from a 7 day-old lamb at 6 dpi and its age-matched control. Representative plot of 3 matched pairs of 7 day-old lambs. The percentages indicated in red represent the percentage of positive cells in the dotted rectangle minus the percentage of non-specific labelling obtained with the isotype control antibody.

Additional file 5: Expression of CD25 on small intestinal NCR1+ lymphocytes. The expression of the activation marker CD25 was analyzed in the NCR1+ population and gated as indicated in Figure 8. The individual percentages and the mean fluorescence intensity (MFI) of CD25+ cells are shown in jejunal Peyer's patches (JPP) and jejunum. Medians are shown with black bars. The matched color symbols correspond to paired lambs; control lambs (open symbols), inoculated lambs (filled symbols). A paired *t* test was performed on CD25-MFI data. Statistically significant differences are indicated with ** $p < 0.01$.

Additional file 6: Cytotoxicity of small intestinal NCR1+ cells from a *C. parvum* infected lamb and its control. At six days post-inoculation, the NCR1+ cells were isolated from Jejunal Peyer's patches (JPP) and jejunum from an inoculated lamb and its age-matched control by magnetic sorting. The isolated cells were cultured for 4 days in the presence of recombinant ovine IL2 and recombinant human IL15. Their cytotoxicity was assessed against the ovine fibroblast line IDO5 as described by Elhmouzi et al. [51].

Abbreviations

MLN: Mesenteric lymph node; GALT: Gut associated lymphoid tissues; IPP: Ileal Peyer's patch; JPPs: Jejunal Peyer's patches; FAE: Follicle associated epithelium; MNC: Mononuclear cell; NK cells: Natural killer cells; IL: Interleukin; IFNγ: Gamma interferon; CD: Cluster of differentiation; dpi: Days post-inoculation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: FD, AE, LO. Performed the experiments: LO, CPA (necropsies, histology), FD (necropsies, flow cytometry), SL and CM (real time PCR). Analysed the data: FD and LO, AE, SL, CM, CPÅ. Contributed to animals/reagents/materials/analysis tools: FD, FL, TC, AS. Wrote the paper: FD and LO, FL, SL, AS, PB, AE, CPÅ, TC. All authors read and approved the final manuscript.

Authors' information

Fabrice Laurent and Françoise Drouet share co-seniorship.

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References

1. Angus KW, Tzipori S, Gray EW (1982) Intestinal lesions in specific-pathogen-free lambs associated with a cryptosporidium from calves with diarrhea. Vet Pathol 19:67–78
2. Snodgrass DR, Angus KW, Gray EW (1984) Experimental cryptosporidiosis in germfree lambs. J Comp Pathol 94:141–152
3. de Graaf DC, Vanopdenbosch E, Ortega-Mora LM, Abbassi H, Peeters JE (1999) A review of the importance of cryptosporidiosis in farm animals. Int J Parasitol 29:1269–1287
4. Chen XM, Keithly JS, Paya CV, LaRusso NF (2002) Cryptosporidiosis. N Engl J Med 346:1723–1731

5. Naciri M, Mancassola R, Fort G, Danneels B, Verhaeghe J (2011) Efficacy of amine-based disinfectant KENOCOX on the infectivity of Cryptosporidium parvum oocysts. Vet Parasitol 179:43–49
6. Lefay D, Naciri M, Poirier P, Chermette R (2001) Efficacy of halofuginone lactate in the prevention of cryptosporidiosis in suckling calves. Vet Rec 148:108–112
7. Tzipori S, Angus KW, Campbell I, Clerihew LW (1981) Diarrhea due to Cryptosporidium infection in artificially reared lambs. J Clin Microbiol 14:100–105
8. Abrahamsen MS, Lancto CA, Walcheck B, Layton W, Jutila MA (1997) Localization of alpha/beta and gamma/delta T lymphocytes in Cryptosporidium parvum-infected tissues in naive and immune calves. Infect Immun 65:2428–2433
9. Fayer R, Gasbarre L, Pasquali P, Canals A, Almeria S, Zarlenga D (1998) Cryptosporidium parvum infection in bovine neonates: dynamic clinical, parasitic and immunologic patterns. Int J Parasitol 28:49–56
10. Wyatt CR, Brackett EJ, Barrett WJ (1999) Accumulation of mucosal T lymphocytes around epithelial cells after in vitro infection with Cryptosporidium parvum. J Parasitol 85:765–768
11. Wyatt CR, Brackett EJ, Perryman LE, Rice-Ficht AC, Brown WC, O'Rourke KI (1997) Activation of intestinal intraepithelial T lymphocytes in calves infected with Cryptosporidium parvum. Infect Immun 65:185–190
12. Wyatt CR, Brackett EJ, Savidge J (2001) Evidence for the emergence of a type-1-like immune response in intestinal mucosa of calves recovering from cryptosporidiosis. J Parasitol 87:90–95
13. McDonald V, Bancroft GJ (1994) Mechanisms of innate and acquired resistance to Cryptosporidium parvum infection in SCID mice. Parasite Immunol 16:315–320
14. McDonald V, Deer R, Uni S, Iseki M, Bancroft GJ (1992) Immune responses to Cryptosporidium muris and Cryptosporidium parvum in adult immunocompetent or immunocompromised (nude and SCID) mice. Infect Immun 60:3325–3331
15. McDonald V, Robinson HA, Kelly JP, Bancroft GJ (1994) Cryptosporidium muris in adult mice: adoptive transfer of immunity and protective roles of CD4 versus CD8 cells. Infect Immun 62:2289–2294
16. Ungar BL, Kao TC, Burris JA, Finkelman FD (1991) Cryptosporidium infection in an adult mouse model. Independent roles for IFN-gamma and CD4+ T lymphocytes in protective immunity. J Immunol 147:1014–1022
17. Korbel DS, Barakat FM, Di Santo JP, McDonald V (2011) CD4+ T cells are not essential for control of early acute Cryptosporidium parvum infection in neonatal mice. Infect Immun 79:1647–1653
18. Lantier L, Lacroix-Lamande S, Potiron L, Metton C, Drouet F, Guesdon W, Gnahoui-David A, Le Vern Y, Deriaud E, Fenis A, Rabot S, Descamps A, Werts C, Laurent F (2013) Intestinal CD103+ dendritic cells are key players in the innate immune control of Cryptosporidium parvum infection in neonatal mice. PLoS Pathog 9:e1003801
19. Barakat FM, McDonald V, Di Santo JP, Korbel DS (2009) Roles for NK cells and an NK cell-independent source of intestinal gamma interferon for innate immunity to Cryptosporidium parvum infection. Infect Immun 77:5044–5049
20. Dann SM, Wang HC, Gambarin KJ, Actor JK, Robinson P, Lewis DE, Caillat-Zucman S, White AC, Jr (2005) Interleukin-15 activates human natural killer cells to clear the intestinal protozoan cryptosporidium. J Infect Dis 192:1294–1302
21. Laurent F, McCole D, Eckmann L, Kagnoff MF (1999) Pathogenesis of Cryptosporidium parvum infection. Microbes Infect 1:141–148
22. McDonald V (2000) Host cell-mediated responses to infection with Cryptosporidium. Parasite Immunol 22:597–604
23. Jung C, Hugot JP, Barreau F (2010) Peyer's patches: the immune sensors of the intestine. Int J Inflam 2010:823710
24. Makala LH, Suzuki N, Nagasawa H (2002) Peyer's patches: organized lymphoid structures for the induction of mucosal immune responses in the intestine. Pathobiology 70:55–68
25. Aleksandersen M, Hein WR, Landsverk T, McClure S (1990) Distribution of lymphocyte subsets in the large intestinal lymphoid follicles of lambs. Immunology 70:391–397
26. Reynolds JD, Morris B (1983) The evolution and involution of Peyer's patches in fetal and postnatal sheep. Eur J Immunol 13:627–635
27. Landsverk T (1984) Is the ileo-caecal Peyer's patch in ruminants a mammalian "bursa-equivalent"? Acta Pathol Microbiol Immunol Scand A 92:77–79
28. Yasuda M, Jenne CN, Kennedy LJ, Reynolds JD (2006) The sheep and cattle Peyer's patch as a site of B-cell development. Vet Res 37:401–415
29. Landsverk T (1987) Cryptosporidiosis and the follicle-associated epithelium over the ileal Peyer's patch in calves. Res Vet Sci 42:299–306
30. Elhmouzi-Younes J, Storset AK, Boysen P, Laurent F, Drouet F (2009) Bovine neonate natural killer cells are fully functional and highly responsive to interleukin-15 and to NKp46 receptor stimulation. Vet Res 40:54
31. Pantenburg B, Castellanos-Gonzalez A, Dann SM, Connelly RL, Lewis DE, Ward HD, White AC, Jr (2010) Human CD8(+) T cells clear Cryptosporidium parvum from infected intestinal epithelial cells. Am J Trop Med Hyg 82:600–607
32. Lacroix-Lamande S, Mancassola R, Naciri M, Laurent F (2002) Role of gamma interferon in chemokine expression in the ileum of mice and in a murine intestinal epithelial cell line after Cryptosporidium parvum infection. Infect Immun 70:2090–2099
33. Olsen L, Boysen P, Akesson CP, Gunnes G, Connelley T, Storset AK, Espenes A (2013) Characterization of NCR1+ cells residing in lymphoid tissues in the gut of lambs indicates that the majority are NK cells. Vet Res 44:109
34. Connelley T, Storset AK, Pemberton A, MacHugh N, Brown J, Lund H, Morrison IW (2011) NKp46 defines ovine cells that have characteristics corresponding to NK cells. Vet Res 42:37
35. Storset AK, Kulberg S, Berg I, Boysen P, Hope JC, Dissen E (2004) NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics. Eur J Immunol 34:669–676
36. Moretta A, Ciccone E, Pantaleo G, Tambussi G, Bottino C, Melioli G, Mingari MC, Moretta L (1989) Surface molecules involved in the activation and regulation of T or natural killer lymphocytes in humans. Immunol Rev 111:145–175
37. Moretta A, Ciccone E, Tambussi G, Bottino C, Viale O, Pende D, Santoni A, Mingari MC (1989) Surface molecules involved in CD3-negative NK cell function. A novel molecule which regulates the activation of a subset of human NK cells. Int J Cancer Suppl 4:48–52
38. Suvarna K, Layton C, Bancroft JD (2012) Bancroft's theory and practice of histological techniques. Elsevier. ISBN: 978-0-7020-4226-3
39. Pakandl M, Sewald B, Drouet-Viard F (2006) Invasion of the intestinal tract by sporozoites of Eimeria coecicola and Eimeria intestinalis in naive and immune rabbits. Parasitol Res 98:310–316
40. Ferret-Bernard S, Remot A, Lacroix-Lamande S, Metton C, Bernardet N, Drouet F, Laurent F (2010) Cellular and molecular mechanisms underlying the strong neonatal IL-12 response of lamb mesenteric lymph node cells to R-848. PLoS One 5:e13705
41. Akesson CP, McL Press C, Espenes A, Aleksandersen M (2008) Phenotypic characterisation of intestinal dendritic cells in sheep. Dev Comp Immunol 32:837–849
42. Tourais-Esteves I, Bernardet N, Lacroix-Lamande S, Ferret-Bernard S, Laurent F (2008) Neonatal goats display a stronger TH1-type cytokine response to TLR ligands than adults. Dev Comp Immunol 32:1231–1241
43. Renaux S, Quere P, Buzoni-Gatel D, Sewald B, Le Vern Y, Coudert P, Drouet-Viard F (2003) Dynamics and responsiveness of T-lymphocytes in secondary lymphoid organs of rabbits developing immunity to Eimeria intestinalis. Vet Parasitol 110:181–195
44. Perez-Cano FJ, Castellote C, Gonzalez-Castro AM, Pelegri C, Castell M, Franch A (2005) Developmental changes in intraepithelial T lymphocytes and NK cells in the small intestine of neonatal rats. Pediatr Res 58:885–891
45. van Elteren PH (1960) On the combination of independent two-sample tests of Wicoxon. Bull Int Statist Inst 37:351–361
46. Theodos CM, Sullivan KL, Griffiths JK, Tzipori S (1997) Profiles of healing and nonhealing Cryptosporidium parvum infection in C57BL/6 mice with functional B and T lymphocytes: the extent of gamma interferon modulation determines the outcome of infection. Infect Immun 65:4761–4769
47. Fuchs A, Colonna M (2011) Natural killer (NK) and NK-like cells at mucosal epithelia: Mediators of anti-microbial defense and maintenance of tissue integrity. Eur J Microbiol Immunol (Bp) 1:257–266
48. Vivier E, Spits H, Cupedo T (2009) Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? Nat Rev Immunol 9:229–234
49. Drouet F, Connelley T, Brunet G, Boysen P, Pende D, Storset AK, Laurent F (2010) Ovine Natural Killer cells from the intestinal mucosal system. In: Boysen ASP (ed) Proceedings of NK cells in veterinary species; satellite

symposium to the 12th meeting of the Society for Natural Immunity & NK 2010: 11th September 2010; Cavtat, Croatia
50. Drouet F, Connelley T, Boysen P, Pende D, Storset AK, Laurent F (2011) Sheep, a model to study intestinal NKp46+ cell subset functions in neonatal infections. In: Immunology S.f.M (ed) Proceedings of 15th International Congress of Mucosal Immunology: 5–9 July 2011; Paris
51. Elhmouzi-Younes J, Boysen P, Pende D, Storset AK, Le Vern Y, Laurent F, Drouet F (2010) Ovine CD16+/CD14- blood lymphocytes present all the major characteristics of natural killer cells. Vet Res 41:4
52. Wyatt CR, Brackett EJ, Perryman LE (1996) Characterization of small intestine mucosal lymphocytes during cryptosporidiosis. J Eukaryot Microbiol 43:66S
53. Li S, Li W, Yang Z, Song S, Yang J, Gong P, Zhang W, Liu K, Li J, Zhang G, Zhang X (2013) Infection of cattle with Cryptosporidium parvum: mast cell accumulation in small intestine mucosa. Vet Pathol 50:842–848
54. Boysen P, Storset AK (2009) Bovine natural killer cells. Vet Immunol Immunopathol 130:163–177
55. Walzer T, Jaeger S, Chaix J, Vivier E (2007) Natural killer cells: from CD3 – NKp46+ to post-genomics meta-analyses. Curr Opin Immunol 19:365–372
56. Cherrier M (2014) [Innate lymphoid cells: new players of the mucosal immune response]. Med Sci (Paris) 30:280–288. (in French)
57. Landsverk T, Halleraker M, Aleksandersen M, McClure S, Hein W, Nicander L (1991) The intestinal habitat for organized lymphoid tissues in ruminants; comparative aspects of structure, function and development. Vet Immunol Immunopathol 28:1–16
58. Mutwiri G, Watts T, Lew L, Beskorwayne T, Papp Z, Baca-Estrada ME, Griebel P (1999) Ileal and jejunal Peyer's patches play distinct roles in mucosal immunity of sheep. Immunology 97:455–461
59. Gregoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, Walzer T (2007) The trafficking of natural killer cells. Immunol Rev 220:169–182
60. Shi FD, Ljunggren HG, La Cava A, Van Kaer L (2011) Organ-specific features of natural killer cells. Nat Rev Immunol 11:658–671
61. Xu X, Weiss ID, Zhang HH, Singh SP, Wynn TA, Wilson MS, Farber JM (2014) Conventional NK cells can produce IL-22 and promote host defense in Klebsiella pneumoniae pneumonia. J Immunol 192:1778–1786
62. McDonald V, Korbel DS, Barakat FM, Choudhry N, Petry F (2013) Innate immune responses against Cryptosporidium parvum infection. Parasite Immunol 35:55–64
63. Drouet F, Lemoine R, El Hmouzi J, Le Vern Y, Lacroix-Lamandé S, Laurent F (2007) Cellules impliquées dans la production précoce d'interféron gamma au cours de l'infection de l'animal nouveau-né par *Cryptosporidium parvum*: participation des cellules NK? In: INRA (ed) Proceedings of Animal Health department conference; Tours, France
64. McDonald V (2011) Cryptosporidiosis: host immune responses and the prospects for effective immunotherapies. Expert Rev Anti Infect Ther 9:1077–1086
65. Akesson CP, McGovern G, Dagleish MP, Espenes A, Mc LPC, Landsverk T, Jeffrey M (2011) Exosome-producing follicle associated epithelium is not involved in uptake of PrPd from the gut of sheep (Ovis aries): an ultrastructural study. PLoS One 6:e22180