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Phenotypic characterisation of intestinal dendritic cells in sheep

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Summary
The present study was undertaken to identify dendritic cells (DCs) in the ileum and rectum of lambs and adult sheep. The distribution of these cells in four different intestinal compartments, i.e. lamina propria, lymphoid follicles, domes and interfollicular areas was assessed, and the presence of these cells in lambs and adult sheep was compared. Specimens were examined by using a number of potential DC markers (CD11c, CD205, MHC class II (MHCII), CD1b and CD209) in immunohistochemical and multicolour immunofluorescent procedures. The ovine ileal and rectal mucosa contain many CD11c+/CD205+ cells with a dendritic morphology, and the majority of these cells co-expressed MHCII. These double-positive cells were also labelled with the CD209 antibody in the lamina propria and interfollicular regions. Only very few cells expressed CD1b. In conclusion, a major DC population in ileum and rectum of sheep co-expressed the CD11c, CD205 and MHCII molecules. The CD209 antibody appeared to be a novel marker for a subpopulation of ovine intestinal DCs.

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells that have the capability to take up, process and present antigen. They provide a major link between innate and adaptive immunity and a defining characteristic is their ability to migrate to and activate naïve T cells in lymphoid organs [1]. This definition is functional rather than phenotypic and a single exclusive marker of DCs has not yet been identified [2,3]. Much research particularly in mice has been directed towards categorising DCs and aligning particular

Abbreviations: DC, dendritic cell; IFA, interfollicular area; PP, Peyer's patch; MHCII, MHC class II; PBS, phosphate-buffered saline; BSA/TBS, bovine serum albumin in Tris-buffered saline; ABC, avidin–biotin complex.

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phenotypes with functional properties [4,5]. However, problems associated with cell isolation in this laboratory species have hindered attempts to characterise intestinal DCs [6,7]. In ruminants such as the sheep, the technique of lymphadenectomy and collection of pseudo-afferent lymph has been used to yield populations of DCs draining from tissues including the skin and gut [8–14]. Data on DCs in sheep have also been gathered from *in vitro* studies of tissue- and monocyte-derived DCs, most recently of pulmonary DCs [15,16]. While information exists on the phenotype and functional properties of DCs draining or isolated from a mucosal tissue such as the intestine in sheep [11,13], few studies have addressed the *in situ* phenotype and distribution of DCs within the intestinal wall [7,17,18]. Knowledge of the *in situ* distribution of intestinal DCs complements functional investigations of isolated cell populations by reconciling the phenotype of cells collected from disrupted tissues or from draining lymph with cell populations present within the intact gut wall. The process of isolation of cells from tissues may alter the cell's phenotype and result in the collection of only a subpopulation of DCs available from the various compartments within the gut wall [19]. Similarly, it can be argued that DCs present in lymph draining the intestine are a subpopulation of DCs at one particular stage of their life history whose compartmental origin from within the gut wall is unknown.

The intestinal immune system is composed of diffuse lymphoid cells in the mucosa and aggregates of lymphoid nodules in organised lymphoid tissue such as the Peyer's patches (PPs) of the small intestine and lymphoid aggregates in colon and rectum. The tissue compartmentalisation of the PP and organised lymphoid tissue at other intestinal sites such as the rectum is similar. In addition to the epithelium and lamina propria of the intestinal mucosa, tissue compartments present in organised intestinal lymphatic tissues include the dome, follicle and interfollicular area (IFA) [20]. Each compartment differs with respect to cellular composition and functions [21–23]. The overall function of the organised lymphoid tissue also differs at sites in the intestine. The large, continuous aggregate of nodules in the ileal PP is responsible for the generation of the vast majority of B cells [24], while other intestinal sites of organised lymphoid tissue such as in the jejunum and rectum have a role in the induction and maintenance of mucosal immunity [25]. The nature of an intestinal site's participation in mucosal immunity can vary and some sites are known to act as a reservoir for pathological agents. The rectum has been shown to harbour enterohaemorrhagic *Escherichia coli* in calves and the scrapie agent in sheep [26,27]. The function at an intestinal site can also differ with the age of the animal. The ileal PP undergoes involution around the age of sexual maturity but the organised lymphoid tissue at other intestinal sites persists throughout life [28].

Cell markers have been used to divide DCs into various subpopulations. The cell markers used and the nature of the defined subpopulations have varied with the species investigated. In sheep, Gupta et al. [29] found that all pseudo-afferent lymph DCs from the drainage area of the subiliac (pre-femoral) lymph node were labelled with antibodies against CD11c, in addition to labelling all alveolar macrophages and a small subpopulation of granulocytes. The CD11c antigen is intimately associated with cell-to-cell and cell-to-matrix adhesion, and in the ovine intestine, CD11c has been reported to be present on interdigitating cells of the PP and on a few villi macrophages [30]. Similarly, Gliddon et al. [31] found in cattle that all pseudo-afferent lymph DCs from the drainage area of the superficial cervical (pre-scapular) lymph node were CD205^{high} but cells of other lineages including T cells and B cells expressed lower levels of this cell surface protein. CD205 is a cell surface protein that belongs to a family of multilectins and probably functions as an endocytic receptor involved in uptake of extracellular antigens [32]. In a study of ovine respiratory tract DCs, McNeilly et al. [16] showed that the majority of parenchymal CD205+ cells exhibited a non-DC-like morphology and did not express MHC class II (MHCII). These authors suggested that single CD205+ cells were not DCs and the observation argues for the use of multiple cell labelling to define DC populations within the gut wall of sheep. In the absence of a single exclusive DC population marker in sheep, Ryan et al. [33] identified three subpopulations of DCs in pseudo-afferent lymph from the drainage area of the subiliac lymph node based on the expression of the CD1b molecule (negative, low or high) and the macrophage marker CD14 (negative or low). Other combinations of cellular markers have been used to define DC subpopulations, adding further evidence of the phenotypic diversity of this cell population in sheep [11,34]. The recent finding that DCs might not be the major lymphatic carriers of certain pathogens from tissues to the lymph node in significant diseases of sheep [35,36] raises the question as to whether ovine DCs express the CD209 (DC-SIGN) molecule, which in humans is a specific marker of DCs that has evoked considerable interest as it also functions as a receptor for a range of pathogens including human immunodeficiency virus types 1 and 2 [37–42].

The aim of the present study was to investigate the phenotypic characterisation of DCs in the ileum and rectum of sheep and lambs. This experimental material was selected to allow a study of the compartmentalisation of DC phenotypes within the gut wall and to enable the comparison of differences in DC phenotype associated with intestinal site and age of animal. Immunohistochemical and immunofluorescence protocols for the multiple labelling of cell populations within tissues were used and the distribution of DCs in various compartments of the organised lymphatic tissue and the diffuse mucosal lymphoid compartments is presented.

Materials and methods

Animals and collection of tissues

Eleven healthy sheep of the Norwegian Dala breed were used in the present study. Tissue samples were collected from six 6-week-old lambs and from five adult ewes aged 2–4 years.

Tissue specimens were taken during pentobarbital anaesthesia (20 mg/kg body weight) or promptly at necropsy. Anaesthetised animals were euthanised with an overdose of pentobarbital before sampling. Specimens for the present study were collected from rectum and ileum at the insertion of the ileocecal ligament. Intestinal tissue samples were

placed with the mucosal side onto thin slices of liver and then frozen in chlorodifluoromethane (Isceon^{TM}) chilled with liquid nitrogen. Liver slices were used to protect the intestinal specimens against mechanical damage and to facilitate cryostat sectioning. The tissues were stored at −70 °C until further preparation.

Procedures have been conducted in accordance with the laws and regulations controlling experiments using live animals in Norway, that is, the Animal Welfare Act of 20 December 1974 and the Regulation of Animal Experimentation of 15 January 1996.

Antibodies

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

Immunohistochemistry

Cryostat sections were cut 7 μm in thickness, mounted onto poly-lysine-coated slides and stored at −70 °C before use. The sections were air dried at room temperature for 1 h, fixed in acetone for 10 min and then air dried for another 10 min. The sections were rinsed and rehydrated in phosphate-buffered saline (PBS) for 5 min.

An indirect immunoperoxidase staining technique was performed on the sections by using an avidin–biotin complex (ABC) method with the aid of a commercial kit (Vector Laboratories, Burlingame, CA, USA). Endogenous peroxidase was inhibited by treatment with 0.05% phenyl hydrazine (Fluka, Bucs, Switzerland) in PBS for 40 min at 37 °C. To avoid non-specific binding of the biotinylated antibody, a blocking solution containing either normal horse serum (for the monoclonal antibodies) or normal goat serum

Table 1 Primary antibodies used in the present study.

Antibody	Specificity	Cellular expression	Source	Cross reactivity[a]	Reference
Mouse anti-ovine MHCII (SBU-II, 28-1)	pan-MHCII	Dendritic cells, macrophages, B lymphocytes	The University of Melbourne, Melbourne, Australia		Puri et al. [66]
Mouse anti-bovine CD11c (BAQ153A)	CD11c	Dendritic cells, some macrophages	VMRD, Inc., Pullman, WA, USA	Sheep, goat	Howard et al. [67]
Mouse anti-bovine CD205 (MCA1651)	CD205	Dendritic cells, some T cells, some B cells, some epithelial cells	Serotec, Ltd., Oxford, UK	Sheep	Gliddon et al. [31]
Mouse anti-human macrophage, (EBM11)	CD68	Macrophages, monocytes	Dako, Glostrup, Denmark	Sheep, cattle and others	Bielefeldt-Ohmann et al. [68]
Polyclonal rabbit anti-human CD209 (DC-SIGN, AHP627)	CD209/ DC-SIGN	Dendritic cells	Serotec, Ltd., Oxford, UK	Not stated	Geijtenbeek et al. [69]
Mouse anti-bovine CD11b (MCA1425, clone CC126)	CD11b	Macrophages, monocytes, granulocytes	Serotec, Ltd., Oxford, UK	Sheep	Gupta et al. [30]
Mouse anti-bovine CD1w2 (MCA2058)	CD1b	Dendritic cells, cortical thymocytes	Serotec, Ltd., Oxford, UK	Sheep, goat, horse, dog	Bujdoso et al. [8]
Polyclonal rabbit anti-human CD3 (A 0452)	CD3	All T cells	Dako, Glostrup, Denmark	Sheep, goat, cattle and others	Jones et al. [70]
Mouse anti-ovine T cells (SBU-T4)	CD4	T-helper cells	The University of Melbourne, Melbourne, Australia		Maddox et al. [71]
Mouse anti-ovine B cells (DU2-74-25)	CD21	B cells, follicular dendritic cells	Kind gift from Dr. W. Hein		Hein et al. [72]
Mouse anti-ovine macrophages (MCA920)	CD14/VPM65	Ovine macrophages	Serotec, Ltd., Oxford, UK		Gupta et al. [73]

[a]For antibodies not raised against sheep antigens.

(for the polyclonal antibodies) diluted 1:50 in 5% bovine serum albumin in Tris-buffered saline (BSA/TBS) was applied to the sections for 20 min at room temperature. The blocking solution was carefully tapped off the slides. The antibodies were diluted in 1% BSA/TBS, added to the slides and incubated overnight at 4 °C. The next day biotinylated horse anti-mouse IgG and goat anti-rabbit IgG were diluted 1:200 in 1% BSA/TBS and added to the slides for 30 min at room temperature. The ABC–horseradish peroxidase complex solution was prepared at least 30 min prior to use, according to kit instructions. The sections were incubated with the complex solution for 30 min. All incubations were done in a slowly rotating humid chamber, and rinsing between each step was in PBS for 5 min. Peroxidase activity was visualised either by using a commercial diaminobenzidine tetrachloride (DAB)-enhancement kit (Immuno-Pure Metal Enhanced DAB Substrate Kit, Pierce, Rockford, IL, USA) or by incubation with a solution consisting of 4 mg 3-amino-9-ethylcarbazole (AEC), 800 μL *N*,*N*-dimethylformamide, 14 mL 0.1 M acetate buffer, pH 5.2, and 150 μL 3% H_2O_2. The reaction was stopped by rinsing in PBS after 5 and 15 min. The slides were counterstained with Mayer's haematoxylin for 1 min, rinsed in PBS and mounted. To control for non-specific binding, all runs included a control section where the primary antibodies were replaced by 1% BSA/TBS. Tissue sections labelled by immunohistochemistry were examined in a Leica DM RXA microscope and digital images collected using a Spot RX slider digital camera.

Immunofluorescence

Frozen tissue sections were made as described for immunohistochemistry. The slides were air dried, fixed in acetone and treated with 20% BSA/TBS at room temperature for 10 min in order to block non-specific binding. The blocking solution was tapped off and a mixture composed of either two or three primary antibodies was made by dilution in 1% BSA/TBS. This mixture was left on the sections for 1 h at room temperature. The mixture of primary antibodies consisted of antibodies produced in rabbit and/or murine monoclonal antibodies of different isotypes. Cocktails of Alexa fluorescent secondary antibodies against rabbit IgG (H+L) (Molecular Probes, Inc., Eugene, OR, USA) and/or against various mouse immunoglobulin classes and isotypes such as IgG_1, IgG_{2a}, IgG_{2b} and IgM (Molecular Probes, Inc.) corresponding to the primary antibodies were applied. The secondary antibodies were diluted 1:400 in 1% BSA/TBS and centrifuged at 1400 rpm for 5 min before application of the supernatants to the sections for 1 h. All incubations were done in a slowly rotating humid chamber, and rinsing between each step was in PBS for 5 min. The slides were mounted in polyvinyl alcohol at pH 8. Control sections included (a) replacement of both primary and secondary antibodies by 1% BSA/TBS, (b) replacement of primary antibodies by 1% BSA/TBS and (c) replacement of secondary antibody with secondary antibody of irrelevant isotype.

Sections labelled by immunofluorescence were stored at 4 °C until examination in a confocal microscope (Zeiss LSM510) equipped with an LSM 510 laser confocal unit (Carl Zeiss, Jena, Germany). Fluorochrome filters corresponding to the fluorochrome-labelled antibodies in the section investigated were used. The emitted signal was recorded in separate monochrome digital images, one for each labelled cell marker. Subsequently, appropriate pseudo-colours were added and the images were merged for identification of double and triple immunolabelling.

Evaluation of immunolabelled slides

The presence of immunolabelled cells was evaluated in the four tissue compartments of the intestinal immune system, namely the dome, follicle, IFA and lamina propria. The presence of labelled cells in these compartments was classified as none (−), few (+), moderate (++) or many/numerous (+++).

Results

General remarks

Many cells in the ileal and rectal wall were immunohistochemically labelled using antibodies against MHCII, CD11c, CD205, CD209, CD11b and CD68 (Table 2, Figure 1A–J). The distribution and phenotypic characteristics of these cell populations did not show any major age-related differences and unless otherwise stated the following observations refer to findings in both lambs and adult sheep. Very few CD1b+ cells were present (Figure 1K), while CD14+ cells were not detected in the intestinal tissues studied.

Table 2 Distribution of cells labelled with dendritic cell markers in different intestinal compartments.

Compartments	Cell markers					
	MHCII	CD11c	CD205	CD68	CD11b	CD209
Dome	+++	+++	+++	+[a]	++[b]	−
Follicle	+++	−	++	++	−	−
Interfollicular area	+++	++	++	+[a]	++	++
Lamina propria	+++	+++	+++	+[a]	++[b]	++

Numbers of labelled cells were subjectively assessed and graded as none (−), few (+), moderate (++) and many/numerous (+++).
[a]The number of labelled cells varied from few to moderate.
[b]The number of labelled cells varied from moderate to numerous.

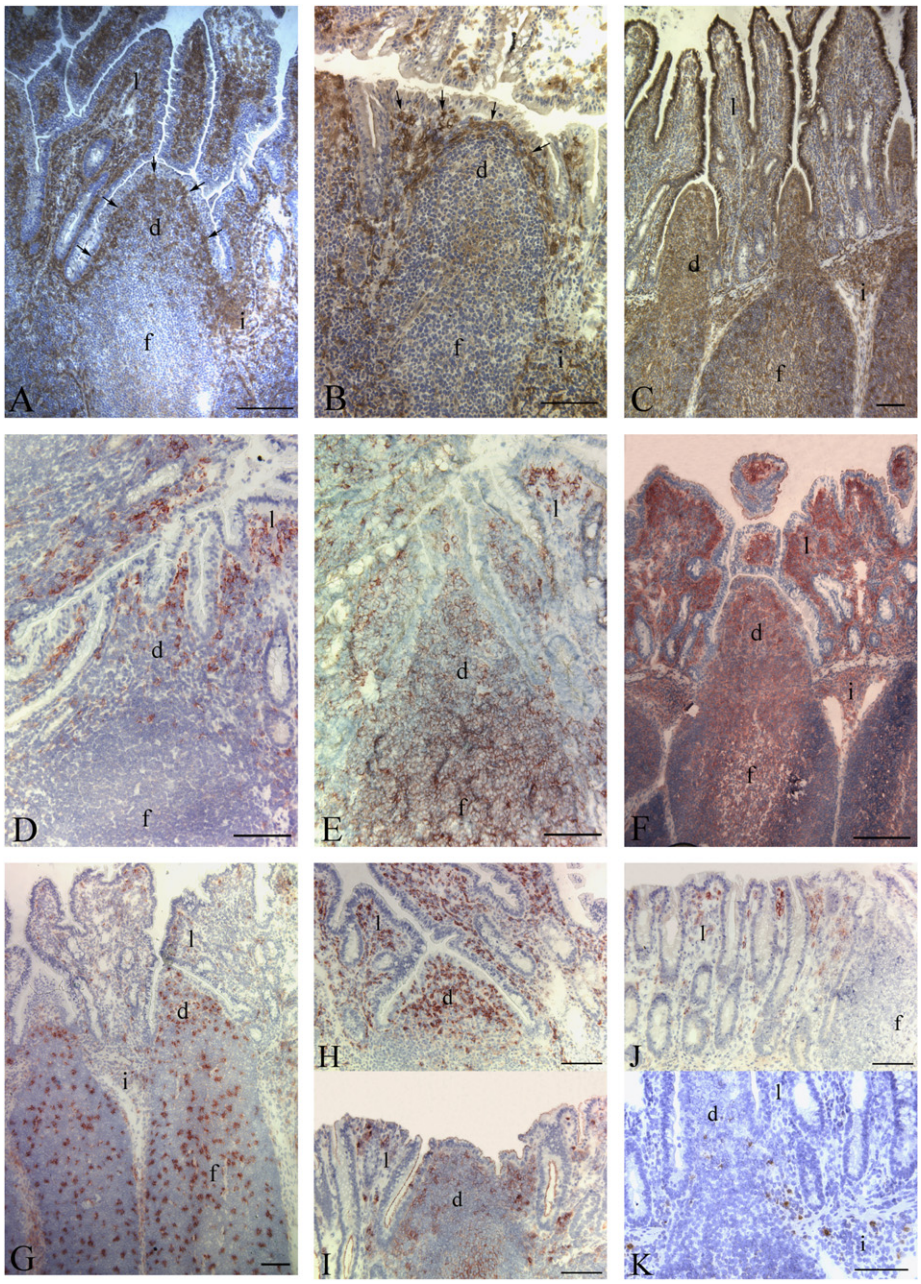

Figure 1 Immunohistochemical labelling (ABC method) of ileum and rectum of sheep. (A and B) Immunolabelling with the BAQ153A antibody against CD11c in ileum (A) and rectum (B) of lambs shows moderate to many labelled cells in the dome, interfollicular area and lamina propria. Note the layer of CD11c+ cells present immediately below the epithelial basement membrane (arrows) and the absence of CD11c+ cells in the follicles. (C) Immunolabelling with the MCA1651 antibody against CD205 in the ileum of a lamb. Numerous CD205+ cells are present in the dome, follicles, IFA and lamina propria. Strong labelling is also found in the intestinal epithelium. (D and E) Immunolabelling for CD11c (D) and CD205 (E) in the rectum of adult sheep (2–4 years). The pattern of immunolabelling is similar to that seen in young animals. (F) Immunolabelling with the SBU-II antibody against MHCII in the ileum of a lamb. Labelled cells are numerous throughout the intestinal mucosa. (G) Immunolabelling with the EBM 11 antibody against CD68 in the ileum of a lamb. Labelling against CD68 is most prominent in the follicles and a moderate number of cells is present in this area. (H and I) Immunolabelling with the MCA1425 antibody against CD11b in the ileum (H) and rectum (I) of a lamb. Note that CD11b+ cells are numerous in the ileum compared with the rectum. (J) Immunolabelling with the AHP627 antibody against CD209 in the rectum of a lamb. CD209+ cells are present in the lamina propria adjacent to the epithelium. (K) Immunolabelling with the MCA2058 antibody against CD1b in the ileum of a lamb. Very few CD1b+ cells are observed in the intestine. When present, these cells are located in the dome and IFA. Lamina propria (l), dome (d), follicle (f), IFA (i). Scale bars (A–K): 100 μm.

MHCII expression was abundant throughout the intestinal wall, while the antibodies against CD11c and CD205 labelled a moderate to large number of cells. In comparison, fewer cells showed expression for the macrophage marker CD68 and the human-specific DC marker CD209. Many of the labelled cells possessed a DC morphology, in that the cells had an irregular shape and often showed cellular extensions (Figure 2A). The molecules CD11c, CD205, CD1b, CD11b and CD68 did not co-localise with the markers CD3, CD4 or CD21 in the ileal and rectal mucosa (data not shown).

CD11c+/CD205+

Many cells were labelled with CD11c antibody in the dome and lamina propria, and these cells were particularly prominent underneath the epithelium, where they formed a layer close to the epithelial basement membrane (Figure 1A and B). The CD205+ cells formed a network of moderately labelled cells in the dome. In addition, many strongly labelled CD205+ cells were distributed throughout the lamina propria and the dome regions, and many were found in the subepithelial region (Figure 3A and B). A moderate number of cells showing labelling for CD11c and CD205 were present in the IFA (Figure 2B). Double-immunofluorescence labelling revealed that CD11c and CD205 were often expressed in the same cells; however, a few single labelled CD11c+ and CD205+ cells were observed (Figure 2B and C). Triple labelling showed that the majority of the CD11c+/CD205+ cells were also MHCII+ (Figure 3A).

CD68

Few to a moderate number of CD68+ cells were present in the dome, lamina propria, IFA and follicles of the ileum and rectum (Table 2, Figure 1G). Only a few CD68+ cells showed labelling with CD11c (Figure 2D). In the dome, the few CD68+/CD11c+ cells present showed cytoplasmic labelling for the CD68-antibody and surface membrane labelling for the CD11c-antibody (Figure 2E). Nearly all CD68+ cells co-expressed CD205. In other words, most CD68+ cells were CD68+/CD205+/CD11c− and only few were CD68+/CD205+/CD11c+ (Figure 3B). CD68+ cells in the lamina propria were an exception and these cells showed surface membrane labelling for CD68, and co-expressed CD205, CD11c and CD209 (Table 3).

CD11b

There were many CD11b+ cells in the dome, IFA and lamina propria of the intestine and CD11b+ cells were also detected in the follicle-associated epithelium and absorptive epithelium. In general, CD11b+ cells were more numerous in the dome and lamina propria of the ileal PP than in comparable regions of the rectum (Figure 1H and I). Most cells of the ileum and rectum that expressed CD11b did not show labelling for the antibody against CD68. Only a few CD11b+ cells co-expressed CD11c, and these cells showed a weak labelling for the CD11c marker.

CD209

In the ileal and rectal mucosa, the cells that were labelled with the human DC-specific marker CD209 also expressed CD11c, CD205 and MHCII (Figure 3C and D). While quadruple immunohistochemical labelling was not performed, the results from the various combinations of double- and triple-labelling experiments suggested that a subpopulation of CD11c+/CD205+/MHCII+ cells was labelled with the CD209 antibody. The CD209+ cell subpopulation was predominantly present in the lamina propria and IFA and very few CD209+ DCs were present in the domes of the sheep ileal and rectal follicles (Figure 3E). None was detected in the lymphoid follicles (Table 2). In the lamina propria of both the ileum and rectum, CD209+ cells showed a sub-epithelial localisation (Figures 1J and 3C).

CD1b

The marker of ovine afferent lymph DCs, CD1b, was not co-expressed on cells of the ileal and rectal mucosa with any of the other markers used in the present study. In general, very few cells showed labelling for CD1b in any of the tissue compartments examined in the ileum or rectum (Figure 1K). Rarely, an occasional dome was found to contain a moderate number of CD1b+ cells. CD1b+ cells were not detected in the lamina propria of any intestinal tissues examined.

Lymphoid follicles

Apart from MHCII and CD205, DC markers were expressed on few follicular cells in the present study. Cells in the lymphoid follicles of the ileum and rectum did not label with the antibodies against CD11c (Figure 1A and B), CD11b, CD209 or CD1b (Table 2). MHCII expression was present in a large proportion of cells in the light central zone of the lymphoid follicles, whereas there was less MHCII expression in cells of the dark peripheral zone (Figure 1F). There was a network of CD205+ cells in the lymphoid follicles (Figure 1C and E), in addition to the CD68+/CD205+ cell population of tingible body macrophages.

Intestinal sites of organised lymphoid tissue

The immunohistochemical examination of tissues from the ileum and rectum of lambs and adult sheep showed that numerous cells labelled with antibodies against markers associated with DCs (Tables 2 and 3). The overall distribution of these markers within the various tissue compartments examined was similar in both the ileum and rectum. The only exception was the CD11b+ cells that were abundant in the ileum, while they were only present in a moderate number in the rectum (Figure 1H and I).

Age of animals

The ileal PP of the older animals was involuted and only a few rudimentary follicles were detected in the submucosa in two of the five adult ewes included in the present study. A few CD11c+/CD205+/MHCII+ cells were found adjacent to

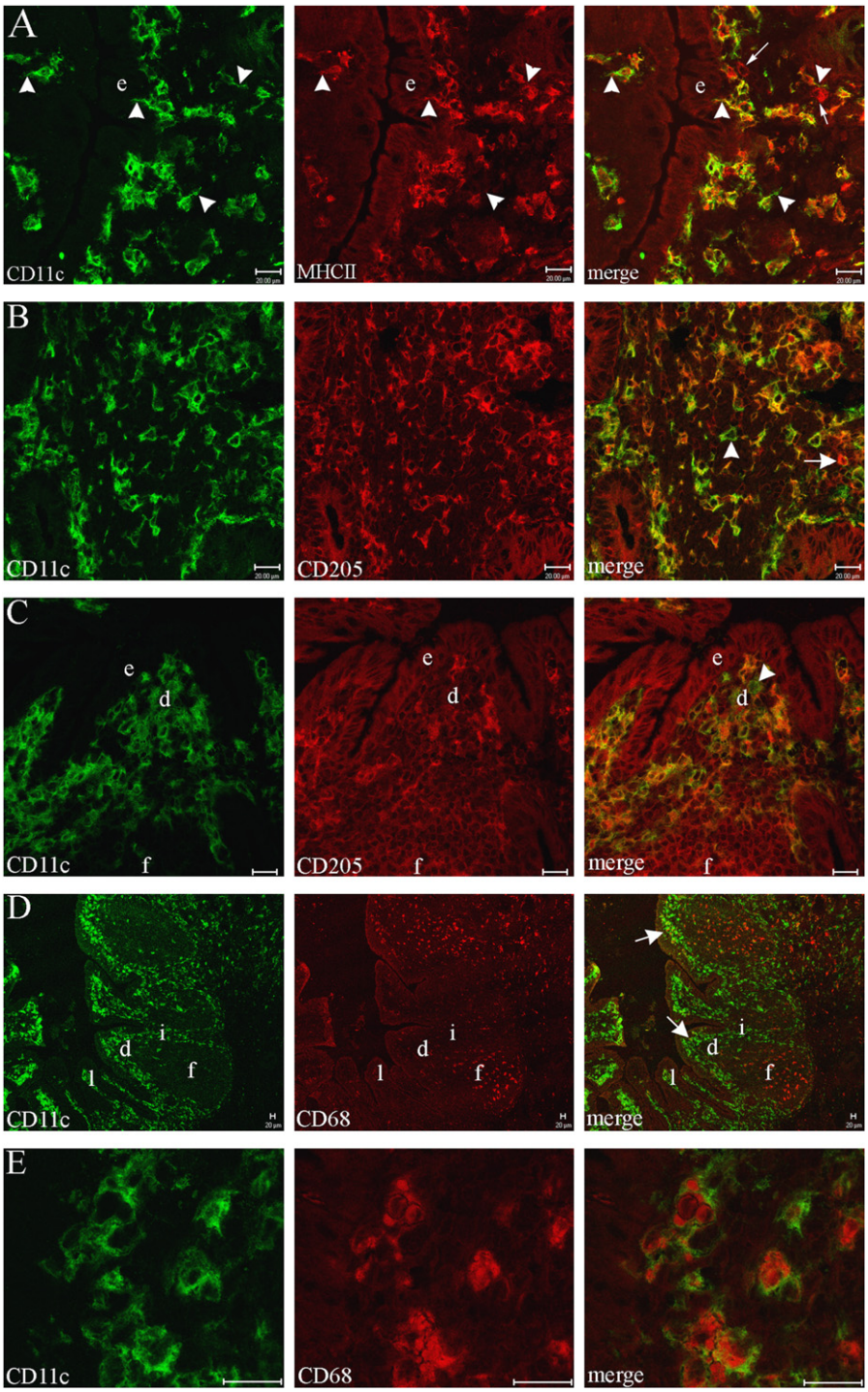

Figure 2 *In situ* characterisation of DCs in ovine intestinal mucosa, represented here by micrographs from the ileum and rectum of lambs. Double immunofluorescence labelling for markers is indicated for each micrograph. Double-labelled cells in merged images appear yellow. (A) Immunolabelling for CD11c and MHCII in lamina propria, rectum. Most CD11c+ cells show co-labelling for MHCII, and in the lamina propria these cells are located underneath the epithelium and have an irregular shape with extensions (arrowheads). There were few single MHCII+ cells (thin arrows). (B and C) Most cells labelled with the BAQ153A antibody against CD11c show co-labelling for CD205 in IFA, rectum (B) and in dome, ileum (C). Few cells are single CD11c+ (arrowheads) or CD205+ (arrow). (D and E) Immunolabelling for CD11c and CD68 in rectum. (D) CD68+ cells are most prominent in the follicles and few to a moderate number of labelled cells are present in the domes, IFA and lamina propria. CD11c+ cells are moderate to numerous in the dome, IFA and lamina propria, but are not present in the follicles. Few of the cells labelled with the CD68-antibody show co-labelling for CD11c (arrows). (E) A few CD11c+/CD68+ double-labelled cells are present in the domes and these show cytoplasmic staining for CD68 and surface membrane labelling for CD11c. Lamina propria (l), dome (d), follicle (f), IFA (i), epithelium (e). Scale bars (A–E): 20 μm.

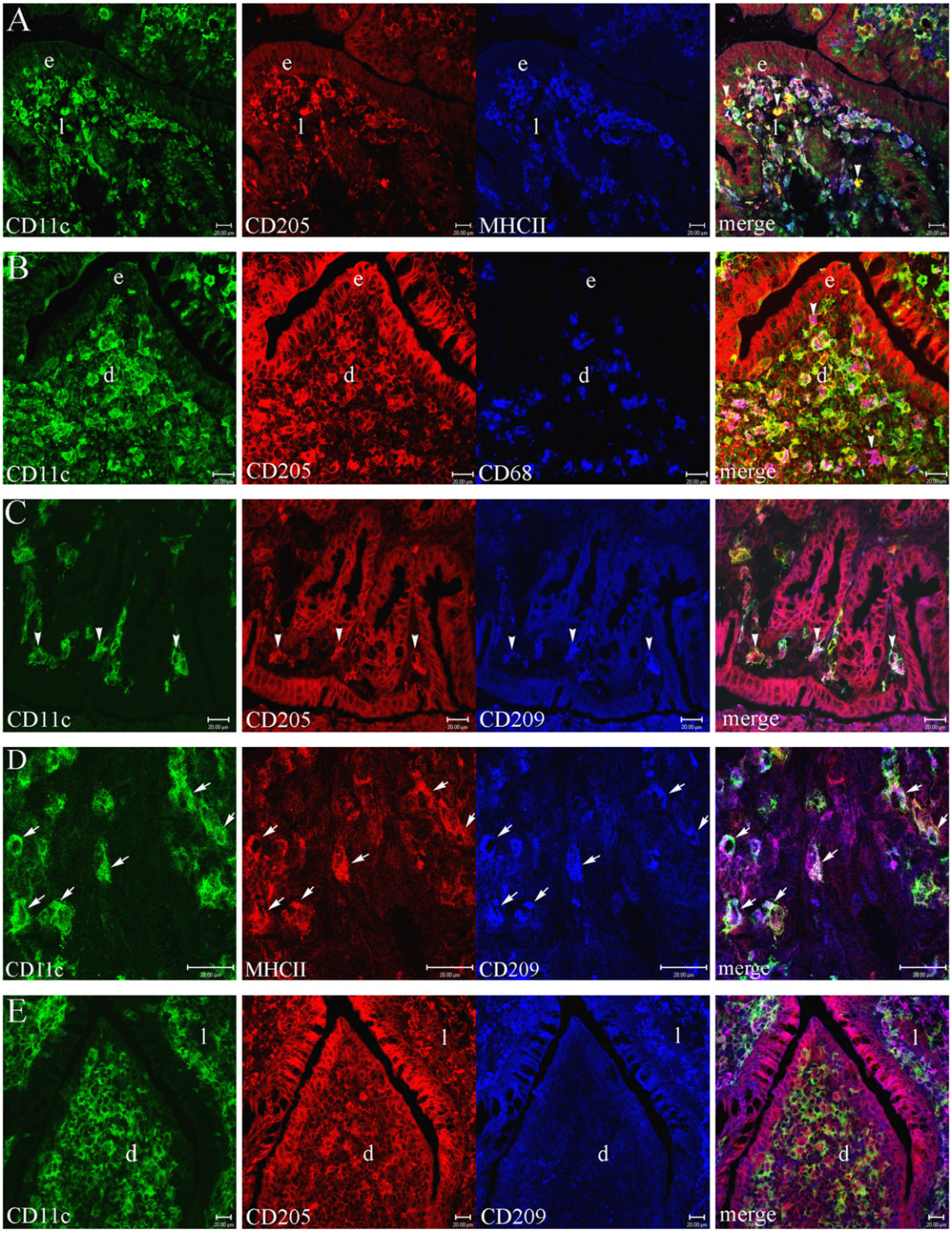

Figure 3 *In situ* characterisation of DCs in ovine intestinal mucosa, represented here with micrographs from the ileum and rectum of lambs. Multicolour immunofluorescence labelling for markers is indicated for each micrograph. Triple-labelled cells in merged images appear white. (A) Immunolabelling for CD11c, CD205 and MHCII in lamina propria, rectum. Most CD11c+/CD205+ cells show co-labelling with MHCII. Only a few cells showed labelling with CD11c and CD205 and not with MHCII (arrowheads, yellow cells). Note the predominantly subepithelial distribution of the triple-labelled CD11c+/CD205+/MHCII+ cells. (B) Immunolabelling for CD11c, CD205 and CD68 in dome, ileum. Many cells show labelling with antibodies against CD11c and CD205, and a moderate number of cells show co-labelling with these two markers (yellow cells in merged image). A moderate number of CD205+ cells show co-labelling for CD68 (arrowheads, pink cells), but few cells were triple labelled (CD11c+/CD205+/CD68+). (C and D) Triple immunolabelling for CD11c/CD205/CD209 in lamina propria, rectum (C) and CD11c/MHCII/CD209 in IFA, ileum (D). CD209+ cells in lamina propria and IFA, to a large extent, show co-labelling for CD11c, CD205 and MHCII (arrowheads/arrows, white cells). (E) Immunolabelling for CD11c, CD205 and CD209 in dome, ileum. CD209+ cells are not detected in the domes. Lamina propria (l), dome (d), follicle (f), IFA (i), epithelium (e). Scale bars (A–E): 20 μm.

Table 3 Co-labelling of dendritic cell (DC) markers in intestinal populations of CD11c+, CD205+ cells and double-positive CD11c+/CD205+ cells.

Compartments	CD11c+ cells co-labelling with DC markers				CD205+ cells co-labelling with DC markers				CD11c+/CD205+ cells co-labelling with DC markers		
	CD205	CD209	CD68	MHCII	CD11c	CD209	CD68	MHCII	CD209	CD68	MHCII
Dome	++	—	+	+++	++	—	++	++[a]	—	+	+++
Follicle	—	—	—	—	—	—	++	+++	—	—	—
Interfollicular area	++	++	+	++[a]	++	+[b]	+[b]	++	++	+	++[a]
Lamina propria	+++	++	++	+++	+++	++	++	+++	++	++	+++

Numbers of labelled cells were subjectively assessed and graded as none (—), few (+), moderate (++) and many/numerous (+++).
[a]The number of labelled cells varied from moderate to numerous.
[b]The number of labelled cells varied from few to moderate.

these rudimentary follicles in the expanded submucosal IFA, but a similar presence of this DC population was to be found in areas lacking follicles at the same level in the submucosa.

Discussion

The present study compared the co-localisation of markers for DCs on cells within the tissue compartments of the ileum and rectum of adult sheep and lambs, and shows that a major DC population co-expressed CD11c, CD205 and MHCII. This CD11c+/CD205+/MHCII+ DC population was widespread within these tissue compartments. To our knowledge, this is the first report on triple labelling of ovine DCs in peripheral tissues. Recent studies in other peripheral tissues such as the ovine lung have shown by double immunolabelling that the majority of lung DCs co-express CD11c and CD205 [15] and many CD11c+ cells were MHCII+ [15,16]. These observations are consistent with the findings of the present study that the majority of DCs in peripheral tissues co-express these three molecules. *In vitro* studies of ovine monocyte-derived DCs have also reported the co-expression of CD11c and MHCII [43]. Significantly, in the present study, CD11c+/CD205+/MHCII+ DCs were often strategically located in the lamina propria underneath the epithelium and in the subepithelial dome that overlies the intestinal lymphoid follicles. The identification of a sub-epithelial layer of DCs in ileum and rectum of sheep suggests an optimal localisation for contact with antigens entering the intestinal mucosa. DCs are believed to function as sentinels at mucosal surfaces where they encounter both infectious agents and innocuous antigens derived from food components and the normal microflora. A similar distribution of intestinal DCs has been reported in mice where DCs are prominent in the dome and IFAs of the murine PPs [17].

Studies in mice have shown that DCs in the dome have the ability to migrate to the IFAs, the follicles or the regional lymph nodes after uptake and processing of antigens [5]. In the present study, CD11c+/CD205+/MHCII+ cells constituted a major cell population in the dome, lamina propria and submucosal interfollicular T cell areas of the ovine ileum and rectum. Studies in mice have focused on differences in the composition of DC populations between intestinal compartments [5]. In the mouse, the PPs harbour three predominant populations of CD11c+ DCs, namely CD11b+, CD8α+ and CD8α—/CD11b— cells. Of these subtypes, CD11b+ DCs were localised in the dome, whereas CD8α+ DCs were exclusively located in the IFA [5]. In the ovine ileal and rectal tissues, only a few CD11c+ cells expressed CD11b irrespective of tissue compartment, indicating that CD11b did not identify a large population of intestinal DCs in sheep. The major DC phenotype identified in the present study (CD11c+/CD205+/MHCII+) was equally represented in dome, lamina propria and submucosal interfollicular T cell areas; however, a consideration of other DC markers revealed evidence for site-specific compartmentalisation of DC populations.

The anti-human CD209 (DC-SIGN) antibody appeared to be a novel marker of ileal and rectal DCs in sheep. In humans, CD209 is a specific marker of DCs and the receptor mediates interactions between DCs and resting T cells [37]. Several studies have focused on CD209 because of its ability to bind human immunodeficiency virus types 1 and 2 at mucosal surfaces and transmit the virus to T cells in humans [38,42,44]. Furthermore, CD209 can function as a receptor for a range of pathogens including *Neisseria meningitidis*, *Helicobacter pylori*, *Candida* sp., *Aspergillus* sp. and *Leishmania* sp. [45–47]. In the human intestine, CD209+ cells localise predominantly in the IFAs, in clusters in the dome and in the lamina propria, and particularly in the rectal lamina propria [39]. In sheep, cells labelled with the CD209 antibody were mainly located in the IFAs and lamina propria of the intestine with no obvious predilection for the rectal mucosa. Interestingly, CD209+ DCs were not detected in the domes at either of the intestinal sites examined in the present study. Whether the CD209 negative DCs of the dome constitute a compartment-specific population in sheep and whether the anti-CD209 antibody identifies a pathogen-recognition molecule in sheep require further investigation.

An additional compartmentalisation of DCs in the ileum and rectum of sheep that differs from observations in the mouse and man was the absence of identifiable DC populations from certain compartments within the gut wall. Cells with DC phenotypes were absent from the submucosal follicles in both the ileum and rectum. While

cell phenotypes consistent with follicular DCs (weak reticular pattern of CD205), tingible body macrophages (CD68+/CD205+) and B cell populations (gradient of MHCII expression towards follicle centre) were identified, a distinct DC population was absent. Germinal centre DCs with a haematopoietic origin and an antigen-transport function, as distinct from the autochthonous antigen-retaining follicular DCs, have been described in mice and humans [48,49]. Evidence for the existence of this DC population in the submucosal intestinal follicles of sheep was not identified. DC populations were also not detected in the epithelium of the intestinal sites investigated, although a significant DC population was present immediately subjacent to the epithelium. The close proximity of lamina propria DCs to the intestinal epithelium could indicate that this is an important site for antigen uptake. Indeed, orally administered proteins have been found to associate with antigen-presenting cells of the lamina propria to a much greater extent than with the antigen-presenting cells of the PPs in mice [50]. Some of these subepithelial DCs have transepithelial dendrites and are able to collect antigens directly from the intestinal lumen [51]. Lamina propria DCs in other species may also have similar characteristics, as has recently been suggested in pigs [52]. CD11c+ dendrites extending from the lamina propria into the overlying epithelium were observed in the present study (data not shown) but whether these dendrites reach into the gut lumen and are able to sample luminal antigen require further investigation in sheep.

Much previous work investigating DCs in sheep has been based on the study of DCs collected from afferent lymph [8,9,11,30,33,53–55]. The present *in situ* study of DCs shows that CD1b, a cell marker useful for identifying afferent lymph DCs, was not expressed on significant populations of intestinal DCs. It should be noted that most studies of afferent lymph DCs are based on the collection of pseudo-afferent lymph after the removal of a surgically accessible lymph node such as the superficial cervical (pre-scapular) or subiliac (pre-femoral) lymph node [56]. Few studies have collected pseudo-afferent lymph from the small intestine of sheep [13] and none has collected lymph from the rectum. A large proportion (60–75%) of DCs in pseudo-afferent lymph of sheep expressed the CD1b molecule [33,54,55] and three subtypes of DCs in lymph were identified based on expression of the CD1b (negative, low or high) and CD14 (negative or low) [33]. In the present study, the CD1b molecule was rarely detected on DCs in the ovine ileal and rectal mucosa, which differs from the 2–3% CD1b+ leucocytes in pseudo-afferent intestinal lymph reported by Hein et al. [8,13]. CD1b+ cells did not show labelling with any of the other DC markers used in the present study. This observation indicates that the phenotype of ileal and rectal DCs differs from that of DCs in afferent lymph. The CD1b molecule has been reported to be present in high numbers on DCs in the thymus, spleen and lymph nodes [57]. The current opinion is that DCs in afferent lymph and the draining lymph node are more mature than the populations in peripheral tissues [3,58,59]. It is postulated that as DCs migrate to the local lymph node after antigen uptake, these cells change their phenotype by up- or down-regulation of certain molecules and/or expression of accessory molecules [58,59]. It could be hypothesised that CD1b is a marker of this more differentiated or mature DC subpopulation. The occasional observation of a dome region containing numerous CD1b+ cells invites speculation as to whether these labelled cells were in the process of emigration.

The CD11c molecule has proved to be a useful marker of DCs in sheep. This marker is expressed by a subpopulation of cells in ovine afferent lymph, and CD11c+ cells constituted 51–85% of DCs in pseudo-afferent lymph collected from the drainage area of ovine subiliac (pre-femoral) lymph node [30]. Gupta et al. [29] reported that a CD11c antibody labelled all afferent lymph DCs enriched from pseudo-afferent subiliac lymph. Intestinal pseudo-afferent lymph in sheep had a lower proportion of CD11c+ cells and this population comprised approximately 16% of the cells [13]. The present study shows that the CD11c+ cells constitute a moderate to high number of cells with DC morphology in the intestinal lamina propria, dome and IFA. Taken together, these findings may indicate that CD11c is expressed by a population of DCs both in the intestinal mucosa and in intestinal afferent lymph, in contrast to the CD1b molecule. A similar distribution of CD11c+ cells to the dome and IFA has been reported in the small intestine of sheep [29]. Expression of CD11c has also been reported in various tissues including the thymus, lymph nodes and lungs [13,15,30], and *in vitro* studies have shown that ovine monocyte-derived DCs express CD11c [43].

CD14+ cells were not detected in the intestinal tissues in this study. This observation is in accordance with studies showing that resident macrophages in intestinal tissues do not express CD14 (reviewed in Smith et al.) [60].

CD205 was present on all DCs in afferent lymph in cattle [31], whereas 65–90% of DCs in sheep afferent lymph expressed this marker [33,61,62]. In the bovine intestine, CD205+ cells were present in the lamina propria and more extensively in the dome region of the PPs [31]. In addition, CD205+ cells were identified in the T cell areas (interdigitating cells) and in the B cell follicles (B cells) of bovine lymph nodes [31]. The present study found that numerous cell types in the ileum and rectum of sheep express CD205. This cellular marker was present with variable labelling intensity on several cell types including DCs, some macrophages, follicle populations of B cells and/or follicular DCs and also intestinal epithelial cells. The presence of a DC marker on a range of cell types illustrates the difficulties of characterising DC phenotypes in the absence of a pan-DC marker. The present study used double and triple labelling of cells to overcome this limitation to a certain extent, but the species variation in molecules expressed by DCs and the existence of subtypes representing different maturation stages or functional status continue to be a challenge for the study of intestinal DCs [5,59].

The challenge of distinguishing between cell types is particularly apparent when considering the relationship between DCs and macrophages. Moderate numbers of CD68+ cells were present in the ileum and rectum particularly intermingled in the sub-epithelial layer of DCs of the lamina propria and dome. In the present study, a subpopulation of the intestinal CD68+ cells was strongly labelled for CD205 and a few of the cells showed labelling for CD11c. These antigen-presenting cell subpopulations could represent macrophages or DCs. Studies in mice have shown that circulating monocytes can differentiate into DCs

in peripheral tissues [63,64], and a recent study of the murine small intestine found that the majority of DCs were derived from monocytes [65]. These findings indicate that expression of monocyte/macrophage markers on differentiating DCs might be possible. Whether subpopulations of cells that co-expressed macrophage markers such as CD68 or CD11b and DC markers represented differentiating DCs of monocyte origin is not known. The relationship between macrophages, DCs expressing macrophage markers and DCs labelled with the anti-CD209 antibody is also not known.

In summary, numerous antigen-presenting cells are present in the ovine ileum and rectum. In the ileal PP and rectal aggregated lymphoid tissue, most DCs were found in the dome, IFA and lamina propria and expressed CD11c, CD205 and MHCII. Double and triple immunolabelling showed co-expression of these markers in DCs. The anti-CD209 antibody appeared to be a novel marker of a population of ovine intestinal DCs.

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