Characterization of CD79αcy+ cells in placentas from ruminants

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ABSTRACT

Previous work carried out to characterise different immune cells in ruminant placentas found strong CD79αcy nuclear labelling in cells histologically resembling trophoblast cells. In the attempt to characterize this cell population, placentomes collected from cattle, sheep and water buffaloes were examined by immunohistochemistry with single and double labelling using monoclonal antibodies (mAb) against B lymphocytes and trophoblast cells. Most CD79αcy + cells co-expressed placental lactogen or cytokeratin and were CD21 and MHC class II negative strongly suggesting they do not have a B cell origin. However, a potential immunological role of these cells cannot be ruled out and it is currently unknown if the findings described may have an impact on physiological knowledge, health, and or diseases pathogenesis in ruminants.

1. Introduction

The placenta is a transitory organ formed by maternal and foetal tissues apposition. The main function of this tissue is to ensure exchange of nutrients and waste products between mother and foetus, while avoiding adverse reactions of the mother's immune system towards the foetus (Kaufmann and Burton, 1994; Steven, 1975; Wooding and Flint, 1994).

Although the placentas from all eutherian species share common organization and functions, there are important variations in their anatomy (Igwebuike, 2006; Schlafer et al., 2000). Ruminant placenta is classified as chorioallantoic, villous and cotyledonary (Sammin et al., 2009). This last term refers to its gross anatomical features, exhibiting discrete areas of attachment, the placentomes, formed by interaction of patches of the chorioallantois (foetal cotyledon) with the endometrium (maternal caruncle). The chorioallantois is lined on its external surface by cells of the trophectodermal epithelium, assuming specialized functions and referred as trophoblast cells (Clark et al., 1990; Igwebuike, 2006).

During previous experiments characterizing the cellular immune response in the placentomes from cattle and water buffalos the presence of CD79 positive cells was described (Cantón et al., 2013, 2014; Maley et al., 2006). Although CD79 has been described to be expressed almost exclusively by B cells Chu and Arber (2001), Cantón et al. (2013, 2014)

described the presence of rare individualised CD79αcy+ mononucleated and binucleated cuboidal cells in ruminant placentomes, diffusely distributed and aligned with the endometrial epithelium in the maternal caruncles and foetal villi. Therefore, the true identity of these CD79αcy+ cells was questioned because morphologically and histologically they resembled trophoblast cells.

The aim of this work is to further characterize this placental cell population in ruminants using immunohistochemistry (IHC) with a panel of different polyclonal (pAb) and monoclonal (mAb) antibodies, previously described as specific for identifying trophoblast and B lymphocytes.

2. Materials and methods

2.1. Tissue sampling

Paraffin-wax embedded ruminant placentome tissue blocks fixed with zinc salts fixative (ZSF) (pH 7.0–7.4) were used for this study. They came from all available uninfected negative controls animals from infectivity studies previously published. Bovine placentomes (n = 110) came from 11 Holstein, Aberdeen Angus cross or Belgian Blue cross dams aged 16 to 48 months at days 84, 98, 112, 126 (Macaldowie et al., 2004), 154, 168, 182 (Maley et al., 2003), 224, 238, 252 and 266 of gestation (Benavides et al., 2012).

Similarly, ovine samples (n = 60) were collected from pregnant Scottish Blackface ewes aged 3–5 years at days 85, 92, 99, 106, 113, 120, 127 of gestation (Maley et al., 2009) and bubaline samples (n = 20) came from Mediterranean water buffalos (*Bubalus bubalis*) cows aged 4 to 14 years old at days 98 and 118 of gestation (Konrad et al., 2012).

2.2. Immunohistochemistry

For each paraffin-wax embedded placentome blocks, 5 μm-sections were cut, mounted on polylysinated glass slides (Superfrost® Plus; Menzel-Gläser, Braunschweig, Germany) and dried overnight at 37 °C. Then they were dewaxed in xylene and hydrated through graded ethanol solutions. Endogenous peroxidase was blocked by incubating with 3% hydrogen peroxide in methanol for 30 min at room temperature. Non-specific labelling was reduced by using 25% normal goat serum in Tris-buffered saline (TBS). Immunohistochemistry was performed using an EnVision + kit (Dako North America Inc, Carpinteria, USA). Tissue sections were incubated overnight at 4 °C with each pAb or mAb antibodies (diluted in TBS) that recognize: pAb raised in rabbits against recombinant bovine placental lactogen (bPL) and mAb raised in mice that specifically recognize $CD79_{\alpha cy}$, CD21, MHC class II (MHC-II) and cytokeratin (CK) (see more details in Table 1). After washes with TBS, anti-rabbit or anti-mouse IgG polymers (Dako EnVision®) were employed as secondary antibodies for pAb or mAb primary antibodies, respectively. The reaction was revealed after incubation with DAB chromogen (brown labelling, Dako EnVision®) during 8 min. Slides were then washed with distilled water and counterstained using Mayer's Haematoxylin for 1 min before being dehydrated and mounted. Sections of ZSF-fixed bovine retropharyngeal lymph nodes were used as positive control tissues.

Double labelling using the combination of the previous pAb and mAb were performed on the same tissue sections, using a two-step IHC protocol. Briefly, immediately after revealing the first labelling reaction using DAB chromogen, slides were washed with distilled water, and the subsequent immune-labelling was preceded by new endogenous peroxidase and non-specific labelling blocking steps. Slides were then incubated overnight at 4 °C with the second primary antibody, as previously described for the single IHC protocol. Sections were then incubated with the appropriate IgG polymers (Dako EnVision®) and labelling was revealed using a different chromogen (purple labelling, Vector VIP®; Vector Labs, Burlingame, USA). After washes with distilled water, slides were counterstained with Methyl Green (Vector Labs), dehydrated and mounted.

3. Results and discussion

The cell-surface B-cell receptor (BCR) is formed by distinct subunits: the ligand-binding portion is surface immunoglobulin (sIg, usually IgM or IgD), and the signal transduction portion are CD79. The latter is present in virtually every mature and immature B cells and

Table 1
Antibodies used to characterize the CD79^{+} cell population in the placentomes from different ruminant species.

Clone	Target	Dilution
HM57[a]	CD79a receptor in B lymphocytes	1:200
CC21[b]	CD21 receptor in mature B lymphocytes and follicular dendritic cells	1:2000
SW73.2[b]	MHC Class II antigens	1:200
AE1/AE3[a]	Cytokeratin	1:100
–	Bovine placental lactogen[c]	1:6000

[a] Dako Cytomation, Glostrup, Denmark (catalogue number R715901).
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encompasses two transmembrane proteins, CD79a and CD79b, which form a disulfide-linked heterodimer. Transcription of CD79a and CD79b can be identified in the majority of B cells and is absent in other cell types. However, transcription of CD79a is not a constant finding in plasma cells. BCR expression is essential for the maturation of precursor B cells and is required for the survival of mature B lymphocytes outside the bone marrow (Chu and Arber, 2001). Clone HM57 was raised against the intracytoplasmic portion of recombinant CD79a (Mason et al., 1991). In normal human cells, cell surface and cytoplasmic CD79 expression is usually restricted to B lymphocytes. In this study, $CD79_{\alpha cy}$ labelled the membrane of cells in cortical lymphoid follicles and germinal centres from bovine positive control lymph nodes. Some $CD79_{\alpha cy}$ positive cells were also observed in the paracortical and medullar region.

Positive nuclear labelling in large $CD79_{\alpha cy}$ labelled mononucleated or binucleated cells were observed in all placentomes collected from cattle, sheep and water buffalos (see Fig. 1**A–C**, respectively). These cells were diffusely distributed, mainly aligned with the endometrial epithelium in the caruncles and in foetal villi. In ovine tissues, a larger number of positive cells were observed when compared with the bovine and bubaline placentomes. No clear differences were observed in the number of $CD79_{\alpha cy}$ cells throughout gestation in bovine and ovine tissues. However, in previous studies $CD79_{\alpha cy}$ + cell were found to be lower in dams carrying non-viable foetuses when compared with the ones carrying viable foetuses (Cantón et al., 2013; Maley et al., 2006). This difference could be due to the large areas of necrosis present in the placentomes from cases with non-viable foetuses. The disruption that these lesions caused to the trophoblasts may explain a decrease in detectable cells.

In the current study fusiform elongated CD21 labelled cells were present in the caruncles and in some cases they were forming small aggregates in the caruncle stalks and in the base of the placentomes of the three studied ruminant species (see Fig. 1**D–F**). No positive cells were observed in fetal villi. Similar number and localization of CD21 positive cells were observed throughout gestation in the bovine and ovine placentomes. Similar labelling to the one observed with the mAb HM57 was observed in the retropharyngeal lymph node after incubation with CC21 mAb (cell membrane labelling), clearly identifying lymphoid follicles.

Positive MHC-II labelling was observed in different cells, morphologically resembling macrophages and fibroblasts chiefly located in the maternal caruncle and in the base of the placentomes in placental tissues from the three species (see Fig. 1**G–I**). No clear differences in the expression of MHC-II antigen was observed throughout gestation in bovine and ovine placentomes. Large number of MHC-II expressing cells (cell membrane labelling) were observed in the retropharyngeal lymph node with different morphology and distributed in cortical, paracortical and medullar region.

When selected placental tissues collected from cattle, sheep and water buffalos were incubated with the mAb AE1/AE3, the trophoblast layer of mononuclear and binucleated cuboidal cells were labelled (see Fig. 1**J–L**). This labelling was consistently observed throughout gestation in the bovine and ovine placentomes.

Large mononucleated and binucleated cuboidal cells were bPL labeled in the placentomes collected from cattle, sheep and water buffalos (cytoplasmatic labelling) (See Fig. 1**M–O**). These positive cells were mainly located in the fetal villi although some of them were located in the maternal caruncle. Mononucleated cells showed a stronger cytoplasmic labelling when compared with the bPL positive binucleated cells. No clear differences in the number of bPL expressing cells were observed throughout gestation in the placental tissues collected from cattle and sheep.

No co-expression of $CD79_{\alpha cy}$ with CD21 or MHC-II was observed in the ruminant placentomes (Fig. 2**A** and **B**). CD21 is an important co-receptor in synergy with the BCR, playing a critical role in B cell responses (Tedder et al., 1997; O'Rourke et al., 1998) and MHC-II
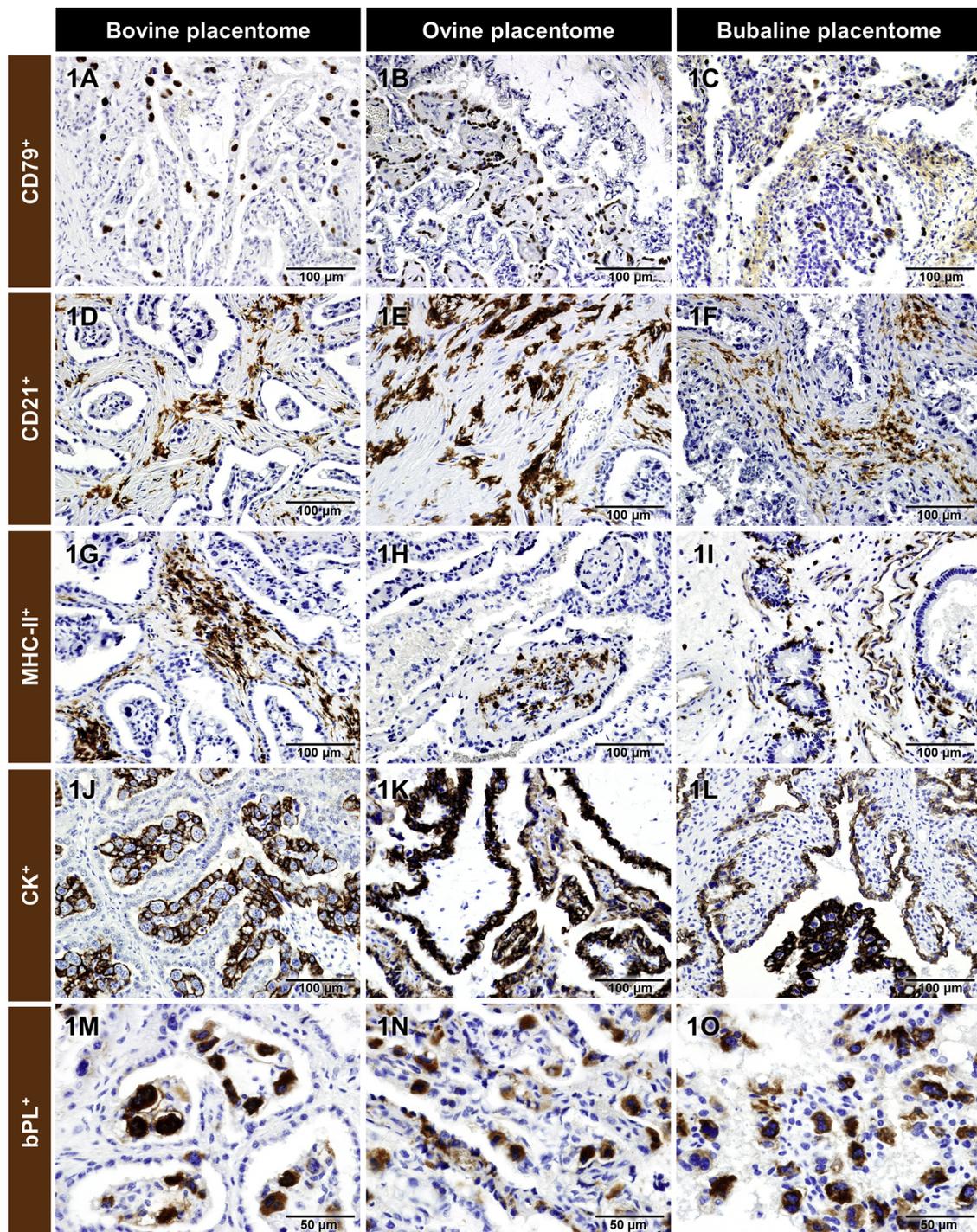
Fig. 1. A–O: Examples of single labelling with the mAb and pAb selected to identify different cell population in the ruminant placentomes. (1A) CD79+ cells in the placentome collected from a cow at day 224 of gestation. (1B) CD79+ cells in the placentome from a ewe at day 127 of gestation. (1C) CD79+ cells in the placentome from a water buffalo at day 98 of gestation. (1D) CD21+ cells in the placentome from a cow at day 224 of gestation. (1E) CD21+ cells in the placentome from a ewe at day 127 of gestation. (1 F) CD21+ cells in the placentome from a water buffalo at day 98 of gestation. (1 G) MHC-II+ cells in the placentome from a cow at day 224 of gestation. (1 H) MHC-II+ cells in the placentome from a ewe at day 127 of gestation. (1I) MHC-II+ cells in the placentome from a water buffalo at day 98 of gestation. (1 J) CK+ cells in the placentome from a cow at day 224 of gestation. (1 K) CK+ cells in the placentome from a ewe at day 127 of gestation. (1 L) CK+ cells in the placentome from a water buffalo at day 98 of gestation. (1 M) bPL+ cells in the placentome from a cow at day 224 of gestation. (1 N) bPL+ cells in the placentome from a ewe at day 127 of gestation. (1O) bPL+ cells in the placentome from a water buffalo at day 98 of gestation.

molecules are expressed selectively on the surfaces of cells involved in immune responses, such as B cells, activated T-cells, macrophages and dendritic cells (Puri et al., 1987; Janeway et al., 1988). The lack of labelling of CD21 and MHC-II in the $CD79_{\alpha cy}$+ cells in the ruminant placentomes, suggests that they are not related to B cells.

Most of the $CD79_{\alpha cy}$ positive cells (mononucleated and binucleated)

were co-expressing CK in their surface in the bovine and bubaline placentomes (Fig. 2C). Large number of $CD79_{\alpha cy}$ cells in the ovine placentomes were not expressing CK. Similarly, CD79 positive nuclear staining was observed in most of the bPL expressing cells (cytoplasmic labelling). An equal number of mononucleated and binucleated cells were $CD79^+/bPL^+$. In accordance with the observation of $CD79^+/CK^+$
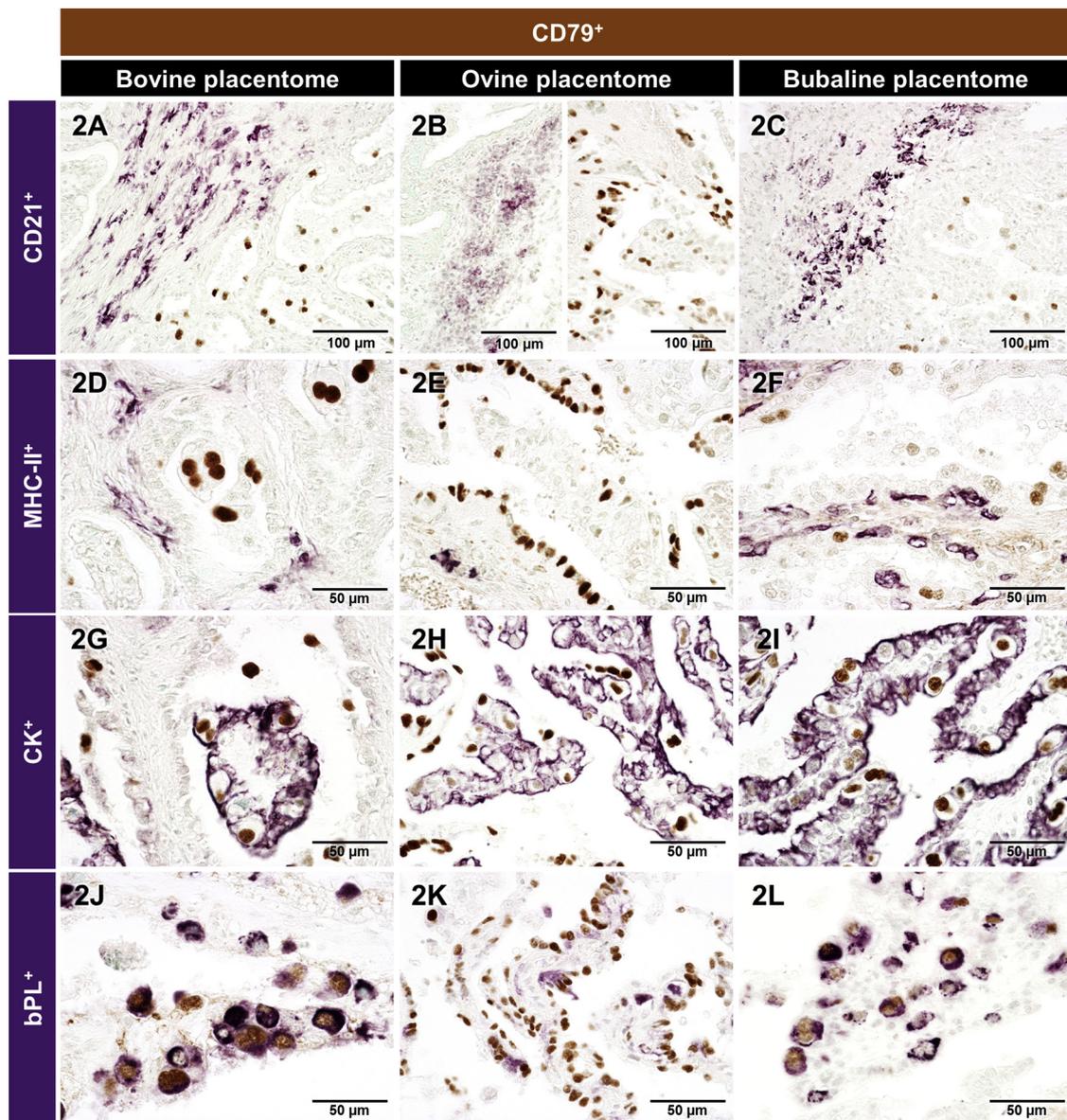
Fig. 2. A-L: Examples of double labelling with mAb against CD79 + cells and other mAb or pAb selected to characterize this population in the ruminant placentomes. CD79 + (brown labelling) and CD21 + (purple labelling) cells in the placentome collected from a cow at day 224 of gestation (2A). CD79 + (brown labelling) and CD21 + (purple labelling) cells in the placentome collected from a ewe at day 127 of gestation (2B). CD79 + (brown labelling) and CD21 + (purple labelling) cells in the placentome collected from a water buffalo at day 98 of gestation (2C). CD79 + (brown labelling) and MHC-II + cells (purple labelling) in the placentome from a cow at day 224 of gestation (2D). CD79 + (brown labelling) and MHC-II + cells (purple labelling) in the placentome from a ewe at day 127 of gestation (2E). CD79 + (brown labelling) and MHC-II + cells (purple labelling) in the placentome from a water buffalo at day 98 of gestation (2 F). CD79 + (brown labelling) and CK + (purple labelling) in the placentome from a cow at day 224 of gestation (2 G). CD79 + (brown labelling) and CK + cells (purple labelling) in the placentome from a ewe at day 127 of gestation (2 H). CD79 + (brown labelling) and CK + cells (purple labelling) in the placentome from a water buffalo at day 98 of gestation (2I). CD79 + (brown labelling) and bPL + cells (purple labelling) in the placentome from a cow at day 224 of gestation (1 J). CD79 + (brown labelling) and bPL + cells (purple labelling) in the placentome from a ewe at day 127 of gestation (2 K). CD79 + (brown labelling) and bPL + cells (purple labelling) in the placentome from a water buffalo at day 98 of gestation (2 L). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cells in ovine placentomes, large number of $CD79^+/bPL^-$ cells were observed in the same tissues. In bovine placentomes, most of the $CD79^+/bPL^-$ cells were located in the maternal caruncle, while most of the $CD79^+/bPL^+$ were present in the fetal villi. Few numbers of $CD21^+/MHC-II^+$ cells were present in the maternal caruncle of placentas from cattle, sheep and water buffalos. No co-expression of CD21 with CK or bPL and co-expression of MHC-II with CK or bPL were observed in the analyzed placentome tissues. Most of the bPL positive cells were CK positive, particularly the ones observed in the fetal villi and few bPL^+/CK^- cells were present in the maternal caruncles.

Placental lactogen is a polypeptide hormone secreted by trophoblastic cells and believed to play an important role in the growth and development of the foetus in some mammalian species (Beckers et al., 1980; Byatt et al., 1992; Handwerger, 1991). Placental lactogen in bovine species have a very particular distribution in maternal and foetal compartments. Maternal concentrations remain under 2 ng/ml during the whole pregnancy period, whereas foetal concentrations are higher, ranging from 25 to 30 ng/ml on day 90 of gestation and decreasing to 5–15 ng/mL near term (Beckers et al., 1982). Most of the $CD79_{\alpha cy}$ + cells observed in the placentas examined were also expressing bPL in their cytoplasm and CK in their surface showing that at least part of this population was composed by trophoblast cells, as

previously hypothesized.

Interestingly, CD79$_{αcy}$ labelling is consistently and exclusively intranuclear in a very specific population of trophoblast-like cells in ruminant placentomes, while it usually stained the cytoplasm or cell surface in lymphoid tissues used as positive controls. The presence of this marker in this trophoblast population may suggest an immunological function, since it has been previously demonstrated that trophoblast cells are able to recognize different pathogens or damaged tissue and demonstrate phagocytic activity (Schlafer et al., 2000; Mor, 2008; Koga et al., 2009). In response they secrete specific cytokines and recruit other immune cells (i.e. macrophages, T regulatory cells, NK cells) supporting a regulatory interaction between the trophoblast and the maternal immune system in order to support the growing foetus (Schlafer et al., 2000; Mor, 2008; Koga et al., 2009). The results obtained in this study do not allow to speculate if the cell population studied has an immunological function and/or if it plays a role in physiology or disease pathogenesis, on ruminants.

Furthermore, the cross-reaction of the mAb HM57 with other proteins is possible and labelling of smooth muscle in different tissues has been shown when using this mAb (Leong et al., 2003). Also, non-specific background staining can often mimic specific labelling due to different interactions (Ward and Rehg, 2014). Antibodies often recognize small peptides that can be present in other antigens (molecular mimicry) in other cells. Moreover, the same antigen may be present in multiple cell types.

Further studies using more techniques and antibodies are required in order to elucidate the significance of the strong positivity of the nuclei from this cell type to CD79$_{αcy}$ antibody.

Acknowledgments

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