Veterinary Immunology and Immunopathology 169 (2016) 79–84

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

NCR1+ cells appear early in GALT development of the ovine foetus and acquire a c-kit+ phenotype towards the end of gestation

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ARTICLE INFO

Article history:
Received 1 June 2015
Received in revised form
21 December 2015
Accepted 22 December 2015

Keywords:
NK cells
Foetus
GALT
Innate lymphoid cells
NCR1
Ovine

ABSTRACT

The amount, distribution and phenotype of ovine NCR1+ cells were investigated during developing GALT from day 70 of gestation. Antibodies against CD3 and CD79 were used to identify the compartments of GALT, and the localization of NCR1+ cells were correlated within these structures. Markers CD34 and c-kit, in addition to Ki67, were used to investigate possible origin and the stage of development of the NCR1+ cells. NCR1+ cells were present as single cells in the subepithelial tissue as early as 70 days of gestation, and were predominantly present in the T cell rich IFAs and domes as these intestinal wall compartments developed. While NCR1+ cells proliferated more intensively at mid-gestation (70–104 days), the number of NCR1+ cells also expressing c-kit, increased at the end of gestation. In conclusion, NCR1+ cells appeared early in T cell areas of the gut and displayed a phenotype consistent with intermediate stages of cNK cells and/or a subpopulation of ILC22.

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 to be inductive sites of the immune response. The lamina propria that is covered with the absorptive epithelium is considered to be an effector site and it is found along the whole length of the gut (Brandtzaeg, 2009).

The gut lymphoid tissue has been described to develop in three stages (Coles et al., 2006; Constantinides et al., 2014). Initially, precursors of hematopoietic innate lymphoid cells (ILC) migrate into the intestinal wall and are evenly distributed where future lymphoid tissue will form (Bando et al., 2015). In the second stage, the precursor ILCs bind to and interact with stromal cells to secrete cytokines and chemokines to promote the further maturation of the lymphoid tissue anlage and the clustering of cells. In the third and final stage, mature lymphocytes migrate into these foetal structures to generate adult lymphoid tissues with B and T cell compartments. Many lymphocytes in these structures are recognized as ILCs, and include conventional natural killer (cNK) cells, which are a recently characterized group of cells that is central in organogenesis as well as mucosal homeostasis and post-natal immunity (Spits et al., 2013). Several ILC subgroups express the natural cytotoxicity receptor 1 (NCR1), also denoted NKp46, but the expression pattern varies between species. In humans, NCR2 (NKp44) is thought to be analogous to murine NCR1+ ILCs and appears in the small intestine early in the second trimester of gestation in human foetuses (Hoorweg et al., 2012), while in mice NCR1+ ILCs first appears after birth (Reynders et al., 2011; Sawa et al., 2010). Apart from humans and mice, little is known about ILCs in mammalian foetal tissues.

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http://dx.doi.org/10.1016/j.vetimm.2015.12.008
0165-2427/

Table 1
Age of the foetuses in each of the four groups used in this study.

Group nr.	1	2	3	4
N foetuses	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

The ovine gestation period is 142–152 days.

Sheep have been used as a model for GALT development in ruminants, and many studies have addressed the difference in lymphopoietic function between the jejunal and ileal PPs (Griebel and Hein, 1996; Landsverk, 1984). The sheep gestation length 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 around 97 days of gestation in the ileum (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 leucocyte 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 revealed at least two subpopulations. More than half of the NCR1+ cells showed high expression of CD16, which is a receptor associated with cytotoxicity whereas the other fraction probably represented other ILCs (Olsen et al., 2015, 2013). Analysis of NCR1+ cells in lamb foetuses can yield information of early events in the formation of PPs. To date, there are no reports on NCR1+ cells in ruminant foetuses.

In the present study, we sought to detect 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 describe the localization of NCR1+ cells in the different areas. Further, the presence of progenitor and/or proliferation markers on NCR1+ cells in the foetal gut 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 × 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 (Supplementary Table 1). The foetuses used in this study were untreated control foetuses used in other studies and described in detail elsewhere (Press et al., 1998, 1996; Renström et al., 1996), thus avoiding to kill more animals in accordance with the principles of the 3Rs (Replacement, Reduction and Refinement).

Supplementary Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2015.12.008.

2.2. Antibodies

The antibodies (Abs) used in this study were directed against: ovine NCR1/NKp46 (EC1.1; IgG1) kind gift from 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., 2015, 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 h 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 without primary or secondary Abs or both Abs. Non-specific (auto)fluorescence was identified by comparing labelled sections with control sections using identical exposure times.

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, 49 Endow GFP and 38 Rhodamine for Alexa Fluor Secondary Abs 350, 488 and 594, 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 different compartments were denoted as –: <1 cell (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 antibodies against the pan-T cell marker CD3 and the B cell marker CD79, we identified structures associated with the development of GALT (Fig. 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 (70–83 days of gestation), the epithelium formed small folds. Small numbers of B cells were observed dispersed in lamina propria under the epithelium. In a few sections of the jejunum, small B cell clusters had started to take form under some epithelial folds at this gestation period, 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 became higher and started to form villi. Recognizable domes were seen above B cell follicles. 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