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REGULAR ARTICLE

Intestinal macrophages in Peyer's patches, sacculus rotundus and appendix of Angora rabbit

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Abstract The largest pool of macrophages in the body is harboured by the intestinal mucosa. As the principal phagocytic component of the immune system, macrophages are essential for maintaining mucosal homeostasis as they prevent commensal bacteria from adhering to mucosal epithelial cells. This study provides a RAM11 immunohistochemical and electron microscopic investigation of the existence, localization and distribution of intestinal macrophages in organized gut-associated lymphoid tissue (GALT), including Peyer's patches (PPs), the sacculus rotundus (SR) and the appendix, in the Angora rabbit. Although rabbit intestinal macrophages did not express the tissue macrophage marker macrosialin (CD68), they expressed RAM11. RAM11-positive intestinal macrophages were mostly localized to the subepithelial dome region, interfollicular area and germinal centres (GCs) of the GALT and the lamina propria or submucosa of the ileum and jejunum devoid of PPs and were also observed in the follicle-associated epithelium of PPs, but not in that of the SR and appendix. RAM11-positive macrophages containing engulfed apoptotic bodies were present in the GCs of the lymphoid follicles in the GALT. Electron microscopy further revealed multiple macrophages containing apoptotic bodies within the GCs of the follicles in the GALT. Some macrophage aggregations were observed in the GC and between the GC and the corona region of the follicles in the SR and appendix. Rabbit intestinal macrophages thus undertake both potent phagocytic activity and the efficient scavenging of apoptotic cells. Immunohistochemical data suggest that RAM11 can be reliably used for the determination of intestinal macrophages in the GALT of rabbits.

Keywords Angora rabbit · GALT · Intestine · Macrophages · RAM11

Introduction

Because of their closer phylogenetic relationship to primates than to rodents, rabbits (Leporidae) are the most common model organism used in basic and clinical research into human diseases (Schnupf and Sansonetti 2012; Haines et al. 2016). Rabbits closely resemble humans both anatomically and physiologically and suffer from several diseases known to have human counterparts. Rabbits were used by Louis Pasteur for the development of the rabies vaccine. Research carried out in rabbits is significant for multiple medical branches, including dermatology, cardiology, opthalmology and internal medicine. Rabbits are also used for antibody production, the detection of the presence or absence of disease and research into immunology and contagious diseases (Leenaars and Hendriksen 2005). Different from other species, the primary antibody repertoire, which develops through the somatic diversification of Ig genes, depends on the development of the gut-associated lymphoid tissue (GALT) in rabbits. Previous research has demonstrated that, in rabbits, the intestinal microbiota has a significant role in the development of both the GALT and humoral immunity (Lanning et al. 2000). In rabbits, the organized GALT comprises aggregated lymphoid follicles, which form Peyer's patches (PPs) along the small intestine, and the sacculus rotundus (SR) and the appendix (Haley 2003; Sinha and Mage 2004; Mage et al. 2006;

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Beyaz et al. 2010a, b; Zhai and Lanning 2013). The small intestinal GALT constitutes over 50% of the total body lymphoid tissue of the rabbit and also accounts for the smaller size of the spleen in this species. PPs in the small and large intestines are very large in the rabbit. Because of their role in transporting luminal antigens and bacteria, PPs are referred to as the immune sensors of the intestine (Makala et al. 2002; Beyaz and Aştı 2004; Cesta 2006; Newberry 2008; Butler and Sinkora 2013; Randall and Mebius 2014).

Rabbits are non-ruminant herbivores and hindgut fermenters. Their rather large caecum contains nearly 40% of the intestinal content and is capable of effectively digesting the high-fibre diet of this species (Kohles 2014; Haines et al. 2016). The rabbit is also known to possess a species-specific lymphoid structure formed by the bulb-like expansion of the terminal ileum, which is connected to the proximal caecum. This lymphoid structure, which is generally referred to as the SR and is composed of aggregated lymphoid follicles, is also known as the iliac tonsil, caecal tonsil or circular PP (Beşoluk et al. 2006; Mage et al. 2006; Beyaz et al. 2010b). The rabbit caecum ends in the appendix, which is a smaller tubular part of the caecum. Reports indicate that, similar to the bursa of Fabricius of avian species, the SR and appendix are the primary lymphoid tissues responsible for B lymphocyte development, particularly in young rabbits, and transform into secondary lymphoid structures such as PPs in adults (Lanning et al. 2000; Weinstein et al. 1994; Pospisil and Mage 1998; Dasso et al. 2000; Hanson and Lanning 2008; Nicholas et al. 2008; Zhai and Lanning 2013).

PPs, SR and appendix, in which IgA-producing cell precursors are formed and clonal B cell production takes place in response to antigen-specific T lymphocyte activation, comprise the inductive sites of the organized GALT. These sites are composed of the same domains, namely, follicles, the interfollicular region, subepithelial dome (SED) and follicle-associated epithelium (FAE; Jung et al. 2010). The FAE shows differences from the epithelium lining the intestinal villi, the most significant difference being the presence of microfold cells (M cells) among the columnar epithelial cells (Brayden et al. 2005; Corr et al. 2008; Mabbott et al. 2013). M cells are membranous specialized cells that transfer the luminal antigens that they encapsulate with their basolateral cytoplasm to lymphoid cells by transcytosis (Elmore 2006; Beyaz et al. 2010a, b; Butler and Sinkora 2013). Apart from the intestinal epithelial cells and M cells, the FAE of PPs includes many intra-epithelial lymphocytes and a few mononuclear phagocytes, including macrophages and dendritic cells (Brayden et al. 2005; Corr et al. 2008). All mucosal tissues contain GALT effector sites in the form of disseminated lymphoid tissue with diffuse distribution throughout the lamina or substantia propria (Yan et al. 2003). The cellular components of these sites comprise T-cells, the majority being $CD4^+$, and IgA plasma cells, fewer IgM and IgG plasma cells and rare B-cells, macrophages and dendritic cells (Pabst 1987; Kelsall and Strober 1999; Makala et al. 2002; MacDonald 2003; Newberry 2008).

The largest pool of macrophages in the body is harboured by the intestinal mucosa. Macrophages are essential for maintaining mucosal homeostasis, as they prevent commensal bacteria from adhering to mucosal epithelial cells (Smith et al. 2005; Platt and Mowat 2008; Sheikh and Plevy 2010; Davies et al. 2013; Bain and Mowat 2014). They are also the predominant type of mononuclear phagocytes found in the steady-state gut lamina propria (Murray and Wynn 2011; Zigmond and Steffen 2013; Mann and Li 2014; Gross et al. 2015). These major effector cells originate from monocytes that migrate to the lamina propria, either in response to the secretion of endogenous chemoattractant substances from the normal and healthy mucosa or upon being induced by inflammatory chemokines and bacterial products generated in the event of inflammation (Bain and Mowat 2011, 2014; Zigmond and Steffen 2013), and are referred to as intestinal macrophages. Intestinal macrophages have two important functions, namely, the protection of the organism from foreign pathogens and the regulation of mucosal responses against commensal bacteria (Bain and Mowat 2011; Smith et al. 2011; Goto and Ivanov 2013; Cerovic et al. 2014). Because of their strong phagocytic and bactericidal activity, intestinal macrophages effectively prevent the systemic invasion of pathogenic microorganisms (Pabs and Bernhardt 2010; Mowat and Bain 2011; Flannigan et al. 2015; Cipriani et al. 2016) and also enable the clearance of both apoptotic cells and foreign debris (Gregory and Devitt 2004; de Almeida and Linden 2005; Kim et al. 2005; Krysko et al. 2006; Hart et al. 2008; Fadeel et al. 2010). Intestinal macrophages are also involved in the removal of sloughed epithelial cells (Muller et al. 2012) and tissue remodelling (Rani et al. 2011) and are known to contribute to the maintenance of the barrier integrity of the intestinal epithelium by producing prostaglandin E2 (Pull et al. 2005). Macrophage-specific markers that have found common use include the macrosialin CD68, lipopolysaccharide-binding protein CD14 and the G-protein-linked TM7-homologous receptor of humans (epidermal growth factor module-containing mucin-like receptor 1; EMR1) or mice (F4/80; Martinez-Pomares et al. 1996; Taylor et al. 2005). Intestinal macrophages are known to have a distinct phenotype. In humans, they express the tissue macrophage marker macrosialin (CD68), which is also found on the majority of intestinal macrophages in mice (Niess and Adler 2010), together with the pan-macrophage markers CD64 (Cluster of Differentiation 64), F4/80, CD11b and the CSF-1 (M-CSF) receptor (CSF-1R; CD115; for reviews, see Mowat and Bain 2011; Tamoutounour et al. 2012). RAM11 is a monoclonal IgG1 antibody produced by immunizing mice with rabbit alveolar macrophage extract. This antibody binds to an unknown cytoplasmic antigen and is used to identify

connective tissue and vascular macrophages in rabbit tissues (Tsukada et al. 1986; Lis et al. 2007; 2010). RAM11 is specifically used for the morphological analysis of normal rabbit arteries and the determination of macrophages in the atherosclerotic lesions of cholesterol-fed rabbits and genetically hyperlipidaemic rabbits (Tsukada et al. 1986; Kinscherf et al. 1999; Lamb et al. 2002). Rabbit pulmonary intravascular macrophages have also been demonstrated to display RAM11 immunoreactivity (Duke-Novakovski et al. 2013). On the other hand, whether rabbit intestinal macrophages show RAM11 immunoreactivity remains unknown.

Previous research on the intestinal macrophages of humans and mice has focussed to a greater extent on macrophages localized to the lamina propria of the intestines (Denning et al. 2007; Kamada et al. 2009; Zigmond and Steffen 2013; Mann and Li 2014; Peterson and Artis 2014). Thus, only a very few studies have been conducted on the localization of intestinal macrophages in the GALT (Iwasaki and Kelsall 2000). Furthermore, to the authors' knowledge, the presence and localization of intestinal macrophages in the ileal and jejunal PPs, SR and appendix constituting the Angora rabbit GALT have not previously been investigated. Earlier studies of the rabbit GALT have, to a greater extent, focused on the development of the primary antibody repertoire (Pospisil and Mage 1998; Mage et al. 2006; Lanning et al. 2000; Zhai and Lanning 2013), rabbit lymphoid microanatomy (Beşoluk et al. 2006; Dasso et al. 2000), the immunophenotypic characterization of T and B cells (Hanson and Lanning 2008; Smith et al. 2011; Haines et al. 2016) and M cell structure and function (Dasso et al. 2000; Hanson and Lanning 2008; Beyaz et al. 2010a, b). Thus, the aim of this study has been to determine the presence and localization of intestinal macrophages in the functional compartments of the GALT, namely, the lymphoid follicles, interfollicular and subepithelial dome regions and the overlying FAE of the ileal and jejunal PPs, SR and appendix by RAM11 immunohistochemistry, in the adult Angora rabbit, which is known as one of the oldest types of domestic rabbit.

Materials and methods

Animals

Ten healthy one-year-old male Angora rabbits with a weight range of 3–3.5 kg and raised at the Research Farm of Erciyes University (Faculty of Veterinary Medicine) were used in this study. The animals were given fresh drinking water and standard laboratory feed and were housed at 23 ± 2 °C. The rabbits were killed by means of the intravenous administration of 0.1 ml/kg sodium pentobarbital. Subsequently, the abdominal cavity of the animals was opened for the collection of tissue samples from the ileal and jejunal PPs, SR and appendix for immunohistochemical and electron microscopic examination. The animals were killed with permission from the Ethics Board of Erciyes University, Faculty of Veterinary Medicine (date of meeting: 09.04.2003, decision no: 25).

Tissue processing and immunostaining

The tissue samples taken for immunohistochemical examination were first fixed in Bouin's solution for 2 h and then passed through a series of graded alcohols, methyl benzoate and benzole and finally embedded in paraffin. Sections (5 μm thick) were cut from the paraffin blocks, transferred to polylysine-coated slides and stained by the streptavidin-ABC immunohistochemical method to determine the immunohistochemical localization of the intestinal macrophages. Endogenous peroxidase activity was blocked by immersing the sections in a solution of 3% hydrogen peroxide, prepared in methanol, for a period of 20 min. Susbequently, while antigen retrieval was performed for the CD68 primary antibodies by using the protease enzyme, no procedure was applied for the RAM11 primary antibody. Later, the sections were rinsed with phosphate-buffered saline (PBS; 0.01 M, pH 7.6) containing 5% bovine serum albumin, 0.5% casein and less than 0.1% sodium azide (Ultra V Block, Thermo Fisher Scientific, LabVision) for 5 min so as to avoid non-specific binding. Next, the sections were incubated for 1 h with monoclonal mouse anti-rabbit macrophage (1:200, Clone: RAM11, DAKO, M0633), monoclonal mouse anti-human CD68 (1:50, Clone: EBM11, DAKO, cat. no. M 0718), monoclonal mouse anti-human CD68 (1:50, Neomarkers, Clone:KP1, cat. no. MS 397) and monoclonal mouse anti-human CD68 (1:50, Clone: PG-M1, Neomarkers, cat. no. MS 1808) primary antibodies. Following incubation, the sections were treated with biotinylated anti-mouse secondary antibody (Thermo Scientific, Ultravision kit, TM-125-HL) for 20 min and with avidin peroxidase solution (Thermo Scientific, Ultravision kit, TM-125-HL) for another 20 min. The sections were first stained with the 3-amino-9-ethylcarbazole (AEC) chromogen (Thermo Sceintific) for 5 to 10 min and then with Gill's haematoxylin for background staining for another 5 min in order to visualize the antigen-antibody reaction. Sections were always washed with PBS and all staining was performed at room temperature in a humidity chamber (Shandon, UK). Rabbit lymph node and tonsil sections incubated with primary antibodies were used as positive controls. For the negative controls, the primary antibodies were replaced by non-immune mouse serum (normal mouse IgG; Santa Cruz Biotechnology, sc-2025) and the remaining procedure was performed as in the standard staining method. The tissue sections were examined by conventional light microscopy (BX51; Olympus, Tokyo, Japan) for the evaluation of RAM11 immunoreactivity and were photographed with a digital camera (Olympus DP72).

Electron microscopy

After being pre-fixed in a glutaraldehyde-paraformaldehyde fixative solution (pH 7.4), the tissue samples taken for electron microscopic examination were fixed for a second time in 1% osmic acid solution for 2 h. Following the second fixation, the tissues were maintained in a 1% uranyl acetate solution for 2 h, passed through graded alcohols and propylene oxide and finally embedded in Araldite M. Semi-thin sections were stained with toluidine blue. Once the targeted areas had been marked on the sections, thin sections were cut at 300–400 Å, transferred to grids and examined and photographed under a Carl Zeiss EM 9S2 model electron microscope.

Results

Immunohistochemical findings

In the present study, examination of the immunohistochemically stained ileal and jejunal PPs demonstrated that RAM11-positive macrophages were localized mostly to the subepithelial dome region, to the corona region separating the dome from the follicles and to the germinal centres (GCs) at the centre of the follicles (Fig. 1a). Whereas many RAM11-positive macrophages were observed in the subepithelial dome region of PPs, particularly in the lamina propria just beneath the FAE, RAM11-positive macrophages became sparser towards the corona region (Fig. 1b). Furthermore, a few intra-epithelial RAM11-positive macrophages were detected in the FAE (Fig. 1b, c). RAM11-positive macrophages were abundant in the GCs of the follicles and some of these cells contained engulfed apoptotic bodies in their cytoplasm (Fig. 1d, e). On the other hand, in the parts of the ileum and jejunum that were devoid of PPs, RAM11-positive intestinal macrophages were widely distributed in the lamina propria and submucosa of the intestinal villi (Fig. 1f).

RAM11-positive macrophages were also localized to the subepithelial dome region, corona region and GC in the SR and appendix but were not observed in their FAE (Fig. 2a, b). However, the enterocytes in the FAE displayed supranuclear RAM11 immunoreactivity in their apical cytoplasm (Fig. 2c, d). The immunoreaction in these enterocytes did not differ with the application of variable antibody dilutions. RAM11-positive macrophages were extensively distributed in the GCs of the SR and appendix and, similar to the case in the ileal and jejunal PPs, some of these cells were observed to contain vacuoles of various sizes and apoptotic cell nuclei and apoptotic bodies (Fig. 2e, f, h). Furthermore, we observed that the RAM11-positive macrophages localized to these tissues formed not only small aggregates that contained fewer cells in the GCs, but also large aggregates that contained many cells in the region in between the GC and the corona (Fig. 2e, g).

Nonetheless, the immunohistochemical stainings performed for all three clones of CD68 in the PPs, SR and appendix of the rabbit yielded negative immunoreactions (not shown).

Electron microscopic findings

In parallel with the immunohistochemical results obtained in the present study, the 1-μm-thick semi-thin sections, which were prepared from the tissue samples taken for electron microscopic examination and were stained with toluidine blue, showed that the macrophages were localized to PPs and GCs of the SR and appendix (Fig. 3a, b). In particular, in the SR and appendix, large macrophages, which had most probably lost their function, were observed in the periphery of the lymphoid follicles (Fig. 3c). Whereas macrophages displayed an intra-epithelial localization in the pocket-like invaginations on the basolateral surface of the M cells in the FAE of PPs (Fig. 3d), they were determined to be localized just below the epithelium in the FAE of the SR and appendix. Conventional transmission electron microsocpy (TEM) demonstrated that, throughout the PPs, SR and appendix, intestinal macrophages possessed the typical ultrastructure of activated macrophages, in that they contained irregular indented euchromatic nuclei and had a relatively abundant cytoplasm with multiple pyknotic nuclear fragments (apoptotic bodies) of various sizes (Fig. 3e, f).

Discussion

Throughout life in both humans and animals, the intestines are continuously exposed to a vast array of food-borne antigens, potential pathogens and commensal micro-organisms. The intestinal mucosal system in association with the GALT constitutes the first line of defence against micro-organisms in the intestinal lumen. In comparison with other mammals, the rabbit has a well-developed GALT, which is localized predominantly to PPs, SR (or cecal tonsil) and appendix (Haley 2003; Sinha and Mage 2004; Mage et al. 2006; Beyaz et al. 2010a, b; Zhai and Lanning 2013). This study was aimed at the systematical examination of the ileal and jejunal PPs, SR and appendix of the Angora rabbit by conventional light and electron microscopy and immunohistochemical labelling by using RAM11 antibodies to determine both the localization and cellular characteristics of intestinal macrophages. Accordingly, intestinal macrophages were determined to share the following characteristics: (1) these cells were localized to the subepithelial dome region, corona region and GCs of the lymphoid tissues of the intestine and to the lamina propria and submucosae of the non-lymphoid regions of the intestine; (2) they contained multiple engulfed apoptotic bodies; (3) they showed peculiar immunohistochemical features that differed

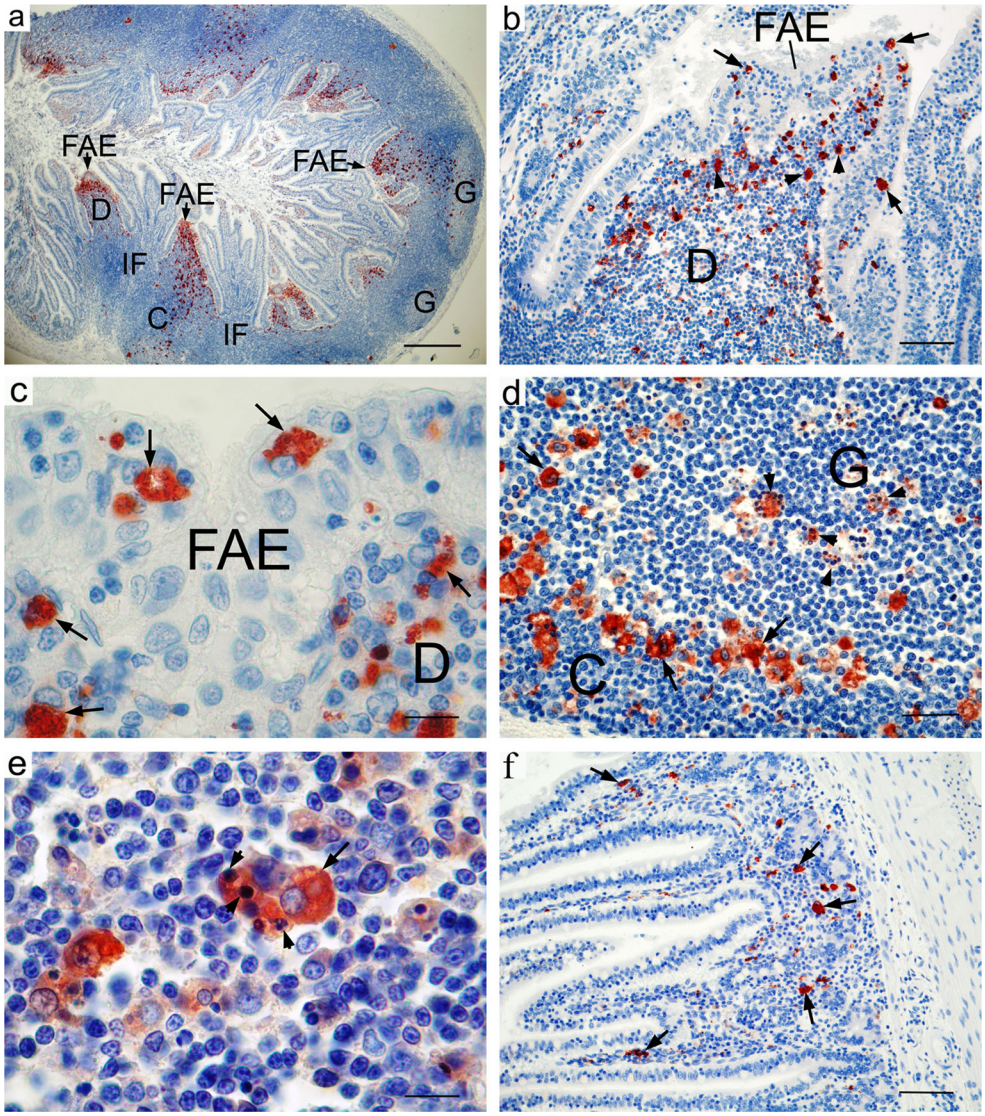


Fig. 1 Immunoreactivity for RAM11 in intestinal macrophages within Peyer's patches (PP). **a** Note the overall appearance of an ileal PP consisting of aggregated lymph follicles (*D* dome region, *FAE* follicle-associated epithelium, *C* corona, *IF* interfollicular region, *G* germinal centre). Immunohistochemical staining, 3-amino-9-ethylcarbazole (AEC). *Bar* 100 μm. **b** Note the RAM11-positive macrophages in the subepithelial dome region (*arrowheads*) and the RAM11-positive intra-epithelial macrophages in the FAE (*arrows*) of a jejunal PP. Immunohistochemical staining, AEC. *Bar* 25 μm. **c** Higher magnification of RAM11-positive macrophages (*arrows*) in the FAE and dome region of an ileal PP. Immunohistochemical staining, AEC. *Bar* 10 μm. **d** Note the RAM11-positive macrophages (*arrowheads*) and RAM11-positive macrophages containing the engulfed nuclei of apoptotic cells (*arrows*) in the GC of an ileal PP. Immunohistochemical staining, AEC. *Bar* 25 μm. **e** Higher magnification of a RAM11-positive macrophage (*arrow*) containing the engulfed nuclei of apoptotic cells (*arrowheads*) in the GC of a jejunal PP. Immunohistochemical staining, AEC, *Bar* 10 μm. **f** RAM11-positive intestinal macrophages (*arrows*) in the lamina propria and submucosa of the parts of the ileum devoid of PP. *Bar* 25 μm

from the characteristics of representative macrophages found in other organs of mammalian species.

Together with dendritic cells and some B lymphocytes, macrophages are part of the population of antigen-presenting cells. These cells have a critical role in the initiation, maintenance and orientation of T-cell-mediated immune responses (Smith et al. 2011; Cerovic et al. 2014; Flannigan et al. 2015). Macrophages and dendritic cells, which are different cell types, have complementary roles in intestinal homeostasis and immune defence (Cerovic et al. 2014; Gross et al. 2015). Because of their migratory capability, dendritic cells can access the mesenteric lymph nodes, which drain the intestines through the lamina propria by means of the lymphoid capillaries, and can eventually present naive T cells with antigens (Pabst and Bernhardt 2010). Furthermore, macrophages are reported also to be involved in the local clearance of bacteria from tissues, the stimulation of other immune cells, the secretion of cytokines to establish a local haemostatic immune cell network and the restimulation of T cells in the lamina propria (Swiatczak and Rescigno 2012; Zigmond and

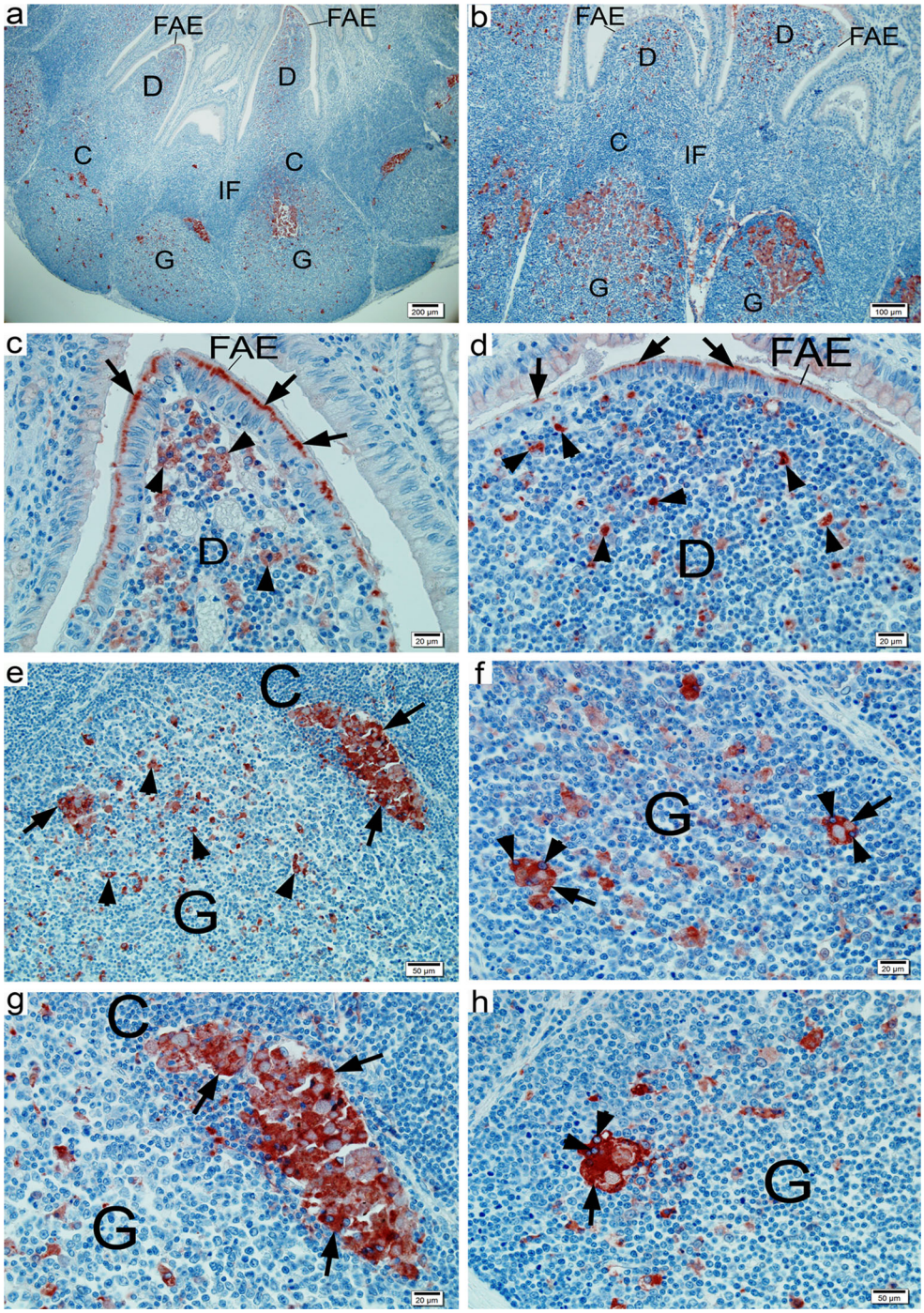


Fig. 2 Immunoreactivity for RAM11 in intestinal macrophages in the sacculus rotundus (SR) and appendix. **a** Note the overall appearance of SR consisting of aggregated lymph follicles (*D* dome region, *FAE* follicle-associated epithelium, *C* corona, *IF* interfollicular region, *G* germinal centre). Immunohistochemical staining, AEC. *Bar* 200 µm. **b** Note the overall appearance of SR consisting of aggregated lymph follicles. Immunohistochemical staining, AEC. *Bar* 100 µm. **c** RAM11-positive macrophages in the subepithelial dome region (*arrowheads*) and RAM11-positive immunoreactivity in the apical cytoplasm of enterocytes (*arrows*) in the FAE of SR. Immunohistochemical staining, AEC. *Bar* 20 µm. **d** RAM11-positive macrophages in the subepithelial dome region (*arrowheads*) and RAM11-positive immunoreactivity in the apical cytoplasm of enterocytes (*arrows*) in the FAE of the appendix. Immunohistochemical staining, AEC. *Bar* 20 µm. **e** Note the RAM11-positive macrophages (*arrowheads*) and the accumulations of RAM11-positive macrophages containing the engulfed nuclei of apoptotic cells (*arrows*) in the GC of the SR. Immunohistochemical staining, AEC. *Bar* 50 µm. **f** Note the RAM11-positive macrophages (*arrows*) containing the engulfed nuclei of apoptotic cells (*arrowheads*) in the GC of the appendix. Immunohistochemical staining, AEC. *Bar* 20 µm. **g** Higher magnification of an accumulation of RAM11-positive macrophages (*arrows*) between the GC and cortex of the SR. Immunohistochemical staining, AEC. *Bar* 10 µm. **h** Higher magnification of an accumulation of RAM11-positive macrophages (*arrow*) containing the engulfed nuclei of apoptotic cells (*arrowheads*) in the GC of the appendix. Immunohistochemical staining, AEC. *Bar* 20 µm

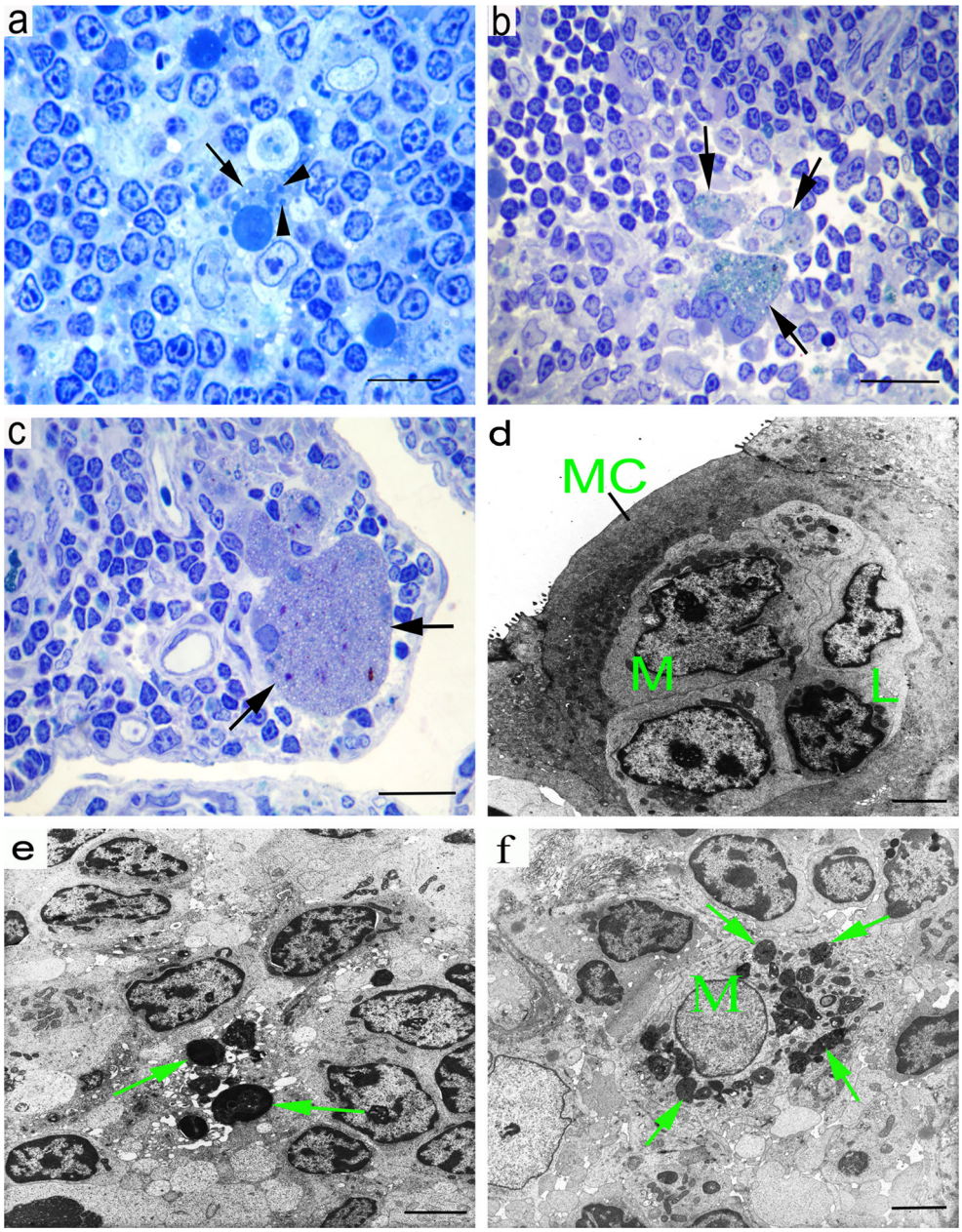


Fig. 3 Semi-thin sections and electron microscopic sections from PPs, SR and appendix. **a** Note a macrophage (*arrow*) containing the engulfed nuclei of apoptotic cells (*arrowheads*) in the GC of an ileal PP. Toluidine blue staining. *Bar* 8 μm. **b** Note macrophages (*arrows*) containing the engulfed nuclei of apoptotic cells in the GC of the SR. Toluidine blue staining. *Bar* 8 μm. **c** Possible inactive giant macrophage (*arrows*) at the periphery of the GC in the appendix. Toluidine blue staining. *Bar* 8 μm. **d** Electron microscopic view of an intra-epithelial macrophage within an M-cell pocket in the FAE of an ileal PP (*MC* M cell, *M* macrophage, *L* lymphocyte). *Bar* 2 μm. **e** Electron microscopic view of apoptotic bodies (*arrows*) in the GC of the SR. *Bar* 2 μm. **f** Electron microscopic view of a macrophage (*M*) containing engulfed nuclei of apoptotic cells (*arrows*) in the GC of the appendix. *Bar* 2 μm

Steffen 2013). Although intestinal macrophages share in common the expression of MHC class II, CD11c and CD11b with dendritic cells, the markers F4/80, CD68, CD14 and CD64 are used to identify the intestinal macrophages of humans and mice (Sagaert et al. 2012). RAM11 is a monoclonal IgG1 antibody produced by means of the immunization of mice with rabbit alveolar macrophage extract. This antibody, which binds to an unknown cytoplasmic antigen, is used for the identification of macrophages in the connective and vascular tissues of the rabbit (Tsukada et al. 1986; Lis et al. 2007, 2010). RAM11 has been specifically used for the morphological analysis of normal rabbit arteries and the immunohistochemical identification of rabbit macrophages in the cellular analysis of the atherosclerotic lesions of cholesterol-fed and genetically hyperlipidaemic rabbits (Tsukada et al. 1986; Kinscherf et al. 1999; Lamb et al. 2002). Furthermore, the pulmonary intravascular macrophages of rabbits have also been determined to display RAM11 immunoreactivity (Duke-Novakovski et al. 2013). To the authors' knowledge, no previous study exists on the RAM11 immunoreactivity of rabbit intestinal macrophages. In view of previous research, RAM11 antibodies were used for the identification of the intestinal macrophages of the Angora rabbit. Although tissue samples were exposed to CD68, a marker of other macrophages, rabbit intestinal macrophages were observed not to react with this antibody.

The small and large intestines, which are characterized by a distinct structure and are known to harbour a rather diverse microbiota, have different functions. Thus, we expected that the small intestinal macrophages would differ from the large intestinal macrophages. As examples, jejunal macrophages highly express the human leukocyte antigen (HLA)-DR but only show low expression of the low-affinity human immunoglobulin (Ig)G receptor CD16 and CD14 (Smythies et al. 2005), whereas colonic macrophages exhibit low expression of CD14 and CD16 and moderately express HLA-DR (Rogler et al. 1998). The immunohistochemical findings obtained in the present study demonstrated that, in the Angora rabbit, all intestinal macrophages localized to the ileal and jejunal PPs, SR and appendix displayed strong immunoreactivity for RAM11. This suggests that RAM11 primary antibodies can be reliably used for the identification of intestinal macrophages in the small and large intestines of the rabbit.

Macrophages show an extensive distribution in both the connective tissue beneath the epithelial layer (lamina propria and submucosa; Pabst and Bernhardt 2010; Swiatczak and Rescigno 2012; Cerovic et al. 2014; Gross et al. 2015; Marikowa et al. 2016) and the GALT, including lymphoid structures such as PPs and isolated lymphoid follicles (Gross et al. 2015; Flannigan et al. 2015). Previous research has shown that the localization of the intestinal macrophages in PPs varies among species. In humans (Spenser et al. 1986) and rats (Cesta 2006), intestinal macrophages have been reported to be localized to the FAE, SED region and interfollicular region of PPs in the small intestine. In their study on the anatomical localization of macrophages in the PPs of mice, Iwasaki and Kelsall (2000) determined that $CD11b^{+}/CD11c^{-}$ macrophages were abundant in the lamina propria of the intestinal villi but were absent in the SED region or the follicles and only occasionally present in the T cell regions of the PPs. Similar to the case in rats (Cesta 2006), although RAM11-positive intestinal macrophages were few in number in the Angora rabbit and displayed an intra-epithelial localization in the FAE of the ileal and jejunal PPs, they were absent in the FAE of the SR and appendix. Electron microscopic examination showed that the few macrophages were localized to the pocket-like invaginations on the surface of the M cells in the FAE of the PPs. Furthermore, we observed that these cells were abundant in the subepithelial dome region, the corona region separating the dome region from the follicles and the GCs at the centre of the follicles in the ileal and jejunal PPs, SR and appendix. We also ascertained that, whereas the RAM11-positive macrophages formed small aggregates composed of a few cells in the GCs of the SR and appendix, they formed large aggregates composed of many cells in the region in between the GC and the corona. According to these results, the localization of macrophages in the SR and appendix does not show complete similarity to that in the ileal and jejunal PPs, the macrophage content differs between the regions of the rabbit GALT, and the differences observed in the Angora rabbit for macrophage localization in the PPs, SR and appendix may arise from the functional differences of the GALT regions (Mowat and Agace 2014). In the present study, the examination of the ileal and jejunal parts devoid of PPs showed that, similar to the case in humans and mice, the intestinal macrophages were distributed within the lamina propria and submucosa (Zigmond and Steffen 2013; Mann and Li 2014; Peterson and Artis 2014). In view of all these findings, we suggest that, in parallel with the general concept of intestinal macrophages, these cells in the rabbit are strategically localized to the mentioned regions in order to capture and eliminate luminal antigens taken up by the M cells and any pathogenic agents that might breach the epithelial barrier.

Lis et al. (2007, 2010) reported the presence of supranuclear RAM11 immunoreactivity in the cells lining the basal layer of the stratified epithelium of the oral mucosa, oesophagus and skin in the rabbit and suggested that the RAM11-positive basal cells were stem cells. Similarly, a supranuclear RAM11 immunoreactivity was determined in the apical cytoplasm of the columnar epithelial cells of the FAE of the SR and appendix. On the other hand, the lack of such immunoreactivity, in the present study, for the stem cells localized to the crypts is not in favour of the hypothesis suggested by Lis et al. (2007, 2010). On the other hand, this finding suggests that the columnar epithelial cells of the FAE of the SR and appendix share a common antigenic epitope with macrophages or that these epithelial cells contain the RAM11 molecule similar to macrophages. Furthermore, this finding, together with the result that the FAE of the SR and appendix do not contain intra-epithelial macrophages, suggests that the RAM11-like molecules found in the apical cytoplasm of the columnar cells have an involvement in the defence function of the epithelial barrier. Macrophages have a major role in the elimination of dying and dead cells and are considered to be the professional phagocytes of apoptotic cells. This conclusion stems from the frequent association of macrophages with apoptotic cells in tissues, such that the apoptotic cell and its fragments are generally clearly visible in phagocytic vacuoles in the cytoplasm of macrophages (Gregory and Devitt 2004). Research in mice and humans has shown both the potent phagocytic activity of intestinal macrophages, as confirmed by the large phagocytic vacuoles found in their cytoplasm, and their strong bactericidal activity (Smythies et al. 2005).

Tingible body macrophages (TBM), a subset of mononuclear phagocytes, are large phagocytic cells that are localized to the GC of secondary lymphoid structures and contain multiple apoptotic bodies in their cytoplasm (Smith et al. 1998; Gregory and Devitt 2004). TBMs are distributed in the dark zones of mature GC and contain apoptotic B cells (Gregory and Devitt 2004). The B cells of the GC dark zone are referred to as centroblasts. B-cell proliferation is induced in the light

zone of the GC, and proliferating cells rapidly move into the dark zone. B cells in the light zone are referred to as centrocytes. Centrocytes are known to be exposed to B cell receptor maturation (BCR) and affinity to the exchange of immunoglobulins (Ig) and isotypes, both of which lead to the formation of memory B cells or plasma cells. Failure in this affinity to maturation brings about the apoptosis of the centrocytes in the GC (Smith et al. 1998; Ninomiya et al. 2003). Phagocytic macrophages enable the clearance of apoptotic B cells localized to the GC. In the present study, we determined that both the RAM11-positive macrophages and the light- and electron-microscopically examined macrophages localized particularly to the GCs in the ileal and jejunal PPs, SR and appendix contained engulfed apoptotic bodies in their cytoplasm. These morphological characteristics clearly demonstrate the strong phagocytic activity of intestinal macrophages in the Angora rabbit and also support the hypothesis that macrophages have a role in the apoptosis of follicular B lymphocytes in the GC of the PPs, SR and appendix in the Angora rabbit.

The proliferation (hyperplasia) of resident macrophages in lymph nodes has been well described. Mesenteric lymph nodes are continuously induced by intra-intestinal antigens and, thus, can harbour high levels of intrasinusoidal macrophages and multifocal aggregates of macrophages in the cortex and paracortex. Macrophage aggregates can show peripheral distribution around the paracortex or within the cortical, paracortical and medullary regions (Elmore 2006). Macrophage aggregates have been reported in the head kidney, spleen and, in some cases, the liver of teleost fish and are considered as the functional equivalents of GCs being actively involved in the storage of exogenous and endogenous waste, the immune response and the storage and recycling of iron (Blazer et al. 1997; Fournie et al. 2001). In the present study, the observation of small and large aggregates of RAM-11-positive macrophages, particularly in the parts of the GCs of the SR and appendix close to the corona region, not only suggests that macrophage aggregates are structures specific to the SR and appendix in the Angora rabbit, but also indicates that these GALT sites are actively involved in protection against intra-intestinal antigens.

In conclusion, the localization of intestinal macrophages in the major inductive sites of the GALT of the Angora rabbit, including PPs, SR and appendix, shows major differences. In this particular species, intestinal macrophages have strong phagocytic and bactericidal activity and, thus, both effectively avoid the systemic dispersal of potentially pathogenic microorganisms and efficiently clear apoptotic cells and foreign debris. On the basis of our immunohistochemical data, RAM11 primary antibodies, produced by immunizing mice with rabbit alveolar macrophage extract, can be reliably used for the identification of intestinal macrophages in the rabbit GALT.

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