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With 6 figures

Summary

Although the tonsils of sheep have gained much attention during the last decade, only few data are available on their lymph vessel architecture. Tonsillar lymph vessels are immunologically important as they form the efferent routes for locally activated immune cells to reach the draining lymph nodes. To gain insight into the tonsillar lymph drainage in the sheep, Indian ink and a casting polymer were injected into the interstitium of the five tonsils present in the heads of slaughtered sheep. This enabled to determine the draining lymph node and to examine the microscopic organization of lymph vessels using light and scanning electron microscopy. No lymph vessels were observed within the tonsillar lymphoid follicles. The corrosion casts demonstrated that the lymphoid follicles are surrounded by numerous sacculated lymph sinuses that drain into a dense interfollicular lymph vessel network. From here the lymph flows into single small lymph vessels that in turn drain into larger lymph vessels extending towards the medial retropharyngeal lymph node. The presented results can be valuable for immunological studies, e.g. during oral or intranasal vaccine development.

Keywords: Sheep; Tonsils; Lymph drainage; Lymph vessels; Corrosion cast

Introduction

Immune responses against ingested and inhaled foreign antigens are initiated in the tonsils since these mucosa-associated lymphoid tissues (MALT) are the body's most proximal line of defence (Casteleyn et al., 2007; Brandtzaeg, 2011; Maunsell et al., 2012). Besides maintaining pharyngeal immunity, locally activated antigen-specific T and B lymphocytes can migrate from the tonsils towards distant MALT sites such as the Peyer's patches (Brandtzaeg and Pabst, 2004; Kuper, 2006). To this purpose, the lymphocytes leave the tonsils via efferent lymph vessels, subsequently drain into the blood circulation and finally enter immunological effector sites by diapedesis through high endothelial venules (HEVs) (Indrasingh et al., 2002; Cesta, 2006; Liebler-Tenorio and Pabst, 2006). As such, lymph vessels and HEVs form the structural basis of the integrated mucosal immune system since they provide the routes for lymphocyte circulation between different MALT sites (Belz and Heath, 1995; Girard and Springer, 1995; Rothkötter et al., 1995; Belz, 1998).

During the past decade the tonsils of sheep have received particular interest within the scope of prion diseases (Schreuder et al., 1998; Van Keulen et al., 2008). To aid the unravelling of the pathogenesis of transmissible spongiform encephalopathies (TSEs), morphological and immunological data on the five ovine tonsils, including the paired palatine tonsil (tonsilla (t.) palatina) located in the oropharynx, the paired paraepiglottic tonsil (t. paraepiglottica) in the laryngopharynx, and the tonsil of the soft palate (t. veli palatina), the pharyngeal tonsil (t. pharyngea) and the paired tubal tonsils (t. tubaria) in the nasopharynx (Casteleyn et al., 2007, 2011a; Breugelmans et al., 2011a), were gathered. The exact anatomical localizations, histological characteristics, volumes and surface areas, distribution of lymphoid cell populations, and innervation of the ovine tonsils have yet been described (Russo et al., 2009; Casteleyn et al., 2007, 2008a, 2010a; Breugelmans et al., 2011a, 2011b, 2011c; Toppets et al., 2012).

60 Remarkably, only few data are available on the lymphatic system of the ovine tonsils.
61 Indeed, many conventional anatomical handbooks do not mention the lymphatic drainage
62 from the tonsils (Martin and Schauder, 1938; Grau-Karlsbad, 1974; Saar and Getty, 1975;
63 Vollmerhaus, 1976; König et al., 2002; Gille, 2008). Moreover, when literature data are
64 available, they are inconsistent or only general in nature, without mentioning species-specific
65 characteristics. For example, according to Berg (1988) the lymph from the tonsils of the
66 common domestic mammals is conveyed to the mandibular lymph centre, while Barone
67 (1996) states that all tonsillar lymph flows directly to the retropharyngeal lymph centre.
68 Detailed, species-specific knowledge of the tonsillar lymphatic system can be valuable for
69 immunological studies, e.g. during oral or intranasal vaccine development investigating the
70 generation of antigen-specific lymphocytes (Casteleyn et al., 2013). The usefulness of such
71 mucosal vaccines is illustrated by the successful eradication of human polio by oral
72 vaccination (Murphy et al., 2008). Veterinary examples of potent intranasal vaccination
73 include the vaccines against kennel cough in the dog, bovine and feline rhinotracheitis,
74 *Streptococcus equi* infections in horses, and infectious bronchitis and Newcastle disease in
75 poultry (Tizard, 2013). Besides the induction of mucosal immunity at the sites of potential
76 infection, immune cells activated in the tonsils by mucosal vaccines use the efferent tonsillar
77 lymph vessels to migrate towards the draining lymph node where serum IgG antibodies,
78 providing systemic immunity, are secreted (Kuper et al., 1992; Zuercher, 2003).
79 In the framework of assessing the effectiveness of a newly developed mucosal vaccine, it
80 is important to identify the lymph nodes draining the ovine tonsils. In line with this, the
81 intratonsillar distribution of the lymph vessels should be investigated to gain insight in the
82 emigration of activated lymphocytes into the tonsillar lymphatic system. In contrast to the
83 well described distribution of lymph vessels within lymph nodes (Heath et al., 1986; Heath
84 and Spalding, 1987; Azzali, 2003), descriptions of the lymphatic systems present in MALT

sites are restricted to the human and canine palatine tonsils, the Peyer's patches of the rabbit, guinea pig, mouse, sheep and pig, and the vermiform appendix of the rabbit (Ohtani and Murakami, 1990; Lowden and Heath, 1992, 1994; Belz and Heath, 1995; Regoli et al., 1995; Fujisaka et al., 1996; Azzali, 1998; Azzali et al., 2002; Azzali, 2003).

Since data on the lymphatic system of the ovine tonsils are largely lacking and ambiguous, the aims of the present study were to identify the draining lymph node(s) and to describe the microstructure of the tonsillar lymphatic system in the sheep. The research methods included interstitial injection of Indian ink and casting polymer into the five ovine tonsils, combined with light and scanning electron microscopy (SEM).

Materials and Methods

Lymph drainage from the tonsils

The heads of five healthy one-year-old Texel sheep were collected at a local abattoir. Using a band saw, they were longitudinally sectioned 1 cm lateral to the median plane in order not to transect the pharyngeal tonsil located at the caudal end of the pharyngeal septum (Casteleyn et al., 2007, 2008a). Indian ink (Pelikan black 17 Fount India, Pelikan Benelux N.V., Groot-Bijgaarden, Belgium) was interstitially injected into the centre of each tonsil (ten palatine, paraepiglottic and tubal tonsils, and five pharyngeal tonsils and tonsils of the soft palate) via a 26 gauge (G) needle inserted just underneath the tonsillar epithelium (Castenholz, 1984, 1986; Lowden and Heath, 1992, 1993; Casteleyn et al., 2007, 2008b). A volume of 0.5 ml ink was injected into the largest ovine tonsil, i.e. the pharyngeal tonsil (Casteleyn et al., 2007). The second largest tonsil, being the palatine tonsil, was injected with 0.3 ml ink, while all other, small, tonsils received 0.1 ml ink (Casteleyn et al., 2007). Subsequently, the heads were dissected to demonstrate black stained lymph vessels emerging from the injected tonsils and coursing towards either the parotid, mandibular or retropharyngeal lymph nodes. These lymph

nodes were examined both macroscopically and histologically using a light microscope (Olympus BX 61, Olympus Belgium, Aartselaar, Belgium) for the appearance of black staining and the presence of carbon particles, respectively (Casteleyn et al., 2008b). For histological analysis, the lymph nodes were fixated in 3.5% neutral buffered formaldehyde for two days at room temperature. They were then routinely processed to a series of 10 histological sections, cut at fixed intervals throughout the entire lymph nodes that were stained with haematoxylin and eosin (HE).

Light microscopy on the tonsils after interstitial injection of Indian ink

The interstitially injected tonsils were excised and fixated in 3.5% neutral buffered formaldehyde for two days at room temperature. The fixated samples were routinely processed to a series of 10 HE-stained histological sections, cut at fixed intervals throughout the entire tonsils. The presence and distribution of carbon particles were examined by light microscopy (Olympus BX 61).

SEM on corrosion casts of the tonsillar lymphatic system

The heads of an additional number of five healthy one-year-old Texel sheep were collected at a local abattoir. Each tonsil was interstitially injected via a 26 G needle inserted just underneath the tonsillar epithelium with, according to the size of the tonsil as described above, 0.1 ml to 0.5 ml Mercox II® (methyl methacrylate-based casting medium, Ladd Research, Williston, USA). After polymerization, which took approximately 15 min, the casted tonsils were excised and macerated overnight in 25% KOH (Carl Roth GmbH, Karlsruhe, Germany). The obtained lymphatic casts were gently rinsed with distilled water, air dried, coated with platinum and examined with SEM (JEOL JSM 5600 LV, Jeol, Zaventem, Belgium).

Results

Lymph drainage from the tonsils

Indian ink that was injected superficially into each of the five ovine tonsils drained directly to the medial retropharyngeal lymph node in all five examined animals. The afferent lymph vessels to this lymph node were easily distinguishable by their black colour (Fig. 1A). At the surface of the lymph node they divided into primary branches, giving off secondary ramifications that in turn divided into tertiary or terminal branches. The latter terminated in the subcapsular sinus of the lymph node (Fig. 1B).

Light microscopy on the tonsils after interstitial injection of Indian ink

After interstitial injection of Indian ink into the tonsils, carbon particles were abundantly present in the lymph vessels located in between neighbouring lymphoid follicles (Fig. 2A). As such, an interfollicular lymph vessel network surrounding the lymphoid follicles could be observed. Only limited amounts of carbon particles were seen within the lymphoid follicles (Fig. 2B). These carbon particles seemed not to be enclosed by endothelium-lined vessels, but appeared as free particles lying in the interstitial spaces between the densely packed lymphocytes composing the lymphoid follicles. The interfollicular lymph vessel network drained into larger lymph vessels that were present in the connective tissue underneath the scatered lymphoid follicles located in the tonsil of the soft palate and in the paraepiglottic and tubal tonsils. Larger lymph vessels also coursed through the connective tissue core of the pharyngeal tonsil, and through the connective tissue septa of the palatine tonsil (Fig. 2A). Blood vessels, containing erythrocytes, contrasted well with the lymph vessels by their lack of carbon particles (Fig. 2B).

SEM on corrosion casts of tonsillar lymph vessels

At low magnification round impressions were visible at the surfaces of the casts from the palatine, paraepiglottic and pharyngeal tonsils (Figs. 3-5). The diameters of these impressions that are located just underneath the tonsillar epithelium amounted to approximately 500 μm. These data combined with the histological images presented in Fig. 2 demonstrate that the round impressions represent the locations of lymphoid follicles in unmacerated tonsils. No indications for the presence of intraepithelial lymph vessels were noticed on the corrosion casts. At higher magnification sacculated lymph sinuses were observed at the bottom and the sides of the lymphoid follicles. In between these follicles a finely-meshed interfollicular lymph vessel network, characterized by numerous cellular impressions, was present (Fig. 5). Some distinct lymph vessels extended through the interfollicular regions and connected to larger efferent lymph vessels that emerged from the tonsils. Deeply indented, V-shaped constrictions were visible at intervals of approximately 500 μm in larger lymph vessels travelling through the connective tissue cores and septa of tonsils and in those running in the subepithelial connective tissue towards the draining lymph node (Figs. 5 and 6).

Since the tubal tonsils and the tonsils of the soft palate only contain scattered lymphoid follicles, impressions could not be recognized on the surfaces of their casts (Fig. 6) (Casteleyn et al., 2007). The interstitial spaces on the nasopharyngeal side of the soft palate and the lateral nasopharyngeal mucosa were recognized on the casts by their numerous cellular impressions. The interstitium drained into small lymph vessels that merged into larger lymph vessels. These vessels extended in rostrocaudal direction towards the medial retropharyngeal lymph nodes and showed deeply indented, often V-shaped constrictions (Fig. 6).

Discussion

184 The present study describes the lymph drainage from the t. palatina, t. paraepiglottica, t.
185 pharyngea, t. tubaria and t. veli palatini. Although an additional, lingual tonsil has been
186 decribed in the sheep (Casteleyn et al., 2007, 2011b), this tonsil was not included since it only
187 consists of small non-organized aggregations of lymphoid cells mainly located in the centres
188 of the vallate papillae at the root of the tongue (Casteleyn et al., 2007; Breugelmans et al.,
189 2011a).

190 The data presented here are morphological in nature, but aim to contribute to the
191 knowledge of the mucosal immune system as well. In order to correspond to the commonly
192 used immunological terminology, the official anatomical terms defining a well-circumscribed
193 aggregation of lymphoid cells, i.e. lymph nodule, lymphonodulus or nodulus lymphaticus,
194 were replaced by the immunological term lymphoid follicle (Casteleyn et al., 2011a).

195 During the interstitial injections with Indian ink in the centres of the tonsils it was
196 macroscopically observed that the injected volumes were sufficient to allow spreading of the
197 ink towards the periphery of the tonsils. The lymph, in our experiments represented by ink,
198 drained directly from each tonsil to the medial retropharyngeal lymph node. Since all
199 experiments were performed on slaughtered animals, this draining was due to the manual
200 pressure by which the liquids were injected. It is plausible that swallowing enhances lymph
201 drainage from the ovine tonsils in vivo. In this respect, the V-shaped indentations that were
202 observed on the casted larger lymph vessels, that are suggestive of lymphatic valves
203 (Castenholz, 1986), are meaningful. The application of manual pressure during the injections
204 of ink might have forced carbon particles into the interstitium of lymphoid follicles, as
205 suggested by Fig. 2B. The high cellular density of lymphoid follicles could explain the
206 presence of only limited amounts of intrafollicular carbon particles. These particles could also
207 be present in small intrafollicular lymph vessels, but this was not be demonstrated on the
208 corrosion casts. In addition, other authors also state that no lymph vessels are present within

lymphoid follicles (Ohtani and Murakami, 1990; Lowden and Heath, 1992, 1994; Belz and Heath, 1995; Regoli et al., 1995; Fujisaka et al., 1996; Azzali, 1998; Azzali et al., 2002; Azzali, 2003).

For the preparation of the corrosion casts, Mercox® was used since its very low viscosity is highly advantageous for interstitial injection and casting of capillary structures (Hodde, 1981; Murakami et al., 1984). Unfortunately, Mercox® casts are very brittle and thus have to be handled with care. Another disadvantage is that Mercox® is approximately twice as expensive as Batson's® (Polysciences, Warrington, USA). The latter casting polymer is also based on methyl methacrylate, but has a much higher viscosity than Mercox®. It is therefore commonly used to cast larger blood vessels and other lumen containing structures such as the avian air sacs (Konerding, 1991; Casteleyn et al., 2010b; Stefanov et al., 2013).

The distribution of the injected Mercox® was similar to that of the Indian ink. Its low viscosity also enabled the polymer to course towards the medial retropharyngeal lymph nodes. The scanning electron microscopic knowledge obtained in the present study concerning the lymph vessel organization of the ovine tonsils correspond with the data presented by other authors investigating the lymph vessel organization of the human and canine palatine tonsils, the Peyer's patches of the rabbit, guinea pig, mouse, sheep and pig, and the rabbit vermiform appendix (Ohtani and Murakami, 1990; Lowden and Heath, 1992, 1994; Belz and Heath, 1995; Regoli et al., 1995; Fujisaka et al., 1996; Azzali, 1998; Azzali et al., 2002; Azzali, 2003).

The parotid and mandibular lymph nodes did not receive Indian ink. Our observation therefore contrasts with the statement of Berg (1988) that all tonsillar lymph flows to the mandibular lymph centre, but corresponds with the data presented by Barone (1996). These lymph nodes therefore seem to play no role in the induction of immunity after antigen uptake at tonsillar epithelia, e.g. by M cell-like cells (Casteleyn et al., 2010a, 2013). Since tonsils

234 lack afferent lymph vessels (Mair et al., 1987; Chen et al., 1989; Indrasingh et al., 2002), it is
235 not surprising that lymph vessels connecting different tonsils were not observed. Thus, no
236 lymphatic connection between the different tonsillar structures of Waldeyer's ring exists (Von
237 Waldeyer-Hartz, 1884).
238 It should be noted that the results presented here have been obtained in Texel sheep. Since
239 this ovine breed is reared for meat production, tissues from Texel sheep are easily collected at
240 the slaughterhouse. This breed is therefore very valuable for ex vivo experiments. To the best
241 of our knowledge, no anatomical studies investigating the lymph drainage from the tonsils in
242 other ovine breeds have yet been performed. As a result, no statement on the existence of
243 breed related anatomical variations can be made. However, it is not plausible that the general
244 pattern of lymph drainage from the tonsils, i.e. collection of interstitial fluid into small lymph
245 vessels that convey into larger lymph vessels flowing towards the medial retropharyngeal
246 lymph node, differs between various ovine breeds.
247 Immunocompetent cells deriving from those tonsils that are considered as the main
248 inductive sites for mucosal immunity in the sheep, i.e. the palatine and pharyngeal tonsils
249 (Breugelmans et al., 2011b) as well as lymphocytes activated in the medial retropharyngeal
250 lymph node by drained antigens all emigrate together with secreted systemic antibodies
251 produced in the draining lymph node (Kuper et al., 1992; Zuercher, 2003) via efferent lymph
252 vessels towards the ipsilateral lateral retropharyngeal lymph nodes (Schummer et al., 1981;
253 Barone, 1996). From here, the lymph flows in the ipsilateral tracheal trunks that extend
254 towards the deep caudal cervical lymph nodes located at the ventral side of the trachea near
255 the median plane (Barone, 1996). Finally, the lymph from these lymph nodes is conveyed in
256 the common duct of the two tracheal trunks which discharge by a variable pattern in the
257 cranialmost segment of the cranial vena cava at the thoracic inlet (Schummer et al., 1981;
258 Barone, 1996). These anatomical structures thus provide the route for antigen-specific

lymphocytes and antibodies to reach the systemic circulation, enabling them to travel to and protect distant MALT sites.

Conclusions

The tonsillar lymphatic system in the sheep is well-developed. It is composed of interfollicular lymph vessel networks draining into single small lymph vessels that join to form larger efferent lymph vessels containing valves. All efferent lymph vessels from each of the five ovine tonsils drain to the retropharyngeal lymph centre. The other cranial lymph centres, i.e. the parotid and mandibular lymph centres, play no role in the induction of immunity against harmful antigens sampled at the tonsillar surfaces.

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424

425 **Figure legends**

426 **Fig. 1.** Paramedian section through the ovine head showing a medial view of the right side of
427 the nasopharynx. After Indian ink was interstitially injected into the tubal tonsil (1), black
428 carbon particles spread through lymph vessels (arrows) that extended towards the medial
429 retropharyngeal lymph node (2). Some anatomical landmarks, such as the soft palate (3) and
430 the epiglottis (4) are additionally indicated. B: Medial retropharyngeal lymph node receiving

carbon particles via three afferent lymph vessels (alv) giving off primary branches (pb) that subsequently divide into secondary branches (sb) which finally give off terminal branches (tb) that enter the subcapsular sinus (ss).

Fig. 2. Histological section through the ovine pharyngeal tonsil after interstitial injection of Indian ink. A: Carbon particles are visible in the interfollicular lymph vessel network (iln) which surrounds the lymphoid follicles (F). Larger lymph vessels (lLV) are present in the connective tissue core of the tonsil. B: Higher magnification showing the abundant presence of carbon particles in the interfollicular lymph vessel network (iln) and lymph sinuses surrounding the lymphoid follicles (F). The interfollicular blood vessels (arrows) are not loaded with carbon particles. Notice the intrafollicular presence of carbon particles that are not enclosed by endothelium-lined vessels (arrowheads).

Fig. 3. SEM views of the lymph vessel architecture of the ovine pharyngeal tonsil. A: Spaces previously occupied by lymphoid follicles (F) are surrounded by numerous sacculated lymph sinuses (sl) that drain into interfollicular lymph vessel networks (iln). Small lymph vessels (sLV) emerge from this network. B: Higher magnification of the interfollicular lymph vessel network (iln) showing the imprints of lymphoid cells (arrows) present in the interfollicular areas. C: The small lymph vessels (sLV) that emerge from the interfollicular lymph vessel network (iln) surrounding the lymphoid follicles (F) drain into large lymph vessels that travel through the connective tissue core of the tonsil. D: A larger lymph vessel (lLV) characterized by the presence of many, often V-shaped, constrictions (arrows) corresponding to lymph vessel valves courses through interstitial spaces (i).

Fig. 4. SEM images of the lymph vessel architecture of the ovine palatine tonsil. A: The locations of macerated lymphoid follicles (F) can be recognized as round impressions. Each lymphoid follicle is surrounded by the interfollicular lymph vessel network (iln). Some lymph vessels (LV) are additionally indicated. B: Higher magnification of the impression of the lymphoid follicle encircled in A. Sacculated lymph sinuses (sl) surrounding the lymphoid follicle are prominent. They drain into the interfollicular lymph vessel network (iln).

Fig. 5. SEM view of the lymph vessel architecture of the ovine paraepiglottic tonsil. Sacculated lymph sinuses (sl) surrounding the lymphoid follicles (F) drain into the interfollicular lymph vessel network (iln) which is connected by small calibre lymph vessels (sLV) to larger lymph vessels (lLV).

Fig. 6. SEM images of the lymph vessel architecture of the ovine tonsil of the soft palate. A: No imprints of lymphoid follicles can be seen in this diffusely organized tonsil. The subepithelial interstitial spaces (i) drain into small lymph vessels (sLV), which in turn flow into large lymph vessels (lLV) that travel through the nasopharyngeal mucosa. B: Higher magnification of the subepithelial interstitial spaces (i) that drain into a small lymph vessel (sLV).