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Genital organ-associated lymphoid tissues arranged in a ring in the mucosa of cow vaginal vestibules

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21

22 **Abstract**

23 Female reproductive tracts are equipped with local and mucosal immune systems; however,
24 structural information remains unclear for farm animals. In this study, the
25 mucosa-associated lymphoid tissue-like structures in cow reproductive tracts were
26 described. Vaginal vestibule (VV) and external parts of the genital organ, including the
27 clitoris and vulva, were morphologically analyzed. Whole-mount specimens revealed
28 several hematoxylin-positive spots arranged in a ring in the mucosa. Histologically, these
29 spots were aggregated immune cells and defined as genital organ-associated lymphoid
30 tissues (GOALTs). GOALTs were composed of lymphatic follicles (LFs) or diffuse
31 lymphoid tissues (DLTs) at different depths of lamina propria. LFs frequently contained
32 germinal centers. Scattered lymphocytes occupied the border area between follicles and
33 epithelium, whereas DLTs had indefinite shapes. GOALTs contained immune cells and
34 high endothelial venules. B cells were dominant both in LFs and DLTs. Abundant
35 collagenous fibers were stretched across VV lamina propria, whereas reticular fibers were
36 primarily observed in the DLT rather than LF. The epithelium covering of GOALTs was
37 partially or fully disrupted by the invasion of immune cells toward the VV lumen. These
38 findings suggest GOALTs function as a “genital lymphoid ring” as in Waldeyer’s
39 pharyngeal ring and act as immunological gate systems in cow reproductive tracts.

40

41 **Keywords**

42 Cow; Genital organ associated lymphoid tissue; Lymphoid ring; Lymphatic follicle;

43 Diffuse lymphoid tissue

44

45 **Introduction**

46 The mucosa-associated lymphoid tissue (MALT) is a local immune system beneath the
47 mucosal epithelium (Kenneth Murphy, 2017; Lanzkowsky et al., 2016). MALTs are located
48 in systemic organs that directly contact the outer environment, including alimentary tracts,
49 salivary gland ducts, conjunctiva, nasopharynx, and respiratory tracts (Forchielli and
50 Walker, 2005; Kenneth Murphy, 2017; Knop and Knop, 2011; Kracke et al., 1997; Kuper
51 et al., 1992; Moyron-Quiroz et al., 2004; Nair and Schroeder, 1986; Randall, 2010).
52 Gut-associated lymphoid tissue (GALT) is a well-known MALT and also known as Peyer's
53 patches (Forchielli and Walker, 2005). In the oropharynx, there are ring-shaped localized
54 MALTs, including the lingual, palatine, and pharyngeal areas and the tubal tonsils
55 (Hellings et al., 2000). Waldeyer's pharyngeal ring is the sentinel for the oral, nasal, and
56 auditory tracts toward to the pharynx and basically functions in antibody production to
57 protect against regular environmental antigens (Liebler-Tenorio et al., 2006; Manesse et al.,
58 1998).

59 MALTs mainly consist of lymphoid tissues (LTs) with two types of morphology.
60 Briefly, the lamina propria of several MALT-forming organs exhibit diffused arrangement
61 of immune cells, and this type is called diffuse LT (DLT). However, in several MALTs, the
62 accumulation of immune cells is separated by connective tissue from surrounding tissues,

63 and these are called lymphatic follicles (LFs) or lymph nodules (Wojciech and Ross, 2020).
64 Furthermore, the MALT is composed of B cells, CD4⁺, or CD8⁺ T-cells, antigen-presenting
65 cells (APCs), macrophages, and occasionally, mast cells and eosinophils. Each cell is
66 well-situated to initiate an immune reaction when encountering antigens passing through
67 the mucosal epithelium (Cesta, 2006).

68 Mammalian female reproductive tracts are also in close contact with the outer
69 environment and maintain innate and adaptive immune systems constructed by the
70 epithelial barrier, the production of antimicrobial agents, cytokines secreted by the
71 epithelial cells, and the innate immune cells (Hickey et al., 2011; Mair et al., 2014).
72 Importantly, the reproductive tracts of humans or other animal species contain MALT with
73 species- or disease-specific variations in morphology. A series of studies on LT have
74 recently been pursued in some female genital tracts, including the vaginal vestibule (VV),
75 vagina, cervix, uterus, and oviducts in non-human primates, pigs, and cows to evaluate the
76 effects of drug administration or mucosal vaccination (Blazquez et al., 1987a; Kathrine and
77 Steen, 2011; Lehner et al., 1995). Specifically, the guiana dolphin (*Sotalia guianensis*)
78 exhibited aggregations of LFs in the lamina propria of their uterine(Silva et al., 2016).
79 Importantly, healthy female mice did not have MALT in their reproductive tracts, but they
80 exhibited a MALT-like structure in their vaginal mucosa after receiving intravaginal
81 immunization with a specific peptide (Wang et al., 2015). Furthermore, locally organized
82 MALT in the VV observed in patients with provoked vulvodynia compared with healthy
83 women, indicated the MALT emerged because of the local alternation of VV
84 morpho-function (Liebler-Tenorio et al., 2006; Tommola et al., 2015).

85 The reproductive management of animals is crucial in veterinary medicine. In cattle,
86 gross anatomically and functionally, MALT in the alimentary and respiratory tract has been
87 well-investigated in several studies (Anderson et al., 1986; Manesse et al., 1998; Parsons et
88 al., 1989). Briefly, several types of MALTs were established according to their anatomical
89 localization, including GALTs, conjunctiva-associated LTs, and bronchus-associated LTs
90 (Anderson et al., 1986; Chodosh et al., 1998). Importantly, epithelium-associated LF
91 aggregations were suggested throughout the reproductive tracts in cattle, including the VV
92 (Blazquez et al., 1987a). The LF aggregations located in the lamina propria of the clitoris
93 and VV could play an important role in immune induction sites because of their appearance
94 during infection with cow reproductive tract pathogens (Blazquez et al., 1987b; Wang et al.,
95 2011); however, structural information on reproductive tract-associated MALTs, in
96 particular their localizations, morphological types, and cellular composition, has received
97 limited attention.

98 In the present study, we found a MALT-like structure arranged in a ring around the
99 cow VV, and the stratified epithelium covering the LT was partially or completely
100 disrupted, which permitted direct passage of immune cells and erythrocytes into the
101 intraluminal space. We named these LTs the “genital lymphoid ring” and discussed the
102 similarities with Waldeyer’s pharyngeal ring, which acts as an immunological gate.

103

104 **Materials and methods**

105 *Animal and sample preparation*

106 Animal experimentation procedures were approved by the Institutional Animal Care and
107 Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval no.
108 19-0097). Investigators adhered to the Guidelines for the Care and Use of Laboratory
109 Animals of Hokkaido University, Faculty of Veterinary Medicine. All animal experimental
110 protocols were approved by the Association for Assessment and Accreditation of
111 Laboratory Animal Care International. Holstein breed, normal non-pregnant cows (total n =
112 8, over 2-year-old) were sedated with xylazine (3.0 mg/kg body weight) through an
113 intramuscular route followed by general anesthesia with pentobarbital sodium (500 mg/kg
114 body weight) via the intravascular route (Supplemental table 1). The cows were sacrificed
115 by intravenous administration of a saturated potassium chloride solution of at least 0.1
116 mL/kg body weight. Then, the jugular vein was exsanguinated. After euthanasia, female
117 genital organs were obtained, and the vagina, VV, and EPGOs, including the clitoris and
118 vulva were separated. They were immediately fixed in 10% neutral buffered formalin for
119 more than 7 d. These specimens were dissected using scissors at the dorsal commissure
120 along the median line of the vulva to the vagina.

121

122 *Whole-mount observation*

123 Separated genital organs were pinned flat, with the mucosa uppermost, and immersed in
124 Mayer's hematoxylin for approximately 10 min (examining at 3–4 min intervals) to
125 visualize LTs. LTs were then visible as small navy-blue spots. To decrease the intensity of

126 the staining background, specimens were rinsed in 70% alcohol containing 1%
127 hydrochloric acid.

128 Counting of hematoxylin-positive spots was performed for the VV and EPGOs.
129 Flattened VV and EPGOs were divided into six regions (**Fig. 1**). Briefly, we divided the
130 VV and EPGOs into two equal regions (cranial and caudal) along the horizontal line (the
131 length of the external urethral orifice to the ventral commissure was divided into two equal
132 lengths). Then, the cranial and caudal regions were subdivided by two vertical lines into
133 three equal subregions. The external urethral orifice was located at the center of the cranial
134 median region (**Fig 1, region II**), and the clitoris was located at the center of the caudal
135 median region (**Fig. 1, region V**). Each area was measured using ImageJ (National
136 Institutes of Health; MD, Bethesda, USA) after taking photos with a DMC-FX3799 device
137 (Panasonic; Osaka, Japan). The number of hematoxylin-positive spots was counted, and
138 their density in each region was calculated.

139

140 *Histoplanimetry*

141 The VV of each cow was cut into more than 15 pieces according to their size. Then,
142 whole-mount specimens with hematoxylin were embedded into paraffin and cut to a
143 thickness of 5 μm . Sections were stained with hematoxylin and eosin (H&E) or PSR for
144 histological analysis. Stained sections were scanned with a NanoZoomer 2.0 RS virtual
145 slide scanner (Hamamatsu Photonics; Shizuoka, Japan). Histological images for
146 microscopic examinations were observed using a model BZ-X710 microscope (Keyence;

147 Osaka, Japan). The number of LFs and DLTs was counted after H&E staining. This
148 measurement was performed for more than 120 sections of eight cows. We also
149 characterized the LFs according to their localization (isolated, migrating, bordering) and the
150 DLTs according to their shape (oval, c-shaped, triangle, amorphous), and their
151 appearance % was calculated.

152

153 *Immunohistochemistry (IHC)*

154 IHC was performed to evaluate the presence of B-cells (CD20), T-cells (CD3),
155 macrophages (IBA1), APCs (MHCII), high endothelial venules (PNAd), and tight junctions
156 (Occludin). The staining conditions of each antibody are listed in Supplementary table 2. In
157 brief, sections were deparaffinized and incubated in 10 mM citrate buffer (pH 6.0) or
158 Tris-HCl buffer (pH 9.0) for 15 min at 110 °C. Thereafter, slides were submerged in
159 methanol containing 0.3% H₂O₂ for 20 min at 20–22 °C and blocked with normal goat
160 serum (SABPO kit, Nichirei Bioscience; Tokyo, Japan) for 1 h at room temperature.
161 Sections were incubated with primary antibody overnight at 4 °C. After rinsing in
162 phosphate-buffered saline (PBS), sections were incubated with biotinylated secondary
163 antibody (goat anti-rabbit or goat anti-rat) for 30 min and streptavidin-horseradish
164 peroxidase (Nichirei Bioscience) for 30 min at room temperature. To develop the color,
165 sections were incubated in a 3,3'-diaminobenzidine tetrahydrochloride-hydrogen peroxide
166 solution for 4 min. Finally, the sections were counterstained with hematoxylin and

167 dehydrated with ascending grades of alcohols. After washing with PBS, the sections were
168 mounted and examined using a BZ-X710 microscope.

169 Next, we performed the histoplanimetical analysis of LTs using the IHC sections for CD20,
170 CD3, IBA1, and MHCII. More than 10 LFs and DLTs were examined. Firstly, total area of
171 LFs or DLTs and the positive reaction area for each marker were measured, and percentage
172 of the latter to the former was calculated.

173

174 *Electron microscopy*

175 For SEM, small pieces of VV were treated with tannic acid and post-fixed with 1% OsO₄
176 (osmium tetroxide) for 1 h. Then, the specimens were dehydrated in ascending grades of
177 alcohol, immersed in 3-methylbutyl acetate, and dried with the HCP-2 critical point dryer
178 (Hitachi; Tokyo, Japan). The specimens were then observed under an S-4100 scanning
179 electron microscope (Hitachi).

180

181 *Statistical analyses*

182 The results are expressed as the mean ± standard error (SE). Multiple comparisons were
183 performed using the Kruskal–Wallis test followed by the Scheffé method when a
184 significant difference was observed ($P < 0.01$).

185

186 **Results**

187 *LT found in the VV and external parts of genital organs (EPGO) mucosa of cows*

188 We investigated the mucosa of the vagina and VV extending from the external urethral
189 orifice to the vulva, and the vulva along with the pudendal labia and clitoris were also
190 examined as EPGOs (**Supplemental figure 1**). Whole-mount specimens were visualized
191 for hematoxylin-positive spots in the examined mucosae; in particular, we focused on VV
192 and EPGOs because these areas are close to the outer environment and have contact with
193 urine and feces. All examined cows had numerous hematoxylin-positive spots in the
194 mucosal surface of the VV and EPGOs, and these spots were distributed at an
195 approximately 1 cm distance from the boundary of the un-haired pudendal labia (**Fig. 1A,**
196 **B**). Furthermore, 37.5% of examined cows showed these spots in EPGOs (three of eight
197 cows), especially in the area close to the clitoris (**Fig. 1C**). Then, these spots were
198 histologically identified as LT (**Fig. 1D**), and classified as genital organ-associated LTs
199 (GOALTs). They were further investigated in subsequent experiments.

200 We also examined the localizations of GOALTs by dividing the VV and EPGOs into
201 six regions (**Fig. 1E**). During the histometry of the density of GOALT spots for the six
202 examined regions, region II and V, located along the ventral median line of the VV,
203 including the external urethral orifice, clitoris, and ventral commissure of the labia, tended
204 to exhibit a higher density without significance (**Fig. 1F**). These results indicated that
205 GOALTs were distributed in the mucosa of the VV and EPGOs in a ring shape; therefore,
206 we denoted the GOALT distributions as a "genital lymphoid ring," based on "Waldeyer's
207 lymphoid pharyngeal ring." In subsequent analysis, we focused on VV mucosa.

208

209 *Histology of GOALTs found in the cow VV mucosa*

210 The histological observation was performed in the VV. The mucosa was covered by
211 non-keratinized and stratified squamous epithelium (**Fig. 2**). The GOALTs were mainly
212 localized in the lamina propria in the VV. Importantly, regarding the LT structures, two
213 different types of GOALTs were found in the VV; LF (**Fig. 2A-D**) and DLT (**Fig. 2E-H**).
214 For LF, three types were primarily observed in the VV mucosa based on their morphology.
215 First, several LFs were localized in the deep portion of the lamina propria and fully
216 surrounded by connective tissues. In this type, the VV epithelium completely maintained
217 the structure (**Fig. 2A**). Second, scattered lymphocytes were observed between the LF and
218 VV epithelium, and these lymphocytes were also thoroughly or partially observed within
219 the VV epithelium structure (**Fig. 2B**). Third, LF directly faced the VV lumen, and the VV
220 epithelium was occupied by lymphocytes (**Fig. 2C**). Further, most of the LFs were
221 solitarily, but paired LFs were also noticed (**Fig. 2D**). Additionally, several shapes of DLTs
222 were observed, such as triangle-, C-shaped-, amorphous-, and oval-types (**Fig. 2E-H**).

223 In the histometry of the VV mucosa, LF- and DLT-types accounted for 35% and 65%,
224 respectively (**Fig. 2I**). Furthermore, as a result of morphometry according to the LF
225 localization, migrating-, isolated-, and bordering-types accounted for 51%, 37%, and 12%,
226 respectively (**Fig. 2J**). Finally, for the DLT shapes, amorphous-, oval-, C-shaped-, and
227 triangle-types accounted for 57%, 30%, 9%, and 4%, respectively (**Fig. 2K**).

228

229 *Cell composition of GOALTs in the cow VV mucosa*

230 Regarding LFs of the GOALT in the VV mucosa, numerous CD20⁺ B cells were found,
231 and the germinal center and an area between the LF and VV epithelium were located (**Fig.**
232 **3A**). Further, CD3⁺ T cells were diffusely distributed in LFs, including the germinal center,
233 and tended to mainly localize along the border area between the LF and VV epithelium
234 (**Fig. 3B**). IBA1⁺ macrophages and MHCII⁺ APCs were similar in localization and were
235 located in the germinal center and the border area between the LF and VV epithelium; the
236 former was also localized in the VV epithelium (**Fig. 3C, D**). Next, PNA⁺ high endothelial
237 venules were localized around the LFs and in the border area between the LF and VV
238 epithelium (**Fig. 3E**). In agreement with histological observations, the percentage of B cells
239 was statistically significantly higher in LFs than the percentage of T cells, macrophages,
240 and APCs ($P < 0.01$, **Fig. 3F**).

241 In the DLT, B cells, T cells, macrophages, APCs, and high endothelial venules were
242 dispersed diffusely (**Fig. 4A-E**). Similar to LFs, DLTs had a statistically significantly
243 higher percentage of B cells than that of other examined immune cells ($P < 0.01$, **Fig. 4F**).

244

245 *Histology of epithelial cells covering the GOALT in the cow VV mucosa*

246 Histologically, the VV mucosa was covered by stratified epithelial cells, wherein the basal
247 layer was smaller with narrow cytoplasm, and their nuclei were located close to one another
248 (**Fig. 5A**). Follicular epithelial cells found in MALTs were not identified. Importantly, in
249 several LTs, the thickness of the epithelium gradually became smaller towards the
250 epithelium covering LTs (**Fig. 5A**). Furthermore, the epithelium covering LTs showed
251 complete destruction of each junction of epithelial cell with numerous lymphocytes and

252 erythrocytes, and several epithelial cells peeled off and created a large junctional gap,
253 which permitted direct passage of immune cells into the intraluminal space of the VV (**Fig.**
254 **5B**). Occludin⁺ reactions were observed in the intercellular region of the epithelial cells, but
255 these were unclear in the epithelium covering LTs (**Fig. 5C, D**).

256 Next, picrosirius red (PSR) staining revealed the developed connective tissue fibers in
257 the lamina propria of the VV mucosa. Under polarized light observation, well-developed
258 collagen fibers were visualized as bright orange to red, whereas thinner reticular fibers
259 appeared yellow to green (**Fig. 5E**). In GOALTs, the DLT-types contained abundant
260 collagen and reticular fibers, but they were scarce in the LF-type (**Fig. 5E**). The VV
261 mucosa exhibited well-developed collagen and reticular fibers beneath the epithelium (**Fig.**
262 **5F**), but they were obscure in the LF (**Fig. 5G**).

263

264 *Ultrastructure of surface of LT in the VV*

265 Next, we observed the GOALT surface in the VV mucosa (**Fig. 6**). Whole-mount staining
266 with hematoxylin revealed the GOALTs as spots (**Fig. 6A**). Using scanning electron
267 microscopy (SEM), the epithelium covering GOALTs was found to partially lack in several
268 areas (**Fig. 6B**). Briefly, at these regions, several polygonal-shaped epithelial cells were
269 peeled off, and lymphocytes appeared to be directly exposed to the intraluminal space
270 through the hole (**Fig. 6C, D**). **Figure 6E** shows the large sized, dome-shaped LF covered
271 by the VV epithelium, which was approximately 350–480 μm in a diameter. Several
272 differently sized destroyed areas of the epithelium fused with each other and formed a
273 complicated cleft. From the inside of the GOALT, erythrocytes were also exposed directly

274 to the intraluminal space (**Fig. 6F, G**). Additionally, small protrusions occurred that
275 appeared to be lymphocytes pushed up to the surface of the epithelium (**Fig. 6F, G**). Some
276 connective tissue fibers were also observed inside of the GOALT and opened to the lumen
277 (**Fig. 6G**). Summary of general quantitative data shown in **supplemental table 3**.

278

279

280 **Discussion**

281 This study clarified the morphological characteristics of GOALTs found in the VV and
282 EPGOs of the cow. In cows, the VV extends from the external urethral orifice to the ventral
283 commissure of the labia and is composed of the caudal part of the reproductive and/or
284 urinary system, localized ventral to the anus, with a length of approximately 7–11 cm
285 (Budras and E.Habel, 2003; Konig and Liebich, 2007). The LTs in the bovine VV were
286 previously suggested in cows (Blazquez et al., 1987b; Cole, 1930; Hammond, 1927), but
287 detailed morphological information was lacking. The high abundance of LTs along the VV
288 may relate to increased antigenic stimulation in this region. In the present study, the ventral
289 median region (II and V containing EPGOs) of the VV and EPGOs tended to have a greater
290 number of GOALTs than other areas. We considered that the developed GOALTs in this
291 area reflected the possibility of high exposure to antigenic substances, such as urine or
292 feces, because of the anatomical localization of the VV. These relationships between
293 antigenic challenge and the localizations of LTs are compatible with statements regarding
294 the amount and type of MALTs being partially dependent on the antigenic challenge
295 (Chuluunbaatar et al., 2020; Jericho et al., 1971; Pollard and Sharon, 1970). Additionally,

296 the age of the cow appeared to be a factor influencing the amount of LT, and the VV of
297 adult cows contained a significantly higher number of LTs than 6–8-week-old calves
298 (Blazquez et al., 1987a).

299 GOALTs were morphologically divided into LFs and DLTs, and the latter was
300 dominant in the VV. LFs were usually dispersed solitarily in a random manner throughout
301 the mucosa of the VV or EPGOs. Most LFs had a germinal center, which initiated a series
302 of events that included lymphocyte activation and proliferation, plasma cell differentiation,
303 and antibody production. Mitotic patterns were frequently seen in the germinal center,
304 indicating that new lymphocytes were proliferating *in situ* (Wojciech and Ross, 2020).
305 Although our histological data showed different morphology of LFs, the dominant was the
306 migrating-type, and sectioning of LFs also could have affected their shape observed during
307 microscopy. SEM analysis revealed both an epithelium-covered area (isolated-type) and
308 non-covered area (migrating-, bordering-type) in the same LF. Furthermore,
309 three-dimensional analysis of LFs could clarify their fine structure.

310 DLTs typically do not have a distinct border with surrounding tissues, rather have an
311 amorphous shape and are tactically localized to intercept antigens and initiate an immune
312 response. In general, after contact with antigen, lymphocytes located in the LT move to
313 regional lymph nodes, where they can sustain proliferation and differentiation. Then, the
314 progenies of these cells return to the lamina propria as B and T lymphocytes with effector
315 functions. Therefore, combined localization of LFs and DLTs in the VV or EPGO mucosa
316 could act as a protector of the reproductive tract against pathogenic substances.
317 Furthermore, our histological data showed the different morphology of LFs; the dominant

318 amorphous-type, with variation in the morphology of DLT that might reflect the difference
319 in the activation phase of GOALTs.

320 Subsequently, the percentage of the positive area for the immune cells calculated in
321 GOALT and both DLTs and LFs was dominated by B cells in comparison with T cells,
322 macrophages, and APCs. This was an expected finding, which is in agreement with other
323 research data, including those for the mice spleen, Peyer's patches, isolated LFs, and cow
324 and rabbit GALT (Hamada et al., 2002; Komban et al., 2019; Parsons et al., 1989;
325 Schneider et al., 2001).

326 The cell components of the cow GOALT showed close similarities to those of other
327 MALTs (GALT and Waldeyer's pharyngeal ring); however, the cell distribution was
328 generally solitarily and randomly localized within the VV and EPGOs (Owen and Jones,
329 1974). One of the most important cell components in MALTs is the APC. In this study,
330 APCs were found around the DLT and LF in different regions of the mucosal epithelium,
331 such as the germinal center, mantle zone, submucosa, and middle layers of stratified
332 squamous epithelium of the VV. These strategically positioned localizations might play
333 important roles in the initiation of immune reactions and maintenance of peripheral
334 tolerance. Therefore, various types of APCs in the LTs have different ways to take up
335 antigens from the lumen, including endocytosis, phagocytosis, or transcytosis (Zhang et al.,
336 2007). Although, in this study, the epithelium that covered GOALTs was thinner than areas
337 not covering GOALTs, and in some areas, the epithelium structure was completely lost, and
338 LT and the intraluminal area were directly bordered without any edge. This formation of
339 LT allows direct passage of antigens to the LT and immune cells from the intraluminal area.

340 As described in the literature (Brandtzaeg et al., 2008), MALT is divided into two specific
341 types, an organized MALT delimited by organized collagen fiber tissue and a disorganized
342 or diffuse MALT, comprising populations of lymphocytes from the lamina propria and the
343 base of the epithelial lining, which are consistent with LFs and DLTs. LFs located in the
344 conjunctiva have a lenticular shape, and occasionally around this area, the basement
345 membrane is mostly discontinuous, and the epithelium is thinner (Crespo-Moral et al.,
346 2020). We classified DLTs by shape, and each shape might reflect the different LT activity
347 in the VV mucosa.

348 We studied the normal histomorphology of the GOALT, and unfortunately, the phase
349 of the estrus cycle was impossible to identify in the examined cows. Importantly, the
350 immune system in the female reproductive tract is precisely influenced and regulated by
351 sex steroid hormones, estradiol and progesterone, which are produced in cyclic variations
352 by the ovary during the estrus cycles. Throughout the estrus cycle, immune cells are present
353 in considerable numbers and inconsistently distributed in the stromal layer and the
354 epithelium of the female reproductive tract (Bulmer and Earl, 1987; Hunt, 1994). Moreover,
355 the epithelial barrier of the reproductive tract, which includes various tight junction
356 conditions, provides immune protection by maintaining a strong physical barrier,
357 transferring antibodies to the mucosa, producing antibacterial composites, and recruiting
358 immune cells, all of which are influenced by hormones (Russell and Mestecky,
359 2002)(Ochiel et al., 2008). Additionally, the various shapes of GOALTs found in the VV
360 and EPGOs may be affected by different phases of the estrus cycle. A limitation of this
361 study was the small sample size available to identify whether or not the estrus cycle

362 influences the amount of GOALT in the VV in cows. Further study is needed to clarify the
363 GOALT amount at every stage of the estrous cycle.

364 In summary, this study morphologically and histologically evaluated GOALTs in the
365 VV and EPGOs of cows, which constituted a “genital lymphoid ring.” Regarding GOALT
366 morphology (**Fig. 7**), the mucosal epithelium covering DLTs and LFs was partially or
367 completely demolished, making a direct passage for immune cells to reach the luminal side.
368 The clarification of the morphology of GOALTs in cow genital tracts would contribute to
369 our understanding of its immune-associated function, the pathogenesis of diseases, and the
370 development of therapeutic strategy by applying mucosal vaccinations targeting GOALTs
371 in farm animals.

372

373 **Declarations of competing interest**

374 The authors declare that they have no known competing financial interest or personal
375 relationships that could have influenced in any way the work reported in this paper.

376

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380

381 **Author Contributions**

382 YK and OI supervised the findings of this study. MAM, YHAE, TENA, TANA, MRI, and
383 YO provided critical feedback and helped shape the research, analysis, and manuscript. TC,
384 OI, and YK analyzed data and wrote the paper.

385

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502

503 **Figure legends**

504 **Figure 1. Distribution of GOALTs in the mucosa of the VV and EPGOs of cows.**

505 (A) Gross anatomical features of the VV and EPGOs with whole-mount staining using
506 hematoxylin. White and red asterisks indicate the external urethral orifice and clitoris,
507 respectively. One scale of the ruler is 1 mm.

508 (B) Magnified image of the VV mucosa squared area in panel A. Scale bars = 1 cm.

509 (C) Magnified image of the EPGO mucosa squared area in panel A.

510 Black arrowheads indicate hematoxylin-positive spots. Scale bars = 1 mm.

511 (D) Histology of hematoxylin-positive spot squared area in panel B. Histological
512 observation revealed the spot is a cell aggregation, the GOALT, part of genital lymphoid
513 ring. H&E staining. Scale bar = 100 μ m.

514 (E) Schematic illustration of cutaway of the VV and EPGO area, including the vulva and
515 clitoris. For morphometry, the mucosal area is divided into six regions. Blue circles indicate
516 GOALTs.

517 (F) The density of GOALTs in the VV mucosa (number/cm²).

518 Values = mean \pm SE. $n = 8$.

519 GOALT: genital organ-associated lymphoid tissue, VV: vaginal vestibule, EPGOs: external
520 parts of genital organs, H&E: hematoxylin & eosin

521

522 **Figure 2. Histological characteristics of GOALTs in the cow VV.**

523 (A) Isolated-type LF. LF localizes in the deep portion of the lamina propria and is fully
524 surrounded by connective tissues.

525 (B) Migrating-type LF. Scattered lymphocytes are observed between LF and VV
526 epithelium.

527 (C) Bordering-type LF. LF directly faces the VV lumen, and the VV epithelium is occupied
528 by lymphocytes.

529 (D) Double migrating-type LF.

530 Dotted lines indicate the border of LFs. H&E staining. Scale bars = 100 μ m.

531 (E) Triangle-type DLT.

532 (F) C-shaped-type DLT.

533 (G) Amorphous-type DLT. Dotted lines indicate the border of the DLT.

534 (H) Oval-type DLT.

535 Scale bars = 100 μ m.

536 (I) Appearance of the percentage of LFs and DLTs in the examined GOALTs of the VV.
537 $n = 8$.

538 (J) Appearance of the percentage of each type of LF in the examined LFs of the VV. $n = 8$.

539 (K) Appearance of the percentage of each type of DLT in the examined DLTs of the VV.
540 $n = 8$.

541 GOALT: genital organ-associated lymphoid tissue, VV: vaginal vestibule, H&E:
542 hematoxylin & eosin, LF: lymphatic follicle, DLT: diffuse lymphoid tissue

543

544 **Figure 3. Immune cells composing the LF-type GOALT in the cow VV.**

545 IHC was performed in the VV mucosa, and immune cells and high endothelial venules
546 were found in the GOALTs.

547 (A) CD20 for B-cells.

548 (B) CD3 for T-cells.

549 (C) IBA1 for macrophages.

550 (D) MHCII for APCs.

551 (E) PNAd for high endothelial venules.

552 Squares denote the magnified area. Black arrowheads indicate positive cells for the IHC.

553 Scale bars = 100 and 25 μm (low and high magnifications, respectively).

554 (F) Percentage of each examined cell occupying the LF. Values = mean \pm SE. $n \geq 4$.

555 Significant differences between the immune cells indicated by *, $P < 0.01$;

556 Kruskal–Wallis test followed by the Scheffé method.

557 GOALT: genital organ-associated lymphoid tissue, VV: vaginal vestibule, LF: lymphatic

558 follicle, IHC: immunohistochemistry, APCs: antigen-presenting cells

559

560 **Figure 4. Immune cells composing the DLT-type GOALT in the cow VV.**

561 IHC was performed in the VV mucosa, and immune cells and high endothelial venules
562 were found in the GOALTs.

563 (A) CD20 for B-cells.

564 (B) CD3 for T-cells.

565 (C) IBA1 for macrophages.

566 (D) MHCII for APCs.

567 (E) PNAd for high endothelial venules.

568 Black arrowheads indicate positive cells for the IHC. Scale bars = 100 μ m.

569 (F) Percentage of each examined cell occupying the DLT. Values = mean \pm SE. $n \geq 4$.

570 Significant differences between the immune cells indicated by *, $P < 0.01$; Kruskal–Wallis
571 test followed by the Scheffé method.

572 GOALT: genital organ-associated lymphoid tissue, VV: vaginal vestibule, DLT: diffuse
573 lymphoid tissue, IHC: immunohistochemistry, APCs: antigen-presenting cells

574

575 **Figure 5. Epithelium covering and connective tissue fibers composing the GOALTs in**
576 **the cow VV.**

577 (A, B) Amorphous-type DLT. Destroyed epithelium located between DLT and intraluminal
578 area. The square indicates the magnified area. Red arrows denote peeled-off area of
579 epithelial cells. H&E staining. Scale bars = 100 μ m.

580 (C, D) Mucosal epithelium features stained using IHC for occludin. Panel C shows the
581 epithelium that does not cover the LT. Panel D shows the epithelium covering the LT.
582 Black arrowheads indicate positive reaction for the occludin. Scale bars = 50 μ m.

583 (E) Connective tissue fibers of LF and DLT stained using PSR under polarized conditions.
584 White dotted line indicates the border between the lamia propria and the epithelium. Blue
585 dotted line denotes the border of the LF. Lower panel is a magnification of the squared area
586 in the upper panel.

587 (F, G) Mucosal epithelium and connective tissue fibers stained using PSR. Panel F shows
588 the mucosa with epithelium that does not cover the DLT. Panel G shows the mucosa with

589 epithelium covering the DLT. Upper and lower panels show optimal and polarized
590 conditions, respectively. Black and white dotted lines indicate the border between the lamia
591 propria and the epithelium.
592 Scale bars = 100 μ m.
593 GOALT: genital organ-associated lymphoid tissue, VV: vaginal vestibule, DLT: diffuse
594 lymphoid tissue, IHC: immunohistochemistry, H&E: hematoxylin & eosin, LF: lymphatic
595 follicle, LT: lymphoid tissue, PSR: picrosirius red

596

597 **Figure 6. Ultrastructure of mucosal epithelium covering the GOALT of the cow VV.**

598 (A) VV mucosa stained in whole-mount with hematoxylin. GOALTs can be observed as
599 hematoxylin-positive spots. Scale bars = 1 mm.

600 (B) VV mucosa examined using SEM. The same area as in panel A was examined. Scale
601 bars = 100 μ m.

602 White arrow heads indicate GOALTs.

603 (C, D) High-magnified areas squared in panel B. Black arrow heads indicate migrating
604 lymphocytes from the GOALT to the lumen of the VV. Red arrow represents small
605 protrusions that could be lymphocytes. Scale bars = 10 μ m.

606 (E) VV mucosa on large GOALTs examined using SEM. Red arrow represents small
607 protrusions that could be lymphocytes. Scale bars = 100 μ m.

608 (F) High-magnified area of panel E. High-magnified areas squared in panel E. Black
609 arrowhead indicates lymphocytes. Scale bars = 10 μ m.

610 (G) High-magnified area of panel F. High-magnified areas squared in panel F. Yellow
611 arrowhead indicates erythrocytes. Scale bars = 2 μ m.

612 GOALT: genital organ-associated lymphoid tissue, VV: vaginal vestibule, SEM: scanning
613 electron microscopy

614

615 **Figure 7. Schematic illustration of GOALTs in the mucosa of the VV and EPGOs in**
616 **cows.**

617 GOALTs consist of various shaped LFs and DLTs that localize in different regions in the
618 lamina propria of the VV. Both LTs contain B-cells, T-cells, macrophages, and APCs.
619 B-cells are dominant. Germinal centers are frequently observed in the center of the LF.
620 High endothelial venules also appeared between LTs and surrounding connective tissues in
621 the lamina propria. Moreover, collagenous fibers are stretched across the lamina propria,
622 whereas reticular fibers are mainly observed in DLTs rather than in LFs. Characteristically,
623 the epithelium covering GOALTs is partially or completely disrupted by the invasion of
624 immune cells composing LFs and DLTs. These GOALTs are arranged as ring in the VV
625 and EPGO mucosa and named the “genital lymphoid ring.”
626 GOALT: genital organ-associated lymphoid tissue, VV: vaginal vestibule, DLT: diffuse
627 lymphoid tissue, LF: lymphatic follicle, LT: lymphoid tissue, APCs: antigen-presenting
628 cells, EPGOs: external parts of genital organs

629

630

631

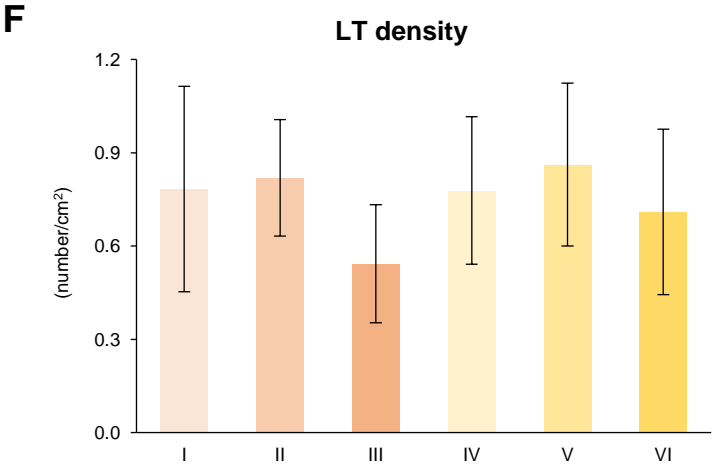
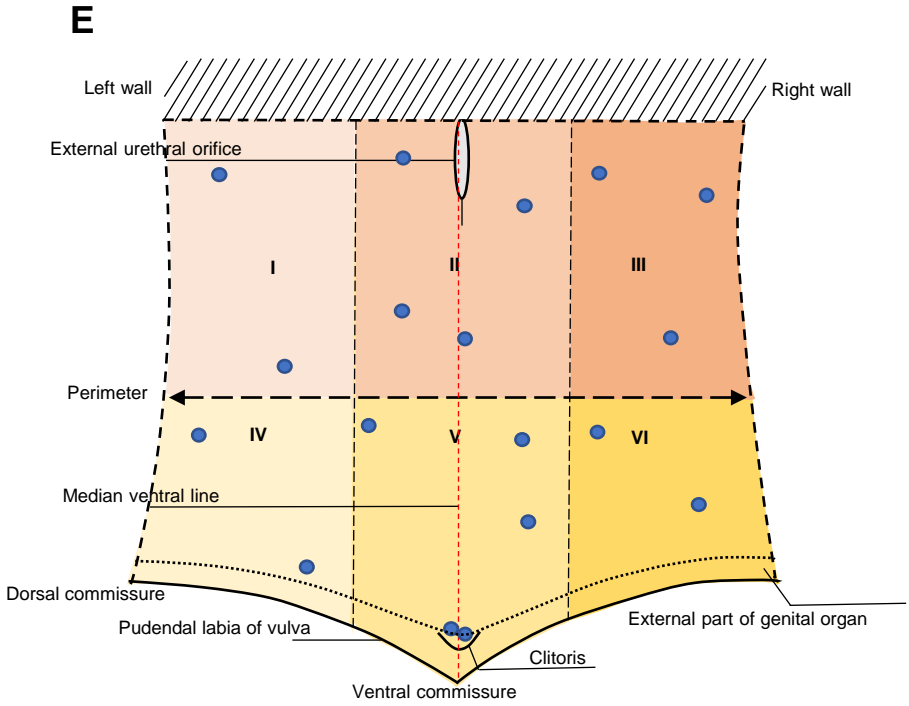
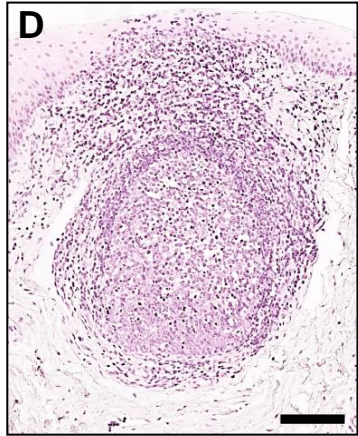
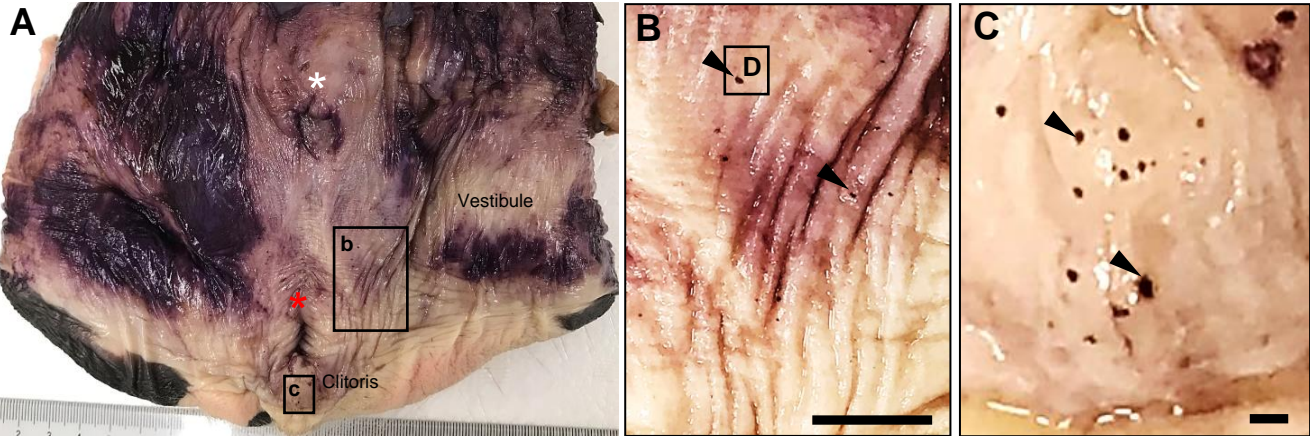
Highlights

- Whole-mount staining revealed lymphoid tissues on the mucosa of vaginal vestibule
- Lymphoid tissues showed two types: lymphatic nodule or diffuse lymphoid tissue
- Disrupted epithelium allowed passage for immune cells into the intraluminal space

Declarations of competing interest

The authors declare that they have no known competing financial interest or personal relationships that could have influenced in any way the work reported in this paper.

Figure 1



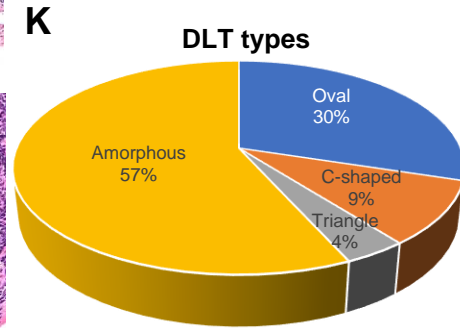
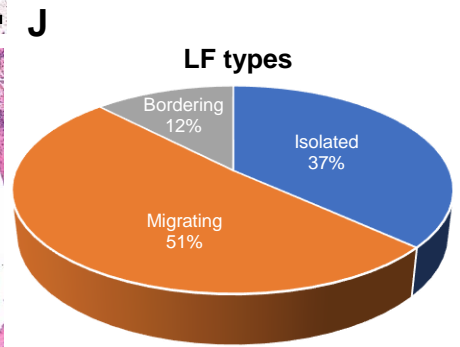
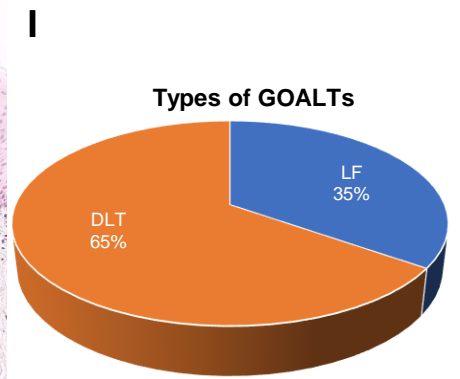
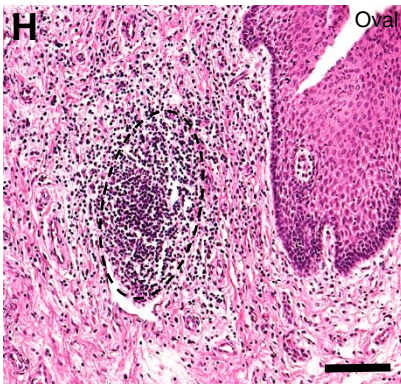
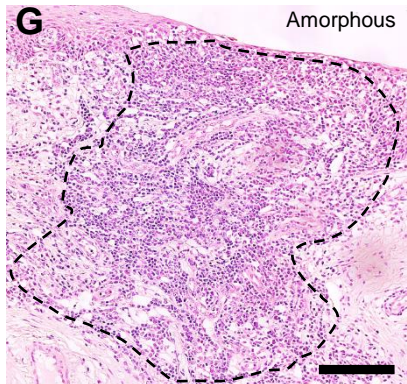
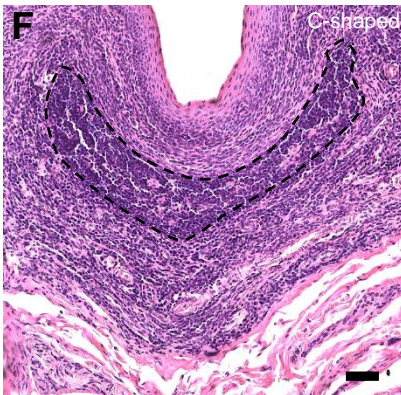
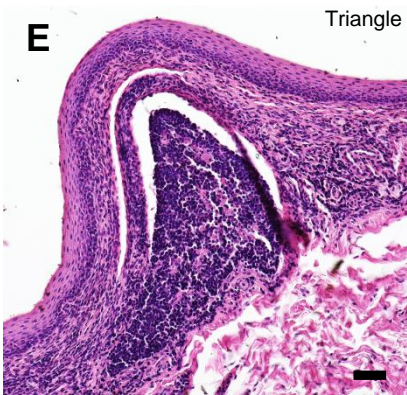
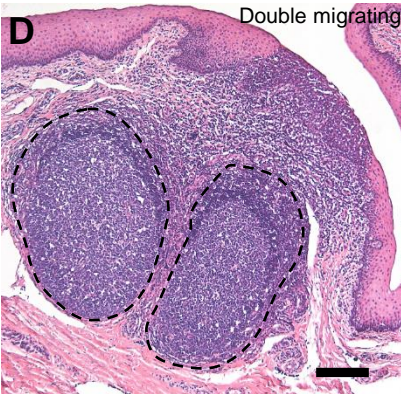
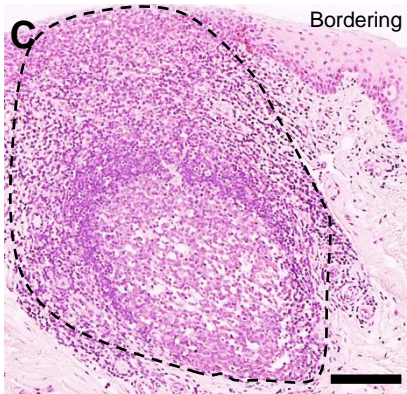
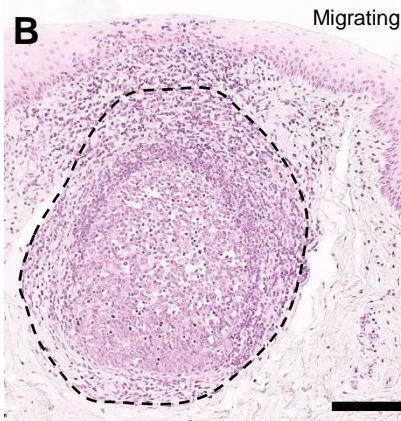
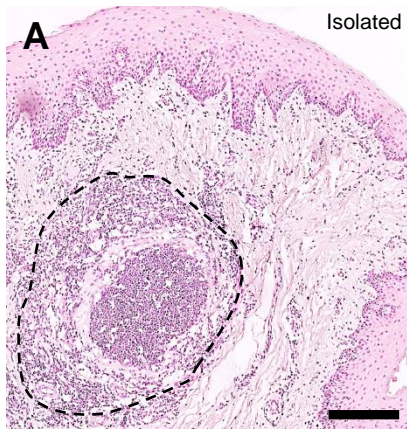


Figure 3

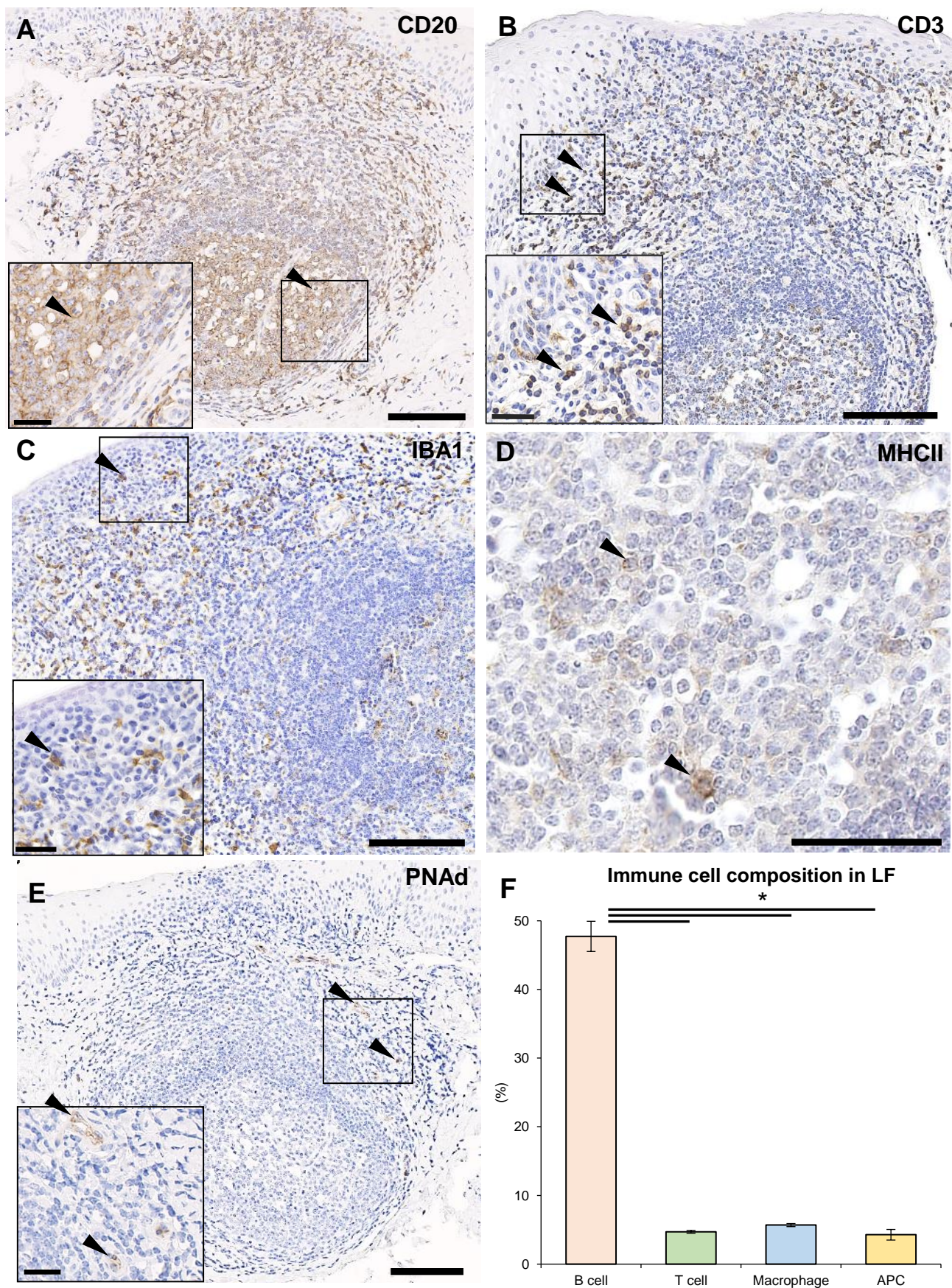


Figure 4

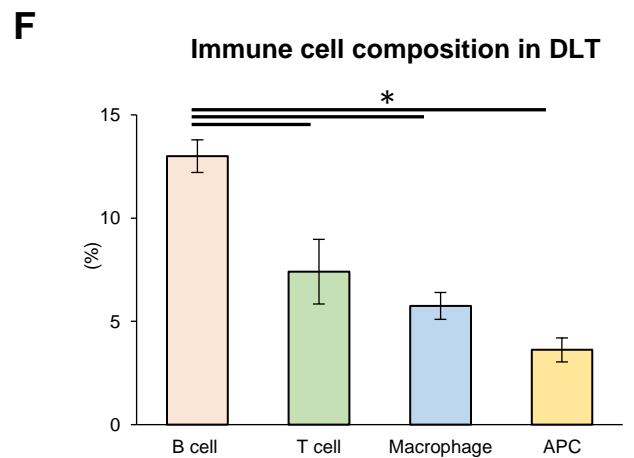
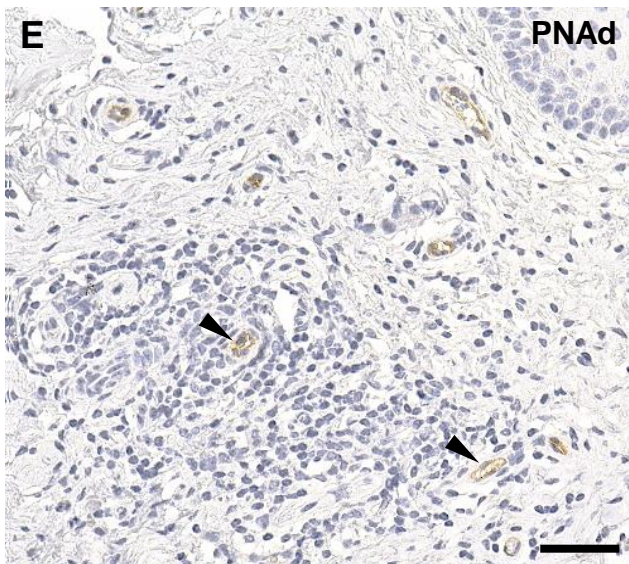
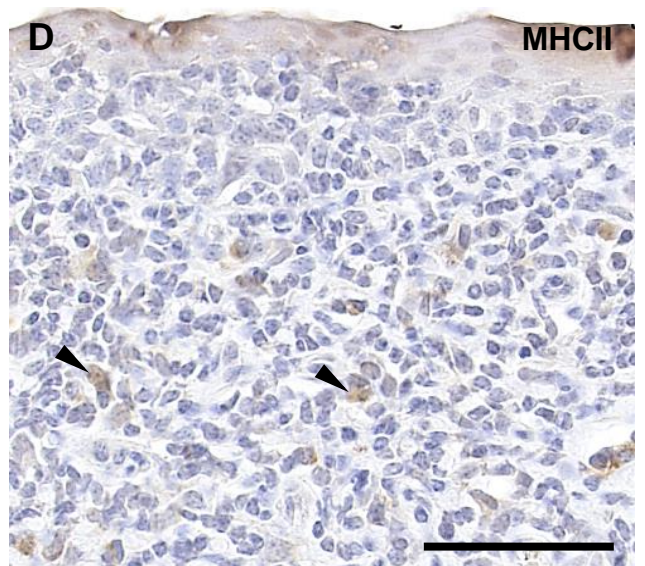
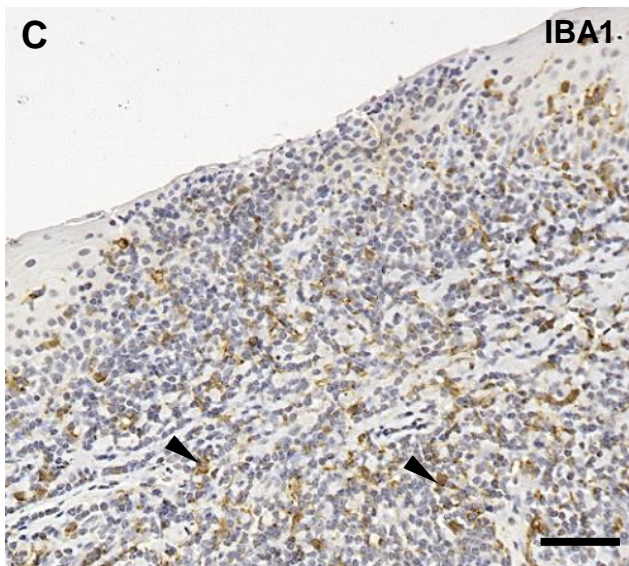
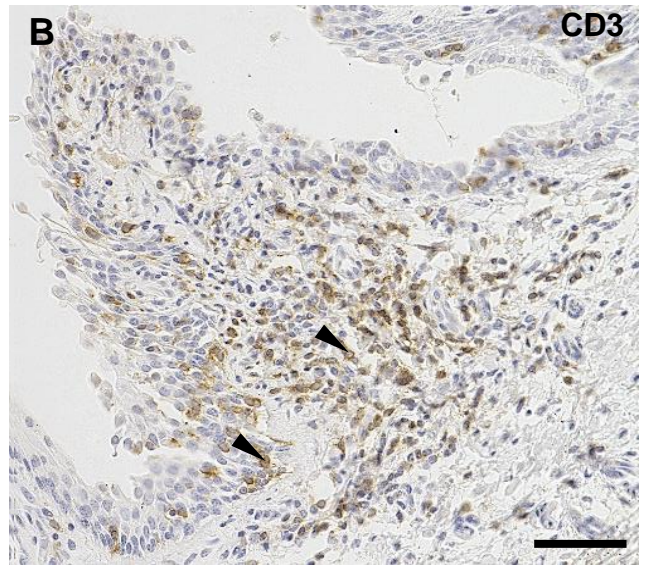
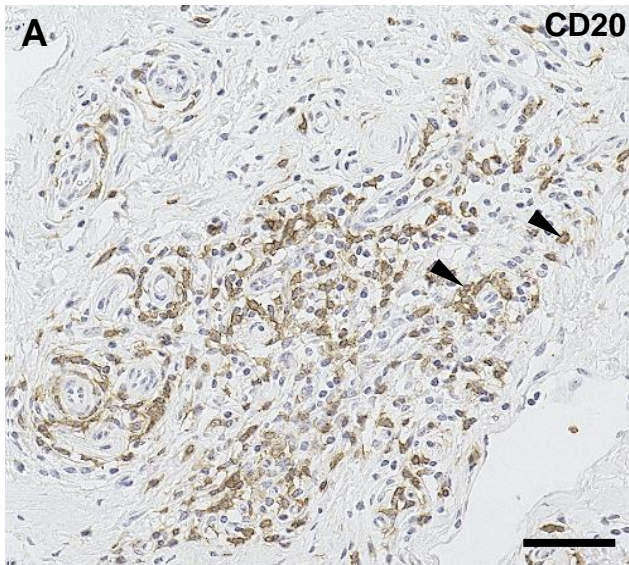


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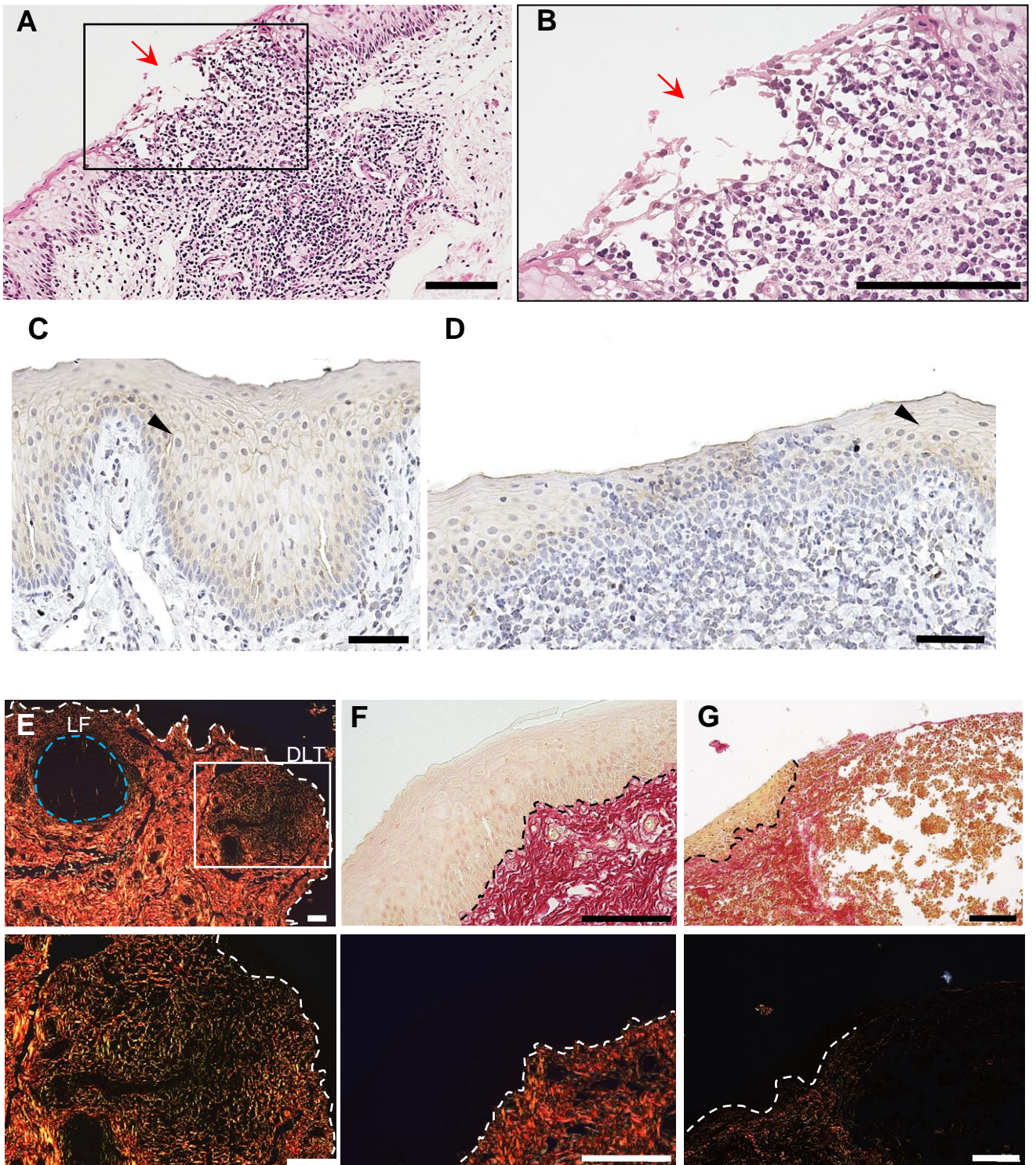


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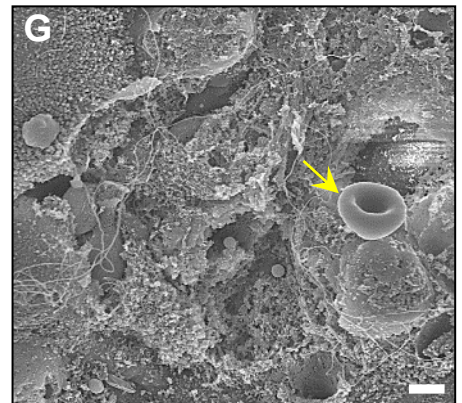
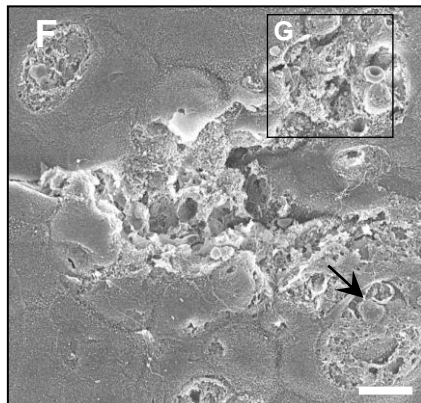
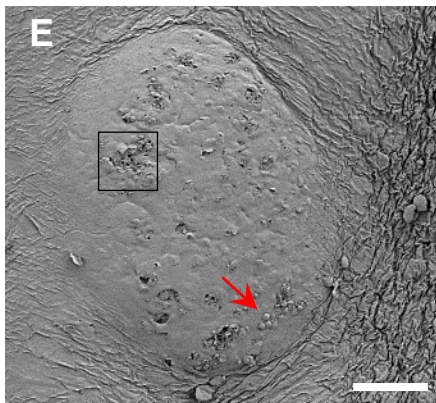
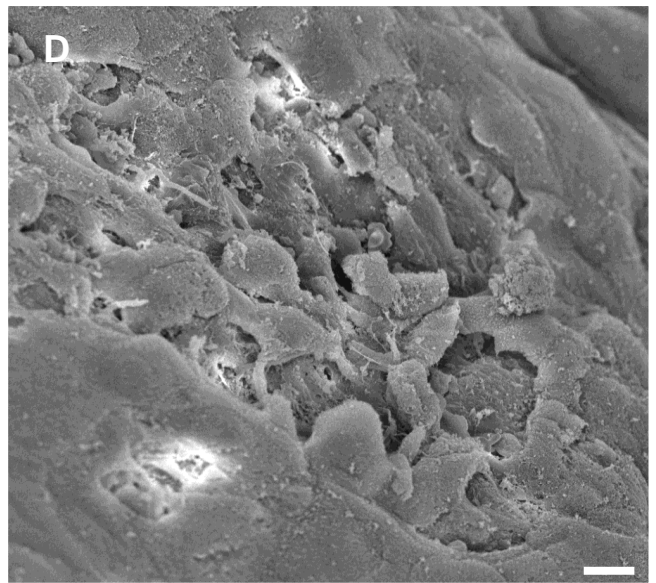
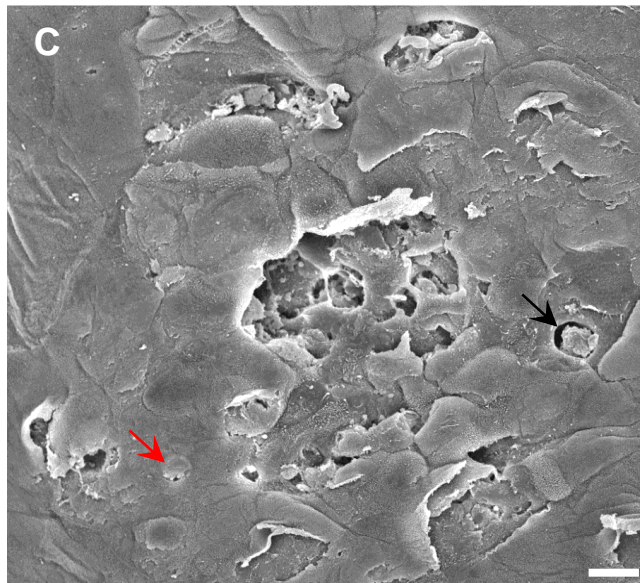
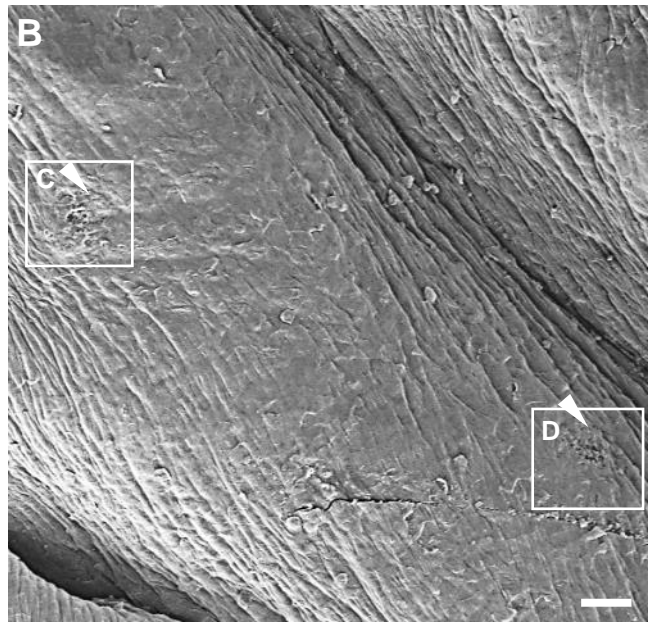
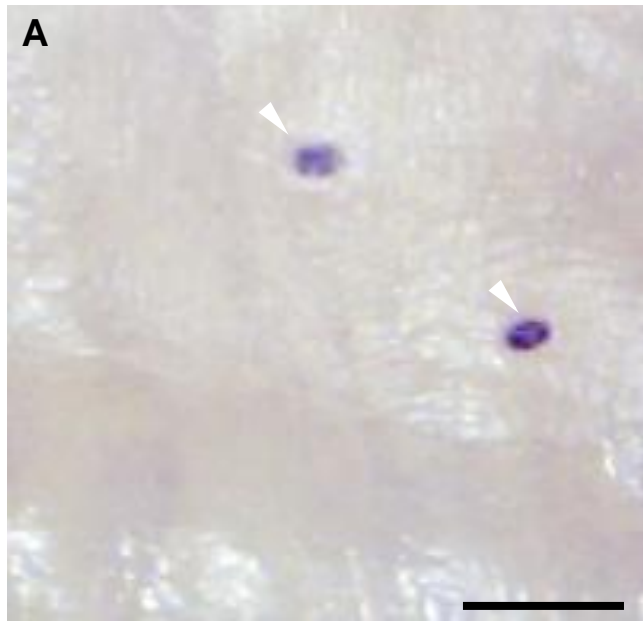


Figure 7

