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1 **Immunohistochemical study of morphology and**
2 **distribution of CD163^{+ve} macrophages in the normal**
3 **adult equine gastrointestinal tract**

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14 **Abstract**

15 Intestinal macrophages are the largest group of mononuclear phagocytes in the
16 body and play a role in intestinal innate immunity, neuroimmune interactions and
17 maintaining intestinal homeostasis. Conversely, they also are implicated in
18 numerous pathologies of the gastrointestinal tract, such as postoperative ileus and
19 inflammatory bowel disease. As a result, macrophages could be potential
20 therapeutic targets. To date, there are limited studies on the morphology and
21 distribution of macrophages in the equine gastrointestinal tract (GIT). The aim of

22 this study was to identify the location and abundance of resident macrophages in
23 the equine GIT using CD163 as an immunohistochemical marker. Tissue samples
24 were obtained post-mortem from 14 sites along the gastrointestinal tracts of 10
25 horses free from gastrointestinal disease; sample sites extended from the stomach
26 to the small colon. CD163^{+ve} cells were present in all regions of the equine GIT
27 from stomach to small colon. CD163^{+ve} cells were also identified in all tissue
28 layers of the intestinal wall; namely, mucosa, submucosa, *muscularis externa*
29 (*ME*), myenteric plexus and serosa. Consistent with a proposed function in
30 regulation of intestinal motility, CD163^{+ve} cells were regularly distributed within
31 the *ME*, with accumulations closely associated with the myenteric plexus and
32 effector cells such as neurons and the interstitial cells of Cajal (ICC).

33 **Keywords: horse; CD163; macrophage; gastrointestinal tract; intestine;**
34 **inflammation**

35 **Abbreviations**

36 BMP bone morphogenetic protein

37 GIT Gastrointestinal tract

38 ICC Interstitial cells of Cajal

39 LpM Lamina propria macrophages

40 ME *muscularis externa*

41 MM *muscularis* macrophages

42 MP myenteric plexus

43 POI Postoperative ileus

44 **Introduction**

45 Resident tissue macrophages are abundant in every organ and adapt in each
46 location to perform tissue-specific roles. Resident intestinal macrophages are a
47 good example of this heterogeneity. They are suitably adapted to maintain
48 intestinal mucosal homeostasis, in addition to playing a role in infection and
49 inflammation within what is considered a unique and difficult environment due to
50 constant antigenic challenge from intestinal luminal contents. It is therefore
51 unsurprising that the gastrointestinal tract (GIT) represents the largest reservoir of
52 macrophages in the body (Lee et al., 1985). Intestinal macrophages are divided
53 into two distinct sub-populations: lamina propria or mucosal macrophages (LpM)
54 and *muscularis* macrophages (MM). Their survival is dependent upon signals
55 from the macrophage colony-stimulating factor receptor (CSF1R), and in mice
56 they are rapidly depleted by treatment with anti-CSF1R antibody (MacDonald et
57 al., 2010). Intestinal macrophages, like most tissue macrophages, are derived
58 from precursors from the yolk sac or fetal liver. However, LpM were shown to
59 turn over relatively rapidly with replacement from blood monocytes in both
60 mouse (Bain et al., 2014) and humans (Bujko et al., 2018). In comparison to
61 LpM, MM have a slower turnover rate and are long-lived (Mikkelsen et al., 2004)
62 although more recent studies in mice suggest the presence of a longer-lived sub-
63 population of LpM defined by expression of the surface markers CD4 and TIM4
64 (Shaw et al., 2018). Long-lived MM are both embryonic and monocyte-derived
65 with a lower turnover than LpM (De Schepper et al., 2018).

66 The abundant LpM population in mice and humans are CD64^{+ve}, MHC Class II^{hi},
67 CD206^{+ve} and CD163^{+ve}. Additionally, in mice LpM are CD11b^{+ve}, CD11c^{+ve},
68 CD14^{+ve} and CX3CR1^{+ve}. This is in contrast to human LpM which express low

69 levels of these markers but instead express CD209 (Bain and Schridde, 2018).
70 LpM are highly phagocytic and bactericidal but exist in a state of ‘hypo-
71 responsiveness’. This anergy can be attributed to the absence of the
72 lipopolysaccharide (LPS) receptor CD14 and the failure of LpM to produce
73 proinflammatory cytokines like tumour necrosis factor- α (TNF α) (Smythies et al.,
74 2005). In pigs, genes for C-type lectins including CLEC7A, CD68 and SIGLEC1
75 were down regulated in the GIT when compared to alveolar macrophages again
76 supporting the expectation that intestinal macrophages tend to be hypo-responsive
77 (Freeman et al., 2012). Additionally, macrophage production and sensing of the
78 anti-inflammatory cytokine interleukin 10 (IL10) is essential to maintain intestinal
79 homeostasis with rodents that are IL10 deficient developing severe colitis
80 (Zigmond et al., 2014). The position of LpM under the epithelium enables them
81 to be ideally situated to sample luminal contents, such as bacteria or dietary
82 antigens, using transepithelial dendrites (Niess et al., 2005). Depletion of LpM
83 with anti-CSF1R antibody revealed an essential function in the control of
84 proliferation and differentiation of intestinal epithelial cells by CD169 expressing
85 cells (Sehgal et al., 2018).

86 MM are considered a phenotypically distinct group from LpM (Gabanyi et al.,
87 2016; Muller et al., 2014) but do express CD64, MHC Class II, CD206 and
88 CD163 as LpM do (Bain and Schridde, 2018). In contrast to LpM, MM express
89 the LPS receptor CD14, meaning they can play a role in endotoxin-mediated
90 responses within the *muscularis* (Kalff et al., 1998b). Whilst their role in the
91 intestine has not been as extensively studied as the LpM population, rodent
92 derived data show that MM interact with neurons to control intestinal motility.
93 MM produce bone morphogenetic protein 2 (BMP2) which acts on the BMP

94 receptor on enteric neurons. Enteric neurons produce CSF1, maintaining the MM
95 population. This bi-directional interaction regulates the smooth muscle
96 contractions of peristalsis (Muller et al., 2014). Although MM appear in the
97 intestinal wall during embryonic development, their development in mice is
98 unaffected by the absence of an enteric nervous system (Avetisyan et al., 2018).

99 Human and rodent studies have described intestinal macrophages in all cross-
100 sectional regions of the intestine with a variation in their morphology dependent
101 upon their location. In the mouse, serosal macrophages are bipolar, slender,
102 orientated parallel to the longitudinal muscle and occasionally have bifurcated
103 processes (Mikkelsen, 2010). Cells within the circular and longitudinal muscle
104 layers are found between the muscular bundles and have an elongated thin shape,
105 with those in the longitudinal muscle being less elongated compared with those
106 within the circular muscle (Kalff et al., 1998b). Their location, morphology and
107 CSF1 responsiveness can be visualised using *Csf1r* reporter transgenes in mice
108 and rats (Hawley et al., 2018; Irvine et al., 2020; Sauter et al., 2014).

109 Macrophages between the circular and longitudinal muscle layers, at the level of
110 the MP (also termed Auerbach's Plexus) are stellate with multiple dendrites (Kalff
111 et al., 1998b; Mikkelsen, 2010).

112 Dysregulation or activation of intestinal macrophages contributes to the
113 pathogenesis of inflammatory bowel disease and postoperative ileus (POI) (Kalff
114 et al., 1998a; Na et al., 2019). In mice, recruited monocytes contribute to the
115 resolution of POI and the administration of CSF1, has therapeutic potential (Farro
116 et al., 2017). POI in horses is a life-threatening complication of abdominal
117 surgery (Lisowski et al., 2018) yet, despite the established influential role of

118 macrophages in the pathogenesis of POI in other species, there have been few
119 studies in the horse.

120 Studies on the distribution of macrophages in the equine GIT are limited, as is the
121 number of species-specific reagents available. This may be partly attributable to
122 limited availability of appropriate immunological markers, such as F4/80 which is
123 commonly used in mice (Hume et al., 1984). A common surface marker used in
124 multiple species, including the horse, is the macrophage scavenger receptor for
125 the haemoglobin-haptoglobin complex (CD163) which distinguishes resident
126 macrophages from recently-recruited monocytes (Chapuy et al., 2019; Sauter et
127 al., 2016; Van den Heuvel et al., 1999; Yamate et al., 2000). Previously used
128 markers for macrophage identification in the horse include the following; CD163
129 to identify lamina, intestinal (mucosal), uveal and alveolar macrophages
130 (Faleiros et al., 2011; Grosche, 2011; Karagianni et al., 2013; Sano et al., 2016;
131 Yamate et al., 2000), CD68 in monocyte-derived macrophages (MDM) and
132 various tissue macrophages including the small intestine (Fidalgo-Carvalho et al.,
133 2009; Siedek et al., 2000), MHC Class II in MDM and uveal macrophages
134 (Fidalgo-Carvalho et al., 2009; Sano et al., 2016), CD14 in monocytes, tendons,
135 alveolar and peritoneal macrophages (Chelvarajan et al., 2015; Dakin et al., 2012;
136 Karagianni et al., 2013), CD172a (Sirp α) in tendon macrophages (Dakin et al.,
137 2012), TLR4 in alveolar, peritoneal and pulmonary macrophages (Karagianni et
138 al., 2013; Singh Suri et al., 2006), CD206 in tendon macrophages (Dakin et al.,
139 2012), MAC387 (MRP-8 and MRP-14) in intestinal (mucosal) macrophages
140 (Packer et al., 2005; Steuer et al., 2018) and lysozyme in mucosal macrophages
141 (Packer et al., 2005) although both MAC387 and lysozyme are non-specific for
142 macrophages since both are also expressed by granulocytes.

143 Significant species-specific responses exist in macrophages. For example, in
144 response to LPS equine bone marrow-derived macrophages do not produce nitric
145 oxide unlike rodents which do (Young et al., 2018). Several studies have
146 highlighted that the horse immune response is more similar to that of human than
147 mouse (Karagianni et al., 2017; Parkinson et al., 2017). Additionally, sequence
148 analysis of human, rodent and horse genes involved in immunity have
149 demonstrated greater synteny between horse and human than rodent and human
150 (Hudgens et al., 2011; Tompkins et al., 2010). The significant role in immunity
151 that macrophages play, and the reported differences in macrophage responses that
152 occur between species, highlights the importance of studying the cells of interest
153 in each species and not always relying on existing data derived from other species
154 (usually rodents). In the GIT, CD163 has been used as a marker to identify both
155 resident and inflammatory equine intestinal macrophages (Grosche et al., 2011;
156 Nielsen et al., 2015; Yamate et al., 2000). The context of the former study
157 focussed on changes in macrophages in the mucosa in response to inflammation
158 and the latter focused on the demonstration of cross-reactivity of a specific anti-
159 CD163 antibody (clone AM-3K) between various species (humans, dogs, cats,
160 cattle, pigs and rabbits) and no quantitation was attempted. In this present study
161 we examine the distribution of CD163⁺ve resident macrophages in the normal
162 equine GIT by immunohistochemistry using an anti-CD163 (Clone EdHu-1)
163 monoclonal antibody (mAb). This will form a baseline for future studies of
164 macrophage populations within the normal equine GIT and help to improve our
165 understanding of the equine innate immune response in the GIT.

166 **Methods**

167 **Animals**

168 Ten horses (median age 17 years, range 6 - 26 years) of various breeds, were
169 admitted to the Equine Hospital at the Royal (Dick) School of Veterinary Studies
170 for elective euthanasia (**Table 1**). Horses were euthanased with secobarbital
171 sodium 400mg/ml and cinchocaine hydrochloride 25mg/ml (SomuloseTM;
172 Arnolds/Dechra), at a dose of 1ml/10kg bodyweight via a pre-placed intravenous
173 catheter in the left jugular vein. None of the animals had any chronic or recent
174 history of gastrointestinal (GI) disease and post -mortem examination confirmed
175 the absence of any gross GI pathology. The study was approved by the University
176 of Edinburgh School of Veterinary Medicine Ethical Review Committee.

177 **Collection of samples from the gastrointestinal tract**

178 Following euthanasia, an incision was made in the ventral midline to expose the
179 abdominal cavity. The GIT was removed from the body by transecting the
180 oesophagus at the level of the diaphragm, removing the mesenteric and body wall
181 attachments and transecting the small colon at the junction with the rectum.
182 Spleen and liver were removed, and the GIT placed on a clean working area.
183 Gross contamination of blood was removed with water. Full thickness 4 cm x 4
184 cm sections were removed from 14 anatomical locations from the stomach to the
185 small colon (**Table 2**) and rinsed in cold phosphate-buffered saline (PBS)
186 (Dulbecco's Phosphate Buffered Saline; Sigma-Aldrich) prior to being trimmed
187 into smaller segments and placed in 10% neutral buffered formalin (4%
188 formaldehyde in neutral buffered solution) (Sigma-Aldrich). Sites were selected
189 such that the distribution of CD163^{+ve} macrophages was representative of the

190 equine GIT and consistently selected from the same region. Areas with
191 immunological activity, such as Peyer's Patches were specifically not selected.

192 **Formalin fixed tissue paraffin embedding**

193 Tissue samples were fixed in formalin for 24-72 hours and processed overnight
194 using an Excelsior tissue processor (Thermo Fisher Scientific), placed in moulds
195 and embedded in paraffin wax. Sections five-micron (5 μ m) in thickness were
196 cut, mounted on slides (Superfrost Plus, Thermo Fisher Scientific), air dried for 1
197 hour and incubated at 55⁰ C for a further hour.

198 **Immunohistochemistry**

199 We elected to use the mouse anti-human mAb (Clone EDHu-1) as previous work
200 confirmed cross reactivity to both pig (Chen et al., 2019) and cattle (Fry et al.,
201 2016). Additional studies within our group demonstrated specific cell surface
202 staining on equine alveolar macrophages, but not on equine bone marrow-derived
203 macrophages by flow cytometry. These findings at protein level were as
204 expected, as indicated by mRNA-Seq analysis (data not shown). After paraffin
205 embedding, tissue sections were deparaffinised in xylene and rehydrated in a
206 graded ethanol series using an automated processor (Leica Autostainer; Leica
207 Biosystems). As a routine, all sections were stained with haematoxylin and eosin
208 (H&E) to allow subsequent confirmation of the absence of any histopathological
209 change indicative of pre-existing underlying pathology. Additionally, heat-
210 mediated antigen retrieval was performed on duplicate sections by placing slides
211 in 0.01M sodium citrate buffer (pH 6.0) in a microwaveable pressure cooker at
212 approximately 110⁰ C for 20 minutes. Endogenous peroxidase activity was
213 inhibited using 3% hydrogen peroxide (Peroxidase-blocking solution; Dako
214 REALTM, Agilent Technologies) in methanol for 30 minutes at room temperature

(RT). Slides were incubated with 100-200 µl blocking buffer (1% normal goat serum [NGS]; Abcam, 5% bovine serum albumin (BSA); Sigma in tris-buffered saline [50 mM Tris-Cl, pH 7.6; Sigma, 150 mM NaCl; Sigma, TBS; Abcam]) for 30 minutes at RT in a humidity chamber. Primary antibody (Mouse anti-human CD163 Clone EDHu1; Bio-Rad) at a dilution of 1:200 in blocking serum was applied and slides incubated for 2 hours at RT in a humidity chamber followed by washing with blocking buffer. Secondary anti-mouse antibody was applied (ImmPRESS HRP Anti-Mouse Ig [peroxidase] Polymer Detection Kit; Vector Labs) for 15-30 minutes at RT, followed by 3,3'-diaminobenzidine (DAB) (3-solution DAB kit; Vector Labs) for 10 minutes. Slides were either dehydrated and mounted with no counterstain or counterstained with haematoxylin. Once dried, slides were scanned using a Nanozoomer (Hamamatsu Photonics) and analysed using NDP.view2 (Hamamatsu Photonics).

Quantification of cells in the equine gastrointestinal tract

Tissue sections were visualised using NDP.view2 (Hamamatsu Photonics). Tissue layers were divided into the following categories; a) mucosa which included the epithelium, lamina propria and *muscularis mucosae* (if present) b) submucosa, c) *muscularis externa* which included both the inner (circular) and outer (longitudinal) muscle, d) myenteric plexus (MP) and e) the serosa. Three areas within a 2 mm region were selected for counting for each layer; 2 mm from the left side edge, the mid-point and 2 mm from the right-side edge of the tissue section. Areas to be analysed were drawn using either the rectangle or freehand region function. All areas were inspected visually prior to counting to evaluate if sections were representative. If areas were selected that were not representative of all slides a new area was selected. Altering areas based on number of space

occupying structures e.g. blood vessels was not performed as this could lead to bias of selecting areas with higher levels of positive staining. For the mucosa, submucosa and muscle layers, a 0.5 mm² area was measured. For the MP and serosa, a 0.1 mm² area was measured. Values were either multiplied by 2 or 10 respectively, to give values per mm². Cells were marked manually, counted and three values for each area averaged for further analysis. See **Supplementary Figure 1** for a summary of the quantification method.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.4.0 for Windows (GraphPad Software) and significance was assumed at $p < 0.05$. Significance in CD163 staining for each tissue layer between individual regions was determined by a Wilcoxon Signed Rank Test. Significance in CD163 staining in the different layers between grouped regions (small intestine vs large intestine) was determined using a Mann–Whitney U test. All graphs were created using GraphPad Prism 8.4.0 for Windows (GraphPad Software).

Results

Presence of macrophages in the equine GIT

Sections from 14 regions of equine GIT collected from 10 horses were stained for CD163. CD163⁺ cells were identified in all intestinal layers (mucosa, submucosa, *muscularis* and serosa) and in all regions of the GIT (stomach to small colon).

261 **Quantification of macrophages in the equine GIT**

262 The distribution of cells across tissue layers was not uniform. **Figure 1** shows the
263 number of CD163⁺ macrophages per mm² of tissue in all layers and regions of
264 the equine GIT. In the distal GIT, from the ileum to the small colon, the highest
265 density of CD163⁺ cells were predominantly in the submucosa. No clear trend
266 was observed from the stomach to the distal jejunum. CD163⁺ cells were less
267 prevalent, but evenly distributed in the *muscularis externa* throughout the full
268 length of the GIT. In the submucosa there was a significant difference ($p \leq$
269 0.0001) in CD163⁺ cells per mm² between the small intestine and large intestine
270 (**Figure 2**). A significant increase in positive cells per mm² was observed
271 between the distal jejunum to the ileum ($p \leq 0.01$) and between the distal jejunum
272 and the remainder of the sections of the distal GIT (**Supplementary Figure 2**).
273 By contrast, the density of CD163⁺ cells in the mucosa was relatively consistent
274 from stomach to small colon. However, as previously noted in humans and dogs
275 (Hume et al., 1987; Wagner et al., 2018), CD163⁺ macrophages in the colonic
276 mucosa were notably concentrated towards the apical regions of villi (**Figure 3c**).

277 **Morphology of macrophages in the equine GIT**

278 Morphological staining characteristics at each location within the GIT are detailed
279 below:

280 **Mucosa**

281 CD163⁺ cells throughout the GIT were predominantly bipolar, each with two to
282 three processes and were located entirely beneath the basement membrane within
283 the lamina propria. No cells or processes were observed crossing the basement
284 membrane and epithelial layer (**Figure 3 and 4**).

285 **Submucosa**

286 CD163^{+ve} macrophages were present in the submucosa of all tissue sections in the
287 small (**Figure 5**) and large intestine (**Figure 6**). In the small intestine,
288 submucosal macrophages were a mix of round and bipolar cells (**Figure 5**); whilst
289 in the large intestine, they were found to be predominantly round (**Figure 6**).
290 Macrophages were also observed adjacent to gut-associated lymphoid tissue
291 (**Figure 7**).

292 ***Muscularis externa*, myenteric plexus and serosa**

293 CD163^{+ve} equine macrophages were present in the four areas; the serosa, within
294 the longitudinal (outer) muscle, at the level of the MP and within the circular
295 (inner) muscle (**Figure 8**). Equine serosal macrophages were relatively regular in
296 their distribution (**Figure 9**). As sections were examined in cross section only,
297 most macrophages appeared bipolar and it was not possible to visualise whether
298 cells were ramified in this view, although occasional ramified cells were observed
299 (**Figure 10**).

300 Equine CD163^{+ve} macrophages were found within the *muscularis*, predominantly
301 lying between intermuscular bundles (**Figure 11**). These cells were uniformly
302 distributed and aligned parallel with the muscle fibres (**Figure 12**). Most cells
303 appeared bipolar or were identified by single areas of circular positive staining,
304 attributable to the orientation of the cross sections (**Figure 13**).

305 Equine CD163^{+ve} macrophages were observed in close proximity to the MP
306 (**Figure 8**), predominantly on the periphery of the MP, although some were also
307 observed in the centre (**Figure 14**). Positively stained cells were bipolar and
308 stellate-shaped in this region (**Figure 15**). The macrophage density (cells per

mm²) was significantly greater ($p \leq 0.0001$) adjacent to the MP compared to that observed within the circular and longitudinal muscle either side of the MP in both the small and large intestine (**Figure 16**).

Discussion

This study aimed to identify and provide an overview of the distribution of macrophages in the normal equine GIT, with a focus on the resident MMs. The densities and morphologies of cells labelled with anti-CD163 are broadly similar to reports of resident macrophages in other species using both CD163 and other markers. In the colon of mice, once Ly6C⁺ monocytes enter tissues, they undergo a differentiation process which involves the loss of Ly6C expression and upregulation of F4/80, CX3CR1, CD163, CD11c and MHC Class II. Similarly, in humans, the differentiation of CD14⁺ monocytes into intestinal macrophages involves the down-regulation of CD14 and upregulation of MHC Class II and CD163 (Bain et al., 2013). The expression of CD163 on human monocytes and macrophages is regulated by both pro- and anti-inflammatory signals. Pro-inflammatory mediators, such as LPS, TNF α and IFN- γ , suppress CD163 expression; whereas, anti-inflammatory signals, such as IL-10, upregulate CD163 expression (Buechler et al., 2000). Equine monocytes also express CD163 (Steinbach et al., 2005), as do equine alveolar and peritoneal macrophages (Karagianni et al., 2013). Our data supports the view that CD163 is expressed by populations of resident intestinal macrophages, regardless of their location in the equine GIT. CD163 plays an important role in gastrointestinal homeostasis in its function as a receptor for the haemoglobin-haptoglobin complex, immune sensing of bacteria and by exerting an anti-inflammatory effect via an IL10 positive

333 feedback loop (Fabriek et al., 2009; Moestrup and Moller, 2004). Therefore, the
334 GIT of the horse is populated with resident intestinal macrophages with an anti-
335 inflammatory phenotype in the steady state.

336 As previously reported in humans and rodents, all macrophages within the mucosa
337 were observed in accumulations below the epithelial layer within the lamina
338 propria (Hume et al., 1983; Nagashima et al., 1996). Other studies have shown
339 CX3CR1^{+ve} cells to sample luminal contents via processes extending through the
340 basement membrane, between epithelial cells into the lumen in mice (Niess et al.,
341 2005) and in dogs CD163^{+ve} cells have been shown to have transepithelial
342 cytoplasmic processes (Wagner et al., 2018). No CD163^{+ve} cells were observed to
343 cross the basement membrane and epithelial layer in the current study. We cannot
344 eliminate the possibility that there are also CD163^{-ve} macrophages in the equine
345 GIT nor can we assess whether the macrophages in the various locations display
346 the same level of heterogeneity demonstrated in other species. This highlights the
347 potential value of a multi-marker approach to identify of intestinal macrophage
348 subsets. Despite the failure to identify processes crossing the epithelial layer, it is
349 feasible that the marker selected failed to identify a subset of CD163^{-ve}
350 macrophages. Attempts to identify such a population, for example via the
351 detection of MHC Class II expression would be an appropriate future objective.
352 This could help to identify CD163^{+ve} MHC Class II^{+ve} macrophage populations
353 and CD163^{-ve} MHC Class II^{+ve} dendritic cell population in the equine GIT.

354 In the submucosa, a significant increase in cells per mm² was observed from the
355 distal jejunum to the ileum and this trend remained consistent for the remainder of
356 the GIT. The increase in submucosal macrophages may reflect the change in
357 bacterial densities of the luminal contents, with a rise in bacterial density from 10¹

358 – 10^3 cfu/ml in the stomach to 10^{11} - 10^{12} cfu/ml in the colon (O'Hara and
359 Shanahan, 2006). Likewise, an accumulation of CD163⁺ cells was observed to
360 accumulate in the apical regions of the villi and not in the crypts, which may also
361 reflect the higher antigenic load at the apex of the villus compared to the crypt.
362 This pattern of expression has also been observed in the dog (Wagner et al.,
363 2018).

364 Submucosal macrophages are thought to be a self-maintaining population of
365 macrophages which help to support the submucosal vasculature and enteric
366 neurons of the submucosal plexus (De Schepper et al., 2018). Although these cells
367 reside distant to luminal signals, depletion of the submucosal macrophages results
368 in abnormalities in the submucosal blood supply, suggesting they may play a
369 stabilising and protective role in remodelling the submucosal vasculature in
370 response to immune cells in the lamina propria.

371 As previously reported in humans (Kalff et al., 2003), equine CD163⁺
372 macrophages were found within the *ME*, predominantly lying between
373 intermuscular bundles. In rodent wholemounts, macrophages can be seen
374 regularly and uniformly distributed throughout the *ME* with no overlap of
375 processes (Mikkelsen et al., 2011; Phillips and Powley, 2012). In equine tissue,
376 the MM were predominantly bipolar but only the occasional CD163⁺ cell was
377 observed. This apparent interspecies difference may be attributable to the
378 relatively greater thickness of the equine *ME* and the associated difficulty in
379 visualising the full network of cells within a single 5µm section. In comparison, it
380 would be possible using light microscopy, to examine the full thickness of the
381 rodent *ME* as a whole mount in future studies.

382 Between the two muscle layers (circular and longitudinal) lies the MP. The MP
383 forms part of the enteric nervous system which is involved in the control of motor
384 function, blood flow and modulates immune and endocrine functions in the
385 intestine. In rodents, the close proximity of macrophages to the MP has been
386 clearly documented (Mikkelsen, 2010; Phillips and Powley, 2012), and the
387 functional importance of this co-localisation has been demonstrated. In mice,
388 MM regulate GI motility via the production of BMP2 that acts on enteric neurons;
389 in addition this neuro-immune communication between the enteric neurons and
390 MM also stimulates tissue protective responses (Gabanyi et al., 2016; Muller et
391 al., 2014). Equine CD163^{+ve} macrophages were also observed near to the MP
392 with macrophage density (cells per mm²) greater adjacent to the MP compared to
393 within the surrounding muscle. Interstitial cells of Cajal (ICC) are also present in
394 the MP of the equine GIT and form a network of cells closely associated to the
395 smooth muscle of the intestine (Hudson et al., 1999), and are considered
396 pacemakers and mediators of neurotransmission of the GIT (Burns et al., 1996).
397 As with the neurons, macrophages in close proximity to the ICC may suggest a
398 functional relationship between them in horses as has been demonstrated in
399 rodents

400 The classification of macrophages into M1 (classical) or M2 (alternative) is
401 widely recognised as an oversimplification of the spectrum of macrophage
402 phenotypes (Hume, 2015). However, proposed M2 markers such as CD163 and
403 CD206 are considered to contribute to an overall anti-inflammatory phenotype
404 and CD206^{+ve} cells are thought to contribute to resolution of inflammation in
405 inflammatory bowel disease in humans (Vos et al., 2012). In humans and rodents
406 there is heterogeneity in phenotype between LpM and MM with regards to the

407 expression of the LPS receptor CD14; MM express higher levels than LpM
408 (Bujko et al., 2018). The activation of CD14^{+ve} MM, such as occurs in surgical
409 manipulation of the intestine results in the development of POI (Kalff et al.,
410 1998a). Intestinal CD14^{+ve} cells contribute to the pathogenesis of Crohns disease
411 in humans (Kamada et al., 2008). These examples of other macrophage markers,
412 that have been previously used in the horse highlight the potential benefits of
413 further studying macrophage subsets in the horse. Ultimately it will further our
414 understanding of the induction and resolution of inflammation in the GIT of the
415 horse and help identify future therapeutic targets.

416 **Conclusion**

417 CD163^{+ve} cells were present in all tissue layers of the equine intestine: mucosa,
418 submucosa, *ME* and the serosa. CD163^{+ve} cells were regularly distributed within
419 the ME, with accumulations adjacent to the MP, and therefore to intestinal
420 motility effector cells such as neurons and the ICC, supporting a potential
421 influential role of macrophages on intestinal motility. Macrophages are not only
422 involved in the regulation of intestinal homeostasis but are implicated in many
423 pathologies of the GIT such as POI and inflammatory bowel disease. Future
424 studies aimed at investigating whether POI or other diseases associated with
425 intestinal dysmotility and inflammation are associated with alterations in the
426 location, morphology or abundance of CD163^{+ve} monocytes and macrophages
427 changes, are therefore warranted.

428

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Authors' contributions

ZML, SP, NPHH and DAH conceptualised, designed and interpreted data in this study. ZML, KAS and LAW contributed to the acquisition of data. ZML analysed the data and compiled the manuscript. All authors contributed to, revised and approved the final manuscript.

Table 1. Horse details

	Age (years)	Sex	Breed
1	26	Gelding	Thoroughbred cross
2	21	Mare	Thoroughbred cross
3	10	Gelding	Thoroughbred
4	9	Gelding	Irish type
5	6	Gelding	Thoroughbred
6	23	Gelding	Highland Pony

7	19	Mare	Thoroughbred cross
8	15	Gelding	Thoroughbred cross
9	11	Gelding	Shire
10	21	Mare	Sports horse

444

445 **Table 2. Description of anatomical locations used for tissue collection of the**
446 **gastrointestinal tract**

447 GC = greater curvature; LC = lesser curvature

448

ID #	Location	Description of location
1	Stomach GC	Greater curvature “at the greatest curve”
2	Stomach LC	Midpoint of lesser curvature on inside of curve
3	Duodenum mid	approx. 50-75cm from pylorus
4	Jejunum proximal	Full circumferential sample and then further samples cut from anti-mesenteric aspect
5	Jejunum mid	
6	Jejunum distal	
7	Ileum mid	Ileum defined by ileocaecal fold, approx. 1m long. Midpoint ileum is midpoint of ileocaecal fold. Anti-mesenteric border.
8	Caecum mid	Sample taken 5cm away from end of ileocaecal fold including taenial band
9	Right ventral colon mid	Midpoint over taenial band

10	Left ventral colon mid	Midpoint over taenial band
11	Pelvic flexure	At apex from the anti-mesenteric aspect
12	Left dorsal colon mid	Midpoint on outer edge
13	Right dorsal colon mid	Midpoint on outer edge over taenial band
14	Small colon	Midpoint overlying anti-mesenteric taenial band

449

450 **Figure 1. Distribution of CD163⁺ve macrophages in the equine**
451 **gastrointestinal tract**

452 Floating box plots showing maximum, minimum and median cells per mm² for
453 each tissue layer (mucosa, submucosa, circular and longitudinal muscle,
454 myenteric plexus and serosa) along the length of the equine gastrointestinal tract
455 from (L-R); stomach lesser curvature (LC), stomach greater curvature(GC),
456 duodenum, jejunum, ileum, caecum, right ventral colon (RVC), left ventral colon
457 (LVC), pelvic flexure (PF), left dorsal colon (LDC), right dorsal colon (RDC) and
458 small colon. n=10

459

460 **Figure 2. Comparison between cells /mm² in the submucosa of the small**
461 **intestine and large intestine.**

462 Floating box plot (minimum, maximum and median) showing individual values
463 (black dots) of cells/mm² in the submucosa of the small intestine (encompassing
464 regions from the stomach to the ileum) and the large intestine (encompassing

regions from the caecum to the small colon). Difference in cells/mm² as measured by a Mann–Whitney U test. **** $p \leq 0.0001$

Figure 3. Lamina propria macrophages in the equine stomach, jejunum and right ventral colon

Staining for CD163 using 3,3'-diaminobenzidine (DAB) as a chromogen in the mucosa of the equine stomach (**A**), jejunum (**B**) and right ventral colon (**C**). Red line represents basement membrane. Magnification X10. Bar=250µm. Images representative of samples taken from 10 horses.

Figure 4 Morphology of lamina propria macrophages in the equine jejunum and right dorsal colon

Staining for CD163 in the lamina propria of the equine jejunum (**A**) and right dorsal colon (**B**) using 3,3'-diaminobenzidine (DAB) as a chromogen with haematoxylin as a counterstain. (**A**) is a cross section. (**B**) is a whole mount. Yellow arrow = bipolar macrophage. Green arrow = macrophages with 3 or more ramifications. (**A**) and (**B**) X20 magnification Bar=100µm. Inset X80 Bar=25µm. Images representative of samples taken from 10 horses.

Figure 5 Morphology of macrophages in the submucosa of the equine small intestine

Staining for CD163 in the submucosa of the equine jejunum (**A**) and ileum (**B**) using 3,3'-diaminobenzidine (DAB) as a chromogen with haematoxylin as a

488 counterstain. Bipolar macrophages = green arrow, blue inset. Round macrophages
489 = yellow arrow, red inset. (A) and (B) X20 magnification; Bar=100µm. Inset X80
490 Bar=25µm. Images representative of samples taken from 10 horses.

491

492 **Figure 6 Morphology of macrophages in the submucosa of equine large**
493 **intestine**

494 Staining for CD163 in the submucosa of the equine pelvic flexure (A) and left
495 dorsal colon (B) using 3,3'-diaminobenzidine (DAB) as a chromogen with
496 haematoxylin as a counterstain. Macrophages in the submucosa are predominantly
497 round (red inset) with occasional bipolar cells (blue inset).

498 (A) and (B) X20 magnification Bar=100µm. Inset X80 Bar=25µm. Images
499 representative of samples taken from 10 horses.

500

501 **Figure 7 Macrophages in gut- associated lymphoid tissues in the equine large**
502 **intestine**

503 Staining for CD163 in the equine right ventral colon using 3, 3'-diaminobenzidine
504 (DAB) as a chromogen with haematoxylin as a counterstain. (A) X2.5
505 magnification; Bar=1mm. Inset (B) X20 Bar=100µm. Images representative of
506 samples taken from 5 horses.

507

508 **Figure 8 Macrophages in the equine *muscularis externa***

509 Staining for CD163 in the equine duodenum using 3, 3'-diaminobenzidine (DAB)
510 as a chromogen with haematoxylin as a counterstain. (A) shows a low power field
511 of view (X5 magnification) of the *muscularis externa* (ME). **CM, MP, LM** and **S**
512 represent high power view (X 20 magnification) of circular muscle (**CM**),
513 myenteric plexus (**MP**), longitudinal muscle (**LM**) and serosa (**S**). (A) X5
514 magnification Bar=500µm. (**CM, MP, LM, S**) X20 magnification Bar=100µm.
515 Images representative of samples taken from 10 horses.

516

517 **Figure 9 Serosal macrophages in the equine gastrointestinal tract**

518 Staining for CD163 in the serosa of the equine duodenum (A), ileum (B) and
519 caecum (C) using 3, 3'-diaminobenzidine (DAB) as a chromogen with
520 haematoxylin as a counterstain. Magnification X40 magnification; Bar=50µm.
521 Images representative of samples taken from 10 horses.

522

523 **Figure 10 Ramified macrophages in the serosa of equine left ventral colon**

524 Staining for CD163 in the serosa of equine left ventral colon using 3, 3'-
525 diaminobenzidine (DAB) as a chromogen with haematoxylin as a counterstain.
526 Black arrow shows macrophage with ramified morphology. Magnification X40
527 magnification. Bar=50µm. Image representative of samples taken from 5 horses.

528

529 **Figure 11 Muscularis macrophages in the equine ileum**

530 Staining for CD163 in the muscularis (circular muscle) of the equine ileum using
531 3, 3'-diaminobenzidine (DAB) as a chromogen with haematoxylin as a

532 counterstain. Macrophages were predominately found between muscle bundles
533 (black arrows) although occasional staining for CD163 was observed within a
534 muscular bundle (red circle). Magnification X10. Bar=250µm. Image
535 representative of small and large intestinal samples in 10 horses.

536

537 **Figure 12 *Muscularis* macrophages in equine duodenum**

538 Staining for CD163 in the muscularis (longitudinal layer) of the equine duodenum
539 using 3, 3'-diaminobenzidine (DAB) as a chromogen with haematoxylin as a
540 counterstain. Macrophages were observed between muscle bundles running
541 longitudinally with the muscle (inset). Magnification X20. Bar=100µm. Inset
542 X40. magnification. Bar=50µm Image representative of small and large intestinal
543 samples in 10 horses.

544 **Figure 13 Morphology of intermuscular *muscularis* macrophages in equine**
545 **ileum**

546 Staining for CD163 in the muscularis (circular muscle) of the equine ileum using
547 3, 3'-diaminobenzidine (DAB) as a chromogen with haematoxylin as a
548 counterstain. Macrophages were predominately bipolar (black arrows) although
549 occasional circular cell bodies were observed (red circle). Magnification X40.
550 Bar=50µm. Image representative of small and large intestinal samples in 10
551 horses.

552

553 **Figure 14 Macrophages associated with the myenteric plexus in the equine**
554 **gastrointestinal tract**

555 Staining for CD163 in the myenteric plexus (**MP**) of the equine jejunum using 3,
556 3'-diaminobenzidine (DAB) as a chromogen with haematoxylin as a counterstain.
557 Macrophages were observed both on the edge of the MP (black arrows) and
558 within the MP (red arrows). Magnification X40. Bar=50µm. CM, cricular muscle.
559 LM, longitudinal muscle. Image representative of small and large intestinal
560 samples in 10 horses.

561

562 **Figure 15 Morphology of myenteric plexus macrophages in the equine**
563 **gastrointestinal tract**

564 Staining for CD163 in the myenteric plexus of the equine jejunum using 3, 3'-
565 diaminobenzidine (DAB) as a chromogen with haematoxylin as a counterstain.
566 The morphology of myenteric plexus macrophages was either round (**A**) or
567 ramified (**B**). Magnification X80. Bar=25µm. Image representative of small and
568 large intestinal samples in 10 horses.

569

570 **Figure 16 Comparison of cells/mm² between the *muscularis externa* and**
571 **myenteric plexus of the small and large intestine.**

572 Floating box plot (minimum, maximum and median) showing individual values
573 (black dots) of cells/mm² in the *muscularis externa* (circular muscle and
574 longitudinal muscle) of the small intestine and the large intestine. Difference in
575 cells/mm² as measured by a Mann–Whitney U test. Significance = **** $p \leq 0.000$.

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864 **Figure 1**

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903 **Figure 15**

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905 **Figure 16**