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1 **Study of Peritoneal Macrophage Immunophenotype in Sheep Experimentally**
2 **Infected with *Fasciola hepatica***

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Abstract

During *Fasciola hepatica* infection, the parasite has the capability to modulate the host immune response towards a non-protector Th2 type instead of Th1. This type of immune response is closely related to the alternative activation of macrophages (M2 profile) as has been shown *in vivo* in murine models. In this study, two similar trials were carried out to evaluate the expression of CD68, CD14, CD206 and iNOS in cells present in the peritoneal fluid of sheep during early stages of infection with *F. hepatica* (1, 3, 9 and 18 days post-infection, dpi) by immunocytochemistry. To the authors' knowledge, this is the first report that studies the *in vivo* immunophenotype of macrophages from the peritoneal fluid of sheep infected with *F. hepatica*. Throughout the experiments the absolute number of leucocytes progressively increased, reaching its highest value at 18 dpi, mainly due to the increase of eosinophils. This immunocytochemical study had two purposes: 1) CD68 expression was assessed with Hansel counterstaining, to optimally identify peritoneal macrophages, eosinophils and lymphocytes; 2) expression of CD14, CD206 and iNOS was evaluated to identify alternative or classical pathways of macrophage activation. In both trials, there was a significant increase in CD14 from day 3 dpi compared with the non-infected group. CD206 expression at all time-points showed a significant and dramatic increase in comparison with the non-infected group. On the other hand, iNOS expression showed little variation, and was significantly decreased at 18 dpi in both trials. These results suggest that *F. hepatica* induces an alternative activation of peritoneal macrophages of sheep from the first day post-infection, which may facilitate parasite survival. This is the first report describing M2 activation of peritoneal macrophages in ruminants infected with *F. hepatica*.

Keywords: *Fasciola hepatica*, macrophages, peritoneal fluid, Sheep, immune response

1.- Introduction

Fasciolosis caused by the liver fluke *Fasciola hepatica* infects millions of ruminants worldwide and is recognised by the World Health Organisation (WHO) as an important zoonosis, which is now emerging or re-emerging in many countries (Gonzalez et al., 2011). The costs incurred due to losses in production and treatment with anthelmintics, as well as the resistance that is now widespread, confirm the urgent need for alternative control methods (Fairweather et al., 2011). During the last two decades, major advances have been made in identifying potential vaccine molecules. Nevertheless, no vaccine candidate has yet reached a commercial or pre-commercial stage. The immune suppression/modulation by *F. hepatica* is one major obstacle to develop a protective vaccine (Toet et al., 2014; Molina-Hernández et al., 2015).

Classical M1 and alternative M2 activation pathways of macrophages, mirroring the Th1-Th2 polarisation of T cells, represent two extremes of a dynamic state of macrophage activation (Wang et al., 2014). Since *F. hepatica* larvae penetrate the intestinal wall of the host and migrate to the liver via the peritoneum, study of the type macrophage activation at this stage plays a critical role in understanding the immune response to parasitic infection and thus for designing an effective vaccine (Molina-Hernández et al., 2015). It has been reported that *F. hepatica* excretion-secretion products (ES) and tegumental coat proteins produce an M2 macrophage phenotype, responsible for host tissue repair, tissue fibrosis and modulation of adaptive immunity, which suppresses a Th1-driven inflammatory pathology in *F. hepatica* infection (Adams et al., 2014; Figueroa-Santiago et al., 2014; Flynn et al., 2007).

In early stages of *F. hepatica* infection, the recruitment and activation of M2 macrophages in the peritoneal cavity of rats occurs within 24h post-infection (Donnelly

72 et al., 2005). To date, peritoneal macrophage activation has not been investigated in *F.*
73 *hepatica* infected ruminants. The aim of this study was to evaluate the macrophage
74 polarisation in peritoneal fluid obtained from sheep experimentally infected with *F.*
75 *hepatica* in the early stages of infection.

76

77 **2.-Materials and Methods**

78 *2.1.-Experimental design*

79 Fifty-eight-month-old male Merino sheep obtained from a liver fluke-free farm were
80 used to study the early stages of infection. Animals were purchased aged one month and
81 housed indoors in the experimental farm of the University of Córdoba until they reached
82 the appropriate age for pathogen challenge. All animals were tested monthly for parasite
83 eggs by faecal sedimentation with negative results in all cases. Moreover, prior to the
84 challenge, all animals were tested for serum IgG specific for *F. hepatica* cathepsin L1
85 (FhCL1) by ELISA, with negative results in all cases. The experiment was carried out
86 in two different trials of 25 sheep in consecutive years. Each trial consisted of five
87 groups composed of five sheep (n=5): an uninfected control group and four infected
88 groups. Sheep were orally infected with one dose of 150 metacercariae of the South
89 Gloucester strain of *F. hepatica* (Ridgeway Research Ltd, UK) and euthanised at 1, 3, 9
90 and 18 days post-infection (dpi). The euthanasia was applied by intravenous injection of
91 T61® (Intervet, Spain). The experiments were approved by the Bioethics Committee of
92 the University of Cordoba (No.1118) and conducted in accordance with European
93 (2010/63/UE) and Spanish (RD 1201/2005) directives on animal experimentation.

94 *2.2.-Recovery of peritoneal fluid*

95 In both trials, peritoneal lavages were conducted immediately after the animals
96 were euthanised. The ventral aspect of the abdomen was shaved and disinfected with

97 10% polyvinylpyrrolidone iodine (AGB, Madrid, Spain). A 2 cm incision was made on
98 the skin over the white line and subcutaneous tissue was dissected, the white line and
99 peritoneum were sectioned with blunt scissors to avoid bleeding. A 40 cm cannula was
100 inserted into the abdominal cavity and connected to a syringe to inject 60 ml sterile
101 DPBS, previously warmed to 37 ºC. In Trial 1, the DPBS contained 6 mM ethylene-
102 diaminetetracetic acid (EDTA) (Sigma-Aldrich, Darmstadt, Germany) as an
103 anticoagulant, whereas 9500 I.U. of heparin (Rovi, Madrid, Spain) were used as the
104 anticoagulant in Trial 2. After softly massaging the abdominal cavity for 1 min, 40 ml
105 of peritoneal fluid was recovered using the syringe connected to the cannula. In cases
106 where residual erythrocytes were present, it was necessary to use an erythrolysis buffer
107 prior to the processing of the cells.

108 *2.3.-Cell populations*

109 The recovered peritoneal lavage fluid was collected and kept at room temperature in
110 Trial 1, whereas in Trial 2, it was cooled on ice until cell processing. The total number
111 of viable peritoneal cells was determined after Trypan Blue staining by counting in a
112 Neubauer haemocytometer. Smear preparations were manually performed on
113 Vectabond®-treated slides (Vector laboratories, California, USA) by centrifuging the
114 recovered peritoneal fluid at 1500 rpm for 10 min. After air draining, these smears were
115 fixed in acetone for 5 min and stored at -80°C for further immunocytochemical studies.
116 For differential cell counting, the Diff-Quick technique was performed in Trial 1,
117 whereas immunocytochemistry using anti-human CD68 monoclonal antibody (Dako,
118 Glostrup, Denmark) in combination with Hansel staining was used in Trial 2 as a novel
119 and more accurate cell counting method. A total of 200 cells per smear were counted in
120 randomly selected fields of 400x magnifications using the software Image Pro-plus 6.0
121 (Media Cybernetics, Silver Spring, USA), and the percentage of lymphocytes (small

122 basophilic nucleus and scanty cytoplasm), macrophages (hyperchromatic nucleus and
123 moderate to large cytoplasm) and eosinophils was obtained. Neutrophils were not
124 included in the cell count since they were only very occasionally observed.

125 *2.4.-Immunocytochemistry (ICC)*

126 An immunocytochemical study was used to assess anti-Human CD68 (M0718, Dako,
127 Glostrup, Denmark), anti-Human CD14 (BOV2027, Monoclonal Antibody Center,
128 Washington State University), anti-Human iNOS (RB-1605-P1, Thermo, Freemont,
129 USA) and anti-Human CD206 (orb4941, Biorbyt, Cambridge, UK) expression in
130 peritoneal fluid macrophages. The dilution used for these primary antibodies was 1:400
131 for CD68; 1:500 for CD14, 1:200 for iNOS; and 1:100 for CD206. The avidin-biotin-
132 peroxidase method described elsewhere (Zafra et al., 2013) was carried out. Briefly,
133 endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide
134 (Panreac, Barcelona, Spain) in phosphate buffered saline (PBS). Then, smears were
135 washed once in PBS and incubated with 10% normal goat serum (MP Biomedicals,
136 Ohio, USA) for 30 min at room temperature. After three 5 min rinses in PBS-Tween
137 (PBST), secondary antibodies were applied for 45 min at 37ºC. A biotinylated goat anti-
138 rabbit immunoglobulin serum (Dako) diluted 1:200 was applied to the smears incubated
139 with the primary polyclonal antibodies (pAbs: iNOS and CD206), whereas a
140 biotinylated goat anti-mouse immunoglobulin serum (Dako) diluted 1:50 was used for
141 the primary monoclonal antibody (mAb: CD68). After two 5 min rinses in PBST, an
142 avidin–biotin-peroxidase complex (Vector, Burlinghame, USA) diluted 1:50 was
143 applied for an hour as a third reagent. Slides were then washed three times in PBST and
144 incubated with Novared® substrate kit peroxidase (Vector) diluted following the
145 manufacturer's instructions, rinsed in water, lightly counterstained with Mayer's
146 haematoxylin and mounted with Eukitt® (Freiburg, Germany). For CD68 antibody,

147 following the haematoxylin stain, eosin was applied to the slides for 1 minute with
148 Hansel stain for the differential cell count in Trial 2. Specific primary antibodies were
149 substituted with PBS or non-immune isotype-matched sera as negative controls.

150 *2.5.-Cell count*

151 Immunoreactive cells were counted in randomly selected fields of 400x magnifications
152 using the Image Pro-plus 6.0 software package. Macros were calibrated for staining
153 intensity and cell size to include all immunostained cells. A total of 200 cells were
154 counted per slide and the percentage of positive and negative cells was obtained.
155 Photomicrographs were taken using an Olympus BX51 photomicroscope with a 400x
156 magnification field. Results were expressed as mean ± SD per animal and per group.
157 The intensity of immunostaining was evaluated semi-quantitatively according to the
158 following score: 1, mild; 2, moderate; 3, severe; 4, very severe.

159 *2.6.-Statistical analysis*

160 Statistical analysis was carried out using the Graphpad Prism 7.0 software package
161 (Graphpad Software, Inc., San Diego, California). The Kolmogorov–Smirnov test was
162 applied to evaluate whether distributions were parametric. Comparisons between groups
163 were made using the Mann–Whitney test for non-parametric distributions. Correlation
164 studies were carried out using the Spearman correlation test for non-parametric
165 distributions. $P<0.05$ was considered significant.

166

167 **3. Results**

168 *3.1. Absolute peritoneal cell count*

169 The results of the absolute peritoneal fluid cell counts in Trials 1 and 2 are shown in
170 Table 1. In both trials, the number of cells increased significantly ($P<0.05$) at 9 and 18
171 dpi compared with the uninfected control group, particularly in Trial 2. A significant

172 decrease in the number of peritoneal leucocytes ($P < 0.05$) was observed in Trial 1 at 1
173 and 3 dpi compared with the uninfected group; however, this finding was not confirmed
174 in Trial 2. It was remarkable that at 9 and 18 dpi the number of peritoneal leucocytes
175 was markedly higher in Trial 2 compared with Trial 1. This difference could be due to
176 partial coagulation of fibrin at 9 and 18 dpi in Trial 1. This was one of the reasons why
177 the experiment was repeated, using heparin in Trial 2 instead of EDTA.

178 *3.2. Differential peritoneal cell count*

179 In Trial 1 routine Diff-Quick staining was used for the differential peritoneal cell count,
180 which was based on cell morphology. Since CD68 has been widely used as a general
181 macrophage marker (Valheim et al., 2004) a CD68 mAb in combination with Hansel
182 stain was used in Trial 2 as a novel and more accurate leucocyte identification method.
183 The differential cell count results (expressed as percentages) from peritoneal fluid smear
184 examinations are shown in Figure 1. No significant differences between Trials 1 and 2
185 were found in the numbers of lymphocytes, macrophages or eosinophils. In the
186 uninfected control group as well as at 1 and 3 dpi in both trials, macrophages
187 represented the majority of the peritoneal leucocytes, followed by lymphocytes, with a
188 small number of eosinophils (Fig. 1). Neutrophils and epithelial cells were only
189 occasionally observed and were not included in the cell count.

190 At 9 and particularly at 18 dpi, there was a very marked increase in the number of
191 eosinophils, which was responsible for the relative decrease in the percentages of
192 macrophages (9 and 18 dpi) and lymphocytes (18 dpi) in comparison with the
193 uninfected control group (Fig. 1). On the other hand, the percentage of lymphocytes
194 showed a significant increase in both trials at 9 dpi with respect to the uninfected
195 control group. This may reflect a stimulation of the recruitment of peritoneal
196 lymphocytes at this time-point when larvae are penetrating or migrating into the liver

197 surface as revealed by the significant increase in the total number of peritoneal
198 leucocytes at 9 and 18 dpi.

199 *3.3. Immunocytochemical study*

200 The anti-CD14 mAb yielded a cytoplasmic immunostaining in peritoneal leucocytes
201 with large cytoplasm and round to ovoid nucleus, this was the same morphology than
202 peritoneal leucocytes expressing CD68. The results of the present study revealed a
203 significant increase of the percentage of CD14+ cells at 3, 9 and 18 dpi in both trials
204 with respect to the uninfected control group (Table 2).

205 Anti-iNOS and anti-CD206 antibodies were used as biomarkers of classical (M1) and
206 alternative (M2) macrophage activation, respectively. The anti-iNOS pAb also showed
207 granular cytoplasmic immunostaining in peritoneal macrophages (Fig. 2) and in some
208 eosinophils, but only the macrophages were counted. The percentage of peritoneal
209 macrophages expressing iNOS varied little during the course of the infection in both
210 trials (Table 2). The intensity of immunolabelling with anti-iNOS was mild in the
211 uninfected control group and at all studied time-points (Table 2).

212 The Anti-human CD206 antibody has been described as a good biomarker of alternative
213 activation of macrophages in sheep (Ampem et al., 2016). In our study, the CD206 mAb
214 yielded weak cytoplasmic immunostaining in peritoneal macrophages from the
215 uninfected control group, whereas the intensity of the immunolabelling was very strong
216 in both trials at 1, 3, 9 and 18 dpi (Fig. 3, Table 2). The percentage of peritoneal
217 macrophages expressing CD206 showed a dramatic and significant increase ($P<0.05$)
218 from 1 dpi onwards, compared with the uninfected groups in both trials (Table 2).

219 The iNOS/CD206 ratio decreased approximately one-fold at 1, 3 and 9 dpi and three-
220 fold at 18 dpi in both trials (Table 2).

221

222 **4. Discussion**

223 The higher percentages of lymphocytes and macrophages in the uninfected control
224 group and at 1 and 3 dpi found in both trials are consistent with previous studies carried
225 out by our group analysing peritoneal leucocytes in goats (Zafra et al., 2013) and sheep
226 (Escamilla et al., 2017) in the early stages of infection. The marked increased of
227 eosinophils in the peritoneal fluid during infection has been also previously reported in
228 goats infected with *F. hepatica* at 7 and 9 dpi (Zafra et al., 2013) and in sheep at 9 and
229 18 dpi (Escamilla et al., 2017).

230 CD14 is a co-receptor for TLR-initiated pro-inflammatory responses in innate immune
231 cells, particularly macrophages. It has been reported that infection by helminths such as
232 *Schistosoma mansoni* in mice (Tundup et al., 2014) and *F. hepatica* in cattle (Garza-
233 Cuartero et al., 2016) induces an increase in CD14 expression in liver macrophages and
234 peripheral blood mononuclear cells (PBMCs), respectively. The present study is the first
235 report analysing the expression of CD14 in peritoneal leucocytes in ruminants infected
236 with *F. hepatica,* and revealed a significant increase at 3, 9 and 18 dpi in both trials with
237 respect to the uninfected control group. This is in concordance with the increased CD14
238 expression in PBMC at 7 and 12 weeks after *F. hepatica* infection in cattle was
239 associated with an alternative activation of macrophages (Garza-Cuartero et al., 2016).
240 In addition, it has been previously shown that CD14 expression increases during sepsis
241 and tissue remodelling processes, and the fact that this increase occurs at 3 dpi, when
242 the parasite is penetrating through the gut and reaching the peritoneal cavity, suggests
243 that it could be an indicator of tissue damage and apoptosis (Devitt et al., 1998).

244 During helminth infections, macrophages that undergo changes to express an M2
245 phenotype have been implicated in the regulation of the cytokine environment. This
246 change leads to preferential induction of the Th2 response, which is ineffective in

247 controlling the parasite infection and results in the chronic stage of the disease (O'Neill
248 *et al.*, 2000; Kreider et al., 2007). Since the host response to *F. hepatica* is thought to be
249 more effective during the intestinal, peritoneal or early hepatic migratory stages (Van
250 Milligen et al., 1999), the rapid M2 polarisation of peritoneal macrophages found in the
251 sheep of the present study may be an important mechanism of modulation, and may
252 facilitate parasite survival during the early stages of infection.
253 In a murine model, very low iNOS gene expression was detected in uninfected controls
254 and at 1, 7, 14 and 21 days after *F. hepatica* infection (Donnelly et al., 2005), while in
255 sheep, a marked decrease in iNOS gene expression was found in PBMC at 7 dpi (Fu et
256 al., 2016), which contrasts with the low level of variation in iNOS expression by
257 immunocytochemistry in both trials of the present study. This difference suggests that
258 iNOS gene and protein expression may differ, with the protein probably remaining
259 active for a longer time than the gene.
260 The ratio iNOS/CD206 suggest M2 polarisation of peritoneal macrophages from 1 dpi
261 onwards in both trials, a finding that is consistent with previous studies in mice
262 (Donnelly et al., 2005), and in PBMC of sheep (Fu et al., 2016) and cattle (Flynn et al.,
263 2007, Garza-Cuartero et al., 2016), that report M2 activation of macrophages induced
264 by *F. hepatica* infection. Further studies should focus on the mechanisms used by the
265 *F. hepatica* parasite to modulate the host response in ruminants, particularly during
266 early stages of infection when the parasite is more vulnerable.
267

268 **Acknowledgments**

269 Work supported by EU Project (H2020-SFS-2014-2-635408- PARAGONE and
270 the Spanish Ministry of Science grant AGL2015-67023-C2-1-R.
271

272 **References**

273 Adams, P.N., Aldridge, A., Vukman, K.V., Donnelly, S., O'Neill, S.M., 2014. *Fasciola*
274 *hepatica* tegumental antigens indirectly induce an M2 macrophage-like phenotype in
275 vivo. Parasite Immunol. 36(10), 531-9.

276 Ampem, G., Azegrouz, H., Árpád, B., Bacsadi, Á., Schmidt, S., Thuróczy, J., Röszer,
277 T., 2016. Adipose tissue macrophages in non-rodent mammals: a comparative study.
278 Cell Tissue Res. 363, 461-478.

279 Devitt, A., Moffatt, O.D., Raykundalia, C., Capra, J.D., Simmons, D.L., Gregory, C.D.,
280 1998. Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature
281 392, 505-509.

282 Donnelly, S, O'Neill, S.M., Sekiya, M., Mulcahy, G., Dalton, J.P., 2005. Thioredoxin
283 peroxidase secreted by *Fasciola hepatica* induces the alternative activation of
284 macrophages. Infect. Immun. 73, 166-173.

285 Escamilla, A., Pérez-Caballero, R., Zafra, R., Bautista, M.J., Pacheco, I.L., Ruiz, M.T.,
286 Martínez-Cruz, M.S., Martínez-Moreno, A., Molina-Hernández, V., Pérez, J., 2017.
287 Apoptosis of peritoneal leucocytes during early stages of *Fasciola hepatica* infections in
288 sheep. Vet. Parasitol. 238, 49-53.

289 Fairweather, I., 2011. Reducing the future threat from (liver) fluke: realistic prospect or
290 quixotic fantasy?. Vet Parasitol. 180, 133-143.

291 Figueroa-Santiago, O., Espino, A.M., 2014. *Fasciola hep*atica fatty acid binding protein
292 induces the alternative activation of human macrophages. Infect. Immun. 82(12), 5005-
293 5012.

294 Flynn, R.J., Irwin, J.A., Olivier, M., Sekiya, M., Dalton, J.P., Mulcahy, G., 2007.
295 Alternative activation of ruminant macrophages by *Fasciola hepatica*. Vet. Immun.
296 Immunopathol. 120, 31-40.

297 Fu, Y., Chryssafidis, A.L., Browne, J.A., O'Sullivan, J., McGettigan, P.A., Mulcahy,
298 G., 2016. Transcriptomic study on ovine immune responses to *Fasciola hepatica*
299 infection. PLOS Negl. Trop. Dis. 10(9), e0005015.
300 Garza-Cuartero, L., O'Sullivan, J., Blanco, A., McNair, J., Welsh, M., Flynn, R.J.,
301 Williams, D., Diggle, P., Cassidy, J. Mucalhy, G., 2016. *Fasciola hepatica* infection
302 reduces *Mycobacterium bovis* burden and mycobacterial uptake and suppresses the pro-
303 inflammatory response. Parasite Immunol. 38, 387-402.
304 González, L.C., Esteban, J.G., Bargues, M.D., Valero, M.A., Ortiz, P., Náquira, C.,
305 Mas-Coma, S., 2011. Hyperendemic human fascioliasis in Andean valleys: An
306 altitudinal transect analysis in children of Cajamarca province, Peru. Acta Tropica 120,
307 119-129.
308 Kreider, T., Anthony, R.M., Urban, J.F. Jr., Gause, W.C., 2007. Alternative activated
309 macrophages in helminth infections. Curr. Opin. Immunol. 19(4), 448-53.
310 Molina-Hernández, V., Mulcahy, G., Pérez, J., Martínez-Moreno, A., Donnelly, S.,
311 O'Neill, S., Dalton, J.P., Cwiklinski, K., 2015. *Fasciola hepatica* vaccine: we may not
312 be there yet but we're on the right road. Vet. Parasitol. 208(1-2), 101-111.
313 Munder, M., Eichmann, K., Modolell, M., 1998. Alternative metabolic states in murine
314 macrophages reflected by the nitric oxide synthase/arginase balance: competitive
315 regulation by CD4+ T cells correlates with Th1/Th2 phenotype. J. Immunol. 160(11),
316 5347-54.
317 O'Neill, S.M., Brady, M.T., Callanan, J.J., Mulcahy, G., Joyce, P., Mills, K.H., Dalton,
318 J.P., 2000. *Fasciola hepatica* infection downregulates Th1 responses in mice. Parasite
319 Immunol. 22(3), 147-55.
320 Toet, H., Piedrafita, D.M., Spithill, T.W., 2014. Liver fluke vaccine in ruminants:
321 strategies, progress and future opportunities. Int. J. Parast. 44, 915-927.

322 Tundup S., Srivasta L., Nagy T., Ham D., 2014. CD14 influences host immune
323 responses and alternative activation of macrophages during *Schistosoma mansoni*
324 infection. Infect. Immun. 82, 3240-3251.
325 Valheim M., Sigurdartóttir O.G., Storset A.K. Aune L.G., Press C.M., 2004.
326 Characterization of macrophages and occurrence of T cells in intestinal lesions of
327 subclinical paratuberculosis in goats. J. Comp. Pathol. 131, 221-232.
328 Van Milligen, F.J., Cornelissen, J.B., Bokhout B.A., 1999. Protection against *Fasciola*
329 *hepatica* in the intestine is highly correlated with eosinophil and immunoglobin G1
330 responses against newly excysted juveniles. Parasite immunol. 21(5), 243-251.
331 Wang, N., Liang, H., Zen, K., 2014. Molecular mechanisms that influence the
332 macrophage M1-M2 polarization balance. Front. Immunol. 5, 1-9.
333 Zafra, R., Pérez-Écija, R.A., Buffoni, L., Moreno, P., Bautista, M.J., Martínez-Moreno,
334 A., Mulcahy, G., Dalton, J.P., Pérez, J., 2013. Early and late peritoneal and hepatic
335 changes in goats immunized with recombinant cathepsin L1 and Infected with *Fasciola*
336 *hepatica*. J. Comp. Path. 148(4), 373-384.
337
338

339 **Figure legends**

340 **Fig. 1.** Differential cell counts in Trials 1 and 2 expressed as percentages of
341 lymphocytes, macrophages and eosinophils in the uninfected control group and in Trials
342 1 and 2 and at 1, 3, 9 and 18 days post-infection. * Significant (P<0.05) with respect to
343 the uninfected control group.

344 **Fig. 2.** Trial 2, peritoneal smear stained with anti-iNOs polyclonal antibody showing
345 mild cytoplasmic immunolabelling (brown colour) in macrophages (arrows) in the
346 uninfected control group (A) and at 3 day post-infection (dpi) (B), 9 dpi (C) AND 18
347 dpi (D). ABC method-haematoxylin counterstain. X400.

348 **Fig. 3.** Trial 2, peritoneal smear stained with anti-CD206 polyclonal antibody showing
349 mild cytoplasmic immunolabelling (brown colour) in macrophages (arrows) from
350 uninfected control (A) and very severe immunolabelling in macrophages at 1 day post-
351 infection (dpi) (B), 9 dpi (C), and 18 dpi (D). ABC method-haematoxylin counterstain.
352 X400.

353

354

355 **Table 1. Absolute peritoneal leucocyte counts in Trials 1 and 2 expressed in 10^6**

356 **cells/ml (mean±SEM)**.

Trial	UC	1 dpi	3 dpi	9 dpi	18 dpi
Trial 1	5.2±1.2	2.0±0.5*	2.1±0.5*	19±8.2*	29.7±6.6*
Trial 2	4.0±0.8	3.3±1.6	7.4±1.4§	74.2±20.1*§	497.9±122*§

357 UC: uninfected control group. dpi: days post-infection.

358 *Significant difference ($P<0.05$) with respect to the UC group.

359 §Significant difference ($P<0.05$) with respect to Trial 1.

360

361

362 **Table 2.** Percentage intensity of immunoreactivity (1-4) of peritoneal macrophages
363 expressed as mean ±SD for the expression of CD14, iNOS, CD206, and the
364 iNOS/CD206 ratio in the uninfected control group (UC) and in the different stages of
365 infection in Trials 1 and 2.

Trial 1	**UC**	**1 dpi**	**3 dpi**	**9 dpi**	**18 dpi**
CD14	16.1±7.8(2)	29.7±13.7(3)	72.0*±10.3(4)	59.7*±10.3(4)	88.7*±2.3(4)
iNOS	47.8±7.8 (1)	59.9±1.9 (1)	70.7*±9.2 (1)	56.5±19.8 (1)	45±4.7(1)
CD206	25.3±2.3 (1)	69.2*±6.3(4)	71.3*±17.3(4)	59.5*±6.3(4)	70.8*±8(4)
iNOS/C206	1.9	0.9	1.0	1.0	0.6
Trial 2					
CD14	51.2±11.1(2)	63.2±19(2)	78.1*±13.1(4)	74.6*±16.9(4)	69.1*±12.9(4)
iNOS	37.3±27.7(1)	64.4±4.1(1)	62.9±17.5(1)	74.4*±9.4(2)	46±21.2(1)
CD206	20.9±4.4(1)	81.5*±8.6(4)	83.9*±15.4(4)	87*±5.7(4)	90.9*±8.02(4)
iNOS/CD206	1.8	0.8	0.8	0.9	0.5

366 § Intensity of immunoreactivity (1: mild, 2: moderate, 3: severe, 4: very severe).
367 *Significant differences with respect to the UC group.
368

Figure
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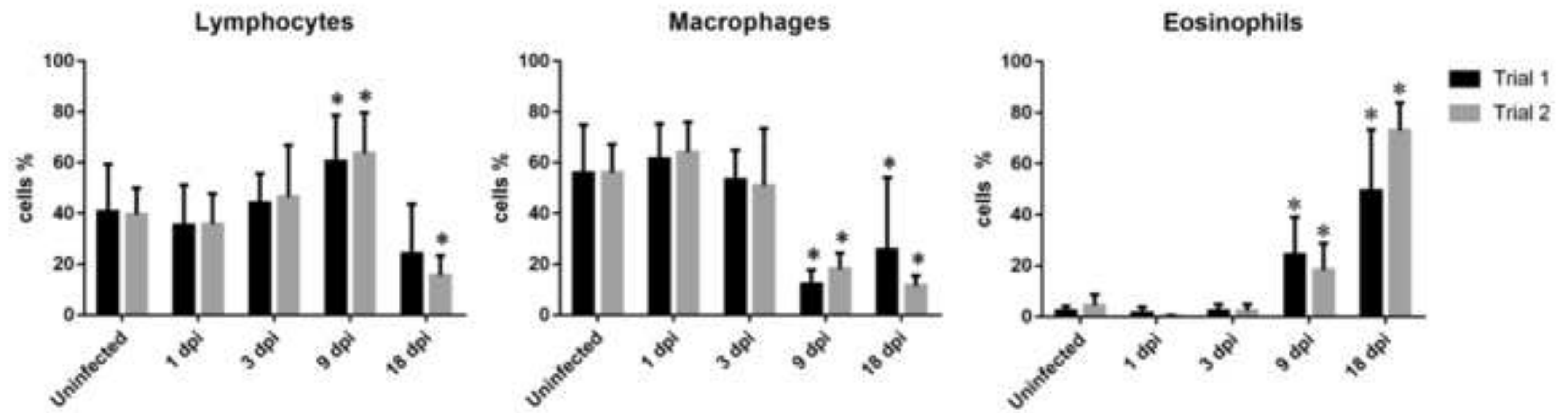

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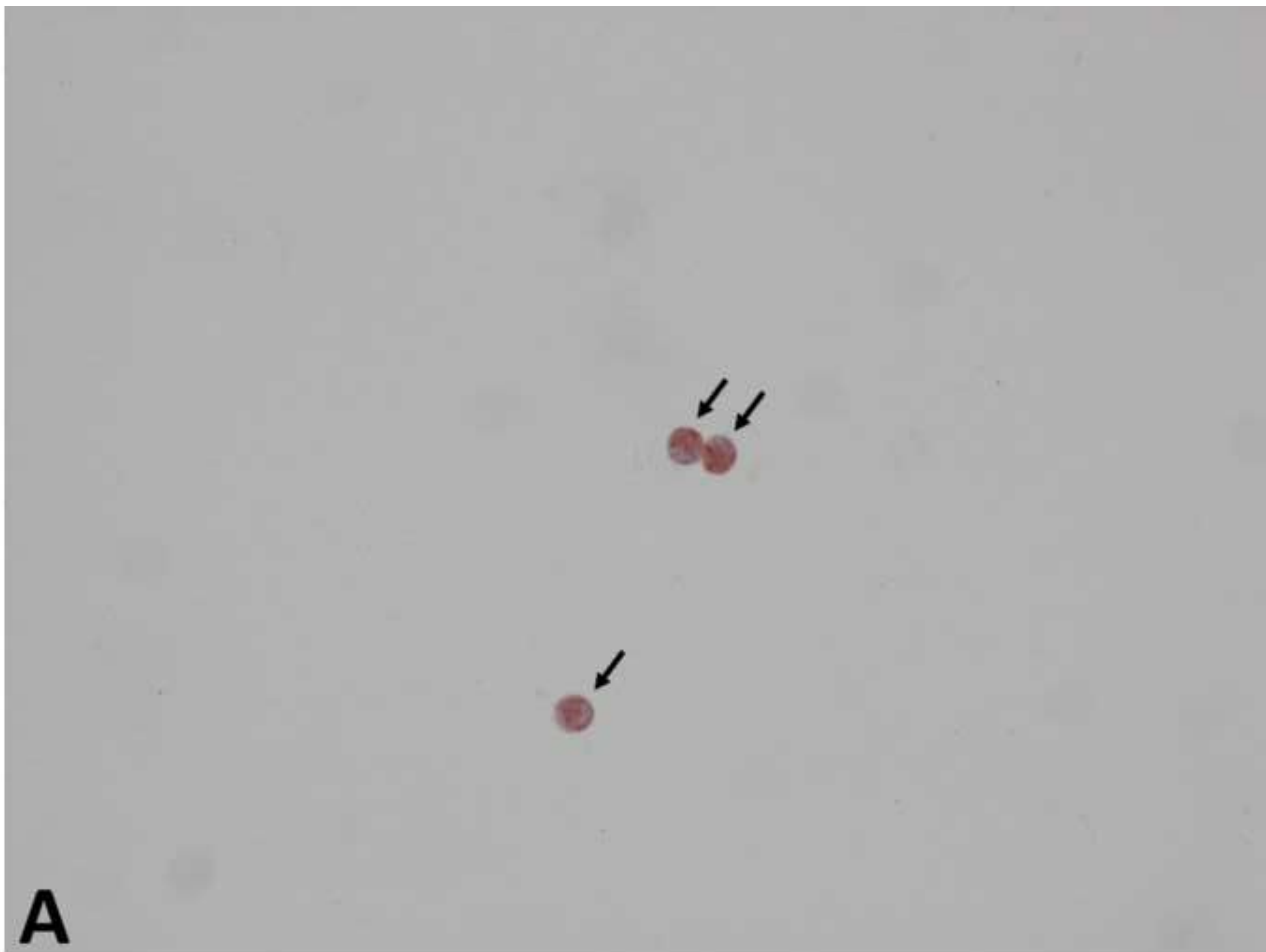

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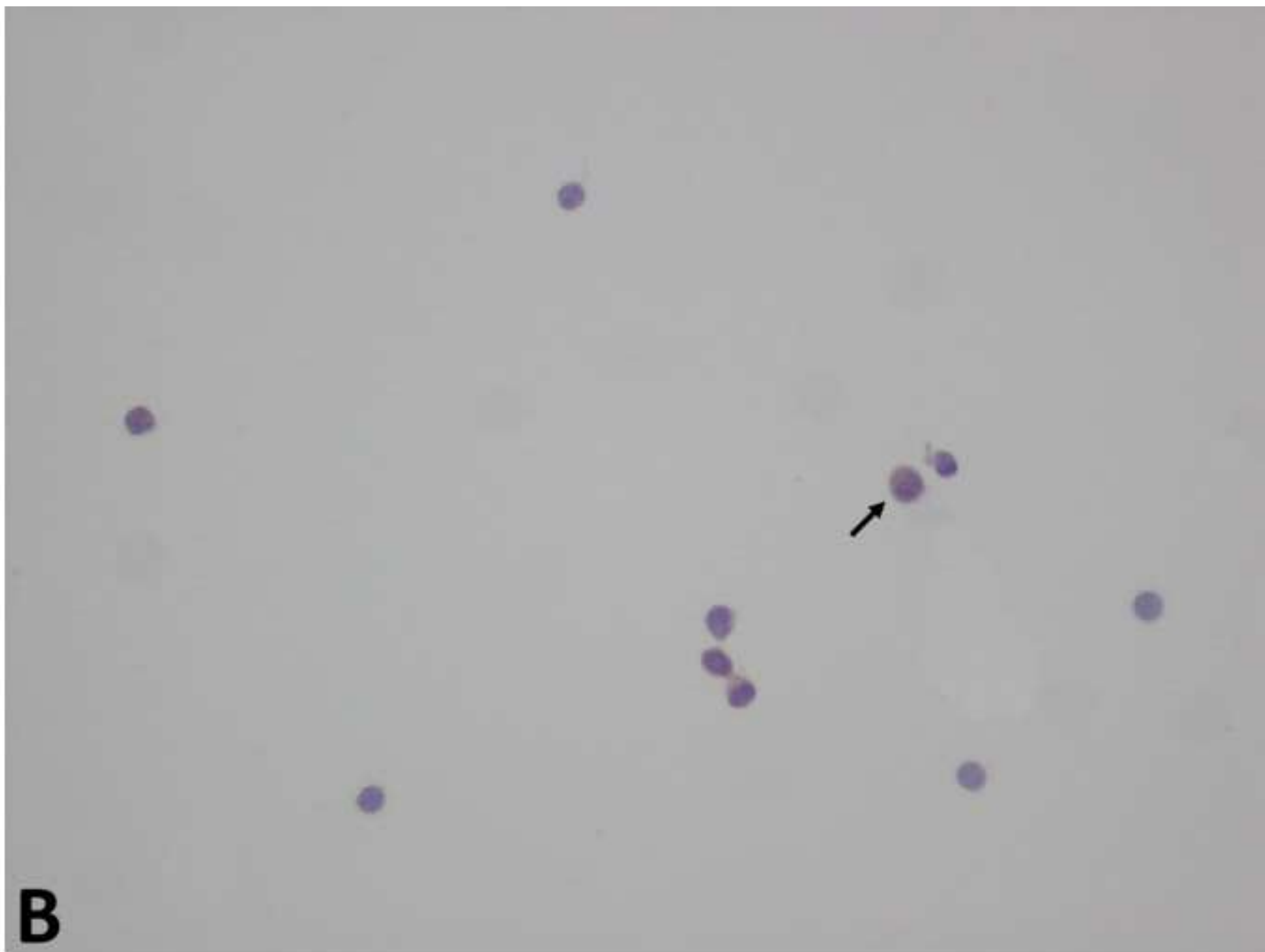

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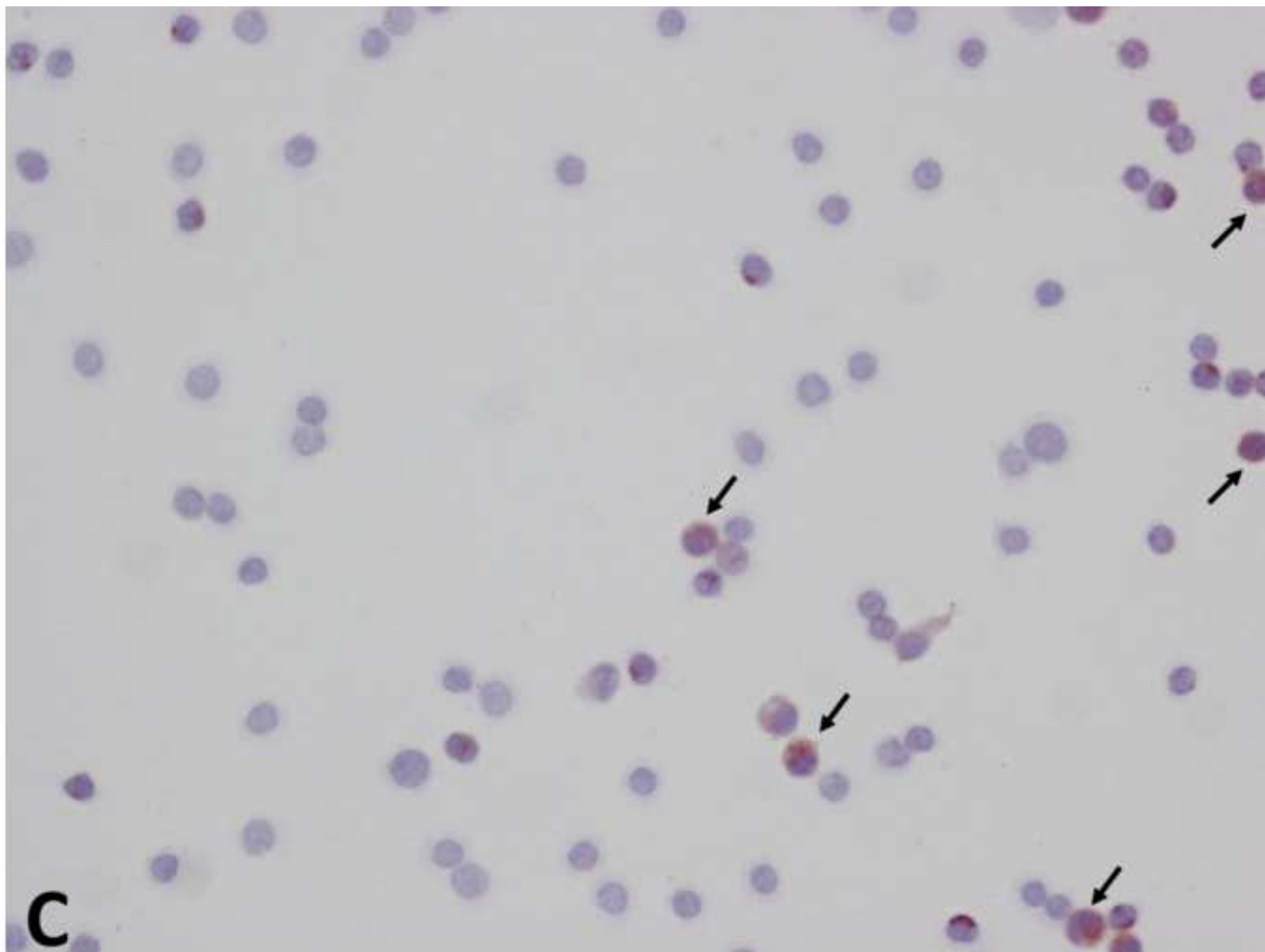

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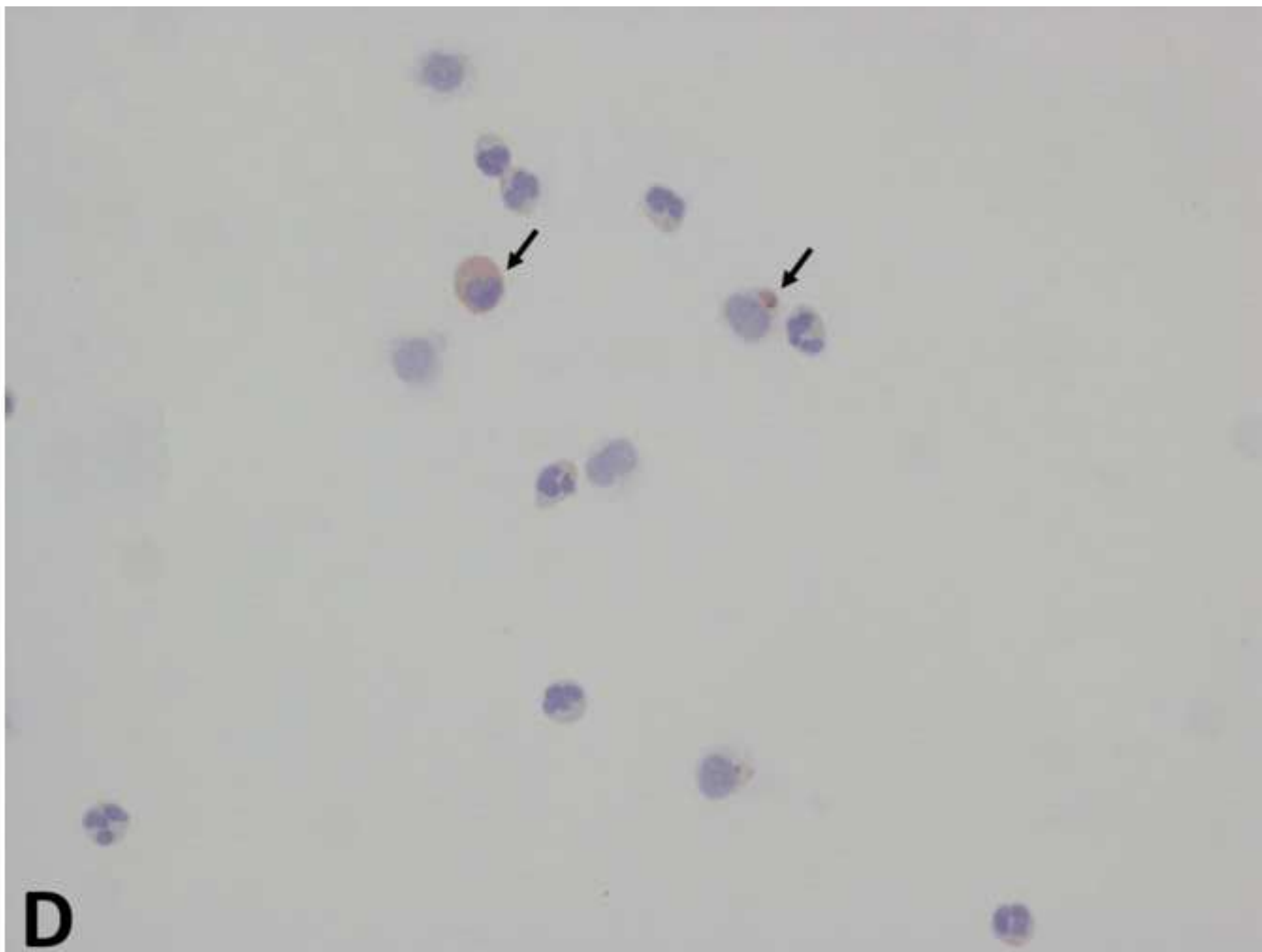

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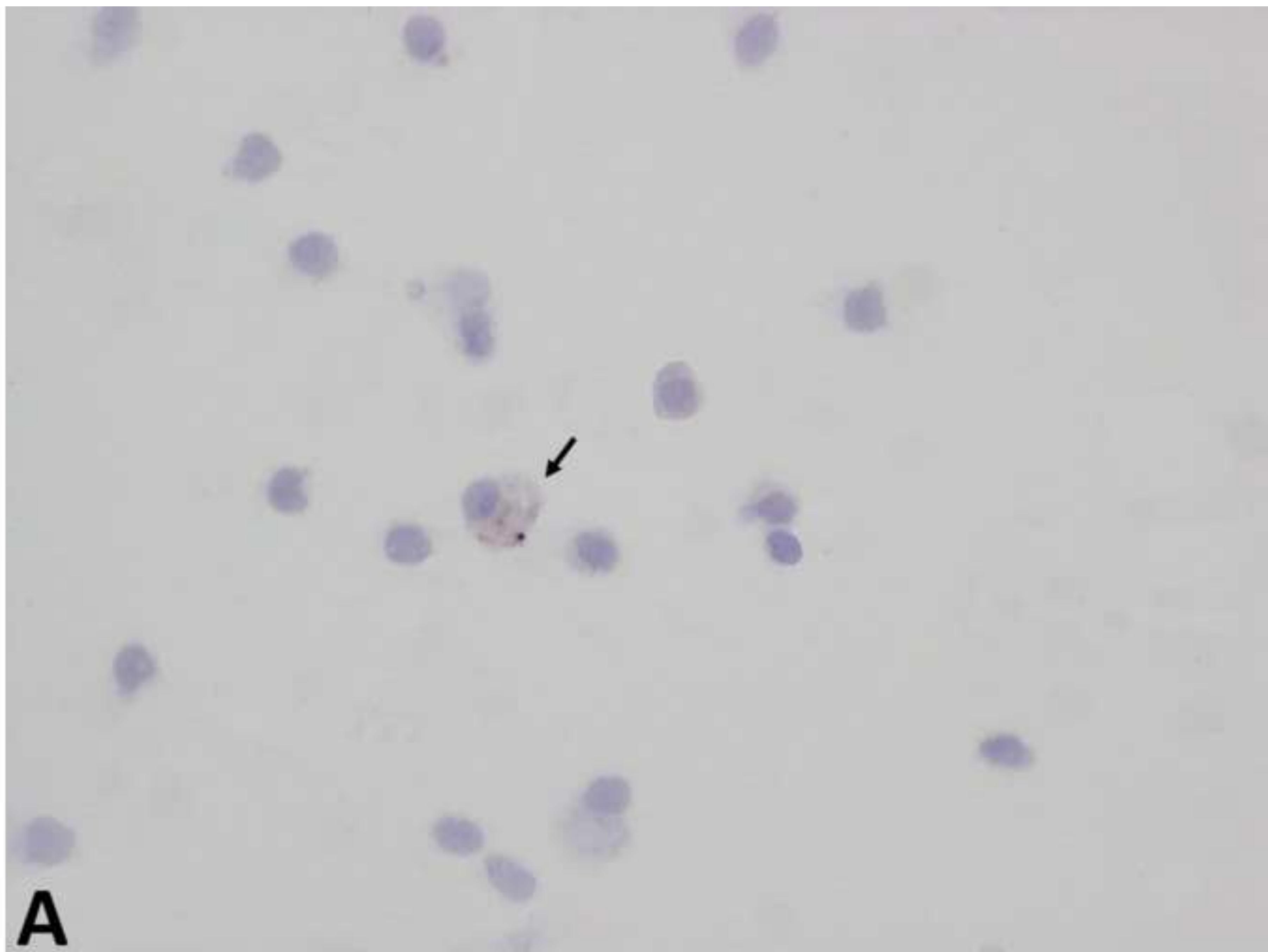

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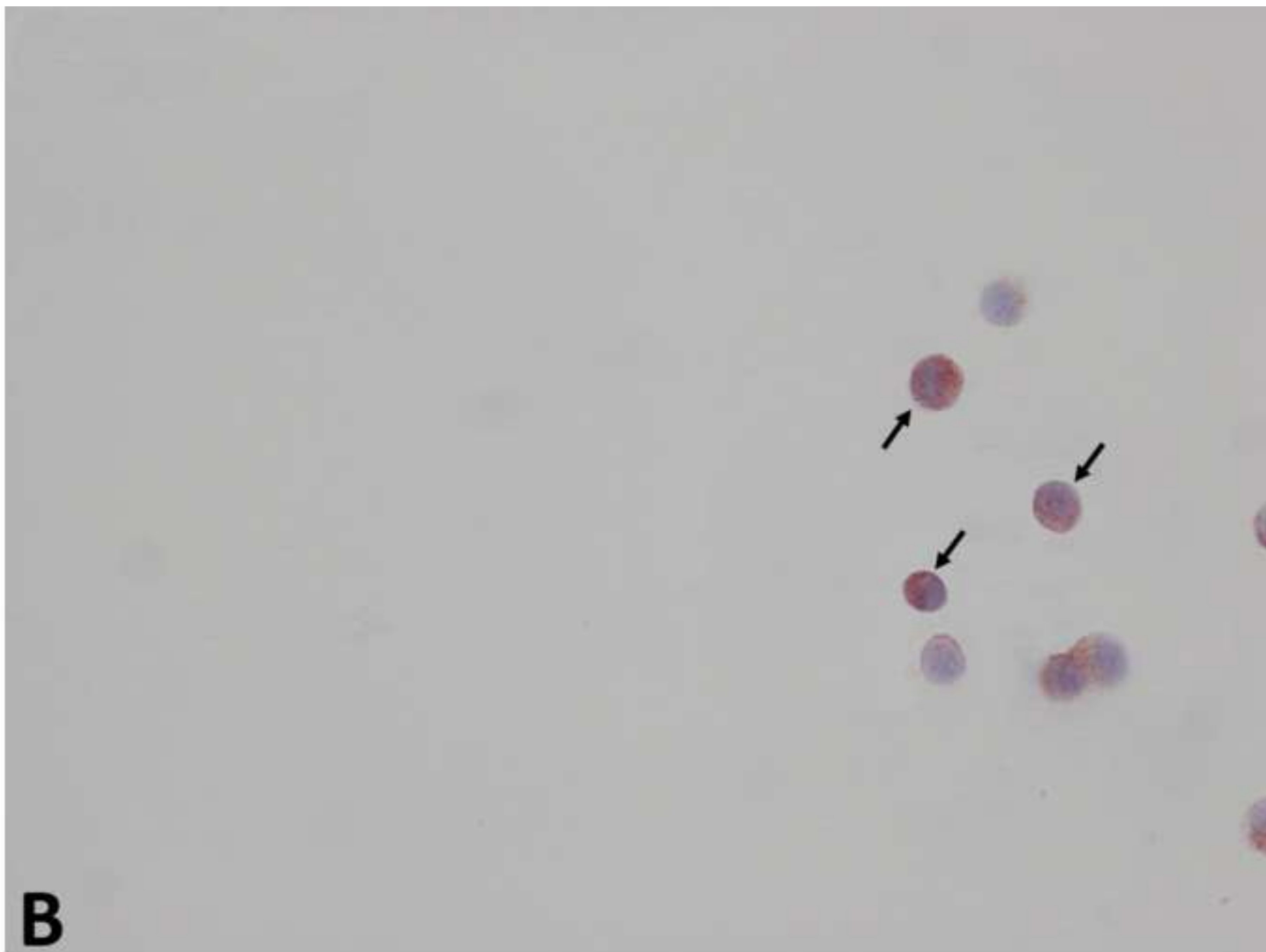

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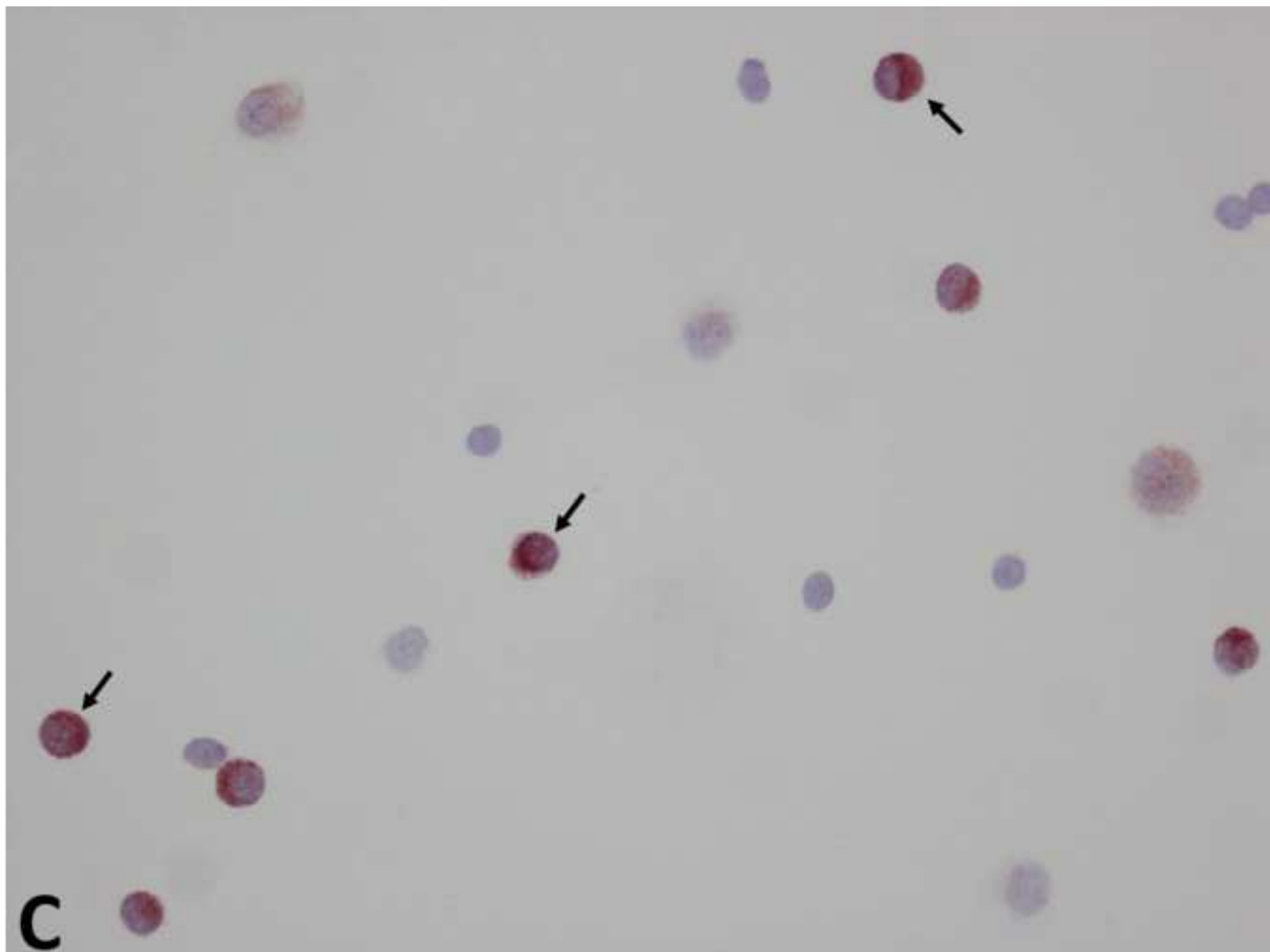

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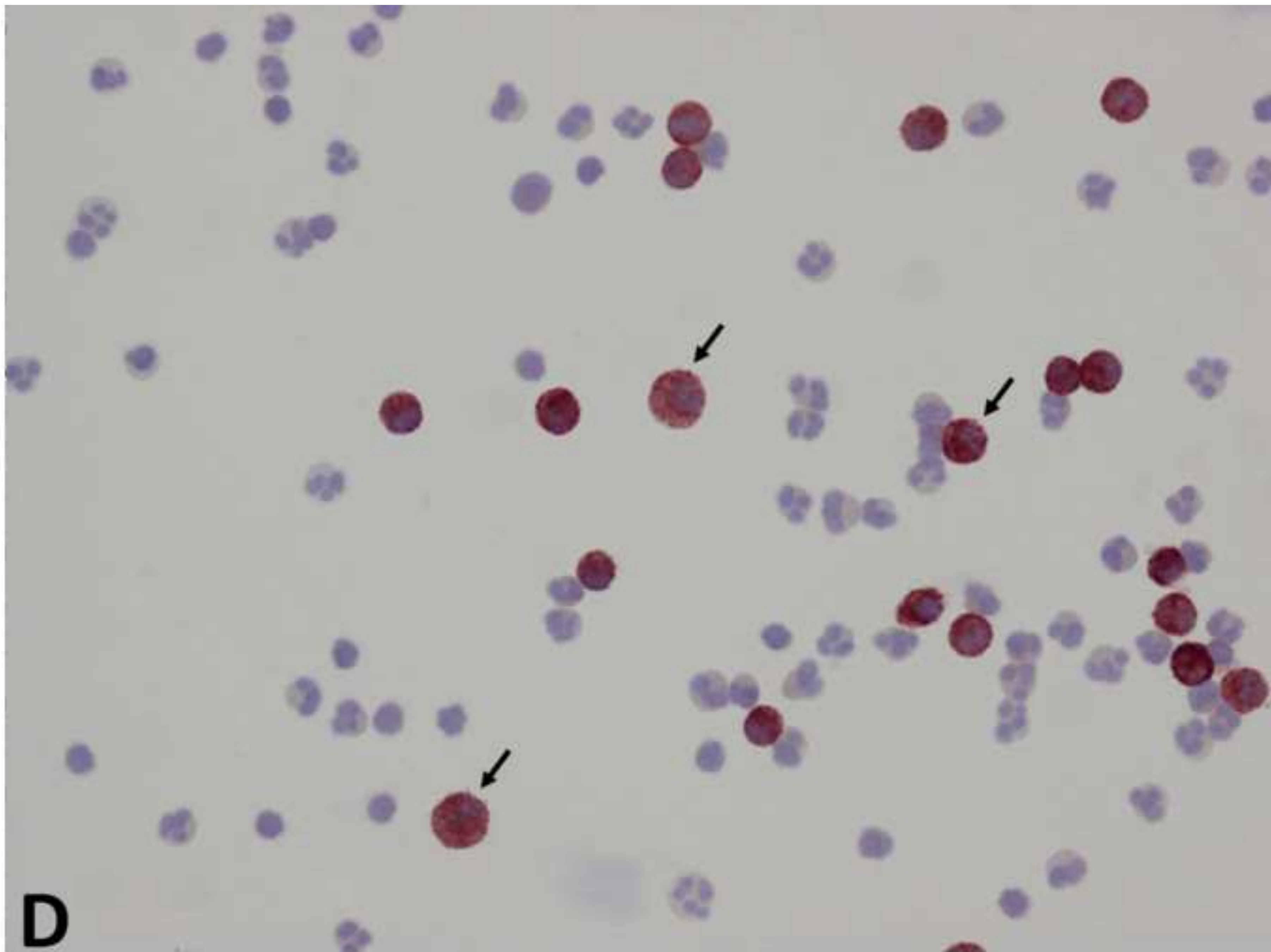

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1 **Study of Peritoneal Macrophage Immunophenotype in Sheep Experimentally
2 Infected with *Fasciola hepatica***

3

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21

22 **Abstract**

23 During *Fasciola hepatica* infection, the parasite has the capability to modulate the host
24 immune response towards a non-protector Th2 type instead of Th1. This type of
25 immune response is closely related to the alternative activation of macrophages (M2
26 profile) as has been shown *in vivo* in murine models. In this study, an experiment was
27 carried out in order to evaluate the expression of CD68, CD14, CD206 and iNOS in
28 cells present in the peritoneal fluid of sheep during early stages of infection with *F.*
29 *hepatica* (1, 3, 9 and 18 days post-infection, dpi) by immunocytochemistry. To the
30 authors' knowledge, this is the first report that studies the *in vivo* immunophenotype of
31 macrophages from the peritoneal fluid of sheep infected with *F. hepatica*. Throughout
32 the experiments the absolute number of leucocytes progressively increased, reaching its
33 highest value at 18 dpi, mainly due to the increase of eosinophils. This
34 immunocytochemical study had two purposes: 1) CD68 expression was assessed with
35 Hansel counterstaining, to optimally identify peritoneal macrophages, eosinophils and
36 lymphocytes; 2) expression of CD14, CD206 and iNOS was evaluated to identify
37 alternative or classical pathways of macrophage activation. The results showed a
38 significant increase in CD14 from day 3 dpi compared with the non-infected group.
39 CD206 expression at all time-points showed a significant and dramatic increase in
40 comparison with the uninfected group. On the other hand, iNOS expression showed
41 little variation, and was significantly decreased at 18 dpi in both trials. These results
42 suggest that *F. hepatica* induces an alternative activation of peritoneal macrophages of
43 sheep from the first day post-infection, which may facilitate parasite survival. This is
44 the first report describing M2 activation of peritoneal macrophages in ruminants
45 infected with *F. hepatica*.

46 **Keywords:** *Fasciola hepatica*, macrophages, peritoneal fluid, Sheep, immune response

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47 **1.- Introduction**

48 Fasciolosis caused by the liver fluke *Fasciola hepatica* infects millions of ruminants
49 worldwide and is recognised by the World Health Organisation (WHO) as an important
50 zoonosis, which is now emerging or re-emerging in many countries (Gonzalez et al.,
51 2011). The costs incurred due to losses in production and treatment with anthelmintics,
52 as well as the resistance that is now widespread, confirm the urgent need for alternative
53 control methods (Fairweather, 2011). During the last two decades, major advances have
54 been made in identifying potential vaccine molecules. Nevertheless, no vaccine
55 candidate has yet reached a commercial or pre-commercial stage. The immune
56 suppression/modulation by *F. hepatica* is one major obstacle to develop a protective
57 vaccine (Toet et al., 2014; Molina-Hernández et al., 2015).

58 Classical M1 and alternative M2 activation pathways of macrophages, mirroring the
59 Th1-Th2 polarisation of T cells, represent two extremes of a dynamic state of
60 macrophage activation (Wang et al., 2014). Since *F. hepatica* larvae penetrate the
61 intestinal wall of the host and migrate to the liver via the peritoneum, study of the type
62 macrophage activation at this stage plays a critical role in understanding the immune
63 response to parasitic infection and thus for designing an effective vaccine (Molina-
64 Hernández et al., 2015). It has been reported that *F. hepatica* excretion-secretion
65 products and tegumental coat proteins produce a M2 macrophage phenotype,
66 responsible for host tissue repair, tissue fibrosis and modulation of adaptive immunity,
67 which suppresses a Th1-driven inflammatory pathology in *F. hepatica* infection (Adams
68 et al., 2014; Figueroa-Santiago et al., 2014; Flynn et al., 2007).

69 In early stages of *F. hepatica* infection, the recruitment and activation of M2
70 macrophages in the peritoneal cavity has been reported in rats at 24h post-infection
71 (Donnelly et al., 2005) and in mice at 48 h post-infection (Guasconi et al. 2011).

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72 Moreover, excretory-secretory products from *F. hepatica* induced M2 activation of
73 peritoneal macrophages in mice (Guasconi et al. 2011). In sheep marked M2 activation
74 has been described by gene expression was in PBMC at 7 dpi (Fu et al., 2016).
75 Recently, proteomic analysis, humoral response and cytokine expression have been
76 studied in sheep experimentally infected with *F. hepatica* at 18 dpi (Ruiz-Campillo et
77 al., 2017), but phenotype of peritoneal macrophages have not been investigated in *F.*
78 *hepatica* infected ruminants. On the other hand, it has been reported that *F. hepatica*
79 induces apoptosis of peritoneal macrophages in rats (Guasconi et al., 2012) and sheep
80 (Escamilla et al., 2017), but the impact of apoptosis in total peritoneal leucocyte count
81 has not been reported in *F. hepatica* infected ruminants. The aim of the present work
82 was to evaluate the dynamic of total and differential leucocyte count and the
83 macrophage polarisation in peritoneal fluid obtained from sheep experimentally infected
84 with *F. hepatica* in the early stages of infection.
85
86 **2.-Materials and Methods**
87 *2.1.-Experimental design*
88 A total of twenty-five eight-month old male Merino sheep obtained from a liver fluke-
89 free farm were used to study the early stages of infection. Animals were purchased aged
90 one month and housed indoors in the experimental farm of the University of Córdoba
91 until they reached the appropriate age for pathogen challenge. All animals were tested
92 monthly for parasite eggs by faecal sedimentation with negative results in all cases.
93 Moreover, prior to the challenge, all animals were tested for serum IgG specific for *F.*
94 *hepatica* cathepsin L1 (FhCL1) by ELISA, with negative results in all cases. The trial
95 consisted of five groups composed of five sheep (n=5): an uninfected control group and
96 four infected groups. Sheep were orally infected with one dose of 150 metacercariae of

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97 the South Gloucester strain of *F. hepatica* (Ridgeway Research Ltd, UK) and
98 euthanised at 1, 3, 9 and 18 days post-infection (dpi). The euthanasia was applied by
99 intravenous injection of T61® (Intervet, Spain). The experiments were approved by the
100 Bioethics Committee of the University of Cordoba (code No. 1118) and conducted in
101 accordance with European (2010/63/UE) and Spanish (RD 1201/2005) directives on
102 animal experimentation.

103 *2.2.-Recovery of peritoneal fluid*

104 Peritoneal lavages were conducted immediately after the animals were euthanised. The
105 ventral aspect of the abdomen was shaved and disinfected with 10%
106 polyvinylpyrrolidone iodine (AGB, Madrid, Spain). A 2 cm incision was made on the
107 skin over the white line and subcutaneous tissue was dissected, the white line and
108 peritoneum were sectioned with blunt scissors to avoid bleeding. A 40 cm cannula was
109 inserted into the abdominal cavity and connected to a syringe to inject 60 ml sterile
110 DPBS (Dulbecco's Phosphate-Buffered Saline, Sigma-Aldrich, Darmstadt, Germany),
111 and 9500 I.U. of heparin (Rovi, Madrid, Spain), previously warmed to 37 ºC. After
112 softly massaging the abdominal cavity for 1 min, 40 ml of peritoneal fluid was
113 recovered using the syringe connected to the cannula. In cases where residual
114 erythrocytes were present, it was necessary to use an erythrolysis buffer prior to the
115 processing of the cells.

116 *2.3.-Cell populations*

117 The recovered peritoneal lavage fluid was collected and cooled on ice until cell
118 processing. The total number of viable peritoneal cells was determined after Trypan
119 Blue staining by counting in a Neubauer haemocytometer. Smear preparations were
120 manually performed on Vectabond®-treated slides (Vector laboratories, California,
121 USA) by centrifuging the recovered peritoneal fluid at 1500 rpm for 10 min. After air

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122 draining, these smears were fixed in acetone for 5 min and stored at -80°C for further
123 immunocytochemical studies. For differential cell counting immunocytochemistry using
124 anti-human CD68 monoclonal antibody (Dako, Glostrup, Denmark) in combination
125 with Hansel staining was used as a novel and more accurate cell counting method. A
126 total of 200 cells per smear were counted in randomly selected fields of 400x
127 magnifications using the software Image Pro-plus 6.0 (Media Cybernetics, Silver
128 Spring, USA), and the percentage of lymphocytes (small basophilic nucleus and scanty
129 cytoplasm), macrophages (round to oval hyperchromatic nucleus and moderate to large
130 cytoplasm stained brownish with the CD68 antibody) and eosinophils (basophilic
131 bilobed nucleus and eosinophilic cytoplasm) was obtained. Neutrophils (basophilic
132 lobulated nucleus and unstained cytoplasm) were not included in the cell count since
133 they were only very occasionally observed.

134 *2.4.-Immunocytochemistry (ICC)*

135 An immunocytochemical study was used to assess anti-Human CD68 (M0718, Dako,
136 Glostrup, Denmark), anti-Human CD14 (BOV2027, Monoclonal Antibody Center,
137 Washington State University), anti-Human iNOS (PA3–030A, Thermo, Freemont,
138 USA) and anti-Human CD206 (orb4941, Biorbyt, Cambridge, UK) expression in
139 peritoneal fluid macrophages. The dilution used for these primary antibodies was 1:400
140 for CD68; 1:500 for CD14, 1:200 for iNOS; and 1:100 for CD206. The CD14
141 monoclonal antibody cross-reacts with ovine tissues according to the manufacturer. The
142 iNOS (Wood et al. 2005), CD68 antibodies (Pinczowski et al., 2017) and anti-human
143 CD206 (Ampem et al., 2016) have shown cross reactivity with ovine tissues. The
144 avidin-biotin-peroxidase method described elsewhere (Zafra et al., 2013a) was carried
145 out. Briefly, endogenous peroxidase activity was blocked by incubation with 3%
146 hydrogen peroxide (Panreac, Barcelona, Spain) in phosphate buffered saline (PBS).

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147 Then, smears were washed once in PBS and incubated with 10% normal goat serum
148 (MP Biomedicals, Ohio, USA) for 30 min at room temperature. After three 5 min rinses
149 in PBS-Tween (PBST), secondary antibodies were applied for 45 min at 37ºC. A
150 biotinylated goat anti-rabbit immunoglobulin serum (Dako) diluted 1:200 was applied
151 to the smears incubated with the primary polyclonal antibodies (pAbs: iNOS and
152 CD206), whereas a biotinylated goat anti-mouse immunoglobulin serum (Dako) diluted
153 1:50 was used for the primary monoclonal antibody (mAb: CD68). After two 5 min
154 rinses in PBST, an avidin–biotin-peroxidase complex (Vector, Burlinghame, USA)
155 diluted 1:50 was applied for an hour as a third reagent. Slides were then washed three
156 times in PBST and incubated with Novared® substrate kit peroxidase (Vector) diluted
157 following the manufacturer's instructions, rinsed in water, lightly counterstained with
158 Mayer's haematoxylin and mounted with Eukitt® (Freiburg, Germany). For CD68
159 antibody, following the haematoxylin stain, eosin was applied to the slides for 1 minute
160 with Hansel stain for the differential cell count. Specific primary antibodies were
161 substituted with PBS or non-immune isotype-matched sera as negative controls. Sheep
162 and human hepatic lymph node lymph node tissue sections were used as positive
163 controls.

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164 *2.5.-Cell count*

165 Immunoreactive cells were counted in randomly selected fields of 400x magnifications
166 using the Image Pro-plus 6.0 software package. Macros were calibrated for staining
167 intensity and cell size to include all immunostained cells. A total of 200 cells were
168 counted per slide and the percentage of positive and negative cells was obtained.
169 Photomicrographs were taken using an Olympus BX51 photomicroscope with a 400x
170 magnification field. Results were expressed as mean ± SD per animal and per group.

171 The intensity of immunostaining was evaluated semi-quantitatively according to the
172 following score: 1, mild; 2, moderate; 3, severe; 4, very severe.
173 *2.6.-Statistical analysis*
174 Statistical analysis was carried out using the Graphpad Prism 7.0 software package
175 (Graphpad Software, Inc., San Diego, California). The Kolmogorov–Smirnov test was
176 applied to evaluate whether distributions were parametric. Comparisons between groups
177 were made using the Mann–Whitney test for non-parametric distributions. Correlation
178 studies were carried out using the Spearman correlation test for non-parametric
179 distributions. $P<0.05$ was considered significant.
180
181 **3. Results**
182 *3.1. Absolute peritoneal cell count*
183 The results of the absolute peritoneal fluid cell counts are shown in Table 1. The
184 number of cells increased significantly ($P<0.05$) at 9 and 18 dpi compared with the
185 uninfected control group. At 1 and 3 dpi no significant differences respect to the
186 uninfected control group were obtained.
187 *3.2. Differential peritoneal cell count*
188 Since CD68 has been widely used as a general macrophage marker (Valheim et al.,
189 2004) a CD68 mAb in combination with Hansel stain was used as a novel and more
190 accurate leucocyte identification method. The differential cell count results (expressed
191 as percentages) from peritoneal fluid smear examinations are shown in Table 2.
192 Neutrophils and epithelial cells were only occasionally observed and were not included
193 in the cell count.
194 At 9 and particularly at 18 dpi, there was a very marked increase in the number of
195 eosinophils, which was responsible for the relative decrease in the percentages of

196 macrophages (9 and 18 dpi) and lymphocytes (18 dpi) in comparison with the
197 uninfected control group Table 2. On the other hand, the percentage of lymphocytes
198 showed a significant increase in both trials at 9 dpi with respect to the uninfected
199 control group. This may reflect a stimulation of the recruitment of peritoneal
200 lymphocytes at this time-point when larvae are penetrating or migrating into the liver
201 surface as revealed by the significant increase in the total number of peritoneal
202 leucocytes at 9 and 18 dpi.

203 *3.3. Immunocytochemical study*

204 The anti-CD14 mAb yielded a cytoplasmic immunostaining in peritoneal leucocytes
205 with large cytoplasm and round to ovoid nucleus, this was the same morphology than
206 peritoneal leucocytes expressing CD68. The results of the present study revealed a
207 significant increase of the percentage of CD14+ cells at 3, 9 and 18 dpi with respect to
208 the uninfected control group (Table 3).

209 Anti-iNOS and anti-CD206 antibodies were used as biomarkers of classical (M1) and
210 alternative (M2) macrophage activation, respectively. The anti-iNOS pAb also showed
211 granular cytoplasmic immunostaining in peritoneal macrophages (Fig. 1) and in some
212 eosinophils, but only the macrophages were counted. The percentage of peritoneal
213 macrophages expressing iNOS varied little during the course of the infection in (Table
214 3). The intensity of immunolabelling with anti-iNOS was mild in the uninfected control
215 group and at all studied time-points (Table 3).

216 The Anti-human CD206 antibody has been described as a good biomarker of alternative
217 activation of macrophages in sheep (Ampem et al., 2016). In our study, the CD206 mAb
218 yielded weak cytoplasmic immunostaining in peritoneal macrophages from the
219 uninfected control group, whereas the intensity of the immunolabelling was very strong
220 at 1, 3, 9 and 18 dpi (Fig. 2, Table 3). The percentage of peritoneal macrophages

221 expressing CD206 showed a dramatic and significant increase (*P*<0.05) from 1 dpi
222 onwards, compared with the uninfected groups (Table 3).
223 The iNOS/CD206 ratio decreased approximately one-fold at 1, 3 and 9 dpi and three-
224 fold at 18 dpi (Table 3).

225 **4. Discussion**

226 It has been reported that *F. hepatica* induces apoptosis of peritoneal leucocytes in sheep
227 (Escamilla et al., 2017) and rat (Guasconi et al., 2012), thus it will be of interest to
228 investigate if *F. hepatica* induces reduction of peritoneal leucocyte during early stages
229 of infection. Differential leucocyte count has been evaluated in *F. hepatica* infected
230 goats (Zafra et al., 2013a) and sheep (Escamilla et al., 2017), but total peritoneal count
231 has not been investigated in *F. hepatica* infected ruminants during early stages of
232 infection. The results of the present study revealed non-significant changes of total
233 peritoneal leucocyte count at 1 and 3 dpi respect to the uninfected control group,
234 suggesting that apoptosis of peritoneal leucocytes do not induce reduction of total
235 peritoneal count at early stages, probably due to leucocyte recruitment. The marked and
236 significant increase of total peritoneal leucocytes found at 9 and 18 dpi is mainly due to
237 the increase of eosinophils, a finding consistent with previous reports in goats (Zafra et
238 al., 2013a) and sheep (Escamilla et al., 2017).

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239 CD14 is a co-receptor for TLR-initiated pro-inflammatory responses in innate immune
240 cells, particularly macrophages. It has been reported that infection by helminths such as
241 *Schistosoma mansoni* in mice (Tundup et al., 2014) and *F. hepatica* in cattle (Garza-
242 Cuartero et al., 2016) induces an increase in CD14 expression in liver macrophages and
243 peripheral blood mononuclear cells (PBMCs), respectively. The present study is the first
244 report analysing the expression of CD14 in peritoneal leucocytes in ruminants infected
245 with *F. hepatica,* and revealed a significant increase at 3, 9 and 18 dpi with respect to

246 the uninfected control group. This is in concordance with the increased CD14
247 expression in PBMC at 7 and 12 weeks after *F. hepatica* infection in cattle was
248 associated with an alternative activation of macrophages (Garza-Cuartero et al., 2016).
249 In addition, it has been previously shown that CD14 expression increases during sepsis
250 and tissue remodelling processes, and the fact that this increase occurs at 3 dpi, when
251 the parasite is penetrating through the gut and reaching the peritoneal cavity, suggests
252 that it could be an indicator of tissue damage and apoptosis (Devitt et al., 1998).
253 During helminth infections, macrophages that undergo changes to express an M2
254 phenotype have been implicated in the regulation of the cytokine environment. This
255 change leads to preferential induction of the Th2 response, which is ineffective in
256 controlling the parasite infection and results in the chronic stage of the disease (O'Neill
257 *et al.*, 2000; Kreider et al., 2007). Since the host response to *F. hepatica* is thought to be
258 more effective during the intestinal, peritoneal or early hepatic migratory stages (Van
259 Milligen et al., 1999), the rapid M2 polarisation of peritoneal macrophages found in the
260 sheep of the present study may be an important mechanism of modulation that may
261 facilitate parasite survival during the early stages of infection.
262 In a murine model, very low iNOS gene expression was detected in uninfected controls
263 and at 1, 7, 14 and 21 days after *F. hepatica* infection (Donnelly et al., 2005), while in
264 sheep, a marked decrease in iNOS gene expression was found in PBMC at 7 dpi (Fu et
265 al., 2016), which contrasts with the low level of variation in iNOS expression by
266 immunocytochemistry in both trials of the present study. This difference suggests that
267 iNOS gene and protein expression may differ, with the protein probably remaining
268 active for a longer time than the gene.
269 The ratio iNOS/CD206 suggest M2 polarisation of peritoneal macrophages from 1 dpi,
270 a finding that is consistent with previous studies in mice (Donnelly et al., 2005), and in

271 PBMC of sheep (Fu et al., 2016) and cattle (Flynn et al., 2007, Garza-Cuartero et al.,
272 2016), that report M2 activation of macrophages induced by *F. hepatica* infection.
273 Further studies should focus on the mechanisms used by the *F. hepatica* parasite to
274 modulate the host response in ruminants, particularly during early stages of infection
275 when the parasite is more vulnerable to the host response. The knowledge of such
276 mechanisms may be used to a more rationale design of new vaccine candidates for
277 blocking immunomodulation and increasing vaccine efficacy.

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279 **Acknowledgments**

280 Work supported by EU Project (H2020-SFS-2014-2-635408- PARAGONE and the
281 Spanish Ministry of Science grant AGL2015-67023-C2-1-R.

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283 **References**

284 Adams, P.N., Aldridge, A., Vukman, K.V., Donnelly, S., O'Neill, S.M., 2014. *Fasciola*
285 *hepatica* tegumental antigens indirectly induce an M2 macrophage-like phenotype in
286 vivo. Parasite Immunol. 36, 531-9.

287 Ampem, G., Azegrouz, H., Bacsadi, Á., Balogh, L., Schmidt, S., Thuróczy, J., Röszer,
288 T., 2016. Adipose tissue macrophages in non-rodent mammals: a comparative study.
289 Cell Tissue Res. 363, 461-478.

290 Devitt, A., Moffatt, O.D., Raykundalia, C., Capra, J.D., Simmons, D.L., Gregory, C.D.,
291 1998. Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature
292 392, 505-509.

293 Donnelly, S, O'Neill, S.M., Sekiya, M., Mulcahy, G., Dalton, J.P., 2005. Thioredoxin
294 peroxidase secreted by *Fasciola hepatica* induces the alternative activation of
295 macrophages. Infect. Immun. 73, 166-173.

296 Escamilla, A., Pérez-Caballero, R., Zafra, R., Bautista, M.J., Pacheco, I.L., Ruiz, M.T.,
297 Martínez-Cruz, M.S., Martínez-Moreno, A., Molina-Hernández, V., Pérez, J., 2017.
298 Apoptosis of peritoneal leucocytes during early stages of *Fasciola hepatica* infections in
299 sheep. Vet. Parasitol. 238, 49-53.
300 Fairweather, I., 2011. Reducing the future threat from (liver) fluke: realistic prospect or
301 quixotic fantasy?. Vet Parasitol. 180, 133-143.
302 Figueroa-Santiago, O., Espino, A.M., 2014. *Fasciola hep*atica fatty acid binding protein
303 induces the alternative activation of human macrophages. Infect. Immun. 82, 5005-
304 5012.
305 Flynn, R.J., Irwin, J.A., Olivier, M., Sekiya, M., Dalton, J.P., Mulcahy, G., 2007.
306 Alternative activation of ruminant macrophages by *Fasciola hepatica*. Vet. Immunol.
307 Immunopathol. 120, 31-40.
308 Fu, Y., Chryssafidis, A.L., Browne, J.A., O'Sullivan, J., McGettigan, P.A., Mulcahy,
309 G., 2016. Transcriptomic study on ovine immune responses to *Fasciola hepatica*
310 infection. PLOS Negl. Trop. Dis. 10, e0005015.
311 Garza-Cuartero, L., O'Sullivan, J., Blanco, A., McNair, J., Welsh, M., Flynn, R.J.,
312 Williams, D., Diggle, P., Cassidy, J. Mucalhy, G., 2016. *Fasciola hepatica* infection
313 reduces *Mycobacterium bovis* burden and mycobacterial uptake and suppresses the pro-
314 inflammatory response. Parasite Immunol. 38, 387-402.
315 González, L.C., Esteban, J.G., Bargues, M.D., Valero, M.A., Ortiz, P., Náquira, C.,
316 Mas-Coma, S., 2011. Hyperendemic human fascioliasis in Andean valleys: An
317 altitudinal transect analysis in children of Cajamarca province, Peru. Acta Trop. 120,
318 119-129.

319 Guasconi, L., Serradell, M.C., Garro, A.P., Iacobelli, L., Masih, D.T. 2011. C-type
320 lectins on macrophages participate in the immunomodulatory response to *Fasciola*
321 *hepatica* products. Immunology. 133, 386-396.
322 Guasconi, L., Serradell, M.C., Masih, D.T. 2012. *Fasciola hepatica* products induce
323 apoptosis of peritoneal macrophages. Vet. Immunol. Immunopathol. 148, 359-363.
324 Kreider, T., Anthony, R.M., Urban, J.F. Jr., Gause, W.C., 2007. Alternative activated
325 macrophages in helminth infections. Curr. Opin. Immunol. 19, 448-453.
326 Molina-Hernández, V., Mulcahy, G., Pérez, J., Martínez-Moreno, A., Donnelly, S.,
327 O'Neill, S., Dalton, J.P., Cwiklinski, K., 2015. *Fasciola hepatica* vaccine: we may not
328 be there yet but we're on the right road. Vet. Parasitol. 208, 101-111.
329 O'Neill, S.M., Brady, M.T., Callanan, J.J., Mulcahy, G., Joyce, P., Mills, K.H., Dalton,
330 J.P., 2000. *Fasciola hepatica* infection downregulates Th1 responses in mice. Parasite
331 Immunol. 22, 147-155.
332 Pinczowski, P., Sanjosé, L., Gimeno, M., Crespo, H., Glaria, I., Amorena, B., de
333 Andrés, D., Pérez, M., Reina, R., Luján, L. 2017. Small ruminant lentiviruses in sheep:
334 pathology and tropism of 2 strains using the bone marrow route. Vet Pathol. 54, 413-
335 424.
336 Ruiz-Campillo, M.T., Molina-Hernandez, V., Escamilla, A., Stevenson, M., Perez, J.,
337 Martinez-Moreno, A., Donnelly, S., Dalton, J.P., Cwiklinski, K., 2017. Immune
338 signatures of pathogenesis in the peritoneal compartment during early infection of sheep
339 with *Fasciola hepatica*. Sci Rep. 7, 2782.
340 Toet, H., Piedrafita, D.M., Spithill, T.W., 2014. Liver fluke vaccine in ruminants:
341 strategies, progress and future opportunities. Int. J. Parasitol. 44, 915-927.

342 Tundup S., Srivastava L., Nagy T., Harn D., 2014. CD14 influences host immune
343 responses and alternative activation of macrophages during *Schistosoma mansoni*
344 infection. Infect. Immun. 82, 3240-3251.
345 Valheim M., Sigurdardóttir O.G., Storset A.K. Aune L.G., Press C.M., 2004.
346 Characterization of macrophages and occurrence of T cells in intestinal lesions of
347 subclinical paratuberculosis in goats. J. Comp. Pathol. 131, 221-232.
348 Van Milligen, F.J., Cornelissen, J.B., Bokhout B.A., 1999. Protection against *Fasciola*
349 *hepatica* in the intestine is highly correlated with eosinophil and immunoglobin G1
350 responses against newly excysted juveniles. Parasite Immunol. 21, 243-251.
351 Wang, N., Liang, H., Zen, K., 2014. Molecular mechanisms that influence the
352 macrophage m1-m2 polarization balance. Front. Immunol. 5, 614.
353 Wood, C.E., Chen, G.F., Keller-Wood, M., 2005. Expression of nitric oxide synthase
354 isoforms is reduced in late-gestation ovine fetal brainstem. Am J Physiol Regul Integr
355 Comp Physiol. 289, R613-R619.
356 Zafra, R., Pérez-Écija, R.A., Buffoni, L., Moreno, P., Bautista, M.J., Martínez-Moreno,
357 A., Mulcahy, G., Dalton, J.P., Pérez, J., 2013a. Early and late peritoneal and hepatic
358 changes in goats immunized with recombinant cathepsin L1 and Infected with *Fasciola*
359 *hepatica*. J. Comp. Path. 148, 373-384.
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361

362 **Figure legends**

363 **Fig. 1**. Peritoneal smear stained with anti-iNOS polyclonal antibody showing mild
364 cytoplasmic immunolabelling (brown colour) in macrophages (arrows) in the uninfected
365 control group (A) and at 3 day post-infection (dpi) (B), 9 dpi (C) and 18 dpi (D). ABC
366 method-haematoxylin counterstain. X400.

367 **Fig. 2.** Peritoneal smear stained with anti-CD206 polyclonal antibody showing mild
368 cytoplasmic immunolabelling (brown colour) in macrophages (arrows) from uninfected
369 control (A) and very severe immunolabelling in macrophages at 1 day post-infection
370 (dpi) (B), 9 dpi (C), and 18 dpi (D). ABC method-haematoxylin counterstain. X400.

371

372

373 **Table 1. Absolute peritoneal leucocyte counts expressed in 10^6 cells/ml**
374 **(mean±SEM)**.

UC	1 dpi	3 dpi	9 dpi	18 dpi
4.0±0.8	3.3±1.6	7.4±1.4	74.2±20.1*	497.9±122*

375 UC: uninfected control group. dpi: days post-infection.

376 *Significant difference (*P*<0.05) with respect to the UC group.

377

378

379

380 **Table 2**. Differential cell count expressed as percentages of lymphocytes, macrophages

381 and eosinophils in the uninfected control group and at 1, 3, 9 and 18 days post-infection.

382

	UC	**1 dpi**	**3 dpi**	**9 dpi**	**18 dpi**
Lymphocytes	39.4±10.6	35.6±12.1	46.4±20.4	63.6±16.0*	15.5±7.8*
Macrophages	56.0±11.1	64.1±11.7	50.9±22.6	18.0±6.3*	11.5±3.9*
Eosinophils	4.4±4.2	0.18±0.3	2.3±2.5	18.1±10.6*	72.9±10.9*

383 *Significant difference ($P<0.05$) with respect to the UC group.

Table 3. Percentage intensity of immunoreactivity (1-4) of peritoneal macrophages expressed as mean ±SD for the expression of CD14, iNOS, CD206, and the iNOS/CD206 ratio in the uninfected control group (UC) and in the different stages of infection.

	UC	1 dpi	3 dpi	9 dpi	18 dpi
CD14	51.2±11.1(2)	63.2±19(2)	78.1*±13.1(4)	74.6*±16.9(4)	69.1*±12.9(4)
iNOS	37.3±27.7(1)	64.4±4.1(1)	62.9±17.5(1)	74.4*±9.4(2)	46±21.2(1)
CD206	20.9±4.4(1)	81.5*±8.6(4)	83.9*±15.4(4)	87*±5.7(4)	90.9*±8.02(4)
iNOS/CD206	1.8	0.8	0.8	0.9	0.5

§ Intensity of immunoreactivity (1: mild, 2: moderate, 3: severe, 4: very severe).

*Significant differences with respect to the UC group.

*Revised Manuscript with NO changes marked (clean)
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1 **Study of Peritoneal Macrophage Immunophenotype in Sheep Experimentally**
2 **Infected with *Fasciola hepatica***

3

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21

22 **Abstract**

23 During *Fasciola hepatica* infection, the parasite has the capability to modulate the host
24 immune response towards a non-protector Th2 type instead of Th1. This type of
25 immune response is closely related to the alternative activation of macrophages (M2
26 profile) as has been shown *in vivo* in murine models. In this study, an experiment was
27 carried out in order to evaluate the expression of CD68, CD14, CD206 and iNOS in
28 cells present in the peritoneal fluid of sheep during early stages of infection with *F.*
29 *hepatica* (1, 3, 9 and 18 days post-infection, dpi) by immunocytochemistry. To the
30 authors' knowledge, this is the first report that studies the *in vivo* immunophenotype of
31 macrophages from the peritoneal fluid of sheep infected with *F. hepatica*. Throughout
32 the experiments the absolute number of leucocytes progressively increased, reaching its
33 highest value at 18 dpi, mainly due to the increase of eosinophils. This
34 immunocytochemical study had two purposes: 1) CD68 expression was assessed with
35 Hansel counterstaining, to optimally identify peritoneal macrophages, eosinophils and
36 lymphocytes; 2) expression of CD14, CD206 and iNOS was evaluated to identify
37 alternative or classical pathways of macrophage activation. The results showed a
38 significant increase in CD14 from day 3 dpi compared with the non-infected group.
39 CD206 expression at all time-points showed a significant and dramatic increase in
40 comparison with the uninfected group. On the other hand, iNOS expression showed
41 little variation, and was significantly decreased at 18 dpi in both trials. These results
42 suggest that *F. hepatica* induces an alternative activation of peritoneal macrophages of
43 sheep from the first day post-infection, which may facilitate parasite survival. This is
44 the first report describing M2 activation of peritoneal macrophages in ruminants
45 infected with *F. hepatica*.

46 **Keywords:** *Fasciola hepatica*, macrophages, peritoneal fluid, Sheep, immune response

47 **1.- Introduction**

48 Fasciolosis caused by the liver fluke *Fasciola hepatica* infects millions of ruminants
49 worldwide and is recognised by the World Health Organisation (WHO) as an important
50 zoonosis, which is now emerging or re-emerging in many countries (Gonzalez et al.,
51 2011). The costs incurred due to losses in production and treatment with anthelmintics,
52 as well as the resistance that is now widespread, confirm the urgent need for alternative
53 control methods (Fairweather, 2011). During the last two decades, major advances have
54 been made in identifying potential vaccine molecules. Nevertheless, no vaccine
55 candidate has yet reached a commercial or pre-commercial stage. The immune
56 suppression/modulation by *F. hepatica* is one major obstacle to develop a protective
57 vaccine (Toet et al., 2014; Molina-Hernández et al., 2015).

58 Classical M1 and alternative M2 activation pathways of macrophages, mirroring the
59 Th1-Th2 polarisation of T cells, represent two extremes of a dynamic state of
60 macrophage activation (Wang et al., 2014). Since *F. hepatica* larvae penetrate the
61 intestinal wall of the host and migrate to the liver via the peritoneum, study of the type
62 macrophage activation at this stage plays a critical role in understanding the immune
63 response to parasitic infection and thus for designing an effective vaccine (Molina-
64 Hernández et al., 2015). It has been reported that *F. hepatica* excretion-secretion
65 products and tegumental coat proteins produce a M2 macrophage phenotype,
66 responsible for host tissue repair, tissue fibrosis and modulation of adaptive immunity,
67 which suppresses a Th1-driven inflammatory pathology in *F. hepatica* infection (Adams
68 et al., 2014; Figueroa-Santiago et al., 2014; Flynn et al., 2007).

69 In early stages of *F. hepatica* infection, the recruitment and activation of M2
70 macrophages in the peritoneal cavity has been reported in rats at 24h post-infection
71 (Donnelly et al., 2005) and in mice at 48 h post-infection (Guasconi et al. 2011).

72 Moreover, excretory-secretory products from *F. hepatica* induced M2 activation of
73 peritoneal macrophages in mice (Guasconi et al. 2011). In sheep marked M2 activation
74 has been described by gene expression was in PBMC at 7 dpi (Fu et al., 2016).
75 Recently, proteomic analysis, humoral response and cytokine expression have been
76 studied in sheep experimentally infected with *F. hepatica* at 18 dpi (Ruiz-Campillo et
77 al., 2017), but phenotype of peritoneal macrophages have not been investigated in *F.*
78 *hepatica* infected ruminants. On the other hand, it has been reported that *F. hepatica*
79 induces apoptosis of peritoneal macrophages in rats (Guasconi et al., 2012) and sheep
80 (Escamilla et al., 2017), but the impact of apoptosis in total peritoneal leucocyte count
81 has not been reported in *F. hepatica* infected ruminants. The aim of the present work
82 was to evaluate the dynamic of total and differential leucocyte count and the
83 macrophage polarisation in peritoneal fluid obtained from sheep experimentally infected
84 with *F. hepatica* in the early stages of infection.
85
86 **2.-Materials and Methods**
87 *2.1.-Experimental design*
88 A total of twenty-five eight-month old male Merino sheep obtained from a liver fluke-
89 free farm were used to study the early stages of infection. Animals were purchased aged
90 one month and housed indoors in the experimental farm of the University of Córdoba
91 until they reached the appropriate age for pathogen challenge. All animals were tested
92 monthly for parasite eggs by faecal sedimentation with negative results in all cases.
93 Moreover, prior to the challenge, all animals were tested for serum IgG specific for *F.*
94 *hepatica* cathepsin L1 (FhCL1) by ELISA, with negative results in all cases. The trial
95 consisted of five groups composed of five sheep (n=5): an uninfected control group and
96 four infected groups. Sheep were orally infected with one dose of 150 metacercariae of

97 the South Gloucester strain of *F. hepatica* (Ridgeway Research Ltd, UK) and
98 euthanised at 1, 3, 9 and 18 days post-infection (dpi). The euthanasia was applied by
99 intravenous injection of T61® (Intervet, Spain). The experiments were approved by the
100 Bioethics Committee of the University of Cordoba (code No. 1118) and conducted in
101 accordance with European (2010/63/UE) and Spanish (RD 1201/2005) directives on
102 animal experimentation.

103 *2.2.-Recovery of peritoneal fluid*

104 Peritoneal lavages were conducted immediately after the animals were euthanised. The
105 ventral aspect of the abdomen was shaved and disinfected with 10%
106 polyvinylpyrrolidone iodine (AGB, Madrid, Spain). A 2 cm incision was made on the
107 skin over the white line and subcutaneous tissue was dissected, the white line and
108 peritoneum were sectioned with blunt scissors to avoid bleeding. A 40 cm cannula was
109 inserted into the abdominal cavity and connected to a syringe to inject 60 ml sterile
110 DPBS (Dulbecco's Phosphate-Buffered Saline, Sigma-Aldrich, Darmstadt, Germany),
111 and 9500 I.U. of heparin (Rovi, Madrid, Spain), previously warmed to 37 ºC. After
112 softly massaging the abdominal cavity for 1 min, 40 ml of peritoneal fluid was
113 recovered using the syringe connected to the cannula. In cases where residual
114 erythrocytes were present, it was necessary to use an erythrolysis buffer prior to the
115 processing of the cells.

116 *2.3.-Cell populations*

117 The recovered peritoneal lavage fluid was collected and cooled on ice until cell
118 processing. The total number of viable peritoneal cells was determined after Trypan
119 Blue staining by counting in a Neubauer haemocytometer. Smear preparations were
120 manually performed on Vectabond®-treated slides (Vector laboratories, California,
121 USA) by centrifuging the recovered peritoneal fluid at 1500 rpm for 10 min. After air

122 draining, these smears were fixed in acetone for 5 min and stored at -80°C for further
123 immunocytochemical studies. For differential cell counting immunocytochemistry using
124 anti-human CD68 monoclonal antibody (Dako, Glostrup, Denmark) in combination
125 with Hansel staining was used as a novel and more accurate cell counting method. A
126 total of 200 cells per smear were counted in randomly selected fields of 400x
127 magnifications using the software Image Pro-plus 6.0 (Media Cybernetics, Silver
128 Spring, USA), and the percentage of lymphocytes (small basophilic nucleus and scanty
129 cytoplasm), macrophages (round to oval hyperchromatic nucleus and moderate to large
130 cytoplasm stained brownish with the CD68 antibody) and eosinophils (basophilic
131 bilobed nucleus and eosinophilic cytoplasm) was obtained. Neutrophils (basophilic
132 lobulated nucleus and unstained cytoplasm) were not included in the cell count since
133 they were only very occasionally observed.

134 *2.4.-Immunocytochemistry (ICC)*

135 An immunocytochemical study was used to assess anti-Human CD68 (M0718, Dako,
136 Glostrup, Denmark), anti-Human CD14 (BOV2027, Monoclonal Antibody Center,
137 Washington State University), anti-Human iNOS (PA3–030A, Thermo, Freemont,
138 USA) and anti-Human CD206 (orb4941, Biorbyt, Cambridge, UK) expression in
139 peritoneal fluid macrophages. The dilution used for these primary antibodies was 1:400
140 for CD68; 1:500 for CD14, 1:200 for iNOS; and 1:100 for CD206. The CD14
141 monoclonal antibody cross-reacts with ovine tissues according to the manufacturer. The
142 iNOS (Wood et al. 2005), CD68 antibodies (Pinczowski et al., 2017) and anti-human
143 CD206 (Ampem et al., 2016) have shown cross reactivity with ovine tissues. The
144 avidin-biotin-peroxidase method described elsewhere (Zafra et al., 2013a) was carried
145 out. Briefly, endogenous peroxidase activity was blocked by incubation with 3%
146 hydrogen peroxide (Panreac, Barcelona, Spain) in phosphate buffered saline (PBS).

147 Then, smears were washed once in PBS and incubated with 10% normal goat serum
148 (MP Biomedicals, Ohio, USA) for 30 min at room temperature. After three 5 min rinses
149 in PBS-Tween (PBST), secondary antibodies were applied for 45 min at 37ºC. A
150 biotinylated goat anti-rabbit immunoglobulin serum (Dako) diluted 1:200 was applied
151 to the smears incubated with the primary polyclonal antibodies (pAbs: iNOS and
152 CD206), whereas a biotinylated goat anti-mouse immunoglobulin serum (Dako) diluted
153 1:50 was used for the primary monoclonal antibody (mAb: CD68). After two 5 min
154 rinses in PBST, an avidin–biotin-peroxidase complex (Vector, Burlinghame, USA)
155 diluted 1:50 was applied for an hour as a third reagent. Slides were then washed three
156 times in PBST and incubated with Novared® substrate kit peroxidase (Vector) diluted
157 following the manufacturer's instructions, rinsed in water, lightly counterstained with
158 Mayer's haematoxylin and mounted with Eukitt® (Freiburg, Germany). For CD68
159 antibody, following the haematoxylin stain, eosin was applied to the slides for 1 minute
160 with Hansel stain for the differential cell count. Specific primary antibodies were
161 substituted with PBS or non-immune isotype-matched sera as negative controls. Sheep
162 and human hepatic lymph node lymph node tissue sections were used as positive
163 controls.

164 *2.5.-Cell count*

165 Immunoreactive cells were counted in randomly selected fields of 400x magnifications
166 using the Image Pro-plus 6.0 software package. Macros were calibrated for staining
167 intensity and cell size to include all immunostained cells. A total of 200 cells were
168 counted per slide and the percentage of positive and negative cells was obtained.
169 Photomicrographs were taken using an Olympus BX51 photomicroscope with a 400x
170 magnification field. Results were expressed as mean ± SD per animal and per group.

171 The intensity of immunostaining was evaluated semi-quantitatively according to the
172 following score: 1, mild; 2, moderate; 3, severe; 4, very severe.

173 *2.6.-Statistical analysis*

174 Statistical analysis was carried out using the Graphpad Prism 7.0 software package
175 (Graphpad Software, Inc., San Diego, California). The Kolmogorov–Smirnov test was
176 applied to evaluate whether distributions were parametric. Comparisons between groups
177 were made using the Mann–Whitney test for non-parametric distributions. Correlation
178 studies were carried out using the Spearman correlation test for non-parametric
179 distributions. $P<0.05$ was considered significant.

180

181 **3. Results**

182 *3.1. Absolute peritoneal cell count*

183 The results of the absolute peritoneal fluid cell counts are shown in Table 1. The
184 number of cells increased significantly ($P<0.05$) at 9 and 18 dpi compared with the
185 uninfected control group. At 1 and 3 dpi no significant differences respect to the
186 uninfected control group were obtained.

187 *3.2. Differential peritoneal cell count*

188 Since CD68 has been widely used as a general macrophage marker (Valheim et al.,
189 2004) a CD68 mAb in combination with Hansel stain was used as a novel and more
190 accurate leucocyte identification method. The differential cell count results (expressed
191 as percentages) from peritoneal fluid smear examinations are shown in Table 2.
192 Neutrophils and epithelial cells were only occasionally observed and were not included
193 in the cell count.

194 At 9 and particularly at 18 dpi, there was a very marked increase in the number of
195 eosinophils, which was responsible for the relative decrease in the percentages of

196 macrophages (9 and 18 dpi) and lymphocytes (18 dpi) in comparison with the
197 uninfected control group Table 2. On the other hand, the percentage of lymphocytes
198 showed a significant increase in both trials at 9 dpi with respect to the uninfected
199 control group. This may reflect a stimulation of the recruitment of peritoneal
200 lymphocytes at this time-point when larvae are penetrating or migrating into the liver
201 surface as revealed by the significant increase in the total number of peritoneal
202 leucocytes at 9 and 18 dpi.

203 *3.3. Immunocytochemical study*

204 The anti-CD14 mAb yielded a cytoplasmic immunostaining in peritoneal leucocytes
205 with large cytoplasm and round to ovoid nucleus, this was the same morphology than
206 peritoneal leucocytes expressing CD68. The results of the present study revealed a
207 significant increase of the percentage of CD14+ cells at 3, 9 and 18 dpi with respect to
208 the uninfected control group (Table 3).

209 Anti-iNOS and anti-CD206 antibodies were used as biomarkers of classical (M1) and
210 alternative (M2) macrophage activation, respectively. The anti-iNOS pAb also showed
211 granular cytoplasmic immunostaining in peritoneal macrophages (Fig. 1) and in some
212 eosinophils, but only the macrophages were counted. The percentage of peritoneal
213 macrophages expressing iNOS varied little during the course of the infection in (Table
214 3). The intensity of immunolabelling with anti-iNOS was mild in the uninfected control
215 group and at all studied time-points (Table 3).

216 The Anti-human CD206 antibody has been described as a good biomarker of alternative
217 activation of macrophages in sheep (Ampem et al., 2016). In our study, the CD206 mAb
218 yielded weak cytoplasmic immunostaining in peritoneal macrophages from the
219 uninfected control group, whereas the intensity of the immunolabelling was very strong
220 at 1, 3, 9 and 18 dpi (Fig. 2, Table 3). The percentage of peritoneal macrophages

221 expressing CD206 showed a dramatic and significant increase ($P<0.05$) from 1 dpi
222 onwards, compared with the uninfected groups (Table 3).
223 The iNOS/CD206 ratio decreased approximately one-fold at 1, 3 and 9 dpi and three-
224 fold at 18 dpi (Table 3).

225 **4. Discussion**

226 It has been reported that *F. hepatica* induces apoptosis of peritoneal leucocytes in sheep
227 (Escamilla et al., 2017) and rat (Guasconi et al., 2012), thus it will be of interest to
228 investigate if *F. hepatica* induces reduction of peritoneal leucocyte during early stages
229 of infection. Differential leucocyte count has been evaluated in *F. hepatica* infected
230 goats (Zafra et al., 2013a) and sheep (Escamilla et al., 2017), but total peritoneal count
231 has not been investigated in *F. hepatica* infected ruminants during early stages of
232 infection. The results of the present study revealed non-significant changes of total
233 peritoneal leucocyte count at 1 and 3 dpi respect to the uninfected control group,
234 suggesting that apoptosis of peritoneal leucocytes do not induce reduction of total
235 peritoneal count at early stages, probably due to leucocyte recruitment. The marked and
236 significant increase of total peritoneal leucocytes found at 9 and 18 dpi is mainly due to
237 the increase of eosinophils, a finding consistent with previous reports in goats (Zafra et
238 al., 2013a) and sheep (Escamilla et al., 2017).
239 CD14 is a co-receptor for TLR-initiated pro-inflammatory responses in innate immune
240 cells, particularly macrophages. It has been reported that infection by helminths such as
241 *Schistosoma mansoni* in mice (Tundup et al., 2014) and *F. hepatica* in cattle (Garza-
242 Cuartero et al., 2016) induces an increase in CD14 expression in liver macrophages and
243 peripheral blood mononuclear cells (PBMCs), respectively. The present study is the first
244 report analysing the expression of CD14 in peritoneal leucocytes in ruminants infected
245 with *F. hepatica,* and revealed a significant increase at 3, 9 and 18 dpi with respect to

246 the uninfected control group. This is in concordance with the increased CD14
247 expression in PBMC at 7 and 12 weeks after *F. hepatica* infection in cattle was
248 associated with an alternative activation of macrophages (Garza-Cuartero et al., 2016).
249 In addition, it has been previously shown that CD14 expression increases during sepsis
250 and tissue remodelling processes, and the fact that this increase occurs at 3 dpi, when
251 the parasite is penetrating through the gut and reaching the peritoneal cavity, suggests
252 that it could be an indicator of tissue damage and apoptosis (Devitt et al., 1998).
253 During helminth infections, macrophages that undergo changes to express an M2
254 phenotype have been implicated in the regulation of the cytokine environment. This
255 change leads to preferential induction of the Th2 response, which is ineffective in
256 controlling the parasite infection and results in the chronic stage of the disease (O'Neill
257 *et al.*, 2000; Kreider et al., 2007). Since the host response to *F. hepatica* is thought to be
258 more effective during the intestinal, peritoneal or early hepatic migratory stages (Van
259 Milligen et al., 1999), the rapid M2 polarisation of peritoneal macrophages found in the
260 sheep of the present study may be an important mechanism of modulation that may
261 facilitate parasite survival during the early stages of infection.
262 In a murine model, very low iNOS gene expression was detected in uninfected controls
263 and at 1, 7, 14 and 21 days after *F. hepatica* infection (Donnelly et al., 2005), while in
264 sheep, a marked decrease in iNOS gene expression was found in PBMC at 7 dpi (Fu et
265 al., 2016), which contrasts with the low level of variation in iNOS expression by
266 immunocytochemistry in both trials of the present study. This difference suggests that
267 iNOS gene and protein expression may differ, with the protein probably remaining
268 active for a longer time than the gene.
269 The ratio iNOS/CD206 suggest M2 polarisation of peritoneal macrophages from 1 dpi,
270 a finding that is consistent with previous studies in mice (Donnelly et al., 2005), and in

271 PBMC of sheep (Fu et al., 2016) and cattle (Flynn et al., 2007, Garza-Cuartero et al.,
272 2016), that report M2 activation of macrophages induced by *F. hepatica* infection.
273 Further studies should focus on the mechanisms used by the *F. hepatica* parasite to
274 modulate the host response in ruminants, particularly during early stages of infection
275 when the parasite is more vulnerable to the host response. The knowledge of such
276 mechanisms may be used to a more rationale design of new vaccine candidates for
277 blocking immunomodulation and increasing vaccine efficacy.

278

279 **Acknowledgments**

280 Work supported by EU Project (H2020-SFS-2014-2-635408- PARAGONE and the
281 Spanish Ministry of Science grant AGL2015-67023-C2-1-R.

282

283 **References**

284 Adams, P.N., Aldridge, A., Vukman, K.V., Donnelly, S., O'Neill, S.M., 2014. *Fasciola*
285 *hepatica* tegumental antigens indirectly induce an M2 macrophage-like phenotype in
286 vivo. Parasite Immunol. 36, 531-9.
287 Ampem, G., Azegrouz, H., Bacsadi, Á., Balogh, L., Schmidt, S., Thuróczy, J., Röszer,
288 T., 2016. Adipose tissue macrophages in non-rodent mammals: a comparative study.
289 Cell Tissue Res. 363, 461-478.
290 Devitt, A., Moffatt, O.D., Raykundalia, C., Capra, J.D., Simmons, D.L., Gregory, C.D.,
291 1998. Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature
292 392, 505-509.
293 Donnelly, S, O'Neill, S.M., Sekiya, M., Mulcahy, G., Dalton, J.P., 2005. Thioredoxin
294 peroxidase secreted by *Fasciola hepatica* induces the alternative activation of
295 macrophages. Infect. Immun. 73, 166-173.

296 Escamilla, A., Pérez-Caballero, R., Zafra, R., Bautista, M.J., Pacheco, I.L., Ruiz, M.T.,
297 Martínez-Cruz, M.S., Martínez-Moreno, A., Molina-Hernández, V., Pérez, J., 2017.
298 Apoptosis of peritoneal leucocytes during early stages of *Fasciola hepatica* infections in
299 sheep. Vet. Parasitol. 238, 49-53.
300 Fairweather, I., 2011. Reducing the future threat from (liver) fluke: realistic prospect or
301 quixotic fantasy?. Vet Parasitol. 180, 133-143.
302 Figueroa-Santiago, O., Espino, A.M., 2014. *Fasciola hep*atica fatty acid binding protein
303 induces the alternative activation of human macrophages. Infect. Immun. 82, 5005-
304 5012.
305 Flynn, R.J., Irwin, J.A., Olivier, M., Sekiya, M., Dalton, J.P., Mulcahy, G., 2007.
306 Alternative activation of ruminant macrophages by *Fasciola hepatica*. Vet. Immunol.
307 Immunopathol. 120, 31-40.
308 Fu, Y., Chryssafidis, A.L., Browne, J.A., O'Sullivan, J., McGettigan, P.A., Mulcahy,
309 G., 2016. Transcriptomic study on ovine immune responses to *Fasciola hepatica*
310 infection. PLOS Negl. Trop. Dis. 10, e0005015.
311 Garza-Cuartero, L., O'Sullivan, J., Blanco, A., McNair, J., Welsh, M., Flynn, R.J.,
312 Williams, D., Diggle, P., Cassidy, J. Mucalhy, G., 2016. *Fasciola hepatica* infection
313 reduces *Mycobacterium bovis* burden and mycobacterial uptake and suppresses the pro-
314 inflammatory response. Parasite Immunol. 38, 387-402.
315 González, L.C., Esteban, J.G., Bargues, M.D., Valero, M.A., Ortiz, P., Náquira, C.,
316 Mas-Coma, S., 2011. Hyperendemic human fascioliasis in Andean valleys: An
317 altitudinal transect analysis in children of Cajamarca province, Peru. Acta Trop. 120,
318 119-129.

319 Guasconi, L., Serradell, M.C., Garro, A.P., Iacobelli, L., Masih, D.T. 2011. C-type
320 lectins on macrophages participate in the immunomodulatory response to *Fasciola*
321 *hepatica* products. Immunology. 133, 386-396.
322 Guasconi, L., Serradell, M.C., Masih, D.T. 2012. *Fasciola hepatica* products induce
323 apoptosis of peritoneal macrophages. Vet. Immunol. Immunopathol. 148, 359-363.
324 Kreider, T., Anthony, R.M., Urban, J.F. Jr., Gause, W.C., 2007. Alternative activated
325 macrophages in helminth infections. Curr. Opin. Immunol. 19, 448-453.
326 Molina-Hernández, V., Mulcahy, G., Pérez, J., Martínez-Moreno, A., Donnelly, S.,
327 O'Neill, S., Dalton, J.P., Cwiklinski, K., 2015. *Fasciola hepatica* vaccine: we may not
328 be there yet but we're on the right road. Vet. Parasitol. 208, 101-111.
329 O'Neill, S.M., Brady, M.T., Callanan, J.J., Mulcahy, G., Joyce, P., Mills, K.H., Dalton,
330 J.P., 2000. *Fasciola hepatica* infection downregulates Th1 responses in mice. Parasite
331 Immunol. 22, 147-155.
332 Pinczowski, P., Sanjosé, L., Gimeno, M., Crespo, H., Glaria, I., Amorena, B., de
333 Andrés, D., Pérez, M., Reina, R., Luján, L. 2017. Small ruminant lentiviruses in sheep:
334 pathology and tropism of 2 strains using the bone marrow route. Vet Pathol. 54, 413-
335 424.
336 Ruiz-Campillo, M.T., Molina-Hernandez, V., Escamilla, A., Stevenson, M., Perez, J.,
337 Martinez-Moreno, A., Donnelly, S., Dalton, J.P., Cwiklinski, K., 2017. Immune
338 signatures of pathogenesis in the peritoneal compartment during early infection of sheep
339 with *Fasciola hepatica*. Sci Rep. 7, 2782.
340 Toet, H., Piedrafita, D.M., Spithill, T.W., 2014. Liver fluke vaccine in ruminants:
341 strategies, progress and future opportunities. Int. J. Parasitol. 44, 915-927.

342 Tundup S., Srivastava L., Nagy T., Harn D., 2014. CD14 influences host immune
343 responses and alternative activation of macrophages during *Schistosoma mansoni*
344 infection. Infect. Immun. 82, 3240-3251.
345 Valheim M., Sigurdardóttir O.G., Storset A.K. Aune L.G., Press C.M., 2004.
346 Characterization of macrophages and occurrence of T cells in intestinal lesions of
347 subclinical paratuberculosis in goats. J. Comp. Pathol. 131, 221-232.
348 Van Milligen, F.J., Cornelissen, J.B., Bokhout B.A., 1999. Protection against *Fasciola*
349 *hepatica* in the intestine is highly correlated with eosinophil and immunoglobin G1
350 responses against newly excysted juveniles. Parasite Immunol. 21, 243-251.
351 Wang, N., Liang, H., Zen, K., 2014. Molecular mechanisms that influence the
352 macrophage m1-m2 polarization balance. Front. Immunol. 5, 614.
353 Wood, C.E., Chen, G.F., Keller-Wood, M., 2005. Expression of nitric oxide synthase
354 isoforms is reduced in late-gestation ovine fetal brainstem. Am J Physiol Regul Integr
355 Comp Physiol. 289, R613-R619.
356 Zafra, R., Pérez-Écija, R.A., Buffoni, L., Moreno, P., Bautista, M.J., Martínez-Moreno,
357 A., Mulcahy, G., Dalton, J.P., Pérez, J., 2013a. Early and late peritoneal and hepatic
358 changes in goats immunized with recombinant cathepsin L1 and Infected with *Fasciola*
359 *hepatica*. J. Comp. Path. 148, 373-384.
360
361

362 **Figure legends**

363 **Fig. 1**. Peritoneal smear stained with anti-iNOS polyclonal antibody showing mild
364 cytoplasmic immunolabelling (brown colour) in macrophages (arrows) in the uninfected
365 control group (A) and at 3 day post-infection (dpi) (B), 9 dpi (C) and 18 dpi (D). ABC
366 method-haematoxylin counterstain. X400.

367 **Fig. 2.** Peritoneal smear stained with anti-CD206 polyclonal antibody showing mild
368 cytoplasmic immunolabelling (brown colour) in macrophages (arrows) from uninfected
369 control (A) and very severe immunolabelling in macrophages at 1 day post-infection
370 (dpi) (B), 9 dpi (C), and 18 dpi (D). ABC method-haematoxylin counterstain. X400.

371

372

373 **Table 1. Absolute peritoneal leucocyte counts expressed in 10^6 cells/ml**
374 **(mean±SEM)**.

UC	1 dpi	3 dpi	9 dpi	18 dpi
4.0±0.8	3.3±1.6	7.4±1.4	74.2±20.1*	497.9±122*

375 UC: uninfected control group. dpi: days post-infection.
376 *Significant difference (*P*<0.05) with respect to the UC group.
377
378
379

380 **Table 2**. Differential cell count expressed as percentages of lymphocytes, macrophages

381 and eosinophils in the uninfected control group and at 1, 3, 9 and 18 days post-infection.

382

	UC	**1 dpi**	**3 dpi**	**9 dpi**	**18 dpi**
Lymphocytes	39.4±10.6	35.6±12.1	46.4±20.4	63.6±16.0*	15.5±7.8*
Macrophages	56.0±11.1	64.1±11.7	50.9±22.6	18.0±6.3*	11.5±3.9*
Eosinophils	4.4±4.2	0.18±0.3	2.3±2.5	18.1±10.6*	72.9±10.9*

383 *Significant difference ($P<0.05$) with respect to the UC group.

384 **Table 3.** Percentage intensity of immunoreactivity (1-4) of peritoneal macrophages
385 expressed as mean ±SD for the expression of CD14, iNOS, CD206, and the
386 iNOS/CD206 ratio in the uninfected control group (UC) and in the different stages of
387 infection.

	UC	1 dpi	3 dpi	9 dpi	18 dpi
CD14	51.2±11.1(2)	63.2±19(2)	78.1*±13.1(4)	74.6*±16.9(4)	69.1*±12.9(4)
iNOS	37.3±27.7(1)	64.4±4.1(1)	62.9±17.5(1)	74.4*±9.4(2)	46±21.2(1)
CD206	20.9±4.4(1)	81.5*±8.6(4)	83.9*±15.4(4)	87*±5.7(4)	90.9*±8.02(4)
iNOS/CD206	1.8	0.8	0.8	0.9	0.5

388 § Intensity of immunoreactivity (1: mild, 2: moderate, 3: severe, 4: very severe).
389 *Significant differences with respect to the UC group.
390

Figure 1A
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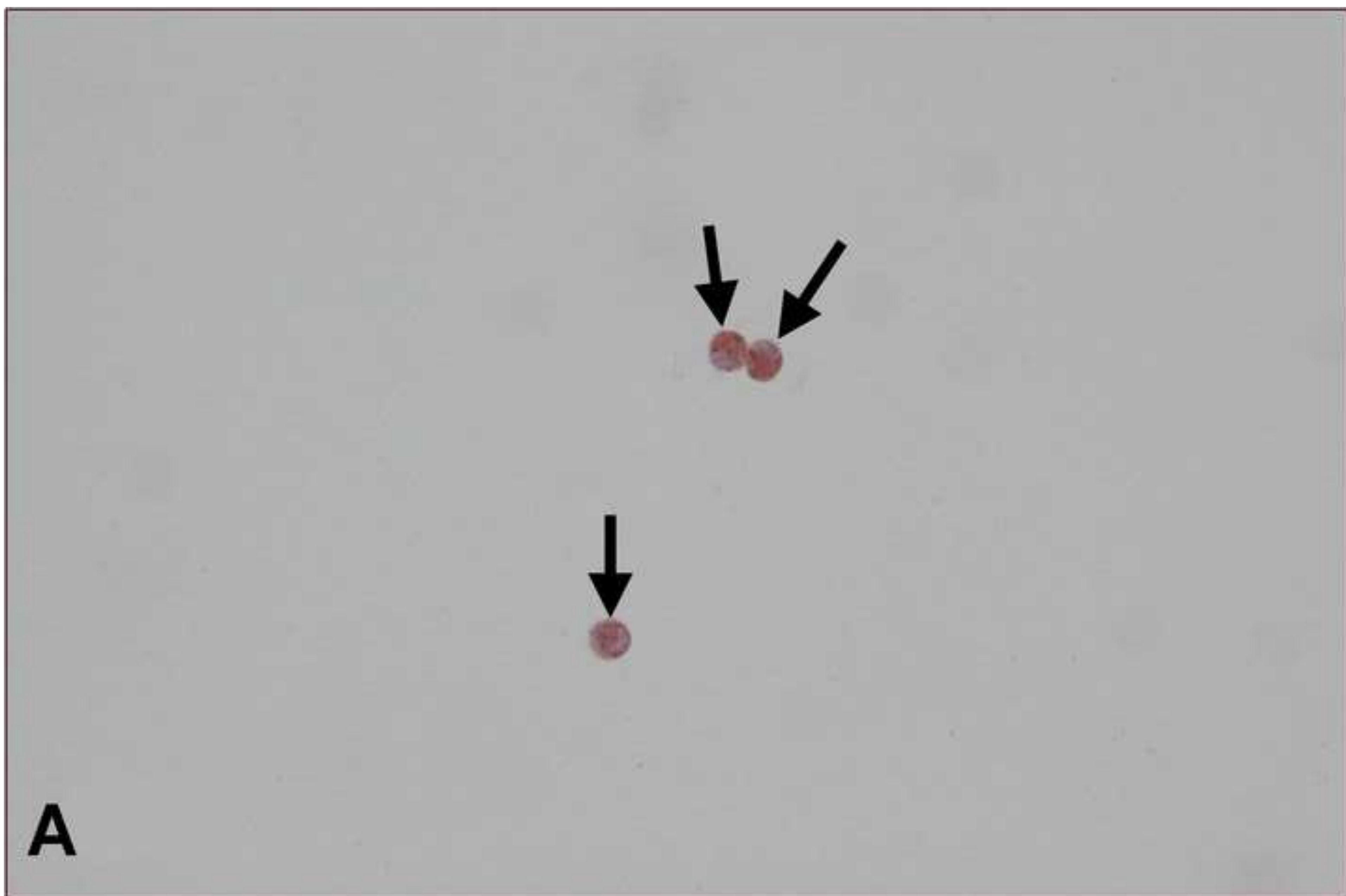

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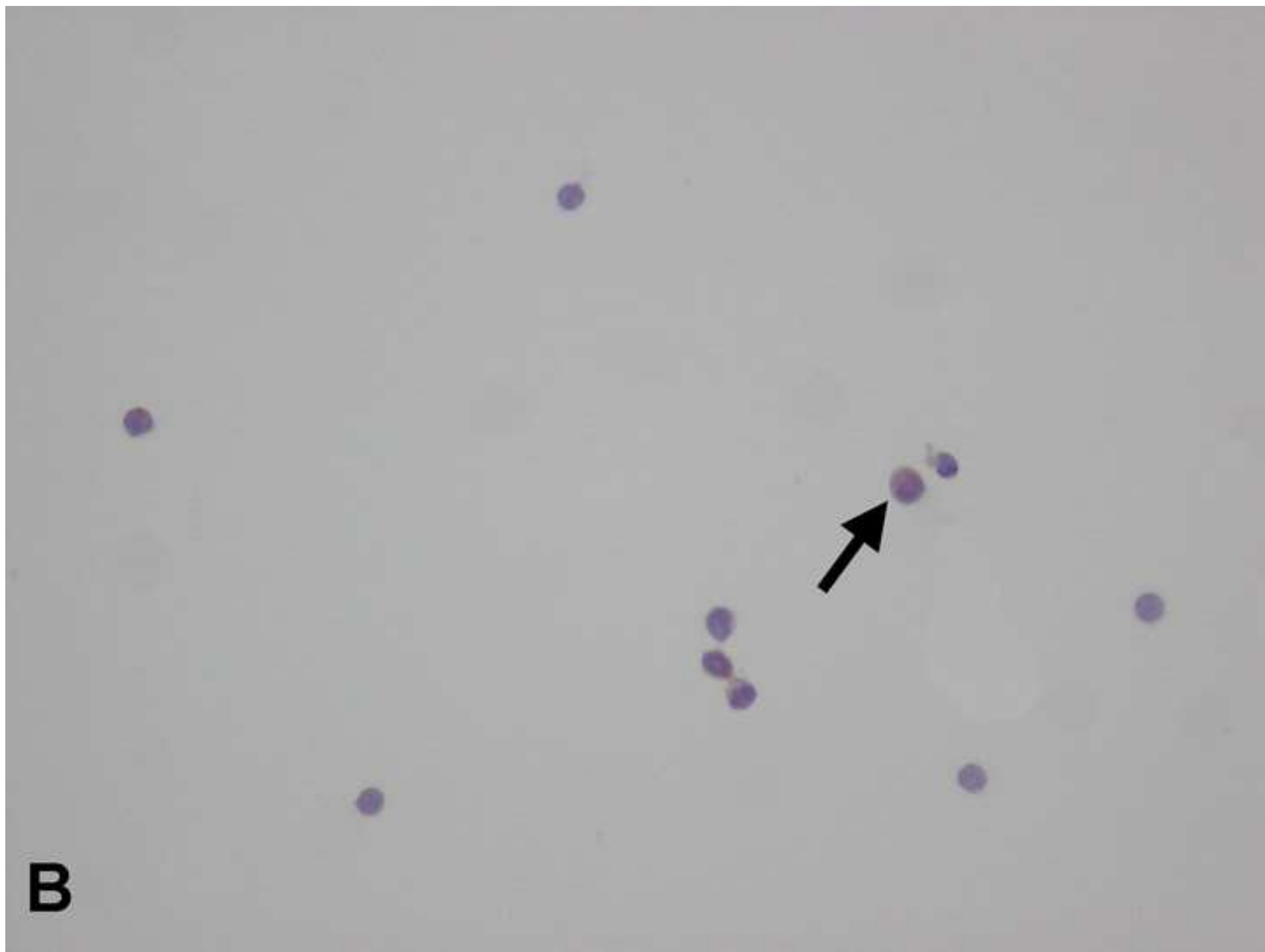

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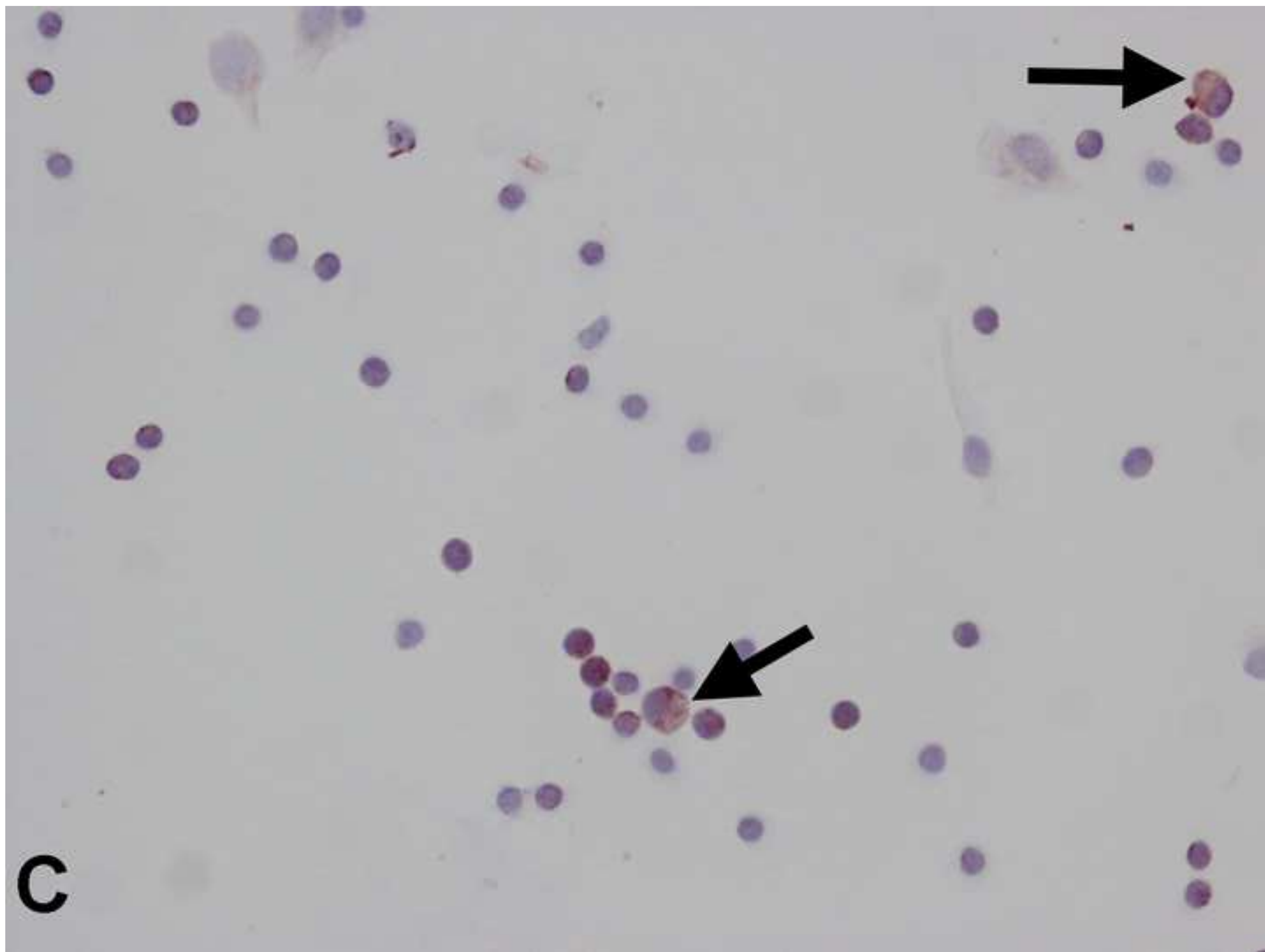

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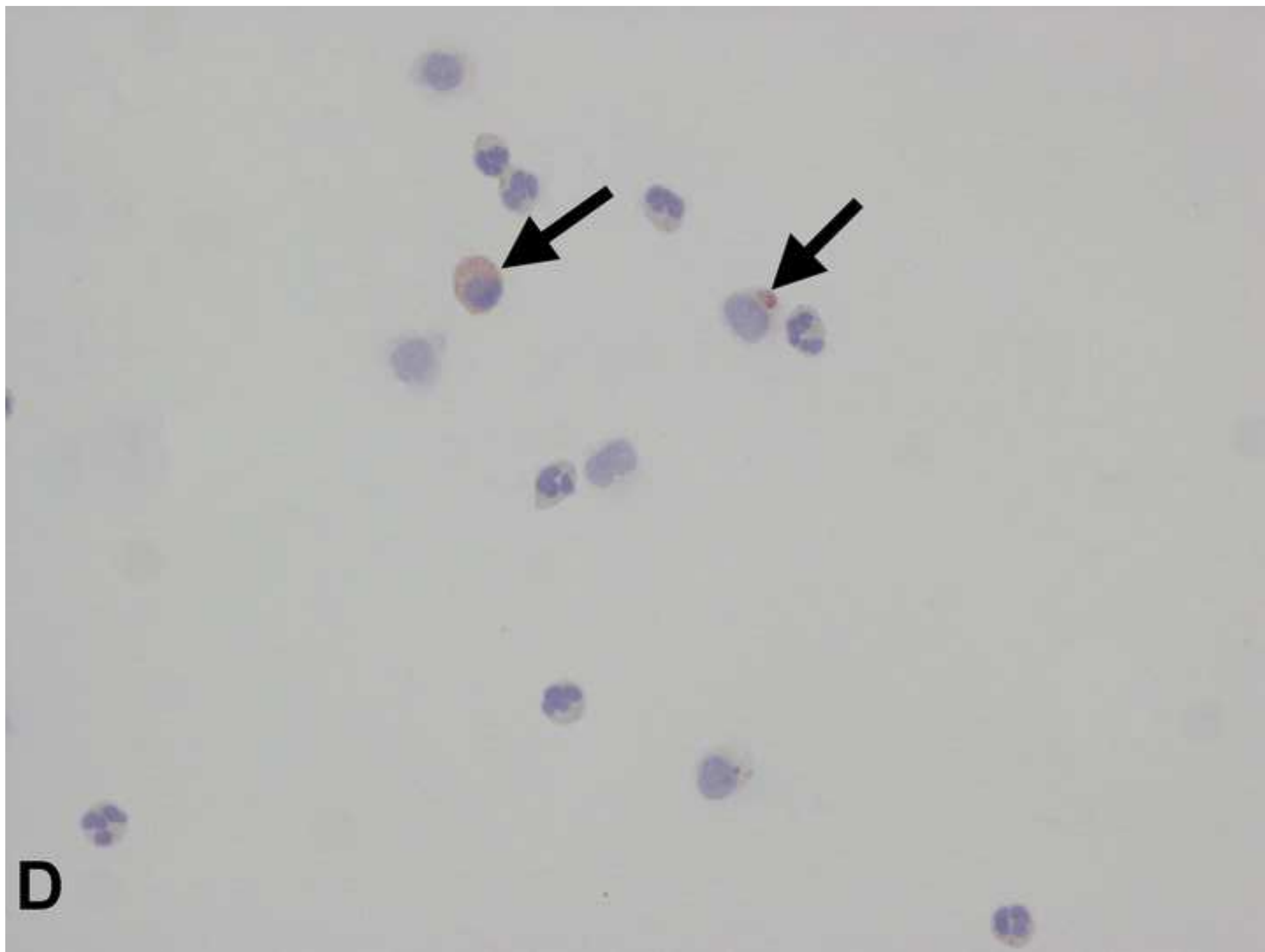

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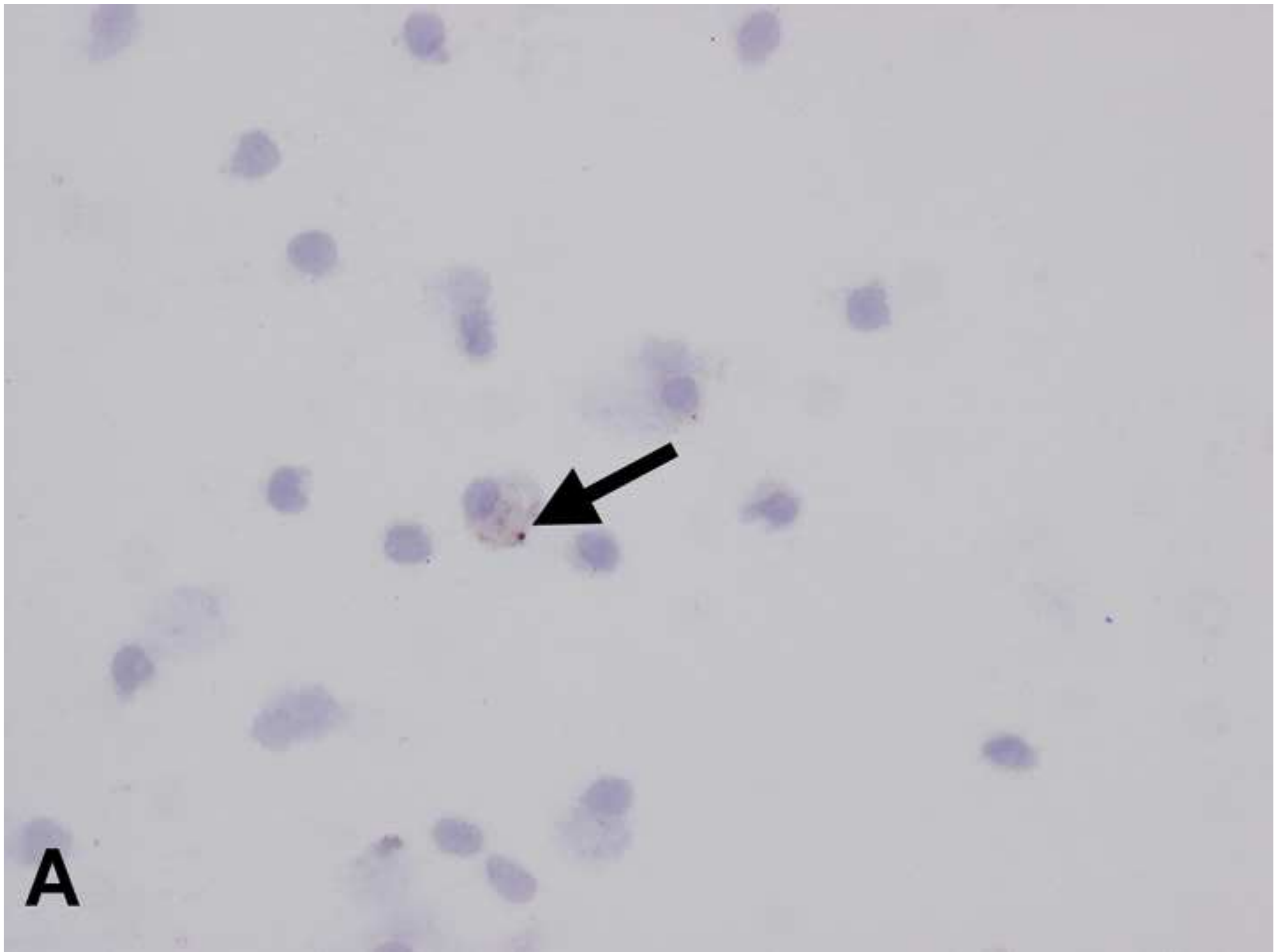

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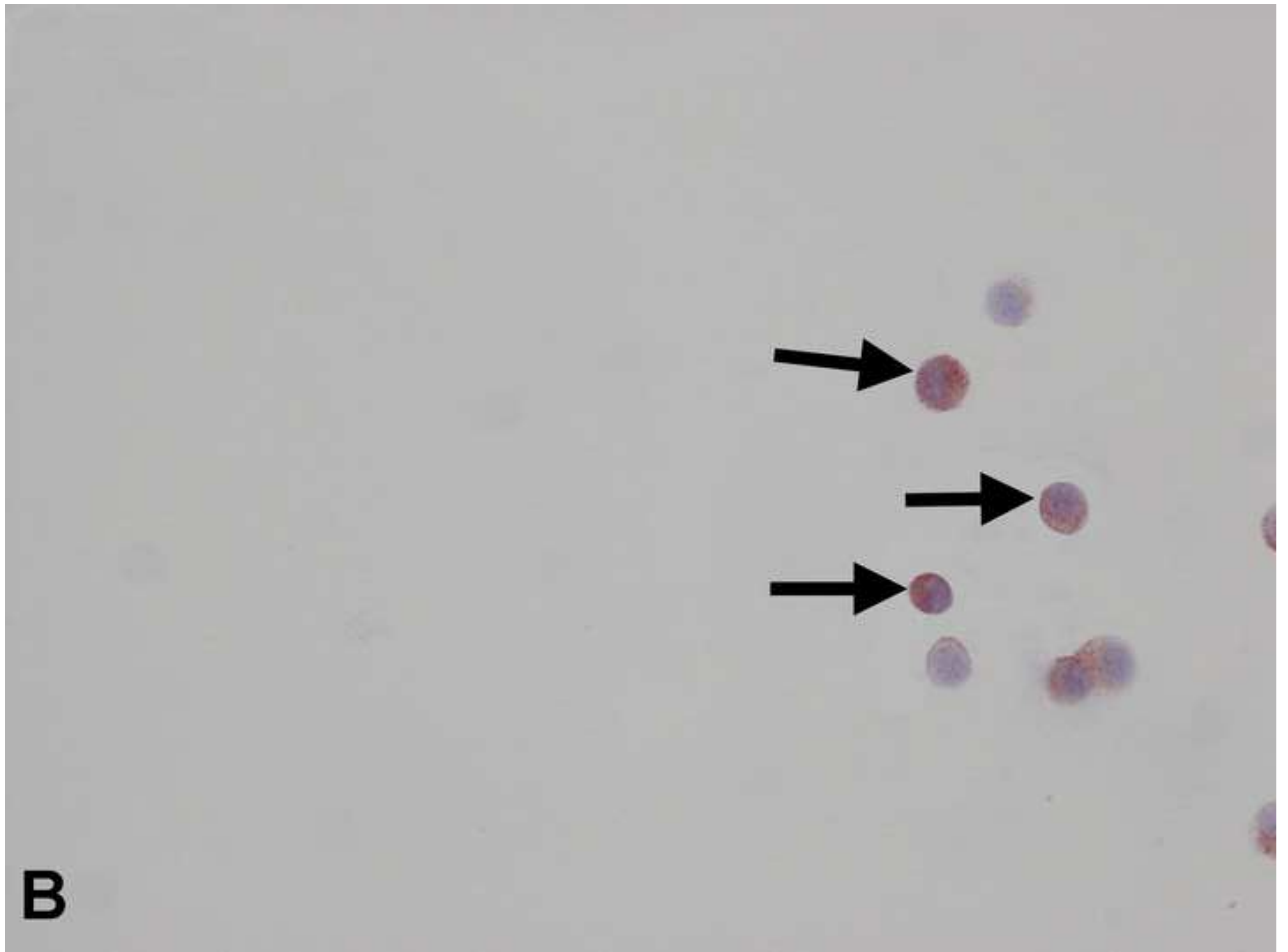

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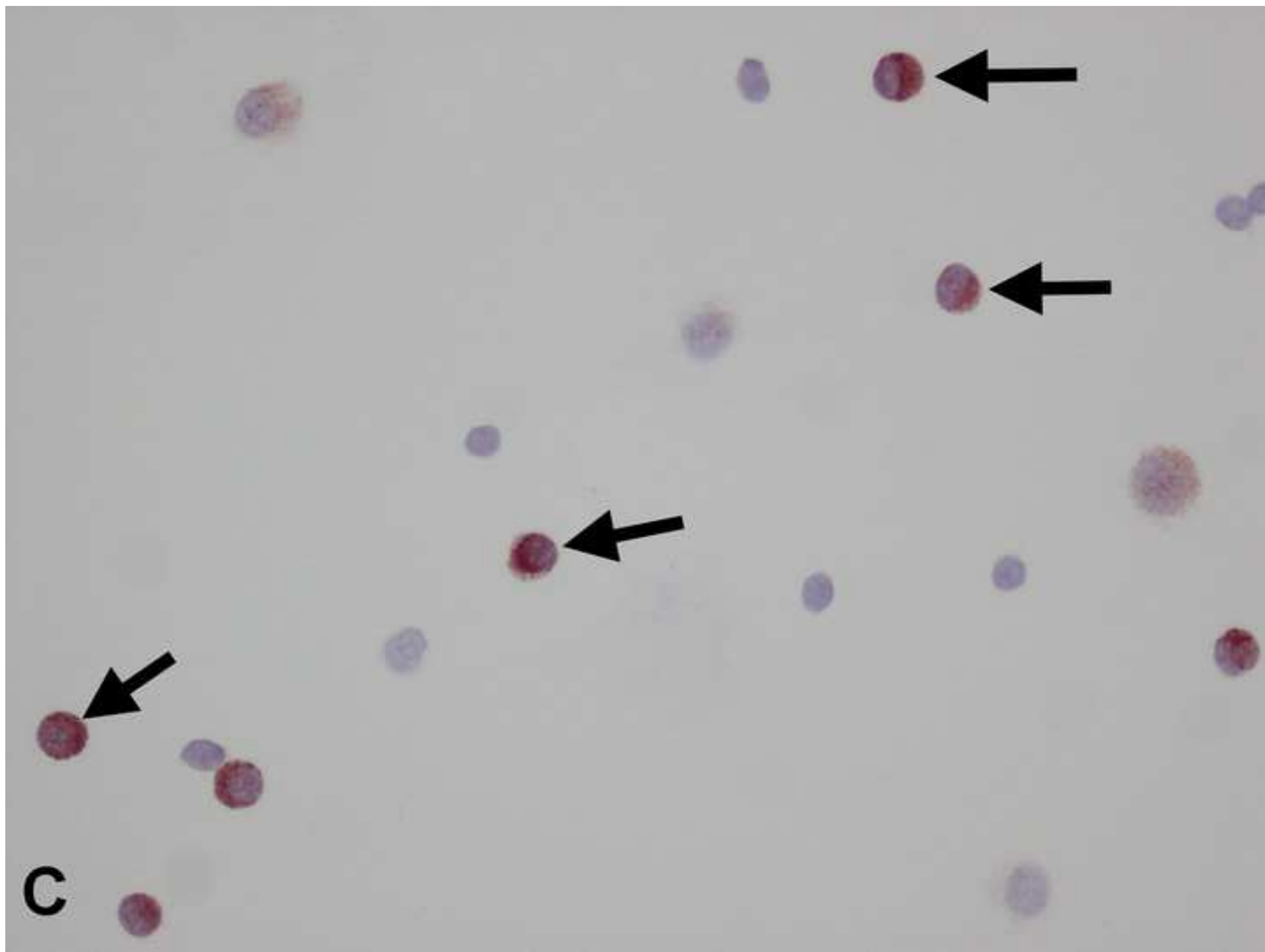

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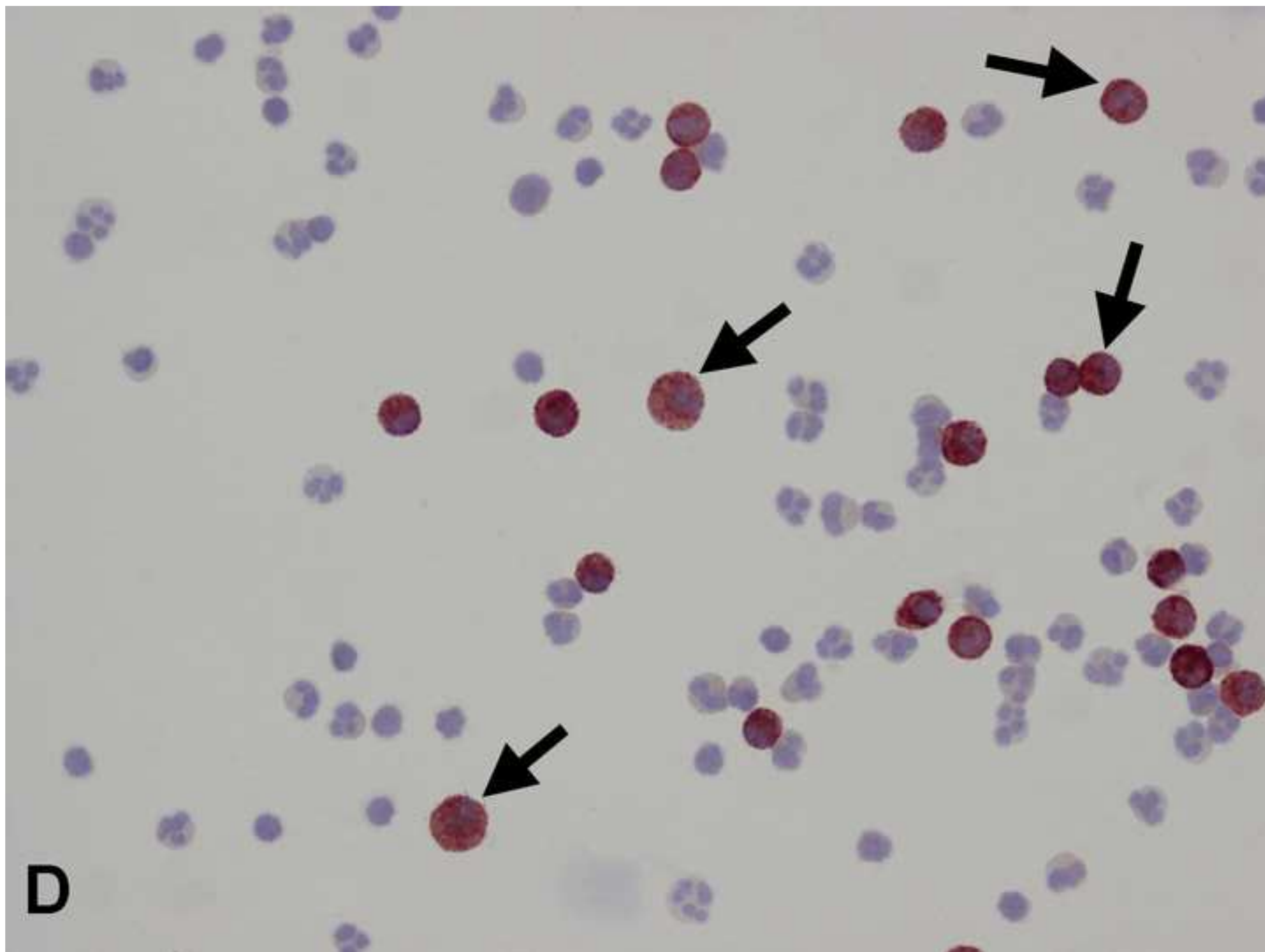

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1 **Study of Peritoneal Macrophage Immunophenotype in Sheep Experimentally**
2 **Infected with *Fasciola hepatica***

3

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22 **Abstract**

23 During *Fasciola hepatica* infection, the parasite has the capability to modulate the host
24 immune response towards a non-protector Th2 type instead of Th1. This type of
25 immune response is closely related to the alternative activation of macrophages (M2
26 profile) as has been shown *in vivo* in murine models. In this study, an experiment was
27 carried out in order to evaluate the expression of CD68, CD14, CD206 and iNOS in
28 cells present in the peritoneal fluid of sheep during early stages of infection with *F.*
29 *hepatica* (1, 3, 9 and 18 days post-infection, dpi) by immunocytochemistry. To the
30 authors' knowledge, this is the first report that studies the *in vivo* immunophenotype of
31 macrophages from the peritoneal fluid of sheep infected with *F. hepatica*. Throughout
32 the experiments the absolute number of leucocytes progressively increased, reaching its
33 highest value at 18 dpi, mainly due to the increase of eosinophils. This
34 immunocytochemical study had two purposes: 1) CD68 expression was assessed with
35 Hansel counterstaining, to optimally identify peritoneal macrophages, eosinophils and
36 lymphocytes; 2) expression of CD14, CD206 and iNOS was evaluated to identify
37 alternative or classical pathways of macrophage activation. The results showed a
38 significant increase in CD14 from day 3 dpi compared with the non-infected group.
39 CD206 expression at all time-points showed a significant and dramatic increase in
40 comparison with the uninfected group. On the other hand, iNOS expression showed
41 little variation, and was significantly decreased at 18 dpi in comparison with the
42 uninfected group. These results suggest that *F. hepatica* induces an alternative
43 activation of peritoneal macrophages of sheep from the first day post-infection, which
44 may facilitate parasite survival. This is the first report describing M2 activation of
45 peritoneal macrophages in ruminants infected with *F. hepatica*.

46 **Keywords:** *Fasciola hepatica*, macrophages, peritoneal fluid, Sheep, immune response

47 **1.- Introduction**

48 Fasciolosis caused by the liver fluke *Fasciola hepatica* infects millions of ruminants
49 worldwide and is recognised by the World Health Organisation (WHO) as an important
50 zoonosis, which is now emerging or re-emerging in many countries (Gonzalez et al.,
51 2011). The costs incurred due to losses in production and treatment with anthelmintics,
52 as well as the resistance that is now widespread, confirm the urgent need for alternative
53 control methods (Fairweather, 2011). During the last two decades, major advances have
54 been made in identifying potential vaccine molecules (Toet et al., 2014; Molina-
55 Hernández et al., 2015; Beesley et al., 2017). Nevertheless, no vaccine candidate has yet
56 reached a commercial or pre-commercial stage. The immune suppression/modulation by
57 *F. hepatica* is one major obstacle to develop a protective vaccine (Toet et al., 2014;
58 Molina-Hernández et al., 2015).

59 Macrophage classical (M1) and alternative (M2) activation phenotypes describe the
60 opposing activities of killing or repairing, and such polarized responses stimulate Th1-
61 or Th2-like responses, respectively. M1 activated macrophages are characterized by
62 high antigen presentation, high production of pro-inflammatory cytokines and nitric
63 oxide. In contrast, M2-type responses are the "resting" phenotype with low production
64 of pro-inflammatory cytokines and high production of anti-inflammatory cytokines such
65 as IL10 (Martínez et al., 2009; Wang et al., 2014). Since *F. hepatica* larvae penetrate
66 the intestinal wall of the host and migrate to the liver via the peritoneum, study of the
67 type macrophage activation at this stage plays a critical role in understanding the
68 immune response to parasitic infection and thus for designing an effective vaccine
69 (Molina-Hernández et al., 2015). It has been reported that *F. hepatica* excretion-
70 secretion products and tegumental coat proteins produce a M2 macrophage phenotype,
71 responsible for host tissue repair, tissue fibrosis and modulation of adaptive immunity,

72 which suppresses a Th1-driven inflammatory pathology in *F. hepatica* infection (Adams
73 et al., 2014; Figueroa-Santiago et al., 2014; Flynn et al., 2007).
74 In early stages of *F. hepatica* infection, the recruitment and activation of M2
75 macrophages in the peritoneal cavity has been reported in rats at 24h post-infection
76 (Donnelly et al., 2005) and in mice at 48 h post-infection (Guasconi et al. 2011).
77 Moreover, excretory-secretory products from *F. hepatica* induced M2 activation of
78 peritoneal macrophages in mice (Guasconi et al. 2011). In sheep marked M2 activation
79 has been described by gene expression was in PBMC at 7 dpi (Fu et al., 2016).
80 Recently, proteomic analysis, humoral response and cytokine expression have been
81 studied in sheep experimentally infected with *F. hepatica* at 18 dpi (Ruiz-Campillo et
82 al., 2017), but phenotype of peritoneal macrophages have not been investigated in *F.*
83 *hepatica* infected ruminants. On the other hand, it has been reported that *F. hepatica*
84 induces apoptosis of peritoneal macrophages in rats (Guasconi et al., 2012) and sheep
85 (Escamilla et al., 2017), but the impact of apoptosis in total peritoneal leucocyte count
86 has not been reported in *F. hepatica* infected ruminants. The aim of the present work
87 was to evaluate the dynamic of total and differential leucocyte count and the
88 macrophage polarisation in peritoneal fluid obtained from sheep experimentally infected
89 with *F. hepatica* in the early stages of infection.
90

91 **2.-Materials and Methods**

92 *2.1.-Experimental design*

93 A total of twenty-five eight-month old male Merino sheep obtained from a liver fluke-
94 free farm were used to study the early stages of infection. Animals were purchased aged
95 one month and housed indoors in the experimental farm of the University of Córdoba
96 until they reached the appropriate age for pathogen challenge. All animals were tested

97 monthly for parasite eggs by faecal sedimentation with negative results in all cases.
98 Moreover, prior to the challenge, all animals were tested for serum IgG specific for *F.*
99 *hepatica* cathepsin L1 (FhCL1) by ELISA, with negative results in all cases. The trial
100 consisted of five groups composed of five sheep (n=5): an uninfected control group and
101 four infected groups. Sheep were orally infected with one dose of 150 metacercariae of
102 the South Gloucester strain of *F. hepatica* (Ridgeway Research Ltd, UK) and
103 euthanised at 1, 3, 9 and 18 days post-infection (dpi). The animals were euthanised by
104 intravenous injection of T61® (Intervet, Spain). The experiments were approved by the
105 Bioethics Committee of the University of Cordoba (code No. 1118) and conducted in
106 accordance with European (2010/63/UE) and Spanish (RD 1201/2005) directives on
107 animal experimentation.

108 *2.2.-Recovery of peritoneal fluid*

109 Peritoneal lavages were conducted immediately after the animals were euthanised. The
110 ventral aspect of the abdomen was shaved and disinfected with 10%
111 polyvinylpyrrolidone iodine (AGB, Madrid, Spain). A 2 cm incision was made on the
112 skin over the white line and subcutaneous tissue was dissected, the white line and
113 peritoneum were sectioned with blunt scissors to avoid bleeding. A 40 cm cannula was
114 inserted into the abdominal cavity and connected to a syringe to inject 60 ml sterile
115 DPBS (Dulbecco's Phosphate-Buffered Saline, Sigma-Aldrich, Darmstadt, Germany),
116 and 9500 I.U. of heparin (Rovi, Madrid, Spain), previously warmed to 37 ºC. After
117 softly massaging the abdominal cavity for 1 min, 40 ml of peritoneal fluid was
118 recovered using the syringe connected to the cannula. In cases where residual
119 erythrocytes were present, it was necessary to use an erythrolysis buffer prior to the
120 processing of the cells.

121 *2.3.-Cell populations*

122 The recovered peritoneal lavage fluid was collected and cooled on ice until cell
123 processing. The total number of viable peritoneal cells was determined after Trypan
124 Blue staining by counting in a Neubauer haemocytometer. Smear preparations were
125 manually performed on Vectabond®-treated slides (Vector laboratories, California,
126 USA) by centrifuging the recovered peritoneal fluid at 1500 rpm for 10 min. After air
127 draining, these smears were fixed in acetone for 5 min and stored at -80°C for further
128 immunocytochemical studies. For differential cell counting immunocytochemistry using
129 anti-human CD68 monoclonal antibody (Dako, Glostrup, Denmark) in combination
130 with Hansel staining was used as a novel and more accurate cell counting method. A
131 total of 200 cells per smear were counted in randomly selected fields of 400x
132 magnifications using the software Image Pro-plus 6.0 (Media Cybernetics, Silver
133 Spring, USA), and the percentage of lymphocytes (small basophilic nucleus and scanty
134 cytoplasm), macrophages (round to oval hyperchromatic nucleus and moderate to large
135 cytoplasm stained brownish with the CD68 antibody) and eosinophils (basophilic
136 bilobed nucleus and eosinophilic cytoplasm) was obtained. Neutrophils (basophilic
137 lobulated nucleus and unstained cytoplasm) were not included in the cell count since
138 they were only very occasionally observed.

139 *2.4.-Immunocytochemistry (ICC)*

140 An immunocytochemical study was used to assess anti-Human CD68 (M0718, Dako,
141 Glostrup, Denmark), anti-Human CD14 (BOV2027, Monoclonal Antibody Center,
142 Washington State University), anti-Human iNOS (PA3–030A, Thermo, Freemont,
143 USA) and anti-Human CD206 (orb4941, Biorbyt, Cambridge, UK) expression in
144 peritoneal fluid macrophages. The dilution used for these primary antibodies was 1:400
145 for CD68; 1:500 for CD14, 1:200 for iNOS; and 1:100 for CD206. The CD14
146 monoclonal antibody cross-reacts with ovine tissues according to the manufacturer. The

147 iNOS (Wood et al. 2005), CD68 antibodies (Pinczowski et al., 2017) and anti-human
148 CD206 (Ampem et al., 2016) have shown cross reactivity with ovine tissues. The
149 avidin-biotin-peroxidase method described elsewhere (Zafra et al., 2013a) was carried
150 out. Briefly, endogenous peroxidase activity was blocked by incubation with 3%
151 hydrogen peroxide (Panreac, Barcelona, Spain) in phosphate buffered saline (PBS).
152 Then, smears were washed once in PBS and incubated with 10% normal goat serum
153 (MP Biomedicals, Ohio, USA) for 30 min at room temperature. After three 5 min rinses
154 in PBS-Tween (PBST), secondary antibodies were applied for 45 min at 37ºC. A
155 biotinylated goat anti-rabbit immunoglobulin serum (Dako) diluted 1:200 was applied
156 to the smears incubated with the primary polyclonal antibodies (pAbs: iNOS and
157 CD206), whereas a biotinylated goat anti-mouse immunoglobulin serum (Dako) diluted
158 1:50 was used for the primary monoclonal antibody (mAb: CD68). After two 5 min
159 rinses in PBST, an avidin–biotin-peroxidase complex (Vector, Burlinghame, USA)
160 diluted 1:50 was applied for an hour as a third reagent. Slides were then washed three
161 times in PBST and incubated with Novared® substrate kit peroxidase (Vector) diluted
162 following the manufacturer's instructions, rinsed in water, lightly counterstained with
163 Mayer's haematoxylin and mounted with Eukitt® (Freiburg, Germany). For CD68
164 antibody, following the haematoxylin stain, eosin was applied to the slides for 1 minute
165 with Hansel stain for the differential cell count. Specific primary antibodies were
166 substituted with PBS or non-immune isotype-matched sera as negative controls. Sheep
167 and human hepatic lymph node lymph node tissue sections were used as positive
168 controls.

169 *2.5.-Cell count*

170 Immunoreactive cells were counted in randomly selected fields of 400x magnifications
171 using the Image Pro-plus 6.0 software package. Macros were calibrated for staining

172 intensity and cell size to include all immunostained cells. A total of 200 cells were
173 counted per slide and the percentage of positive and negative cells was obtained.
174 Photomicrographs were taken using an Olympus BX51 photomicroscope with a 400x
175 magnification field. Results were expressed as mean ± SD per animal and per group.
176 The intensity of immunostaining was evaluated semi-quantitatively according to the
177 following score: 1, mild; 2, moderate; 3, severe; 4, very severe.

178 *2.6.-Statistical analysis*

179 Statistical analysis was carried out using the Graphpad Prism 7.0 software package
180 (Graphpad Software, Inc., San Diego, California). The Kolmogorov–Smirnov test was
181 applied to evaluate whether distributions were parametric. Comparisons between groups
182 were made using the Mann–Whitney test for non-parametric distributions. Correlation
183 studies were carried out using the Spearman correlation test for non-parametric
184 distributions. $P<0.05$ was considered significant.

185

186 **3. Results**

187 *3.1. Absolute peritoneal cell count*

188 The results of the absolute peritoneal fluid cell counts are shown in Table 1. The
189 number of cells increased significantly ($P<0.05$) at 9 and 18 dpi compared with the
190 uninfected control group. At 1 and 3 dpi no significant differences respect to the
191 uninfected control group were obtained.

192 *3.2. Differential peritoneal cell count*

193 Since CD68 has been widely used as a general macrophage marker (Valheim et al.,
194 2004) a CD68 mAb in combination with Hansel stain was used as a novel and more
195 accurate leucocyte identification method. The differential cell count results (expressed
196 as percentages) from peritoneal fluid smear examinations are shown in Table 2.

197 Neutrophils and epithelial cells were only occasionally observed and were not included
198 in the cell count.
199 At 9 and particularly at 18 dpi, there was a very marked increase in the number of
200 eosinophils, which was responsible for the relative decrease in the percentages of
201 macrophages (9 and 18 dpi) and lymphocytes (18 dpi) in comparison with the
202 uninfected control group Table 2. On the other hand, the percentage of lymphocytes
203 showed a significant increase at 9 dpi with respect to the uninfected control group. This
204 may reflect a stimulation of the recruitment of peritoneal lymphocytes at this time-point
205 when larvae are penetrating or migrating into the liver surface as revealed by the
206 significant increase in the total number of peritoneal leucocytes at 9 and 18 dpi.
207 *3.3. Immunocytochemical study*
208 The anti-CD14 mAb yielded a cytoplasmic immunostaining in peritoneal leucocytes
209 with large cytoplasm and round to ovoid nucleus, this was the same morphology than
210 peritoneal leucocytes expressing CD68. The results of the present study revealed a
211 significant increase of the percentage of CD14+ cells at 3, 9 and 18 dpi with respect to
212 the uninfected control group (Table 3).
213 Anti-iNOS and anti-CD206 antibodies were used as biomarkers of classical (M1) and
214 alternative (M2) macrophage activation, respectively. The anti-iNOS pAb also showed
215 granular cytoplasmic immunostaining in peritoneal macrophages (Fig. 1) and in some
216 eosinophils, but only the macrophages were counted. The percentage of peritoneal
217 macrophages expressing iNOS varied little during the course of the infection in (Table
218 3). The intensity of immunolabelling with anti-iNOS was mild in the uninfected control
219 group and at all studied time-points (Table 3).
220 The Anti-human CD206 antibody has been described as a good biomarker of alternative
221 activation of macrophages in sheep (Ampem et al., 2016). In our study, the CD206 mAb

222 yielded weak cytoplasmic immunostaining in peritoneal macrophages from the
223 uninfected control group, whereas the intensity of the immunolabelling was very strong
224 at 1, 3, 9 and 18 dpi (Fig. 2, Table 3). The percentage of peritoneal macrophages
225 expressing CD206 showed a dramatic and significant increase (P<0.05) from 1 dpi
226 onwards, compared with the uninfected groups (Table 3).
227 The iNOS/CD206 ratio decreased approximately one-fold at 1, 3 and 9 dpi and three-
228 fold at 18 dpi (Table 3).

229 **4. Discussion**

230 It has been reported that *F. hepatica* induces apoptosis of peritoneal leucocytes in sheep
231 (Escamilla et al., 2017) and rat (Guasconi et al., 2012), thus it will be of interest to
232 investigate if *F. hepatica* induces reduction of peritoneal leucocyte during early stages
233 of infection. Differential leucocyte count has been evaluated in *F. hepatica* infected
234 goats (Zafra et al., 2013a) and sheep (Escamilla et al., 2017), but total peritoneal count
235 has not been investigated in *F. hepatica* infected ruminants during early stages of
236 infection. The results of the present study revealed non-significant changes of total
237 peritoneal leucocyte count at 1 and 3 dpi respect to the uninfected control group,
238 suggesting that apoptosis of peritoneal leucocytes do not induce reduction of total
239 peritoneal count at early stages, probably due to leucocyte recruitment. The marked and
240 significant increase of total peritoneal leucocytes found at 9 and 18 dpi is mainly due to
241 the increase of eosinophils, a finding consistent with previous reports in goats (Zafra et
242 al., 2013a) and sheep (Escamilla et al., 2017).
243 CD14 is a co-receptor for TLR-initiated pro-inflammatory responses in innate immune
244 cells, particularly macrophages. It has been reported that infection by helminths such as
245 *Schistosoma mansoni* in mice (Tundup et al., 2014) and *F. hepatica* in cattle (Garza-
246 Cuartero et al., 2016) induces an increase in CD14 expression in liver macrophages and

247 peripheral blood mononuclear cells (PBMCs), respectively. The present study is the first
248 report analysing the expression of CD14 in peritoneal leucocytes in ruminants infected
249 with *F. hepatica,* and revealed a significant increase at 3, 9 and 18 dpi with respect to
250 the uninfected control group. This is in concordance with the increased CD14
251 expression in PBMC at 7 and 12 weeks after *F. hepatica* infection in cattle was
252 associated with an alternative activation of macrophages (Garza-Cuartero et al., 2016).
253 In addition, it has been previously shown that CD14 expression increases during sepsis
254 and tissue remodelling processes, and the fact that this increase occurs at 3 dpi, when
255 the parasite is penetrating through the gut and reaching the peritoneal cavity, suggests
256 that it could be an indicator of tissue damage and apoptosis (Devitt et al., 1998).
257 During helminth infections, macrophages that undergo changes to express an M2
258 phenotype have been implicated in the regulation of the cytokine environment. This
259 change leads to preferential induction of the Th2 response, which is ineffective in
260 controlling the parasite infection and results in the chronic stage of the disease (O'Neill
261 *et al.*, 2000; Kreider et al., 2007). Since the host response to *F. hepatica* is thought to be
262 more effective during the intestinal, peritoneal or early hepatic migratory stages (Van
263 Milligen et al., 1999), the rapid M2 polarisation of peritoneal macrophages found in the
264 sheep of the present study may be an important mechanism of modulation that may
265 facilitate parasite survival during the early stages of infection.
266 In a murine model, very low iNOS gene expression was detected in uninfected controls
267 and at 1, 7, 14 and 21 days after *F. hepatica* infection (Donnelly et al., 2005), while in
268 sheep, a marked decrease in iNOS gene expression was found in PBMC at 7 dpi (Fu et
269 al., 2016), which contrasts with the low level of variation in iNOS expression by
270 immunocytochemistry in both trials of the present study. This difference suggests that

271 iNOS gene and protein expression may differ, with the protein probably remaining
272 active for a longer time than the gene.
273 The ratio iNOS/CD206 suggest M2 polarisation of peritoneal macrophages from 1 dpi,
274 a finding that is consistent with previous studies in mice (Donnelly et al., 2005), and in
275 PBMC of sheep (Fu et al., 2016) and cattle (Flynn et al., 2007, Garza-Cuartero et al.,
276 2016), that report M2 activation of macrophages induced by *F. hepatica* infection.
277 Further studies should focus on the mechanisms used by the *F. hepatica* parasite to
278 modulate the host response in ruminants, particularly during early stages of infection
279 when the parasite is more vulnerable to the host response. The knowledge of such
280 mechanisms may be used to a more rationale design of new vaccine candidates for
281 blocking immunomodulation and increasing vaccine efficacy.
282

283 **Acknowledgments**

284 Work supported by EU Project (H2020-SFS-2014-2-635408- PARAGONE and the
285 Spanish Ministry of Science grant AGL2015-67023-C2-1-R.
286

287 **References**

288 Adams, P.N., Aldridge, A., Vukman, K.V., Donnelly, S., O'Neill, S.M., 2014. *Fasciola*
289 *hepatica* tegumental antigens indirectly induce an M2 macrophage-like phenotype in
290 vivo. Parasite Immunol. 36, 531-9.
291 Ampem, G., Azegrouz, H., Bacsadi, Á., Balogh, L., Schmidt, S., Thuróczy, J., Röszer,
292 T., 2016. Adipose tissue macrophages in non-rodent mammals: a comparative study.
293 Cell Tissue Res. 363, 461-478.
294 Beesley N.J., Caminade, C., Charlier, J., Flynn, R.J., Hodgkinson, J.E., Martinez-
295 Moreno, A., Martinez-Valladares, M., Perez, J., Rinaldi, L., Williams, D.J.L., 2017.

296 Fasciola and fasciolosis in ruminants in Europe: Identifiying research needs.
297 Transbound Emerg Dis. doi: 10.1111/tbed.12682
298 Devitt, A., Moffatt, O.D., Raykundalia, C., Capra, J.D., Simmons, D.L., Gregory, C.D.,
299 1998. Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature
300 392, 505-509.
301 Donnelly, S, O'Neill, S.M., Sekiya, M., Mulcahy, G., Dalton, J.P., 2005. Thioredoxin
302 peroxidase secreted by *Fasciola hepatica* induces the alternative activation of
303 macrophages. Infect. Immun. 73, 166-173.
304 Escamilla, A., Pérez-Caballero, R., Zafra, R., Bautista, M.J., Pacheco, I.L., Ruiz, M.T.,
305 Martínez-Cruz, M.S., Martínez-Moreno, A., Molina-Hernández, V., Pérez, J., 2017.
306 Apoptosis of peritoneal leucocytes during early stages of *Fasciola hepatica* infections in
307 sheep. Vet. Parasitol. 238, 49-53.
308 Fairweather, I., 2011. Reducing the future threat from (liver) fluke: realistic prospect or
309 quixotic fantasy? Vet Parasitol. 180, 133-143.
310 Figueroa-Santiago, O., Espino, A.M., 2014. *Fasciola hep*atica fatty acid binding protein
311 induces the alternative activation of human macrophages. Infect. Immun. 82, 5005-
312 5012.
313 Flynn, R.J., Irwin, J.A., Olivier, M., Sekiya, M., Dalton, J.P., Mulcahy, G., 2007.
314 Alternative activation of ruminant macrophages by *Fasciola hepatica*. Vet. Immunol.
315 Immunopathol. 120, 31-40.
316 Fu, Y., Chryssafidis, A.L., Browne, J.A., O'Sullivan, J., McGettigan, P.A., Mulcahy,
317 G., 2016. Transcriptomic study on ovine immune responses to *Fasciola hepatica*
318 infection. PLOS Negl. Trop. Dis. 10, e0005015.
319 Garza-Cuartero, L., O'Sullivan, J., Blanco, A., McNair, J., Welsh, M., Flynn, R.J.,
320 Williams, D., Diggle, P., Cassidy, J. Mucalhy, G., 2016. *Fasciola hepatica* infection

321 reduces *Mycobacterium bovis* burden and mycobacterial uptake and suppresses the pro-
322 inflammatory response. Parasite Immunol. 38, 387-402.
323 González, L.C., Esteban, J.G., Bargues, M.D., Valero, M.A., Ortiz, P., Náquira, C.,
324 Mas-Coma, S., 2011. Hyperendemic human fascioliasis in Andean valleys: An
325 altitudinal transect analysis in children of Cajamarca province, Peru. Acta Trop. 120,
326 119-129.
327 Guasconi, L., Serradell, M.C., Garro, A.P., Iacobelli, L., Masih, D.T. 2011. C-type
328 lectins on macrophages participate in the immunomodulatory response to *Fasciola*
329 *hepatica* products. Immunology. 133, 386-396.
330 Guasconi, L., Serradell, M.C., Masih, D.T. 2012. *Fasciola hepatica* products induce
331 apoptosis of peritoneal macrophages. Vet. Immunol. Immunopathol. 148, 359-363.
332 Kreider, T., Anthony, R.M., Urban, J.F. Jr., Gause, W.C., 2007. Alternative activated
333 macrophages in helminth infections. Curr. Opin. Immunol. 19, 448-453.
334 Martinez, F.O., Helming, L., Gordon, S. 2009. Alternative activation of macrophages:
335 an immunologic functional perspective. Annu. Rev. Immunol. 27, 451–483.
336 Molina-Hernández, V., Mulcahy, G., Pérez, J., Martínez-Moreno, A., Donnelly, S.,
337 O'Neill, S., Dalton, J.P., Cwiklinski, K., 2015. *Fasciola hepatica* vaccine: we may not
338 be there yet but we're on the right road. Vet. Parasitol. 208, 101-111.
339 O'Neill, S.M., Brady, M.T., Callanan, J.J., Mulcahy, G., Joyce, P., Mills, K.H., Dalton,
340 J.P., 2000. *Fasciola hepatica* infection downregulates Th1 responses in mice. Parasite
341 Immunol. 22, 147-155.
342 Pinczowski, P., Sanjosé, L., Gimeno, M., Crespo, H., Glaria, I., Amorena, B., de
343 Andrés, D., Pérez, M., Reina, R., Luján, L. 2017. Small ruminant lentiviruses in sheep:
344 pathology and tropism of 2 strains using the bone marrow route. Vet Pathol. 54, 413-
345 424.

346 Ruiz-Campillo, M.T., Molina-Hernandez, V., Escamilla, A., Stevenson, M., Perez, J.,
347 Martinez-Moreno, A., Donnelly, S., Dalton, J.P., Cwiklinski, K., 2017. Immune
348 signatures of pathogenesis in the peritoneal compartment during early infection of sheep
349 with *Fasciola hepatica*. Sci Rep. 7, 2782.
350 Toet, H., Piedrafita, D.M., Spithill, T.W., 2014. Liver fluke vaccine in ruminants:
351 strategies, progress and future opportunities. Int. J. Parasitol. 44, 915-927.
352 Tundup S., Srivastava L., Nagy T., Harn D., 2014. CD14 influences host immune
353 responses and alternative activation of macrophages during *Schistosoma mansoni*
354 infection. Infect. Immun. 82, 3240-3251.
355 Valheim M., Sigurdardóttir O.G., Storset A.K. Aune L.G., Press C.M., 2004.
356 Characterization of macrophages and occurrence of T cells in intestinal lesions of
357 subclinical paratuberculosis in goats. J. Comp. Pathol. 131, 221-232.
358 Van Milligen, F.J., Cornelissen, J.B., Bokhout B.A., 1999. Protection against *Fasciola*
359 *hepatica* in the intestine is highly correlated with eosinophil and immunoglobin G1
360 responses against newly excysted juveniles. Parasite Immunol. 21, 243-251.
361 Wang, N., Liang, H., Zen, K., 2014. Molecular mechanisms that influence the
362 macrophage m1-m2 polarization balance. Front. Immunol. 5, 614.
363 Wood, C.E., Chen, G.F., Keller-Wood, M., 2005. Expression of nitric oxide synthase
364 isoforms is reduced in late-gestation ovine fetal brainstem. Am J Physiol Regul Integr
365 Comp Physiol. 289, R613-R619.
366 Zafra, R., Pérez-Écija, R.A., Buffoni, L., Moreno, P., Bautista, M.J., Martínez-Moreno,
367 A., Mulcahy, G., Dalton, J.P., Pérez, J., 2013a. Early and late peritoneal and hepatic
368 changes in goats immunized with recombinant cathepsin L1 and Infected with *Fasciola*
369 *hepatica*. J. Comp. Path. 148, 373-384.
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371

372 **Figure legends**

373 **Fig. 1**. Peritoneal smear stained with anti-iNOS polyclonal antibody showing mild
374 cytoplasmic immunolabelling (brown colour) in macrophages (arrows) in the uninfected
375 control group (A) and at 3 day post-infection (dpi) (B), 9 dpi (C) and 18 dpi (D). ABC
376 method-haematoxylin counterstain. X400.

377 **Fig. 2.** Peritoneal smear stained with anti-CD206 polyclonal antibody showing mild
378 cytoplasmic immunolabelling (brown colour) in macrophages (arrows) from uninfected
379 control (A) and very severe immunolabelling in macrophages at 1 day post-infection
380 (dpi) (B), 9 dpi (C), and 18 dpi (D). ABC method-haematoxylin counterstain. X400.

381

382

383 **Table 1. Absolute peritoneal leucocyte counts expressed in 10^6 cells/ml**
384 **(mean±SEM)**.

UC	1 dpi	3 dpi	9 dpi	18 dpi
4.0±0.8	3.3±1.6	7.4±1.4	74.2±20.1*	497.9±122*

385 UC: uninfected control group. dpi: days post-infection.

386 *Significant difference (*P*<0.05) with respect to the UC group.

387

388

389

390 **Table 2**. Differential cell count expressed as percentages of lymphocytes, macrophages
391 and eosinophils in the uninfected control group and at 1, 3, 9 and 18 days post-infection.
392

	UC	**1 dpi**	**3 dpi**	**9 dpi**	**18 dpi**
Lymphocytes	39.4±10.6	35.6±12.1	46.4±20.4	63.6±16.0*	15.5±7.8*
Macrophages	56.0±11.1	64.1±11.7	50.9±22.6	18.0±6.3*	11.5±3.9*
Eosinophils	4.4±4.2	0.18±0.3	2.3±2.5	18.1±10.6*	72.9±10.9*

393 *Significant difference (P<0.05) with respect to the UC group.

394 **Table 3.** Percentage intensity of immunoreactivity (1-4) of peritoneal macrophages
395 expressed as mean ±SD for the expression of CD14, iNOS, CD206, and the
396 iNOS/CD206 ratio in the uninfected control group (UC) and in the different stages of
397 infection.

	UC	1 dpi	3 dpi	9 dpi	18 dpi
CD14	51.2±11.1(2)	63.2±19(2)	78.1*±13.1(4)	74.6*±16.9(4)	69.1*±12.9(4)
iNOS	37.3±27.7(1)	64.4±4.1(1)	62.9±17.5(1)	74.4*±9.4(2)	46±21.2(1)
CD206	20.9±4.4(1)	81.5*±8.6(4)	83.9*±15.4(4)	87*±5.7(4)	90.9*±8.02(4)
iNOS/CD206	1.8	0.8	0.8	0.9	0.5

398 § Intensity of immunoreactivity (1: mild, 2: moderate, 3: severe, 4: very severe).
399 *Significant differences with respect to the UC group.
400

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1 **Study of Peritoneal Macrophage Immunophenotype in Sheep Experimentally
2 Infected with *Fasciola hepatica***

3

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Abstract

During *Fasciola hepatica* infection, the parasite has the capability to modulate the host immune response towards a non-protector Th2 type instead of Th1. This type of immune response is closely related to the alternative activation of macrophages (M2 profile) as has been shown *in vivo* in murine models. In this study, an experiment was carried out in order to evaluate the expression of CD68, CD14, CD206 and iNOS in cells present in the peritoneal fluid of sheep during early stages of infection with *F. hepatica* (1, 3, 9 and 18 days post-infection, dpi) by immunocytochemistry. To the authors' knowledge, this is the first report that studies the *in vivo* immunophenotype of macrophages from the peritoneal fluid of sheep infected with *F. hepatica*. Throughout the experiments the absolute number of leucocytes progressively increased, reaching its highest value at 18 dpi, mainly due to the increase of eosinophils. This immunocytochemical study had two purposes: 1) CD68 expression was assessed with Hansel counterstaining, to optimally identify peritoneal macrophages, eosinophils and lymphocytes; 2) expression of CD14, CD206 and iNOS was evaluated to identify alternative or classical pathways of macrophage activation. The results showed a significant increase in CD14 from day 3 dpi compared with the non-infected group. CD206 expression at all time-points showed a significant and dramatic increase in comparison with the uninfected group. On the other hand, iNOS expression showed little variation, and was significantly decreased at 18 dpi in comparison with the uninfected group. These results suggest that *F. hepatica* induces an alternative activation of peritoneal macrophages of sheep from the first day post-infection, which may facilitate parasite survival. This is the first report describing M2 activation of peritoneal macrophages in ruminants infected with *F. hepatica*.

Keywords: *Fasciola hepatica*, macrophages, peritoneal fluid, Sheep, immune response

47 **1.- Introduction**

48 Fasciolosis caused by the liver fluke *Fasciola hepatica* infects millions of ruminants
49 worldwide and is recognised by the World Health Organisation (WHO) as an important
50 zoonosis, which is now emerging or re-emerging in many countries (Gonzalez et al.,
51 2011). The costs incurred due to losses in production and treatment with anthelmintics,
52 as well as the resistance that is now widespread, confirm the urgent need for alternative
53 control methods (Fairweather, 2011). During the last two decades, major advances have
54 been made in identifying potential vaccine molecules (Toet et al., 2014; Molina-
55 Hernández et al., 2015; Beesley et al., 2017). Nevertheless, no vaccine candidate has yet
56 reached a commercial or pre-commercial stage. The immune suppression/modulation by
57 *F. hepatica* is one major obstacle to develop a protective vaccine (Toet et al., 2014;
58 Molina-Hernández et al., 2015).

59 Macrophage classical (M1) and alternative (M2) activation phenotypes describe the
60 opposing activities of killing or repairing, and such polarized responses stimulate Th1-
61 or Th2-like responses, respectively. M1 activated macrophages are characterized by
62 high antigen presentation, high production of pro-inflammatory cytokines and nitric
63 oxide. In contrast, M2-type responses are the "resting" phenotype with low production
64 of pro-inflammatory cytokines and high production of anti-inflammatory cytokines such
65 as IL10 (Martínez et al., 2009; Wang et al., 2014). Since *F. hepatica* larvae penetrate
66 the intestinal wall of the host and migrate to the liver via the peritoneum, study of the
67 type macrophage activation at this stage plays a critical role in understanding the
68 immune response to parasitic infection and thus for designing an effective vaccine
69 (Molina-Hernández et al., 2015). It has been reported that *F. hepatica* excretion-
70 secretion products and tegumental coat proteins produce a M2 macrophage phenotype,
71 responsible for host tissue repair, tissue fibrosis and modulation of adaptive immunity,

72 which suppresses a Th1-driven inflammatory pathology in *F. hepatica* infection (Adams
73 et al., 2014; Figueroa-Santiago et al., 2014; Flynn et al., 2007).
74 In early stages of *F. hepatica* infection, the recruitment and activation of M2
75 macrophages in the peritoneal cavity has been reported in rats at 24h post-infection
76 (Donnelly et al., 2005) and in mice at 48 h post-infection (Guasconi et al. 2011).
77 Moreover, excretory-secretory products from *F. hepatica* induced M2 activation of
78 peritoneal macrophages in mice (Guasconi et al. 2011). In sheep marked M2 activation
79 has been described by gene expression was in PBMC at 7 dpi (Fu et al., 2016).
80 Recently, proteomic analysis, humoral response and cytokine expression have been
81 studied in sheep experimentally infected with *F. hepatica* at 18 dpi (Ruiz-Campillo et
82 al., 2017), but phenotype of peritoneal macrophages have not been investigated in *F.*
83 *hepatica* infected ruminants. On the other hand, it has been reported that *F. hepatica*
84 induces apoptosis of peritoneal macrophages in rats (Guasconi et al., 2012) and sheep
85 (Escamilla et al., 2017), but the impact of apoptosis in total peritoneal leucocyte count
86 has not been reported in *F. hepatica* infected ruminants. The aim of the present work
87 was to evaluate the dynamic of total and differential leucocyte count and the
88 macrophage polarisation in peritoneal fluid obtained from sheep experimentally infected
89 with *F. hepatica* in the early stages of infection.
90

91 **2.-Materials and Methods**

92 *2.1.-Experimental design*

93 A total of twenty-five eight-month old male Merino sheep obtained from a liver fluke-
94 free farm were used to study the early stages of infection. Animals were purchased aged
95 one month and housed indoors in the experimental farm of the University of Córdoba
96 until they reached the appropriate age for pathogen challenge. All animals were tested

97 monthly for parasite eggs by faecal sedimentation with negative results in all cases.
98 Moreover, prior to the challenge, all animals were tested for serum IgG specific for *F.*
99 *hepatica* cathepsin L1 (FhCL1) by ELISA, with negative results in all cases. The trial
100 consisted of five groups composed of five sheep (n=5): an uninfected control group and
101 four infected groups. Sheep were orally infected with one dose of 150 metacercariae of
102 the South Gloucester strain of *F. hepatica* (Ridgeway Research Ltd, UK) and
103 euthanised at 1, 3, 9 and 18 days post-infection (dpi). The animals were euthanised by
104 intravenous injection of T61® (Intervet, Spain). The experiments were approved by the
105 Bioethics Committee of the University of Cordoba (code No. 1118) and conducted in
106 accordance with European (2010/63/UE) and Spanish (RD 1201/2005) directives on
107 animal experimentation.

108 *2.2.-Recovery of peritoneal fluid*

109 Peritoneal lavages were conducted immediately after the animals were euthanised. The
110 ventral aspect of the abdomen was shaved and disinfected with 10%
111 polyvinylpyrrolidone iodine (AGB, Madrid, Spain). A 2 cm incision was made on the
112 skin over the white line and subcutaneous tissue was dissected, the white line and
113 peritoneum were sectioned with blunt scissors to avoid bleeding. A 40 cm cannula was
114 inserted into the abdominal cavity and connected to a syringe to inject 60 ml sterile
115 DPBS (Dulbecco's Phosphate-Buffered Saline, Sigma-Aldrich, Darmstadt, Germany),
116 and 9500 I.U. of heparin (Rovi, Madrid, Spain), previously warmed to 37 ºC. After
117 softly massaging the abdominal cavity for 1 min, 40 ml of peritoneal fluid was
118 recovered using the syringe connected to the cannula. In cases where residual
119 erythrocytes were present, it was necessary to use an erythrolysis buffer prior to the
120 processing of the cells.

121 *2.3.-Cell populations*

122 The recovered peritoneal lavage fluid was collected and cooled on ice until cell
123 processing. The total number of viable peritoneal cells was determined after Trypan
124 Blue staining by counting in a Neubauer haemocytometer. Smear preparations were
125 manually performed on Vectabond®-treated slides (Vector laboratories, California,
126 USA) by centrifuging the recovered peritoneal fluid at 1500 rpm for 10 min. After air
127 draining, these smears were fixed in acetone for 5 min and stored at -80°C for further
128 immunocytochemical studies. For differential cell counting immunocytochemistry using
129 anti-human CD68 monoclonal antibody (Dako, Glostrup, Denmark) in combination
130 with Hansel staining was used as a novel and more accurate cell counting method. A
131 total of 200 cells per smear were counted in randomly selected fields of 400x
132 magnifications using the software Image Pro-plus 6.0 (Media Cybernetics, Silver
133 Spring, USA), and the percentage of lymphocytes (small basophilic nucleus and scanty
134 cytoplasm), macrophages (round to oval hyperchromatic nucleus and moderate to large
135 cytoplasm stained brownish with the CD68 antibody) and eosinophils (basophilic
136 bilobed nucleus and eosinophilic cytoplasm) was obtained. Neutrophils (basophilic
137 lobulated nucleus and unstained cytoplasm) were not included in the cell count since
138 they were only very occasionally observed.

139 *2.4.-Immunocytochemistry (ICC)*

140 An immunocytochemical study was used to assess anti-Human CD68 (M0718, Dako,
141 Glostrup, Denmark), anti-Human CD14 (BOV2027, Monoclonal Antibody Center,
142 Washington State University), anti-Human iNOS (PA3–030A, Thermo, Freemont,
143 USA) and anti-Human CD206 (orb4941, Biorbyt, Cambridge, UK) expression in
144 peritoneal fluid macrophages. The dilution used for these primary antibodies was 1:400
145 for CD68; 1:500 for CD14, 1:200 for iNOS; and 1:100 for CD206. The CD14
146 monoclonal antibody cross-reacts with ovine tissues according to the manufacturer. The

147 iNOS (Wood et al. 2005), CD68 antibodies (Pinczowski et al., 2017) and anti-human
148 CD206 (Ampem et al., 2016) have shown cross reactivity with ovine tissues. The
149 avidin-biotin-peroxidase method described elsewhere (Zafra et al., 2013a) was carried
150 out. Briefly, endogenous peroxidase activity was blocked by incubation with 3%
151 hydrogen peroxide (Panreac, Barcelona, Spain) in phosphate buffered saline (PBS).
152 Then, smears were washed once in PBS and incubated with 10% normal goat serum
153 (MP Biomedicals, Ohio, USA) for 30 min at room temperature. After three 5 min rinses
154 in PBS-Tween (PBST), secondary antibodies were applied for 45 min at 37ºC. A
155 biotinylated goat anti-rabbit immunoglobulin serum (Dako) diluted 1:200 was applied
156 to the smears incubated with the primary polyclonal antibodies (pAbs: iNOS and
157 CD206), whereas a biotinylated goat anti-mouse immunoglobulin serum (Dako) diluted
158 1:50 was used for the primary monoclonal antibody (mAb: CD68). After two 5 min
159 rinses in PBST, an avidin–biotin-peroxidase complex (Vector, Burlinghame, USA)
160 diluted 1:50 was applied for an hour as a third reagent. Slides were then washed three
161 times in PBST and incubated with Novared® substrate kit peroxidase (Vector) diluted
162 following the manufacturer's instructions, rinsed in water, lightly counterstained with
163 Mayer's haematoxylin and mounted with Eukitt® (Freiburg, Germany). For CD68
164 antibody, following the haematoxylin stain, eosin was applied to the slides for 1 minute
165 with Hansel stain for the differential cell count. Specific primary antibodies were
166 substituted with PBS or non-immune isotype-matched sera as negative controls. Sheep
167 and human hepatic lymph node lymph node tissue sections were used as positive
168 controls.

169 *2.5.-Cell count*

170 Immunoreactive cells were counted in randomly selected fields of 400x magnifications
171 using the Image Pro-plus 6.0 software package. Macros were calibrated for staining

172 intensity and cell size to include all immunostained cells. A total of 200 cells were
173 counted per slide and the percentage of positive and negative cells was obtained.
174 Photomicrographs were taken using an Olympus BX51 photomicroscope with a 400x
175 magnification field. Results were expressed as mean ± SD per animal and per group.
176 The intensity of immunostaining was evaluated semi-quantitatively according to the
177 following score: 1, mild; 2, moderate; 3, severe; 4, very severe.

178 *2.6.-Statistical analysis*

179 Statistical analysis was carried out using the Graphpad Prism 7.0 software package
180 (Graphpad Software, Inc., San Diego, California). The Kolmogorov–Smirnov test was
181 applied to evaluate whether distributions were parametric. Comparisons between groups
182 were made using the Mann–Whitney test for non-parametric distributions. Correlation
183 studies were carried out using the Spearman correlation test for non-parametric
184 distributions. $P<0.05$ was considered significant.

185

186 **3. Results**

187 *3.1. Absolute peritoneal cell count*

188 The results of the absolute peritoneal fluid cell counts are shown in Table 1. The
189 number of cells increased significantly ($P<0.05$) at 9 and 18 dpi compared with the
190 uninfected control group. At 1 and 3 dpi no significant differences respect to the
191 uninfected control group were obtained.

192 *3.2. Differential peritoneal cell count*

193 Since CD68 has been widely used as a general macrophage marker (Valheim et al.,
194 2004) a CD68 mAb in combination with Hansel stain was used as a novel and more
195 accurate leucocyte identification method. The differential cell count results (expressed
196 as percentages) from peritoneal fluid smear examinations are shown in Table 2.

197 Neutrophils and epithelial cells were only occasionally observed and were not included
198 in the cell count.
199 At 9 and particularly at 18 dpi, there was a very marked increase in the number of
200 eosinophils, which was responsible for the relative decrease in the percentages of
201 macrophages (9 and 18 dpi) and lymphocytes (18 dpi) in comparison with the
202 uninfected control group Table 2. On the other hand, the percentage of lymphocytes
203 showed a significant increase at 9 dpi with respect to the uninfected control group. This
204 may reflect a stimulation of the recruitment of peritoneal lymphocytes at this time-point
205 when larvae are penetrating or migrating into the liver surface as revealed by the
206 significant increase in the total number of peritoneal leucocytes at 9 and 18 dpi.
207 *3.3. Immunocytochemical study*
208 The anti-CD14 mAb yielded a cytoplasmic immunostaining in peritoneal leucocytes
209 with large cytoplasm and round to ovoid nucleus, this was the same morphology than
210 peritoneal leucocytes expressing CD68. The results of the present study revealed a
211 significant increase of the percentage of CD14+ cells at 3, 9 and 18 dpi with respect to
212 the uninfected control group (Table 3).
213 Anti-iNOS and anti-CD206 antibodies were used as biomarkers of classical (M1) and
214 alternative (M2) macrophage activation, respectively. The anti-iNOS pAb also showed
215 granular cytoplasmic immunostaining in peritoneal macrophages (Fig. 1) and in some
216 eosinophils, but only the macrophages were counted. The percentage of peritoneal
217 macrophages expressing iNOS varied little during the course of the infection in (Table
218 3). The intensity of immunolabelling with anti-iNOS was mild in the uninfected control
219 group and at all studied time-points (Table 3).
220 The Anti-human CD206 antibody has been described as a good biomarker of alternative
221 activation of macrophages in sheep (Ampem et al., 2016). In our study, the CD206 mAb

222 yielded weak cytoplasmic immunostaining in peritoneal macrophages from the
223 uninfected control group, whereas the intensity of the immunolabelling was very strong
224 at 1, 3, 9 and 18 dpi (Fig. 2, Table 3). The percentage of peritoneal macrophages
225 expressing CD206 showed a dramatic and significant increase (P<0.05) from 1 dpi
226 onwards, compared with the uninfected groups (Table 3).
227 The iNOS/CD206 ratio decreased approximately one-fold at 1, 3 and 9 dpi and three-
228 fold at 18 dpi (Table 3).

229 **4. Discussion**

230 It has been reported that *F. hepatica* induces apoptosis of peritoneal leucocytes in sheep
231 (Escamilla et al., 2017) and rat (Guasconi et al., 2012), thus it will be of interest to
232 investigate if *F. hepatica* induces reduction of peritoneal leucocyte during early stages
233 of infection. Differential leucocyte count has been evaluated in *F. hepatica* infected
234 goats (Zafra et al., 2013a) and sheep (Escamilla et al., 2017), but total peritoneal count
235 has not been investigated in *F. hepatica* infected ruminants during early stages of
236 infection. The results of the present study revealed non-significant changes of total
237 peritoneal leucocyte count at 1 and 3 dpi respect to the uninfected control group,
238 suggesting that apoptosis of peritoneal leucocytes do not induce reduction of total
239 peritoneal count at early stages, probably due to leucocyte recruitment. The marked and
240 significant increase of total peritoneal leucocytes found at 9 and 18 dpi is mainly due to
241 the increase of eosinophils, a finding consistent with previous reports in goats (Zafra et
242 al., 2013a) and sheep (Escamilla et al., 2017).
243 CD14 is a co-receptor for TLR-initiated pro-inflammatory responses in innate immune
244 cells, particularly macrophages. It has been reported that infection by helminths such as
245 *Schistosoma mansoni* in mice (Tundup et al., 2014) and *F. hepatica* in cattle (Garza-
246 Cuartero et al., 2016) induces an increase in CD14 expression in liver macrophages and

247 peripheral blood mononuclear cells (PBMCs), respectively. The present study is the first
248 report analysing the expression of CD14 in peritoneal leucocytes in ruminants infected
249 with *F. hepatica,* and revealed a significant increase at 3, 9 and 18 dpi with respect to
250 the uninfected control group. This is in concordance with the increased CD14
251 expression in PBMC at 7 and 12 weeks after *F. hepatica* infection in cattle was
252 associated with an alternative activation of macrophages (Garza-Cuartero et al., 2016).
253 In addition, it has been previously shown that CD14 expression increases during sepsis
254 and tissue remodelling processes, and the fact that this increase occurs at 3 dpi, when
255 the parasite is penetrating through the gut and reaching the peritoneal cavity, suggests
256 that it could be an indicator of tissue damage and apoptosis (Devitt et al., 1998).
257 During helminth infections, macrophages that undergo changes to express an M2
258 phenotype have been implicated in the regulation of the cytokine environment. This
259 change leads to preferential induction of the Th2 response, which is ineffective in
260 controlling the parasite infection and results in the chronic stage of the disease (O'Neill
261 *et al.*, 2000; Kreider et al., 2007). Since the host response to *F. hepatica* is thought to be
262 more effective during the intestinal, peritoneal or early hepatic migratory stages (Van
263 Milligen et al., 1999), the rapid M2 polarisation of peritoneal macrophages found in the
264 sheep of the present study may be an important mechanism of modulation that may
265 facilitate parasite survival during the early stages of infection.
266 In a murine model, very low iNOS gene expression was detected in uninfected controls
267 and at 1, 7, 14 and 21 days after *F. hepatica* infection (Donnelly et al., 2005), while in
268 sheep, a marked decrease in iNOS gene expression was found in PBMC at 7 dpi (Fu et
269 al., 2016), which contrasts with the low level of variation in iNOS expression by
270 immunocytochemistry in both trials of the present study. This difference suggests that

271 iNOS gene and protein expression may differ, with the protein probably remaining
272 active for a longer time than the gene.
273 The ratio iNOS/CD206 suggest M2 polarisation of peritoneal macrophages from 1 dpi,
274 a finding that is consistent with previous studies in mice (Donnelly et al., 2005), and in
275 PBMC of sheep (Fu et al., 2016) and cattle (Flynn et al., 2007, Garza-Cuartero et al.,
276 2016), that report M2 activation of macrophages induced by *F. hepatica* infection.
277 Further studies should focus on the mechanisms used by the *F. hepatica* parasite to
278 modulate the host response in ruminants, particularly during early stages of infection
279 when the parasite is more vulnerable to the host response. The knowledge of such
280 mechanisms may be used to a more rationale design of new vaccine candidates for
281 blocking immunomodulation and increasing vaccine efficacy.
282

283 **Acknowledgments**
284 Work supported by EU Project (H2020-SFS-2014-2-635408- PARAGONE and the
285 Spanish Ministry of Science grant AGL2015-67023-C2-1-R.
286

287 **References**
288 Adams, P.N., Aldridge, A., Vukman, K.V., Donnelly, S., O'Neill, S.M., 2014. *Fasciola*
289 *hepatica* tegumental antigens indirectly induce an M2 macrophage-like phenotype in
290 vivo. Parasite Immunol. 36, 531-9.
291 Ampem, G., Azegrouz, H., Bacsadi, Á., Balogh, L., Schmidt, S., Thuróczy, J., Röszer,
292 T., 2016. Adipose tissue macrophages in non-rodent mammals: a comparative study.
293 Cell Tissue Res. 363, 461-478.
294 Beesley N.J., Caminade, C., Charlier, J., Flynn, R.J., Hodgkinson, J.E., Martinez-
295 Moreno, A., Martinez-Valladares, M., Perez, J., Rinaldi, L., Williams, D.J.L., 2017.

296 Fasciola and fasciolosis in ruminants in Europe: Identifiying research needs.
297 Transbound Emerg Dis. doi: 10.1111/tbed.12682
298 Devitt, A., Moffatt, O.D., Raykundalia, C., Capra, J.D., Simmons, D.L., Gregory, C.D.,
299 1998. Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature
300 392, 505-509.
301 Donnelly, S, O'Neill, S.M., Sekiya, M., Mulcahy, G., Dalton, J.P., 2005. Thioredoxin
302 peroxidase secreted by *Fasciola hepatica* induces the alternative activation of
303 macrophages. Infect. Immun. 73, 166-173.
304 Escamilla, A., Pérez-Caballero, R., Zafra, R., Bautista, M.J., Pacheco, I.L., Ruiz, M.T.,
305 Martínez-Cruz, M.S., Martínez-Moreno, A., Molina-Hernández, V., Pérez, J., 2017.
306 Apoptosis of peritoneal leucocytes during early stages of *Fasciola hepatica* infections in
307 sheep. Vet. Parasitol. 238, 49-53.
308 Fairweather, I., 2011. Reducing the future threat from (liver) fluke: realistic prospect or
309 quixotic fantasy? Vet Parasitol. 180, 133-143.
310 Figueroa-Santiago, O., Espino, A.M., 2014. *Fasciola hep*atica fatty acid binding protein
311 induces the alternative activation of human macrophages. Infect. Immun. 82, 5005-
312 5012.
313 Flynn, R.J., Irwin, J.A., Olivier, M., Sekiya, M., Dalton, J.P., Mulcahy, G., 2007.
314 Alternative activation of ruminant macrophages by *Fasciola hepatica*. Vet. Immunol.
315 Immunopathol. 120, 31-40.
316 Fu, Y., Chryssafidis, A.L., Browne, J.A., O'Sullivan, J., McGettigan, P.A., Mulcahy,
317 G., 2016. Transcriptomic study on ovine immune responses to *Fasciola hepatica*
318 infection. PLOS Negl. Trop. Dis. 10, e0005015.
319 Garza-Cuartero, L., O'Sullivan, J., Blanco, A., McNair, J., Welsh, M., Flynn, R.J.,
320 Williams, D., Diggle, P., Cassidy, J. Mucalhy, G., 2016. *Fasciola hepatica* infection

321 reduces *Mycobacterium bovis* burden and mycobacterial uptake and suppresses the pro-
322 inflammatory response. Parasite Immunol. 38, 387-402.
323 González, L.C., Esteban, J.G., Bargues, M.D., Valero, M.A., Ortiz, P., Náquira, C.,
324 Mas-Coma, S., 2011. Hyperendemic human fascioliasis in Andean valleys: An
325 altitudinal transect analysis in children of Cajamarca province, Peru. Acta Trop. 120,
326 119-129.
327 Guasconi, L., Serradell, M.C., Garro, A.P., Iacobelli, L., Masih, D.T. 2011. C-type
328 lectins on macrophages participate in the immunomodulatory response to *Fasciola*
329 *hepatica* products. Immunology. 133, 386-396.
330 Guasconi, L., Serradell, M.C., Masih, D.T. 2012. *Fasciola hepatica* products induce
331 apoptosis of peritoneal macrophages. Vet. Immunol. Immunopathol. 148, 359-363.
332 Kreider, T., Anthony, R.M., Urban, J.F. Jr., Gause, W.C., 2007. Alternative activated
333 macrophages in helminth infections. Curr. Opin. Immunol. 19, 448-453.
334 Martinez, F.O., Helming, L., Gordon, S. 2009. Alternative activation of macrophages:
335 an immunologic functional perspective. Annu. Rev. Immunol. 27, 451–483.
336 Molina-Hernández, V., Mulcahy, G., Pérez, J., Martínez-Moreno, A., Donnelly, S.,
337 O'Neill, S., Dalton, J.P., Cwiklinski, K., 2015. *Fasciola hepatica* vaccine: we may not
338 be there yet but we're on the right road. Vet. Parasitol. 208, 101-111.
339 O'Neill, S.M., Brady, M.T., Callanan, J.J., Mulcahy, G., Joyce, P., Mills, K.H., Dalton,
340 J.P., 2000. *Fasciola hepatica* infection downregulates Th1 responses in mice. Parasite
341 Immunol. 22, 147-155.
342 Pinczowski, P., Sanjosé, L., Gimeno, M., Crespo, H., Glaria, I., Amorena, B., de
343 Andrés, D., Pérez, M., Reina, R., Luján, L. 2017. Small ruminant lentiviruses in sheep:
344 pathology and tropism of 2 strains using the bone marrow route. Vet Pathol. 54, 413-
345 424.

346 Ruiz-Campillo, M.T., Molina-Hernandez, V., Escamilla, A., Stevenson, M., Perez, J.,
347 Martinez-Moreno, A., Donnelly, S., Dalton, J.P., Cwiklinski, K., 2017. Immune
348 signatures of pathogenesis in the peritoneal compartment during early infection of sheep
349 with *Fasciola hepatica*. Sci Rep. 7, 2782.
350 Toet, H., Piedrafita, D.M., Spithill, T.W., 2014. Liver fluke vaccine in ruminants:
351 strategies, progress and future opportunities. Int. J. Parasitol. 44, 915-927.
352 Tundup S., Srivastava L., Nagy T., Harn D., 2014. CD14 influences host immune
353 responses and alternative activation of macrophages during *Schistosoma mansoni*
354 infection. Infect. Immun. 82, 3240-3251.
355 Valheim M., Sigurdardóttir O.G., Storset A.K. Aune L.G., Press C.M., 2004.
356 Characterization of macrophages and occurrence of T cells in intestinal lesions of
357 subclinical paratuberculosis in goats. J. Comp. Pathol. 131, 221-232.
358 Van Milligen, F.J., Cornelissen, J.B., Bokhout B.A., 1999. Protection against *Fasciola*
359 *hepatica* in the intestine is highly correlated with eosinophil and immunoglobin G1
360 responses against newly excysted juveniles. Parasite Immunol. 21, 243-251.
361 Wang, N., Liang, H., Zen, K., 2014. Molecular mechanisms that influence the
362 macrophage m1-m2 polarization balance. Front. Immunol. 5, 614.
363 Wood, C.E., Chen, G.F., Keller-Wood, M., 2005. Expression of nitric oxide synthase
364 isoforms is reduced in late-gestation ovine fetal brainstem. Am J Physiol Regul Integr
365 Comp Physiol. 289, R613-R619.
366 Zafra, R., Pérez-Écija, R.A., Buffoni, L., Moreno, P., Bautista, M.J., Martínez-Moreno,
367 A., Mulcahy, G., Dalton, J.P., Pérez, J., 2013a. Early and late peritoneal and hepatic
368 changes in goats immunized with recombinant cathepsin L1 and Infected with *Fasciola*
369 *hepatica*. J. Comp. Path. 148, 373-384.
370

371

372 **Figure legends**

373 **Fig. 1**. Peritoneal smear stained with anti-iNOS polyclonal antibody showing mild
374 cytoplasmic immunolabelling (brown colour) in macrophages (arrows) in the uninfected
375 control group (A) and at 3 day post-infection (dpi) (B), 9 dpi (C) and 18 dpi (D). ABC
376 method-haematoxylin counterstain. X400.

377 **Fig. 2.** Peritoneal smear stained with anti-CD206 polyclonal antibody showing mild
378 cytoplasmic immunolabelling (brown colour) in macrophages (arrows) from uninfected
379 control (A) and very severe immunolabelling in macrophages at 1 day post-infection
380 (dpi) (B), 9 dpi (C), and 18 dpi (D). ABC method-haematoxylin counterstain. X400.

381

382

383 **Table 1. Absolute peritoneal leucocyte counts expressed in 10^6 cells/ml**
384 **(mean±SEM)**.

UC	1 dpi	3 dpi	9 dpi	18 dpi
4.0±0.8	3.3±1.6	7.4±1.4	74.2±20.1*	497.9±122*

385 UC: uninfected control group. dpi: days post-infection.
386 *Significant difference (P<0.05) with respect to the UC group.
387
388
389

390 **Table 2**. Differential cell count expressed as percentages of lymphocytes, macrophages
391 and eosinophils in the uninfected control group and at 1, 3, 9 and 18 days post-infection.
392

	UC	1 dpi	3 dpi	9 dpi	18 dpi
Lymphocytes	39.4±10.6	35.6±12.1	46.4±20.4	63.6±16.0*	15.5±7.8*
Macrophages	56.0±11.1	64.1±11.7	50.9±22.6	18.0±6.3*	11.5±3.9*
Eosinophils	4.4±4.2	0.18±0.3	2.3±2.5	18.1±10.6*	72.9±10.9*

393 *Significant difference (P<0.05) with respect to the UC group.

394 **Table 3.** Percentage intensity of immunoreactivity (1-4) of peritoneal macrophages
395 expressed as mean ±SD for the expression of CD14, iNOS, CD206, and the
396 iNOS/CD206 ratio in the uninfected control group (UC) and in the different stages of
397 infection.

	UC	1 dpi	3 dpi	9 dpi	18 dpi
CD14	51.2±11.1(2)	63.2±19(2)	78.1*±13.1(4)	74.6*±16.9(4)	69.1*±12.9(4)
iNOS	37.3±27.7(1)	64.4±4.1(1)	62.9±17.5(1)	74.4*±9.4(2)	46±21.2(1)
CD206	20.9±4.4(1)	81.5*±8.6(4)	83.9*±15.4(4)	87*±5.7(4)	90.9*±8.02(4)
iNOS/CD206	1.8	0.8	0.8	0.9	0.5

398 § Intensity of immunoreactivity (1: mild, 2: moderate, 3: severe, 4: very severe).
399 *Significant differences with respect to the UC group.
400

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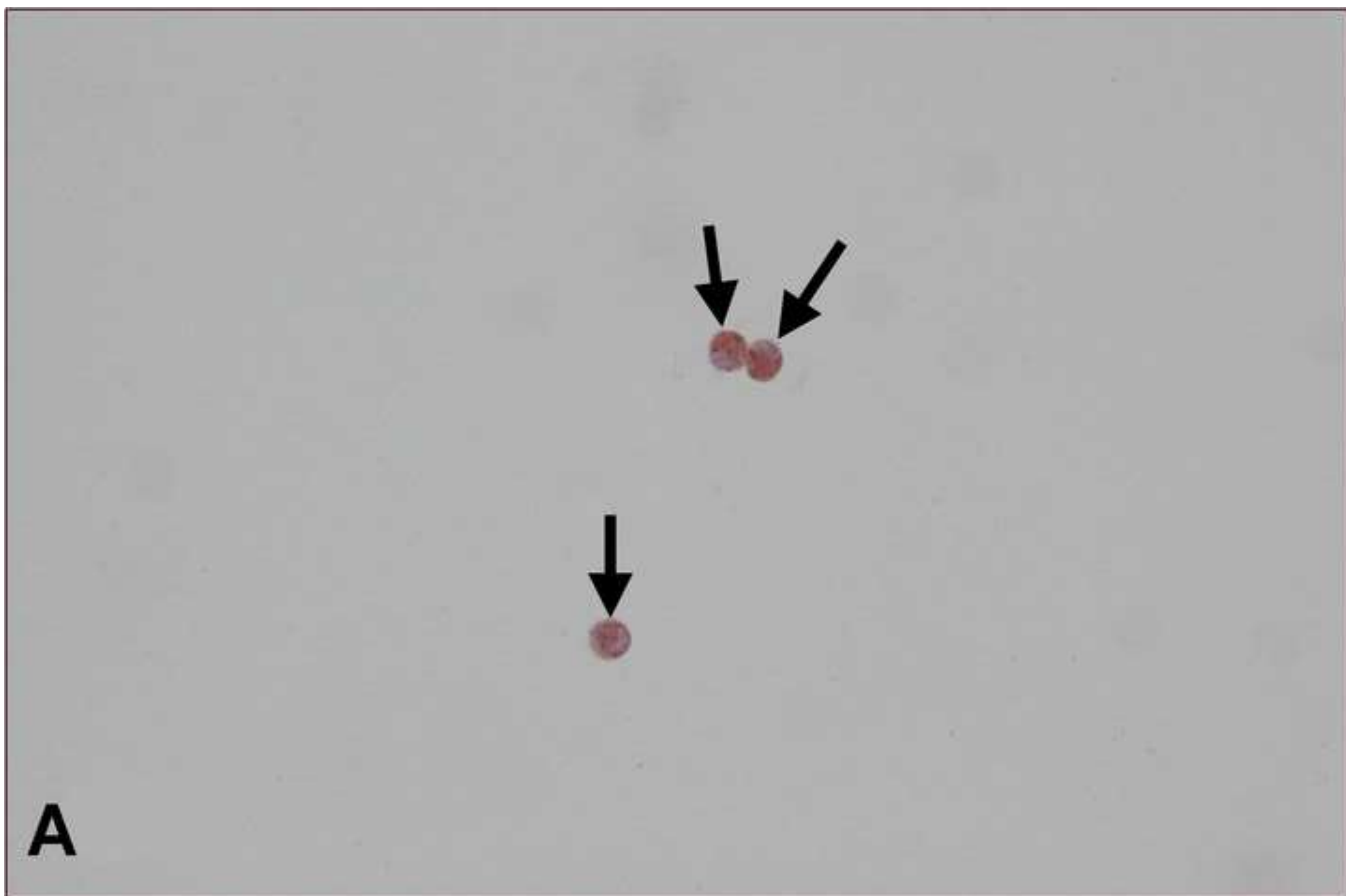

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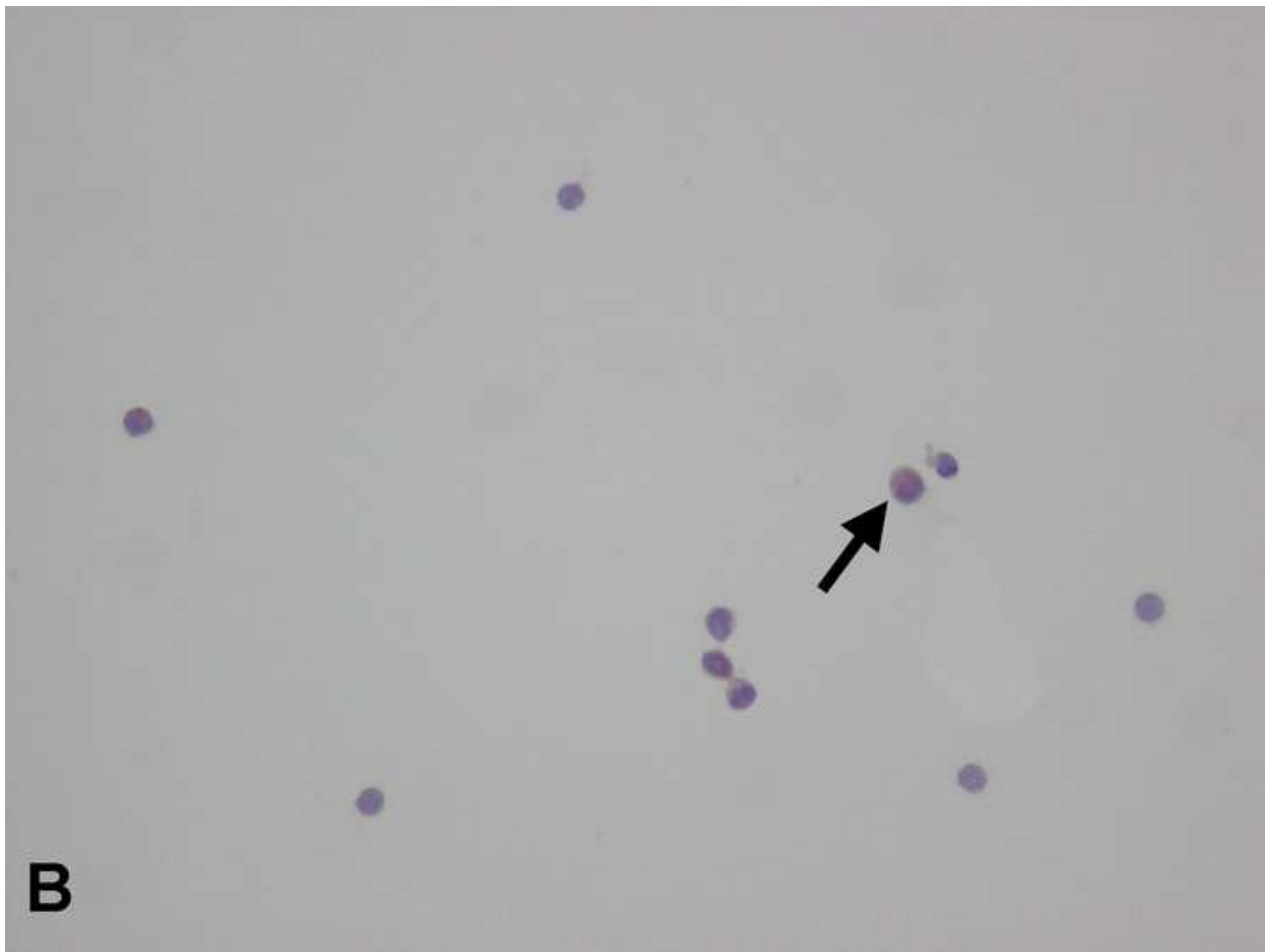

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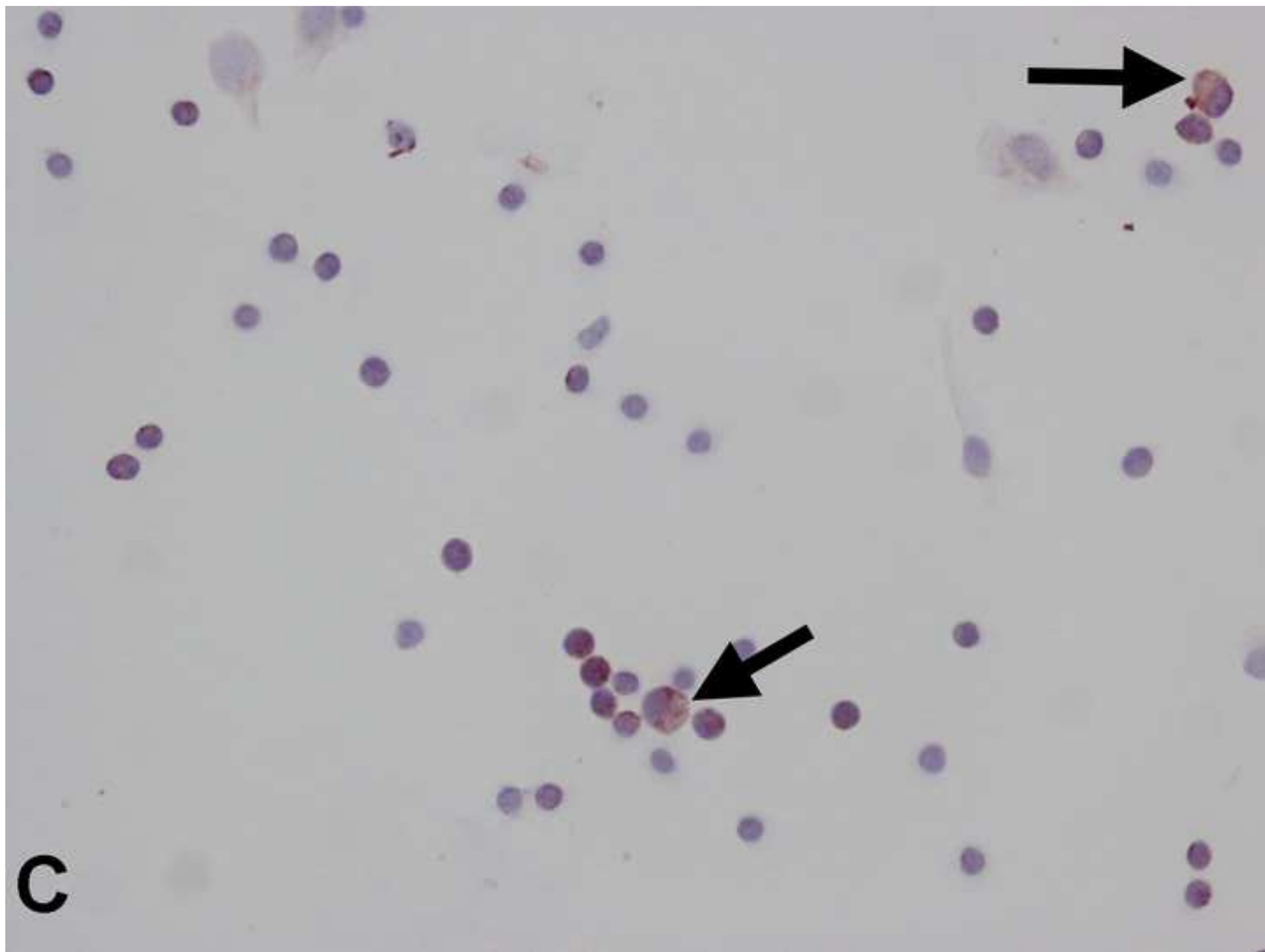

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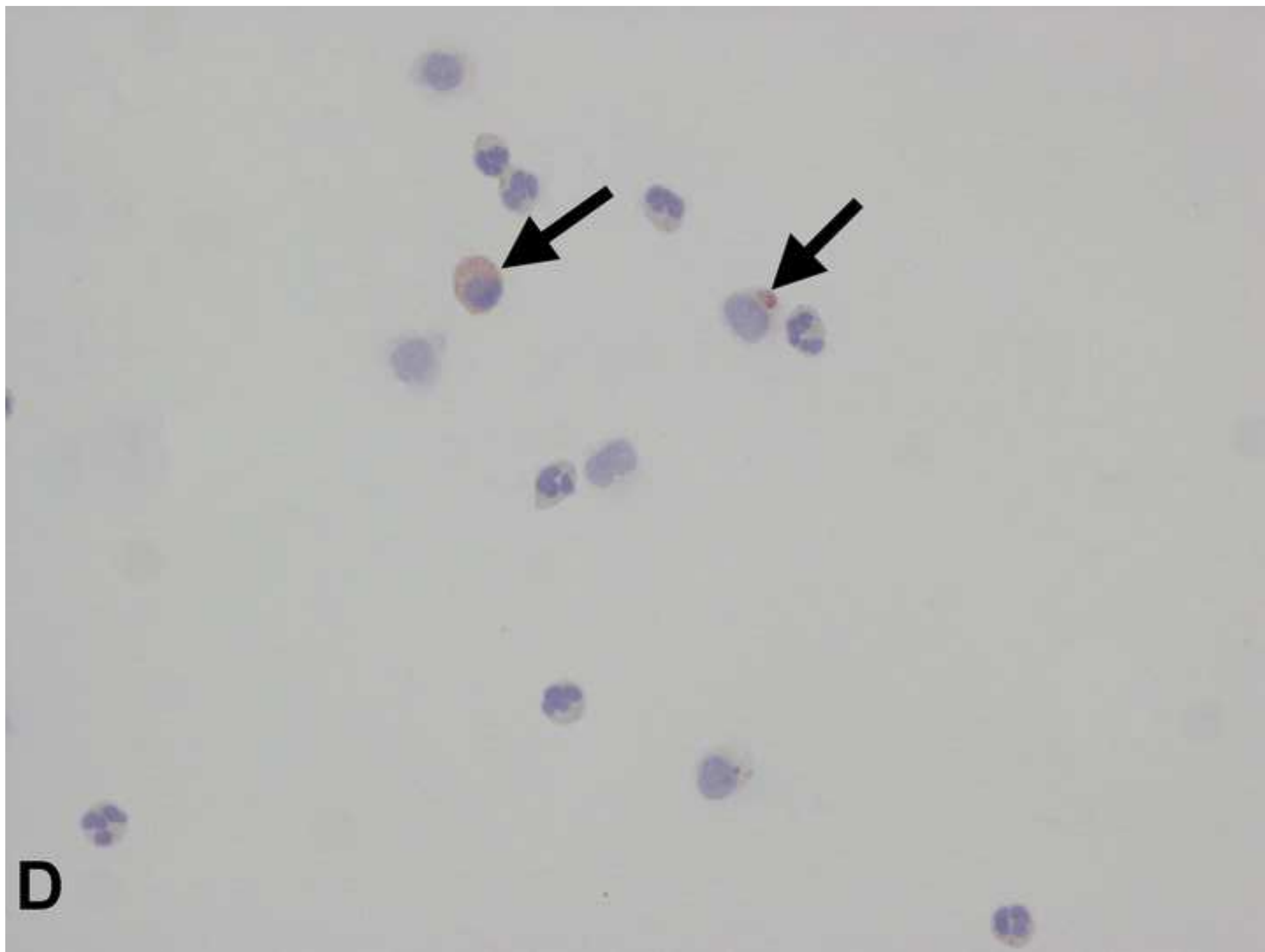

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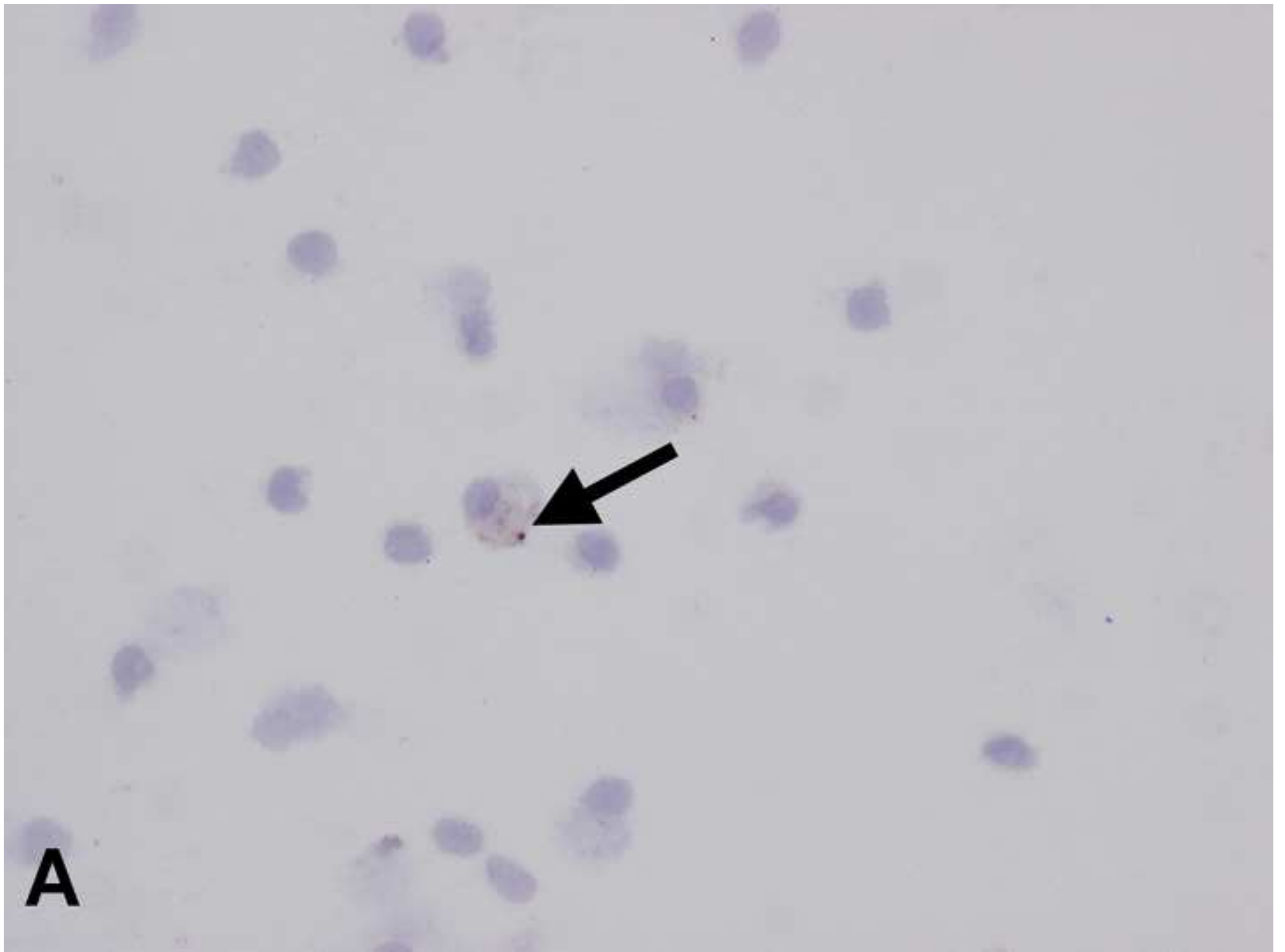

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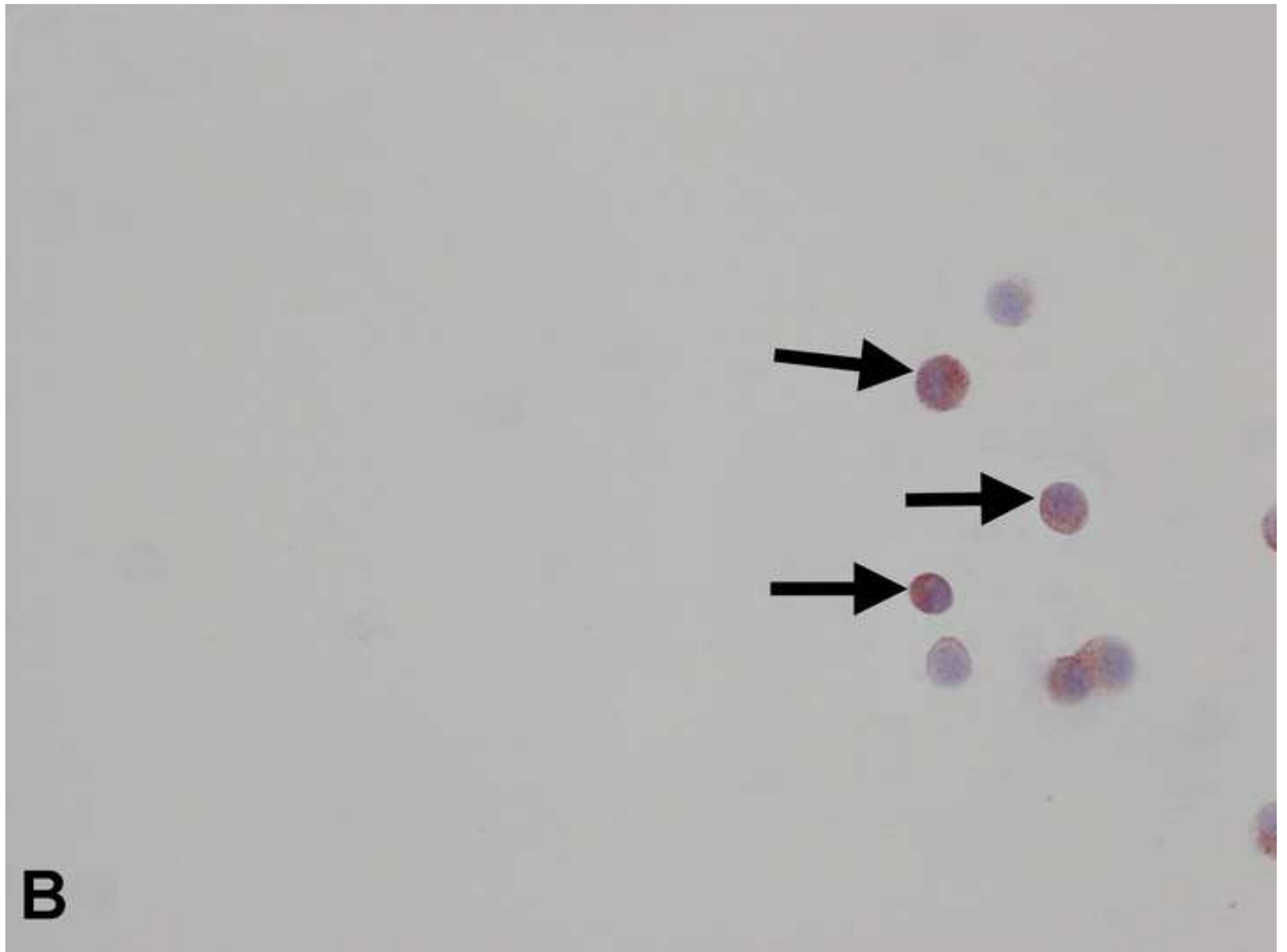

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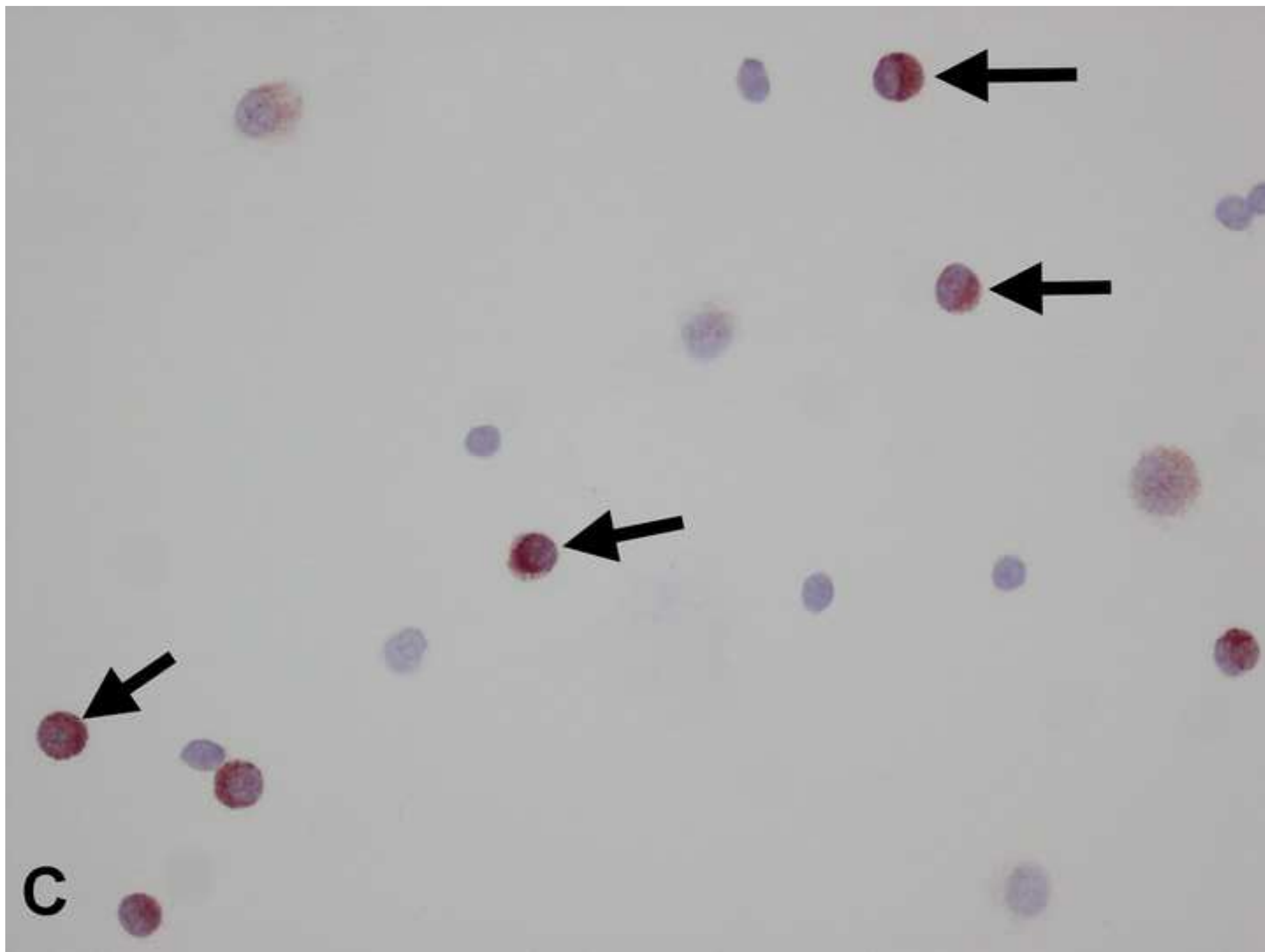

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