Study of Peritoneal Macrophage Immunophenotype in Sheep Experimentally 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

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

2.-Materials and Methods

2.1.-Experimental design

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

2.2.-Recovery of peritoneal fluid

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

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

2.3.-Cell populations

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

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

2.4.-Immunocytochemistry (ICC)

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

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

2.5.-Cell count

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

2.6.-Statistical analysis

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

3. Results

3.1. Absolute peritoneal cell count

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

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

3.2. Differential peritoneal cell count

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

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

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

3.3. Immunocytochemical study

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

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

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

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

4. Discussion

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

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

During helminth infections, macrophages that undergo changes to express an M2 phenotype have been implicated in the regulation of the cytokine environment. This 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**
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337
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Figure legends

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

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

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

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.

	UC	1 dpi	3 dpi	9 dpi	18 dpi
Trial 1					
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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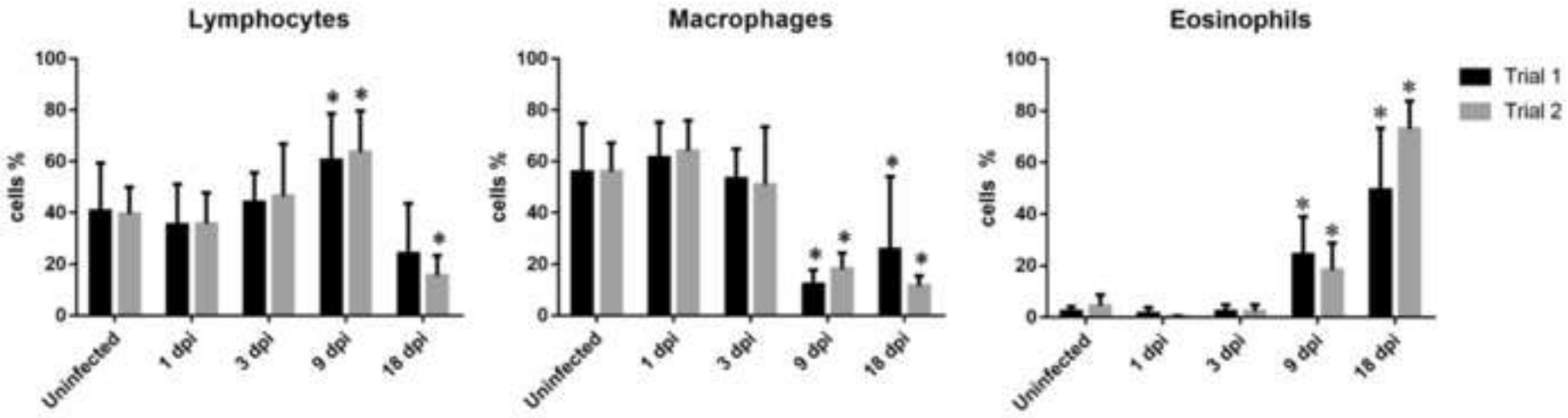

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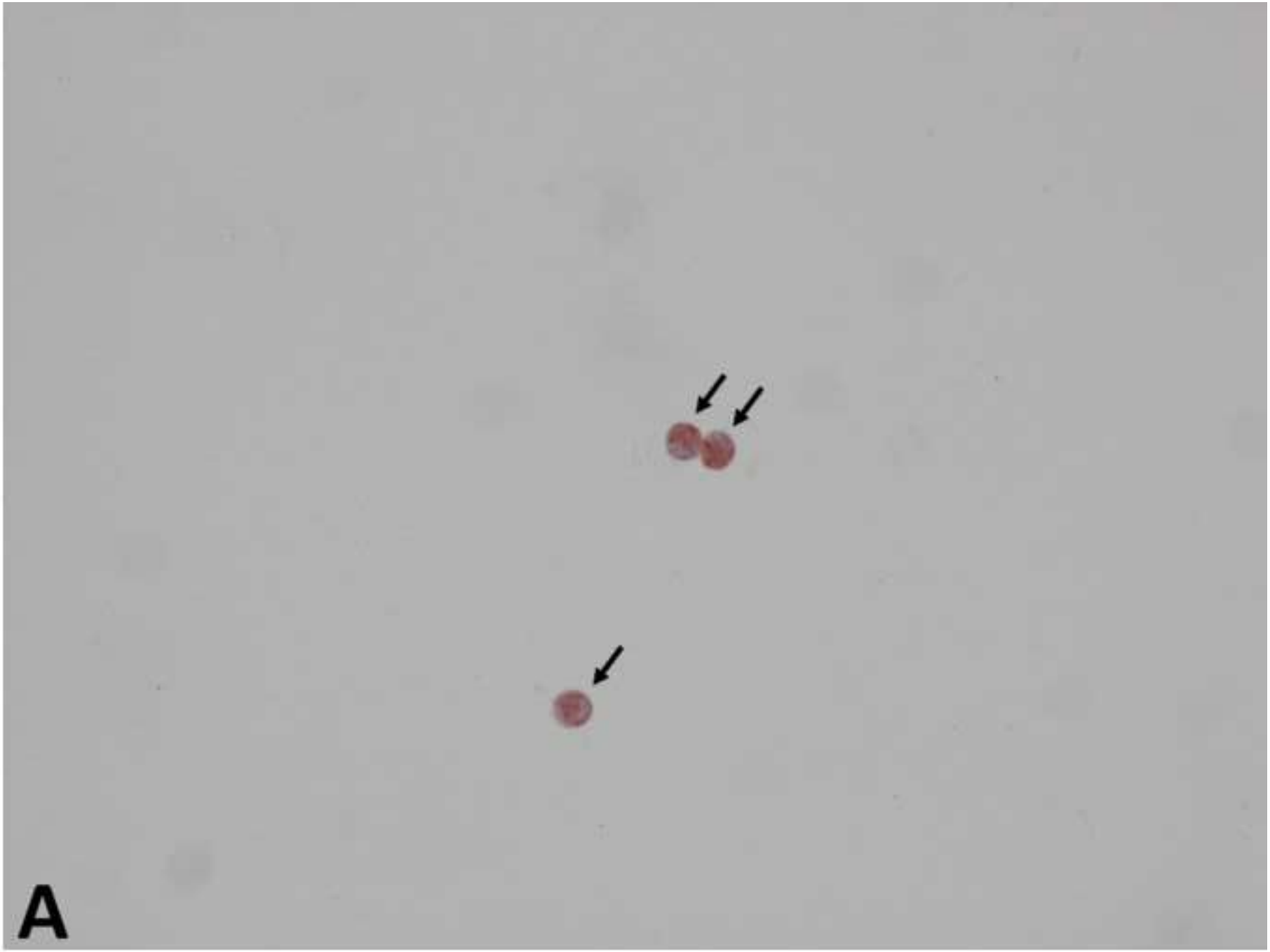

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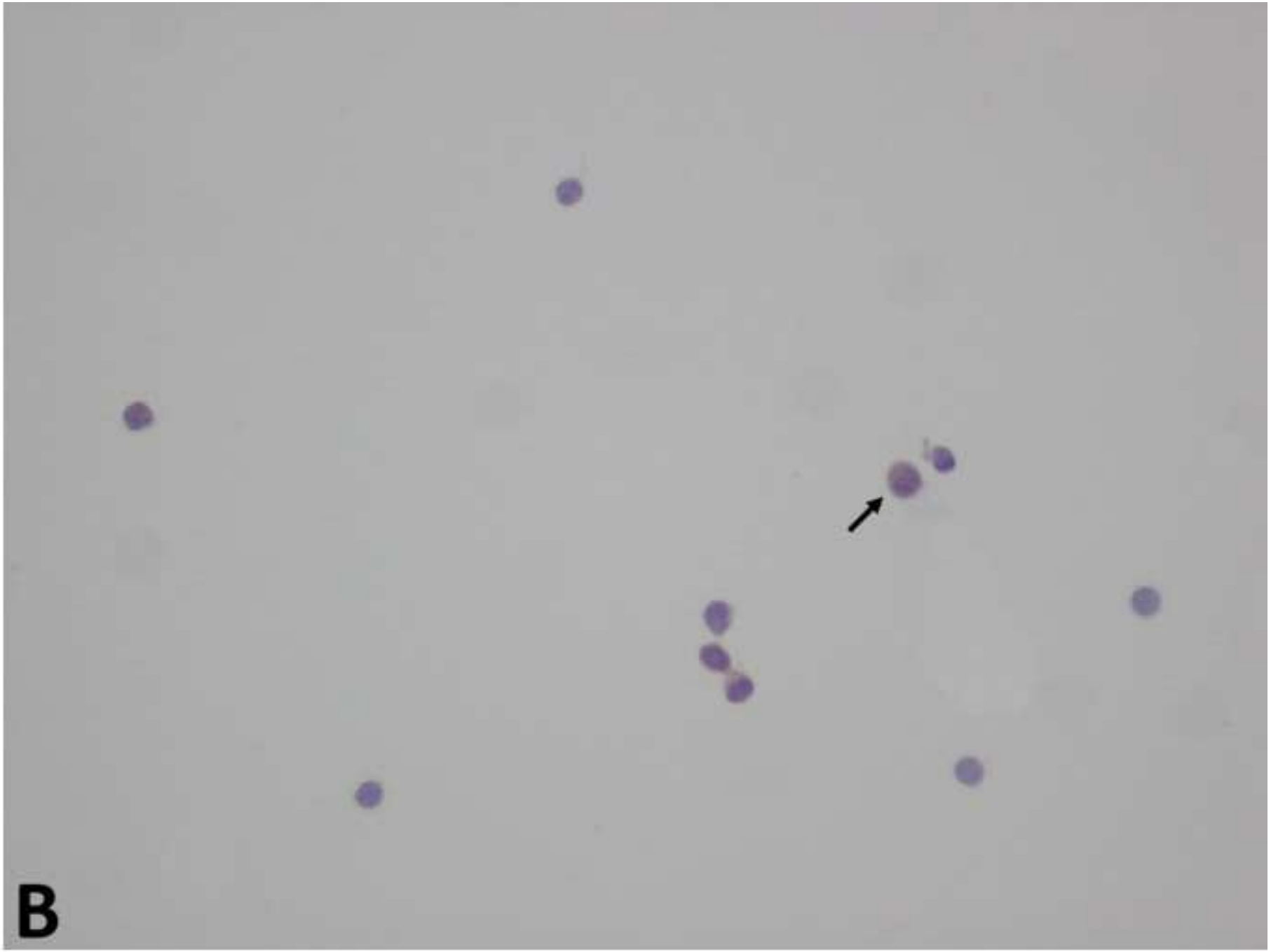

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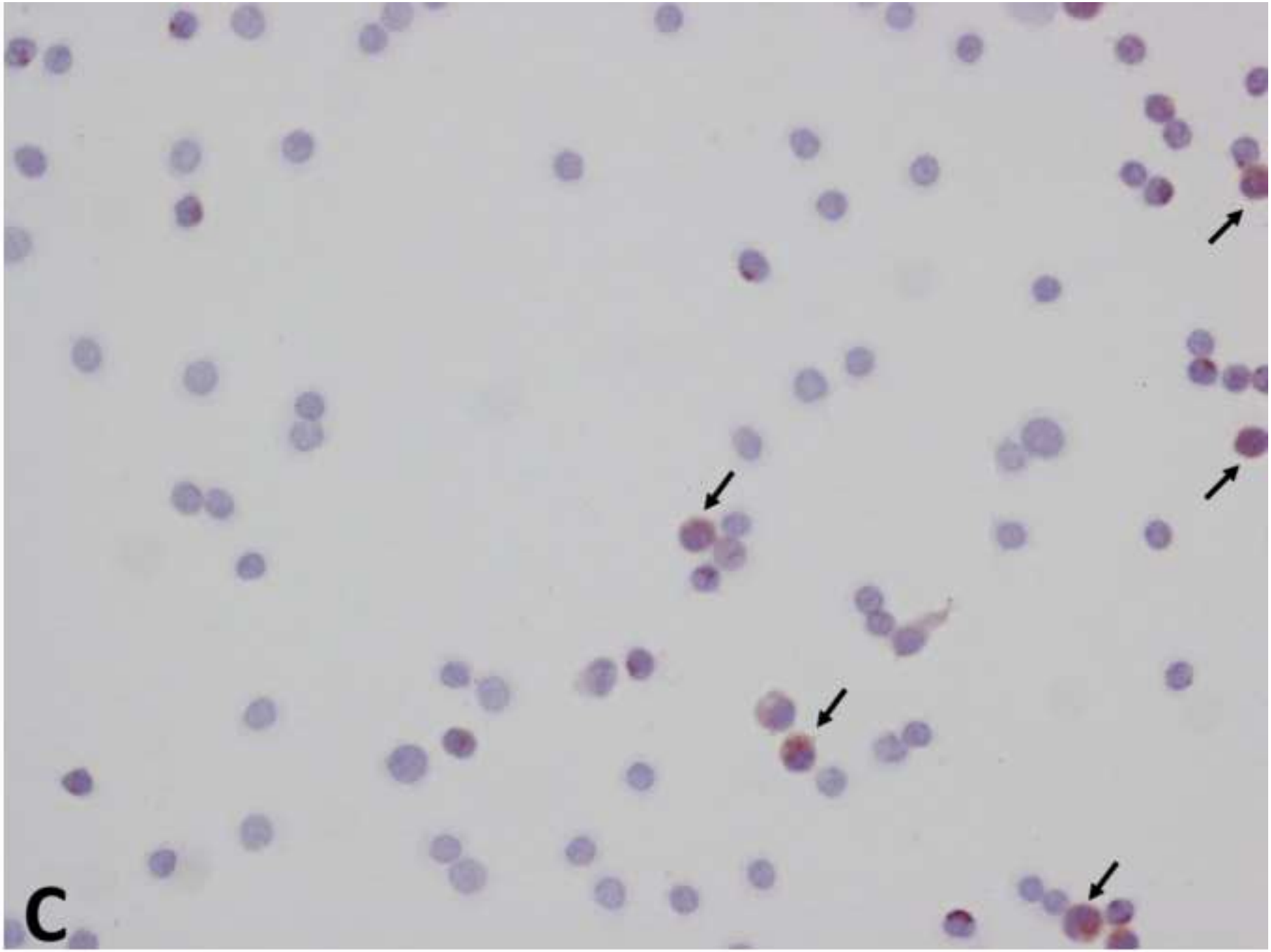

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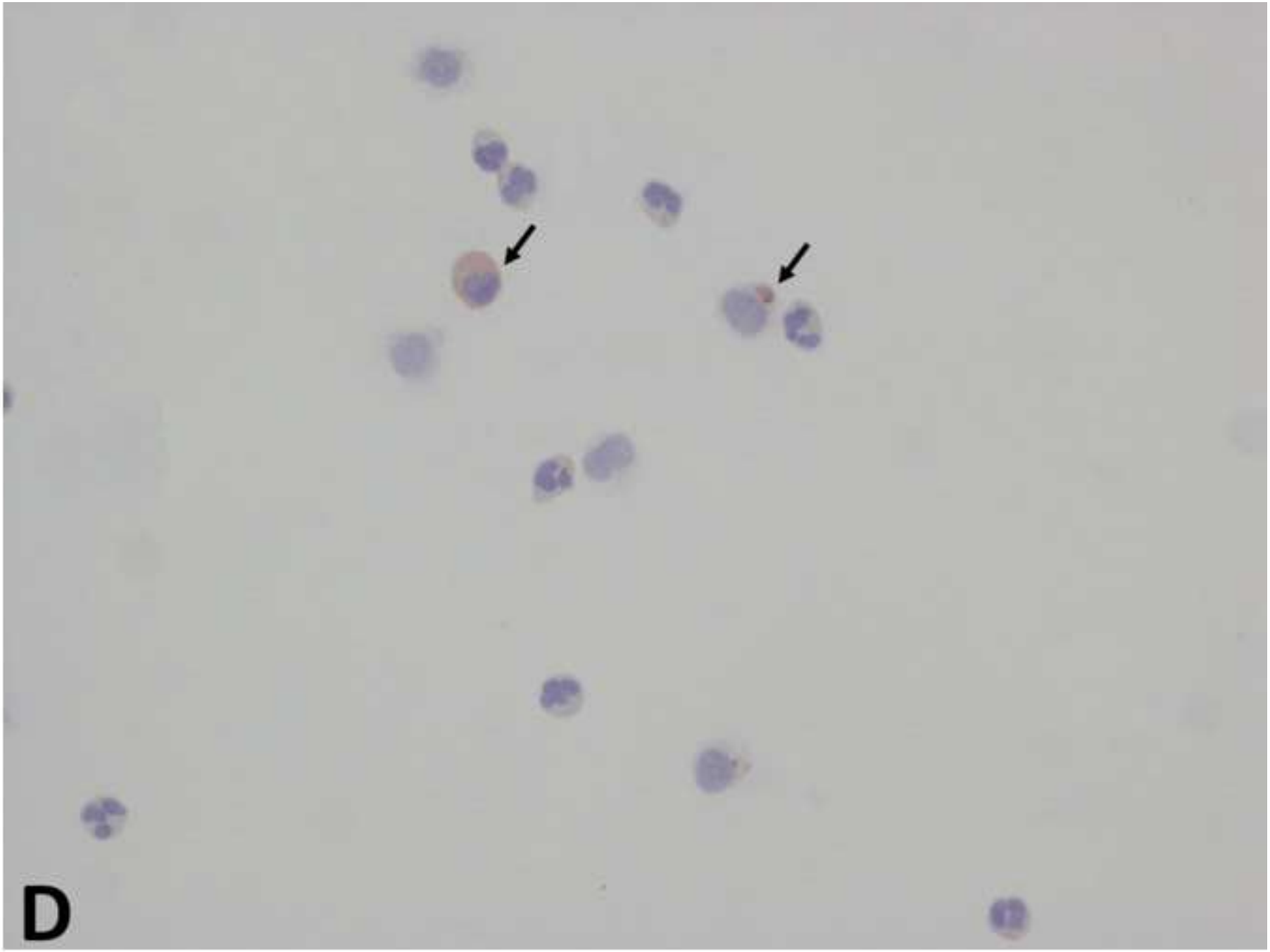

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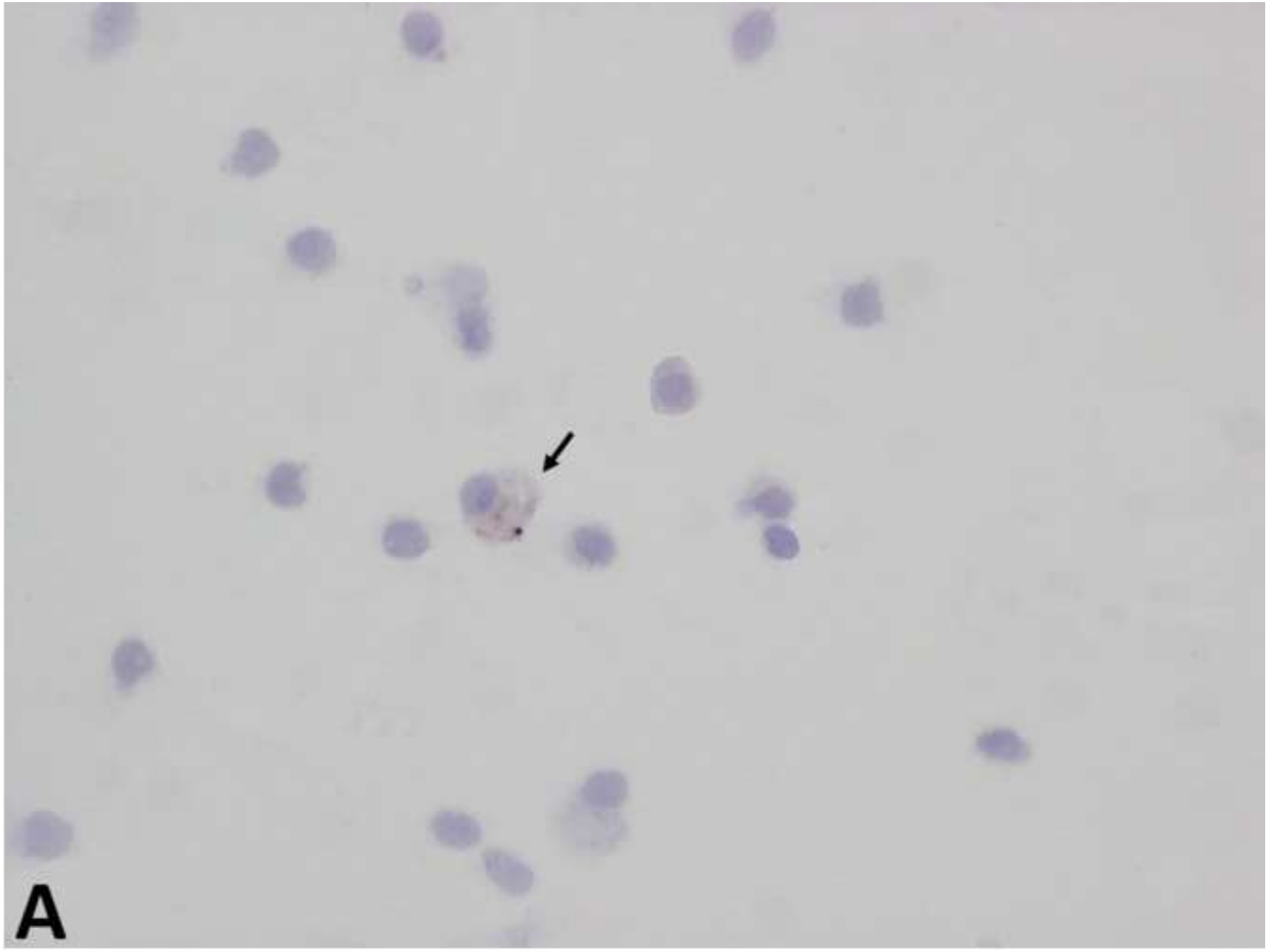

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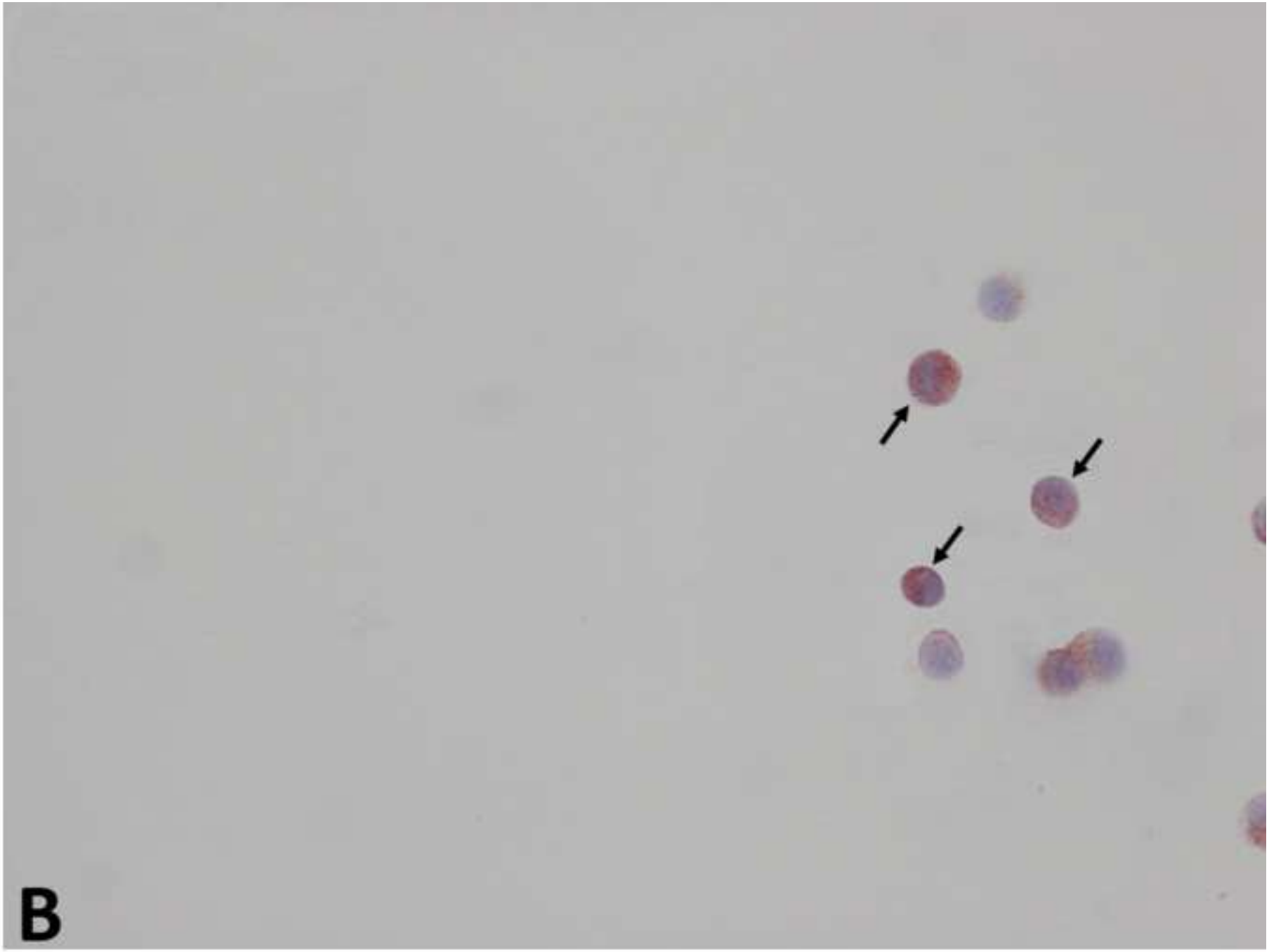

Figure

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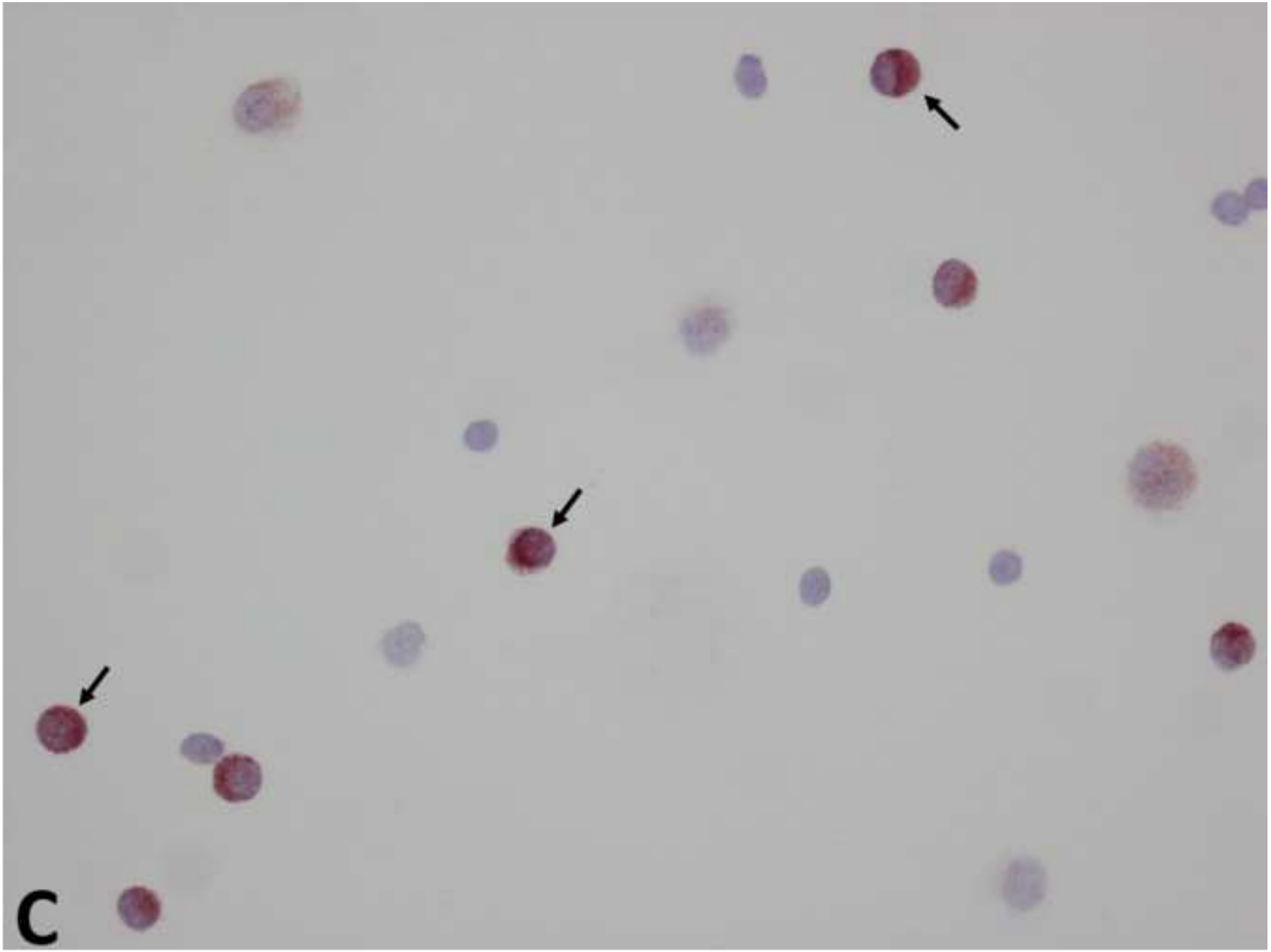

Figure

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