

1 THE CYTOLOGY OF RESTING AND REACTIVE NK CELLS OF CHICKENS

4 ABSTRACT

5 Lymphoid cells are composed of 3 distinct series; those developing in the bursa of Fabricius are B-cells,
6 and those of the thymus are T-cells. The former concern humoral immunity and the latter cell-mediated
7 immunity (CMI). A third lineage, the natural killer cells (**NK**) develop independently. A study of chicken
8 blood films stained with Wright-Giemsa obtained from a Marek's disease project provided an
9 opportunity to study NK cells. These lymphoid cells were recognized because of their characteristic
10 cytoplasmic granules. NK size varied from small cells similar in radius (R_C) to resting T-cells ($R_C \sim 3 \mu\text{m}$) to
11 larger types ($R_C \sim 5 \mu\text{m}$). Granules also varied in size and number. NKs were often in fields also
12 containing bacteria either free swimming or attached to RBC or other cells. The total white cell count
13 (**TWBC**) of the study population ranged from leukopenia (very low) to leukemoid reaction (very high)
14 levels (TWBC 5K/ μL - 200K/ μL ; ave. $\sim 50\text{K}/\mu\text{L}$). NKs comprised 380/45134 ($\sim 1.7\%$) of all leukocytes. The
15 study population heterophil/lymphocyte ratio (**H/L**) ave. (~ 0.4) appears to give a false impression of a
16 low stress status. It is concluded as would be the case for plasmacytes or other forms of reactive cells,
17 multiple forms of NK cells appearing in a hemogram indicate inflammation or infection. Therefore, the
18 simple H/L ratio is an inappropriate stress indicator when accompanied by a high TWBC.

19
20 Key words: NK cell, lymphocyte, stress, H/L ratio

21 **Abbreviations:** cm cell membrane, CMI cell-mediated immunity, CD cluster of differentiation, BCR B cell
22 receptor, TCR T cell receptor, IFN interferon, H/L heterophil/lymphocyte ratio, L_S/L_M small and medium
23 lymphoid cells, NK natural killer cell, L_S/NK small NK cell, L_M/NK medium and large NK cell, MD Marek's
24 disease, MDV Marek's disease virus, R_C cell radius, PR ploidy ratio,

25 INTRODUCTION

26 Lymphoid cells are composed of 3 distinct series; those developing in the bursa of Fabricius are B-cells,
27 and those of the thymus are T-cells. The former concern humoral immunity and the latter are concerned
28 with cell-mediated immunity (CMI). A third lineage, the natural killer cells (NK) develop independently
29 (Lam and Linna, 1979). Chicken NK cells of the spleen and intestinal epithelium express CD8 while
30 lacking BCR and TCR. NK cells of chicken blood express Fc receptors, CD3, low levels of CD4, and are
31 capable of releasing IFN γ (Neulen, et al, 2015). Functionally, NK cells are innate immune cells capable of
32 destroying virally infected or transformed cells (Straub, et al 2013).

33 Marek's disease, an important cause of morbidity and mortality of chickens, is caused by infection with a
34 highly contagious alphaherpesvirus (MDV). T-cell tumors are a hallmark of MDV infection. Resistance
35 to MD can depend on NK cell activity (Sharma, 1981). Recently it has been shown *in vitro* that NK cells
36 are readily infected by MDV (Bertzbach, et al. 2019). The same authors demonstrated an increased
37 expression of CD107 of a small portion of MDV infected NK cells and hypothesized that successful
38 infection of NK cells is a consequence of their activation.

39 Thus, NK cells, like other lymphocytes, may become activated under an appropriate stimulus. For
40 example, members of the B-cell derived plasmacyte series have been shown to display an array of forms
41 described as “reactive plasmacytosis” (Cotter, 2022a). These were found in the blood and bone marrow
42 of lame ducklings from which *Streptococcus* and *E. coli* were isolated.

43 Here the purpose is to describe an array of NK cells in experimental chickens from a study of Marek’s
44 disease. Blood smears from four groups, controls, vaccinated, challenged, and both vaccinated and
45 challenged were studied. For the purpose of this description, NK cells are defined as lymphocytes
46 containing cytoplasmic azurophilic granules detected with light microscopy in Wright-Giemsa stained
47 samples. The presumption is that azurophilic granules correspond to the electron-dense granules of NK
48 cells detected by electron microscopy (Gobel, et al 2015). Active NK cells are recognized by cytology as
49 as well as CD expression. When activated NK cells are in hemograms they indicate stress, inflammation,
50 and disease.

51 MATERIALS AND METHODS

52 *Experimental Chickens*

53 The specific pathogen-free chickens (**SPF**) in this study were from two highly inbred lines of
54 chickens. Line 6.3 is MD-resistant and line 7.2 is MD-susceptible. The study chickens were from
55 unvaccinated breeder hens and carried no maternal antibodies to MDV or herpesvirus of turkeys.

57 *Study Location*

58
59 The chicks were housed in modified Horsfall-Bauer isolation units located at the ADOL (USDA)
60 Laboratory, 3606 E Mt Hope Rd., East Lansing, MI 48823 (USA).

62 *Blood Samples*

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64 Wing-vein blood was drawn at day of age 19, 24, 34, and 49. Vaccination (Rispens) was on day 7
65 and MD challenge (rMd5, a virulent strain) was on day 14. Slides were prepared immediately, air-dried,
66 and post-fixed in 100% MeOH. Staining was with Wright-Giemsa.

68 *Standard Differential Count*

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70 Two counts of 200 leukocytes/slide were sorted using criteria as described by Lucas and Jamroz
71 (1961) and Cotter (2015a). The designation “typical heterophil” (HT) as used here was assigned to the
72 most frequent type seen in earlier studies (Cotter, 2015a, b, c). Classic heterophils (HC) resemble those
73 most often illustrated in the literature. Rare variant heterophils (HV) are distinct from both HT and HV,
74 Cotter and Heller (2016). Ls and Lm are small and medium sized lymphocytes. Total white blood counts
75 (TWBC) were determined by a modified microscopic method as described in Campbell and Ellis (p.26,
76 2007). Standard Differential Count (SDC) was determined at 40x magnification.

78 *Heterophil/Lymphocyte Ratios (H/L 1; H/L 2) and Total White Blood Count (TWBC)*

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80 Two counts of 200 leukocytes gave the total white count (TWBC) by the method of Campbell
81 and Ellis (2007) and 2 heterophil/- lymphocyte ratios. $H/L\ 1 = (HT + HC + HV) / Ls$; and $H/L\ 2 = (HT + HC +$
82 $HV) / (Ls + Lm)$.

83 84 *Light Microscopy and Photomicrographs*

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86 Olympus CX-41(Olympus America, Center Valley, PA 18034-0610) equipped with Plan N 40x,
87 0.65 numerical aperture dry, and Plan N, 1.25 numerical aperture 100x oil objectives. All images were
88 captured at 100x with an Infinity-2 1.4-megapixel charge-coupled device Universal Serial Bus 2.0
89 Camera, and processed with Infinity Analyze software that was also used for pixelation (Release 6.5)
90 (Lumenera, Inc., Ottawa, ON, Canada)

91 92 *Welfare*

93
94 These experiments were approved in accordance with the guidelines set forth by the ADOL
95 Institutional Animal Care and Use Committee and the Guidelines for Care and Use of Laboratory Animals
96 published by the Institute for Laboratory Animal Research.

97 98 RESULTS

99 The strategy for presentation will begin with examples of small and medium size lymphocytes as
100 companions to NK cells. Some cytological features of NK cells are shown with the aid of differential
101 pixelation. Descriptions of reactive NK cells will follow. The SDCs for the study figures are given as % in
102 Table 1. **The emphasis is on NK cytology rather than treatment/group effects.**

103 *NK Cells of Non-Vaccinated Non-Challenged (NV/NC) Controls*

104 Insert figure 1 A here; figure 1B here

105 **Figure 1A. Examples of NK cells in control chickens. Additional descriptions are in the text.**

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107 **Figure 1B. Enhancement of the cytoplasmic features of a reactive Lm/NK of panel A by differential**
108 **pixelation.**

109
110 Figure 1A. shows 2 examples of NK cells in a NV/NC control chicken of the susceptible line (7.2) obtained
111 at 49d. The small cell (*Ls/NK) at the lower left is likely a resting NK; its few small azurophilic granules
112 are restricted to the cytoplasm immediately internal to the cm. The dimensions [$A_C \sim 48\ \mu m^2$; $A_N \sim 30$
113 μm^2] represent diploid conditions [PR 1.0], and the N/C of 0.6 represents an early reactive cell. The
114 cytoplasm located immediately outside of the slightly indented (reniform) nucleus is clearer than the
115 cytoplasm near the cm edge suggesting a Hof (Golgi). The patchy chromatin is typical of an NK at this
116 stage of activation. The larger cell (Lm/NK) at the top right [$A_C \sim 67\ \mu m^2$] is at a further reactive stage,
117 and the slightly larger eccentric nucleus [$A_N \sim 34\ \mu m^2$] is also diploid [PR 1.1]. The greater number and
118 size of the cytoplasmic granules is clear evidence of heightened reactivity. As is the further elaboration
119 of the ER. The chromatin is patchy, and a few azurophilic granules are located directly over the nucleus.
120 Free swimming and cell-associated bacteria (CAB) are encircled and a *Hemomyces avium* (Ha) shard
121 (mycelium fragment) (Cotter, 2015c, 2022c) is located at the arrow. Although a study control, this

122 chicken appears to be infected with both bacteria and a fungus. The smear in which these cells were
123 found was “thin” an indication of rheological changes in plasma were in progress when the blood was
124 drawn. Thus, the TWBC was estimated at 37K/ μ L and H/L ratios (H/L 1 0.23; H/L 2 0.19) were affected by
125 plasma quality.

126 Cytoplasmic features of the small *Ls/NK cell of Figure 1A are enhanced by differential
127 pixelation in Figure 1B. Cytoplasmic and nuclear granule locations are enhanced with fuchsia pixels
128 (panel B; vacuole locations are in yellow (panel C). The reactive Lm/NK is enlarged without pixelation in
129 Panel C, and its vacuoles’ locations are enhanced by red pixels in panel D.

130 A Lm/NK found in an NV/NC of the susceptible line (7.2) at 49d cell is in Figure 2A. It has an
131 eccentric nucleus and characteristic azure cytoplasmic granules and clear vacuoles. A Ls in the same
132 field is likely a B-cell and is diploid because of the dimensions [$A_C \sim 53 \mu\text{m}^2$; $A_N \sim 41 \mu\text{m}^2$] and ploidy [PR
133 1.3]. The patchy chromatin arrangement of the Ls contrasts with the finer chromatin of the Lm/NK
134 whose dimensions [$A_C \sim 113 \mu\text{m}^2$; $A_N \sim 55 \mu\text{m}^2$] and ploidy [PR 1.8] represent a > diploid condition (2N+).
135 About 20 large granules, ~10 small granules, and ~6 clear vacuoles are distributed throughout the
136 cytoplasm of the Lm/NK, and a few are located directly over the nucleus. A group of 3 bacteria and
137 separate encapsulated bacteria are located by arrows. The TWBC of this sample, at 50K/ μ L, indicates
138 (mild) leukocytosis. In contrast the H/L 1 (~0.2) and H/L 2 (~0.15) suggest homeostasis.

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Insert Figure 2A, B here

142 Figure 2A. A reactive NK in a field with a small lymphocyte (Ls) in a control 49 d MD susceptible chick.

143 Panel B. A large reactive NK with small faint cytoplasmic granules from the MD resistant line at 24 d.

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147 A Lm/NK with an eccentric nucleus and condensed patchy chromatin is from a resistant line (6.3)
148 at 27d is in Figure 2B. The cytoplasm contains many clear vacuoles and others with a faint pink
149 substance; possibly incomplete or degraded perforin. A few magenta granules are at the cm edge
150 (arrow). A group of 3 encapsulated bacteria is circled. Several of the RBCs are not fully hemoglobinized
151 and are irregularly shaped (pRBC). A solitary thrombocyte (Th) containing 6 specific granules a reactive
152 cell (Cotter, 2022c).

153

154 NK cells of NV/NC resistant chickens (Line 6.3) at 24d are in Figure 3. A Lm/NK cell with an
155 eccentric nucleus and characteristic azure cytoplasmic granules and clear vacuoles is in a field with a Ls;
156 likely a B-cell [$A_C \sim 32 \mu\text{m}^2$; $A_N \sim 28 \mu\text{m}^2$; PR 1.0]. The patchy chromatin arrangement of the Ls contrasts
157 with the finer chromatin of the Lm/NK whose size [$A_C \sim 66 \mu\text{m}^2$; $A_N \sim 36 \mu\text{m}^2$] and ploidy ratio [PR 1.3]
158 represent a diploid condition (2N). About 20 large granules, ~ 10 small granules, and ~6 clear vacuoles
159 are distributed throughout the cytoplasm of the Lm/NK, and a few are located directly over the nucleus.
160 A group of 3 bacteria and separate encapsulated bacteria are located by arrows. The TWBC of this
161 sample, at 50K/ μ L, indicates (mild) leukocytosis.

162

163 Enhancement of the cytoplasmic features of a reactive Lm/NK of panel A by differential
164 pixelation is in Figure 3B and C. Cytoplasmic and nuclear granule locations are enhanced with fuchsia
165 pixels (panel B) vacuole locations are in yellow pixels (panel C).

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Insert figure 3 here

Figure 3. NK cells of NV/NC resistant chickens (Line 6.3) at 24d.

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171 An array of NK variants of the MD resistant line (6.3) is in Figure 4. Panel A. A large granular
172 Lm/NK has a half-moon eccentric nucleus with patchy chromatin. It contains many magenta granules
173 and has clear cytoplasmic vacuoles. The ruffled cm (zeiosis) indicates heightened reactivity. Its overall
174 dimensions suggest polyploidy [$A_C \sim 99\mu\text{m}^2$, $A_N \sim 43\mu\text{m}^2$, N/C 0.4, PR 1.7]. Panel B. A small Ls/NK with a
175 patchy nucleus [$A_C \sim 67\mu\text{m}^2$, $A_N \sim 41\mu\text{m}^2$, N/C 0.6, PR 1.3] is diploid at an early reactive state. A Ls (T-cell)
176 is at the left of Panel C whose dimensions [$A_C \sim 36\mu\text{m}^2$, $A_N \sim 30\mu\text{m}^2$, N/C 0.8] are of a resting diploid cell.
177 3 NK cells at various reactive states are in panel D. The NK cells appear somewhat distorted possibly due
178 to damage from toxins released by many small encapsulated bacteria located throughout the field.
179 Some bacteria have formed clusters (arrows).

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Insert Figure 4 here

182 Figure 4. Examples of NK cells from MD the resistant (Line 6.3) at 5d post MD challenge (Panels A, B) and
183 34 d post-challenge (Panels C, D).

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186 A Lm/NK with an eccentric nucleus with patchy chromatin is in Figure 5, Panel A. The cytoplasm
187 contains granules and clear vacuoles. The dimensions [$A_C \sim 121\mu\text{m}^2$, $A_N \sim 64\mu\text{m}^2$, N/C 0.5, PR 2.1]
188 indicate a tetraploid cell. A pair of toxic heterophils (HT) and reactive thrombocytes (Th) are at the lower
189 right (Cotter, 2022). A smaller Ls/NK cell in a field with bacteria (arrows) is in panel B. The dimensions
190 [$A_C \sim 69\mu\text{m}^2$, $A_N \sim 34\mu\text{m}^2$, N/C 0.5, PR 1.1] indicate a diploid reactive type. A giant NK cell with a reniform
191 nucleus is surrounded by cytoplasm differentiated into an endoplasmic zone immediately surrounding
192 the nucleus and a deeper stained ectoplasmic zone. An accumulation of clear vacuoles at the edge of
193 the nucleus suggests the formation of a Hof (Golgi) an indication of a secretory potential. Magenta
194 granules are also scattered throughout the cytoplasm. The giant cell dimensions [$A_C \sim 113\mu\text{m}^2$, $A_N \sim 43$
195 μm^2 , N/C 0.4, PR 1.7] indicate a tetraploid. A solitary Lm/NK with an irregular cm is in a field containing
196 many small encapsulated bacteria enhanced by fuchsia pixelation. Some of the bacteria have attached
197 to the deep folds of the cm also fuchsia enhanced. The giant cell dimensions [$A_C \sim 85\mu\text{m}^2$, $A_N \sim 34\mu\text{m}^2$,
198 N/C 0.5, PR 1.1] indicate a diploid. A few bacteria have attached to cytoplasmic streams emanating from
199 the cm (arrows).

200
201

Insert Figure 5 here

202 Figure 5. Examples of NK cells from MD susceptible from a single chick of line 7.2 at 22d post MD
203 challenge (A, B).

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206 *Hemomyces avium* (Ha)

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209 The fungus *Hemomyces avium* (Ha) was initially described in a blood smear from a laying hen
210 (LSL 56 wk) housed in an enriched cage whose TWBC was in the leukemoid reaction range ($\sim 200 \text{ K}/\mu\text{L}$)
211 with low (~ 0.25) H/L ratios (Cotter, 2015c). Bacteria and atypical leukocytes were also observed in the
212 same sample. A more detailed description of the microscopic characteristics of Ha appears in (Cotter

212 2021). This fungus was detected at all sample dates and both chicken lines in blood smears from the
213 current study. 3 (Line 6.3) examples seen in blood and 1 bone marrow observation (Line 7.2) are in
214 Figure 6. Bacteria also were commonly observed in the same fields as Ha.

215

216 Insert Figure 6 here

217 Figure 6. Examples of the fungus *Hemomyces avium* (Ha) in 3 blood films (Panels A, B, C) and 1 bone
218 marrow sample from the current study (D). Bacteria are located by arrows.

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DISCUSSION

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223 Control of Marek's disease remains an important concern to the poultry industry despite the
224 availability of vaccines for nearly a half-century. Certain chickens are resistant others are susceptible as a
225 result of their peculiar genetics. It has long been established that the expression of genes at the major
226 histocompatibility locus (B-complex) contributes to differential resistance (Bacon and Witter, 1993).
227 Early *in vitro* studies implicated the role of NK cells in MD resistance (Sharma and Okazaki, 1981). NK
228 cells are known for anti-viral and anti-tumor activity by directly killing target cells through the release of
229 several cytokines. Therefore, the study of NK cell features should be of interest to Marek's disease
230 investigators and those concerned with other infectious diseases. The occurrence of NK cells along with
231 bacteria (Figures 1, 2, 4, 5) and fungi (Figure 6) reinforces this notion.

232 Here the variety of NK cells is demonstrated. NK forms differ in size and cytoplasmic condition.
233 It is presumed that some of the variations described here represent the transition from small resting
234 cells with few granules to larger reactive types with more granules and vacuoles. Some NKs may be
235 phagocytic (Figure 5D) but this possibility needs further study

236 It is also demonstrated that the reniform nuclear shape and patchy chromatin of some NK's
237 coupled with granulation aids in their differentiation from other lymphocytes, especially plasmacytes.
238 Even Mott cells, atypical plasmacytes, whose cytoplasm is filled with immune globulin (Ig) containing
239 vacuoles should not be confused with NK's (Cotter, 2022; Cotter and Bakst, 2017).

240 NKs are typically rare in the blood (Straub et al. 2013) here they represented ~ 1.7% of all
241 leukocytes in this study (380/~45,000). They were as frequent in control chickens as in the other groups.
242 A maximum of 19 NKs were in 1 blood smear but no NKs were found in ~ 46% (127/277) of the SDCs
243 obviating the ability to associate NK forms with individual treatments. Therefore, the focus is placed on
244 a description of variation in NK cytology.

245 The occurrence of the fungus *Hemomyces avium* (Ha) is a remarkable feature of these
246 samples. Ha in blood and bone marrow is a clear sign of immunosuppression. Surprisingly Ha and
247 bacteria occurred in control samples as well as vaccinated or challenged chickens. This observation
248 suggests the possibility of superinfection with an unidentified virus. The nature of the responsible agent
249 remains unknown.

250 An indirect objective of this study is to suggest that when diverse NK forms occur in a hemogram
251 they should be given similar weight as other atypia in deciding the appropriateness of a simple H/L ratio
252 as a stress determinant (Cotter, 2015a, 2022c). This aspect is clearly indicated when reactive NKs occur
253 simultaneously with low H/Ls. It is also obvious that when low H/Ls are accompanied by high TWBC the
254 stress question had already been decided.

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CONCLUSION

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258 It is concluded that as would be the case for plasmacytes or other forms of reactive cells, multiple types
259 of NK cells appearing in a hemogram indicate inflammation. They are likely indicators of viral infection
260 and coincide with the fungus *Hemomyces avium*. In such circumstances, the simple H/L ratio is an
261 inappropriate stress indicator when accompanied by a high TWBC.

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280 Figure Legends:
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282 Figure 1A. Examples of NK cells in control chickens. Additional descriptions are in the text.
283
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299 marrow sample from the current study (D). Bacteria are located by arrows.

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343

344 DECLARATION

345 The author declares that the research was conducted in the absence of any commercial or financial
346 relationships that could be construed as a potential conflict of interest

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Table 1. Standard differential counts (% SDC) based on 2 x 200 total cells for samples of Figures 1- 6

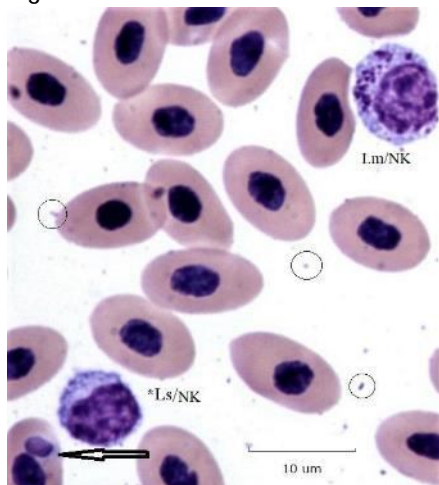
Figure	HT ¹	HV	HC	Ls	Lm	NK	Bst	Mn	Ba	Eo	TWBC(K)	H/L 1	H/L 2	ΔH/L
1	6.8	1.3	10.6	62.8	13.0	0.2	0.4	0.0	4.8	0.0	38.0	0.3	0.3	0.1
2	6.1	6.1	0.0	62.3	19.1	0.2	0.0	0.2	5.9	0.0	50.0	0.2	0.2	0.0
3	5.8	0.2	1.2	74.3	10.8	0.0	0.2	0.7	6.7	0.0	20.0	0.1	0.1	0.0
4A	5.1	0.0	1.9	71.0	18.1	0.0	0.0	0.5	3.4	0.0	15.0	0.1	0.1	0.0
4C	14.3	0.0	0.5	51.7	25.1	0.0	0.0	0.0	8.4	0.0	20.0	0.3	0.2	0.1
4D	39.4	0.2	1.2	40.8	15.5	0.0	1.0	0.2	1.7	0.0	40.0	1.1	0.7	0.3
5A, B	22.7	1.2	0.2	52.7	16.9	1.7	1.0	0.0	3.6	0.0	73.0	0.5	0.3	0.1
5C	9.0	0.0	19.1	54.5	13.1	1.3	1.5	0.2	1.3	0.0	100.0	0.5	0.4	0.1
5D	22.7	1.2	0.2	52.7	16.9	1.7	1.0	0.0	3.6	0.0	76.0	0.5	0.4	0.1
6B	17.5	0.5	0.0	65.5	12.1	0.0	1.5	1.0	1.9	0.0	40.0	0.3	0.2	0.0
6C	5.2	2.0	0.5	77.8	7.9	0.2	0.2	0.5	5.7	0.0	40.0	0.3	0.2	0.0
4B, 6A	No SDC													
6D	Bone marrow													

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¹Abbreviations: Cells: H, heterophil (HC, classic, HV, variant, HT, typical) Ls small lymphocyte ~6 μm diameter, Lm medium lymphocyte ~8 μm diameter, Mn, monocyte, NK natural killer cell, Bst granuloblast, Ba, basophil, Eo, eosinophil. TWBC(K), total white blood cells per cubic mL in thousands (K). H/L 1 = (HC + HT + HV) / Ls; H/L 2 = (HC + HT + HV) / (Ls + Lm); ΔH/L= H/L1 – H/L2.

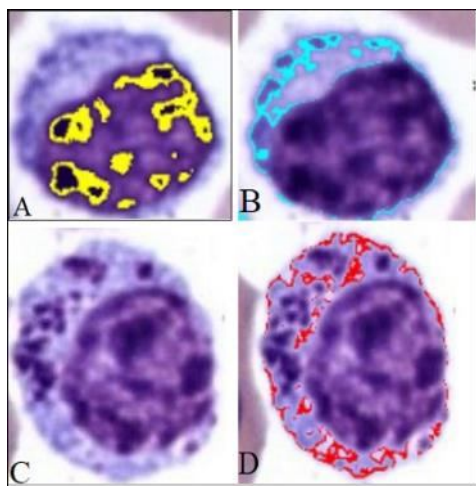
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Figure 1 A



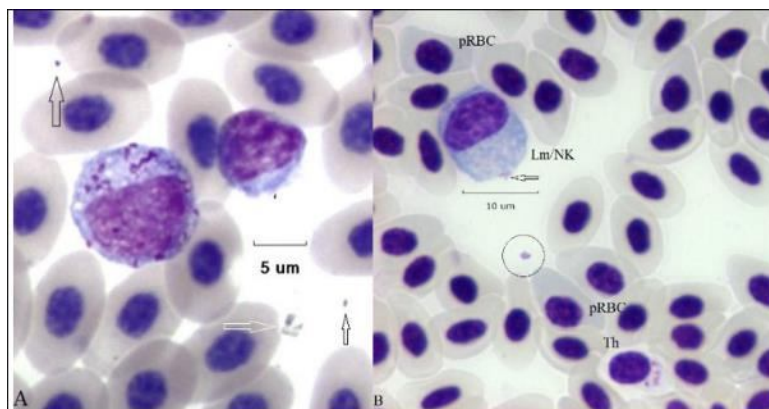
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Figure 1 B



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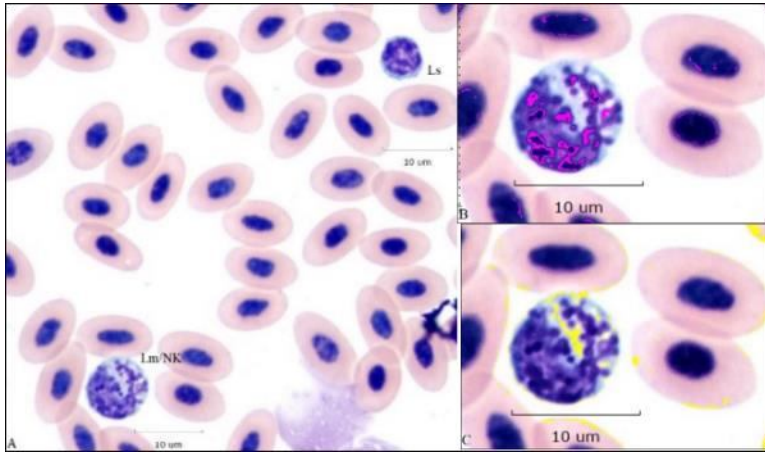
Figure 2A, B



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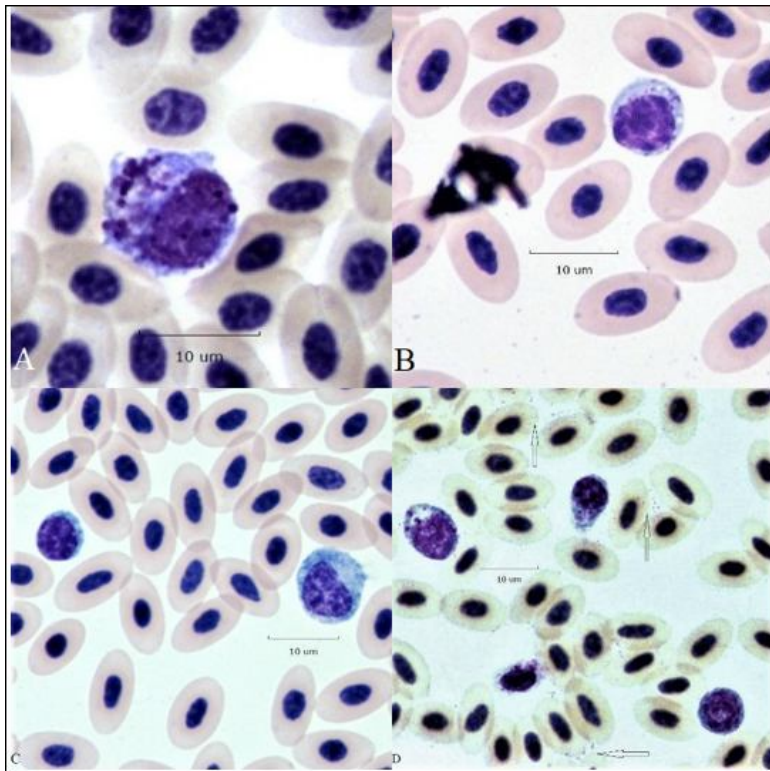
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Figure 3.



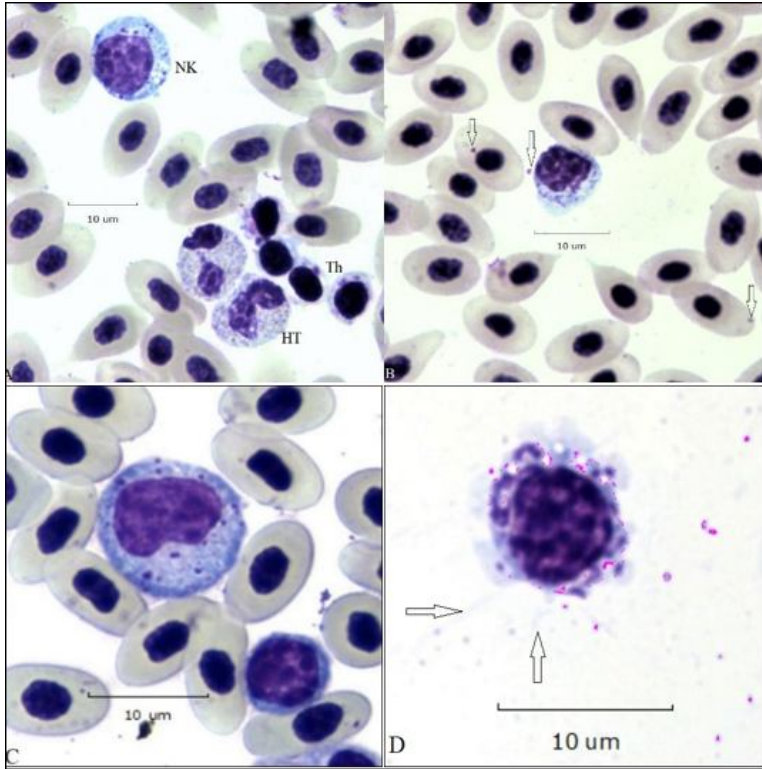
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Figure 4.



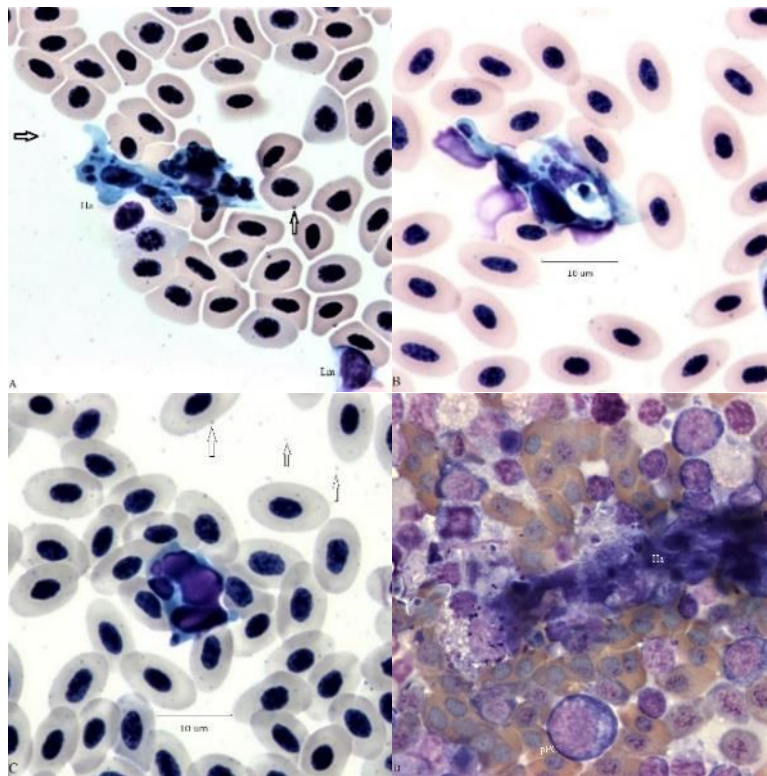
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Figure 5



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Figure 6



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