Flow cytometry is a standard tool to detect protein expression, DNA content, and other cell physiological parameters at single cell level (1–3). Since flow cytometers are capable of processing single cell suspensions, they are excellent instruments to study fluid tissue samples like blood or bone marrow aspirates. This makes flow cytometry a very useful tool in hematology and immunology.

In human blood samples, the cells of interest are usually the leukocytes, therefore the largest portion of the formed elements, the anucleated erythrocytes, and platelets, are undesired in the samples. To remove these cells from the samples, often hypotonic lysis is used in routine hematology. Afterwards, the lysed sample can be measured without further steps (lyse-no wash protocols) or can be washed and centrifuged (lyse-wash protocols). Both methods have advantages and disadvantages. In lyse-no wash samples the cell loss is minimalized, but due to the lack of the washing the background fluorescence can be high, which decreases the resolution of the method. Furthermore, the final volume of sample is usually large, which makes the acquisition longer. On the contrary, lyse-wash samples are smaller in volume, resulting faster measurement, and due to the washing the resolution can be better, but it can increase the cell loss (4). Rarely no lyse-no wash protocols are also used, but these are less common in routine diagnostics (5).

Observing an average blood sample that was analyzed with routine flow cytometry (Fig. 1), it is clear that most of the different leukocyte subsets can be easily separated. What is more, very rare events, like circulating progenitor cells (Fig. 1B and D) can be identified. However, to achieve this, we removed more than the 99.9% of the formed elements with a hypotonic lysis and a washing step. Without the lysis steps the rare events could not have been resolved, since the instrument was not able to record sufficient number of leukocytes together with red blood cells. Other methods, for instance microscopic investigation are also unusable due to the lack of morphological differences between leukocytes and the low number of numerable white blood cells (Fig. 1G).

In the case of several human hematologic, lymphatic or immunologic diseases the examination of the blood cannot provide enough evidence for proper diagnosis. In these cases, samples of other tissues, like bone marrow, lymph nodes or other fluid samples (ascites or other exsudatum, cerebrospinal fluid) should be investigated. Since single cell suspensions can be prepared relatively easily from these samples and tissues (6), they are also suitable for flow cytometry investigation. Several lymphomas not necessarily appear in the blood, but can be identified in a lymph node sample (Fig. 2A–F). Needless to say that in human hematology and immunology, the results of flow cytometric measurements should be evaluated together with the results of several other methods. These include histology, immunohistochemistry, cytogenetics, molecular genetics, and biochemical tests. Besides, several diseases cannot be identified by flow cytometry, since the pathologic cells have no specific immunophenotype, or they cannot be extracted from the tissue samples (Fig. 2G–K).

Flow cytometry can be used not only in human science and medicine. There are several published methods aimed to investigate the blood and tissue samples of other mammals.
These methods concern mainly the most widely used laboratory animals, especially mice, rats, and other rodents (7), but there are many methods for primate, canine, and feline samples. The preparation of their samples is similar to the human ones, since they similarly have anucleated blood cells with increased osmotic sensitivity (8–10). Although the preparation of blood and other hematological or immunological samples of the mammals can be similar, and hypotonic lysis is applicable in almost all cases where it is required, the situation can be completely different with samples from other vertebrate classes. For instance nucleated red blood cells can make the blood samples of birds, reptiles, and amphibians more resistant to hypotonic lysis (11,12). There are several important species among the mentioned groups, but chickens deserve special attention due to their role as model animal in science and their importance in economy. Investigation of their leukocytes can provide useful hematological and immunological information even in research and veterinary medicine. Since in chicken blood the leukocytes are also present in small quantities, flow cytometric analysis of their white blood cell subsets without lysis is challenging (13). On the other hand, determining the percentage or the accurate concentration of the different leukocytes of the birds with other methods is also problematic compared to the ones

Figure 1. Investigation of normal human peripheral blood with flow cytometry and microscopy. The flow cytometric measurement was performed using a two tubes, nine color per tube, lyse-wash protocol, and 100,000 events were recorded. The method is suitable to resolve the main leukocyte subsets based on side scattered light and CD45 expression (A) and for further characterization based on different antigen expressions: Mature (CD14+) and premature (CD14−) monocytes (B), circulating blasts (CD117+, CD34+) (D), B (CD19+) and T (CD3+) lymphocytes (E), CD8+ and CD8− NK cells (CD56+) (H), and basophils (CD33+, HLA-DR− cells in lymphocyte gate) (I). Presence of near equal number of Igκ and Igλ light chain expressing B-lymphocytes, which exclude the clonal restriction (C) also the presence of the CD4+ and CD8+ T-lymphocytes (F). In contrast to these observations only a few leukocytes can be identified in the view field (G) by microscopic investigation. On the microphotograph two neutrophils (white arrows), a monocyte (black arrow) and a lymphocyte (asterisk) can be seen. May Grünwald–Giemsa staining, bar: 20 μm.

Flow Cytometry of Hematological and Immunological Samples
Figure 2. Lymph node samples containing pathologic cells that can (A–F) or cannot be identified (G–K) with flow cytometry. Sample from an excised lymph node (A), which contains normal B-lymphocytes and T-lymphocytes (B and C) beside a huge amount of aberrant cells (indicated with black). These cells express CD5 and CD8, but no CD56, so they are not NK cells (D and F). Intracellular expression of CD3 that is not present on the cell surface proves that the cells are aberrant T-lymphocytes. Based on the results of the flow cytometry, histology, and immunohistochemistry, the patient was diagnosed with a peripheral T-cell lymphoma. In another lymph node sample (G), the T and B lymphocytes show neither clonal restriction nor aberrant phenotype in the flow cytometry experiment (H–J). In contrast to flow cytometric measurements, histology revealed the presence of Reed-Sternberg and Hodgkin cells (K, white arrows). The patient was diagnosed with classic Hodgkin lymphoma. Hematoxylin–eosin staining, bar: 20 μm.
which can be used with human samples. Automated hematological counters that differentiate blood cells based on their electrical impedance showed increased inaccuracy, while manual methods, like counting in hemocytometer are labor-intensive and affected by human factors (14). Thus, flow cytometric methods, despite its difficulties seems ideal to determine the ratios of the avian leukocyte subsets. In case of avian, especially chicken blood, several staining protocols have been published. In these, unlike the mammal protocols, no lysing step is used and density-based methods or dedicated antibodies are responsible for separating the erythrocytes and thrombocytes from white blood cells (15,16). Considering all mentioned methods, we can say the separation and analysis of lymphocyte subsets of chicken blood can be considered solved.

However, similarly to several human hematologic, lymphatic, or immunologic diseases, there are avian conditions where investigation of the blood cannot provide enough information about the immunological and hematological status of the animals. In contrast with mammals, avian species including hens, lack encapsulated lymph nodes, so their immune processes take place mostly in their spleen and in specialized lymphatic tissues among their gastrointestinal tract. Since preparation of single cell suspensions of these tissues are also solved, investigation of the lymphocyte subsets with flow cytometry may provide valuable extra information.

In this issue, on pages 289-300, Hofmann and Schmucker published a staining method, which is suitable to determine the number and ratio of several lymphocyte subsets from chicken spleen and cecal tonsils. Their method is able to differentially exclude the red blood cells, thrombocytes, and other nonleukocytes. Then, the leukocytes can be further divided into monocytes, its derived cells, and different lymphocyte subsets. The method can be well used to assess the immune status and the number of the common leukocyte and lymphocyte subsets of the chickens. On the other hand, considering that the measurement requires the slaughter of the animals, the flow cytometric investigation should be associated with other conventional or novel methods, like histology, immunohistochemistry or imaging cytometry to associate the phenotypic information with structure (17).

Taken together, the antibody panel presented in the paper of Hofmann and Schmucker can make a valuable contribution to the avian research and veterinary medicine, and can be the basis of further improvements.

As Hofmann and Schmucker also stated in their paper, the number of the commercially available fluorescently labeled monoclonal antibodies is limited and this sets a barrier to the expansion of the antibody panels. This is true not only for the avian but to other nonmammal monoclonal antibodies as well. Hopefully in the future the supply of these antibodies will increase, which would make it possible to design new, more versatile panels. By that, these panels could take advantage of the newer flow cytometers, which offer over 10 fluorescent channels and facilitate the discrimination of leukocyte subsets better. This way, these improved panels will help us to understand the hematological and immune processes and diseases better in several different species.

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AUTHOR CONTRIBUTIONS

Gábor Szalóki: Conceptualization; visualization; writing-original draft; writing-review and editing. Ágnes Czeti: Writing-review and editing.

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