SYSMEX EDUCATIONAL ENHANCEMENT
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SEED HAEMATOLOGY

Lymphocyte differentiation update – helpful or dispensable?

One of the biggest challenges in cytology is the differentiation of lymphocytes in blood films because they appear in many varieties and diseases are manifold. Aside from the experience of the examiner, the basis for qualified cytological blood findings is the availability of a standardised nomenclature, especially in cases where there are lymphocyte alterations. In order to have a uniform lymphocyte differentiation in Germany and harmonise it with the European consensus, the Working Committee 'Laboratory' of the DGHO (Deutsche Gesellschaft für Hämatologie und Onkologie, or German Association for Haematology and Oncology) has published and recommended a revised lymphocyte classification in 2011 [3].

The essential results and modifications from this update will be presented below in a compact form. The intention of this article is to make the update accessible and understandable to as many lab technologists in the field of cytology as possible. In addition, it provides experience from the practical application of the update and therefore helps to draw first conclusions.

Lymphocyte development

Lymphocytes can be classified into B, T and NK (natural killer) cells [4, 6]. They originate from the lymphoid stem cell, which forms the basis for all following development stages. Lymphocyte maturation takes place in the primary lymphoid organs (T cells = thymus, B cells = bone marrow) [1, 5]. Effector cells, which ensure the adapted immunity and can be identified by immunophenotyping methods using specific antigen expression patterns, have been activated in the secondary lymphoid tissues (lymph nodes, spleen, etc.) by antigen contact.

The most differentiated cell of the B cell line is the plasma cell, which, at the same time, is the only non-dividing cell in the entire lymphatic system [7]. Plasma cells produce immunoglobulin and are therefore carriers of humoral immunity. The peripheral T cells express not only the T cell receptor and the pan-T cell markers, but also either CD4 or CD8 [13, 15].

Typical lymphocytes in blood smears

The blood of healthy adults contains approx. 1,000 – 2,800 lymphocytes/µL. 55 – 83 % of these are naive T lymphocytes, 6 – 19 % are naive B lymphocytes and 7 – 31 % are NK cells [8]. In this context, 'naive' means that antigen contact has not taken place yet.

When smears are stained cytomorphologically using the May Grünwald Giemsa (MGG) staining method, these normal lymphocytes are classified together as 'typical' lymphocytes. Here, the morphological differentiation of naive T and B lymphocytes is often not possible. Rather, they present as standard lymphocytes with the following characteristics:

- Size about 8 – 10 µm
- Diameter of the nucleus comparable with the size of a normal red blood cell
- Nuclear chromatin heterogeneous, flaky
- Cytoplasm narrow, light basophilic with smooth margins

In addition to standard lymphocytes, a maximum of 10 % of 'large granular lymphocytes' (in short, LGL) can be seen in the smear. Biologically, these cells are either NK cells or cytotoxic T cells with CD8 positivity. Cytological characteristics are as follows: a little larger than standard lymphocytes, cytoplasm a little wider and pale basophilic, plus dotted azurophilic granulation.

LGL cells may only account for up to 10 % of all white blood cells in healthy individuals. If they exceed this level they are classified as 'atypical lymphocytes'. To complete matters, it should be mentioned that some activated lymphocytes with a somewhat wider cytoplasm margin may also be found in healthy persons. Their percentage, however, can be neglected (Fig. 1; image 4).

Pathological changes, atypical lymphoid cells

Lymphocytes having an unusual appearance can be released into peripheral blood by both reactive diseases (e.g., viral infections) and neoplasms (e.g., lymphomas). According to the new lymphocyte classification 2011, these lymphocytes are described as 'atypical', regardless of their cause. Here, 'atypical' means that, when stained according to the Pappenheim method, these lymphocytes have an appearance that is different from that of the standard lymphocytes or LGL cells. Usually, reactive changes show a 'colourful' picture

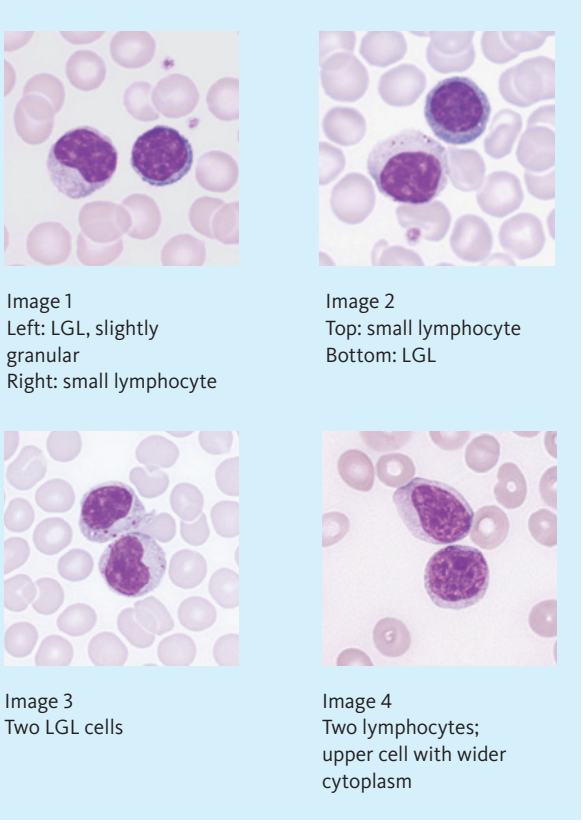

Image 1
Left: LGL, slightly granular
Right: small lymphocyte

Image 2
Top: small lymphocyte
Bottom: LGL

Image 3
Two LGL cells

Image 4
Two lymphocytes; upper cell with wider cytoplasm

Fig. 1 *Examples of standard lymphocytes*

and, at the same time, different lymphocyte forms. Malignant diseases of the lymphatic system (malignant lymphomas of the B, T or NK cell series) representing clonal diseases can display characteristic, repeating cytological atypias as well as lymphocyte proliferation, and so, in contrast to reactive diseases, this results in uniformity, i.e., a monomorphic lymphocyte picture [10]. According to clinical-morphological criteria, lymphomas are furthermore grouped into aggressive and indolent types, the latter progressing slowly in most cases. Lymphoma cells of the indolent type generally appear mature, while those of the aggressive type are immature and blast-like in most cases. Immunophenotyping characterisation, histological diagnostics and complementary cytogenetic and molecular genetic testing are essential for further cell classification and prognostic assessment [9].

Lymphocyte differentiation

Until 2011, there has not been any standardised nomenclature and classification of lymphocytes in Germany. Especially the term 'atypical lymphocytes' was used and interpreted in two different ways. Since the beginning of the 1990s, external quality assessment (EQA) providers have, for example,

insisted on using this term exclusively for malignant lymphoid cells, strictly separate from reactive lymphocytes (according to a DGHO recommendation). In routine, however, the term 'atypical lymphocyte' was used by some examiners for all lymphocytes with a morphologically irregular appearance, regardless of the cause. This diversity in the interpretation of the term 'atypical' was misleading when reading and transferring or communicating cytological findings, which in the worst case might have resulted in a wrong diagnosis. Therefore a clear definition of these terms and a guideline for standardised classification was the top objective in the revision of lymphocyte differentiation.

Table 1 *Comparison of old and revised differentiation*

Old nomenclature

Classification
Normal lymphocytes
Reactive forms = virocytes
Atypical lymphocytes = all neoplastic forms

Revised nomenclature 2011

Primary differentiation	Secondary classification
Typical lymphocytes	Standard lymphocytes LGL cells (<10 % of all cells)
Atypical lymphocytes	Atypical lymphocytes, suspect reactive
	Atypical lymphocytes, suspect neoplastic

Summary of the new lymphocyte differentiation 2011 in ten take-home messages [3]

1. Lymphocytes are divided into 1. 'typical' and 2. 'atypical' lymphocytes.
The classification is purely cytological. Here, 'typical' means that the lymphocytes are morphologically normal; the term applies to the standard lymphocytes (see Fig. 1) and the LGL cells for a maximum of 10 % of all white blood cells. All other lymphoid cells are referred to as 'atypical' [14].

2. Atypical lymphocytes are grouped into 'suspect reactive' and 'suspect neoplastic'.
Taking into account the type and frequency of the detected atypia as well as the overall cell picture, atypical lymphocytes are further subdivided into 'atypical, suspect reactive' and 'atypical, suspect neoplastic'. This is followed by a description with comments using acknowledged, cytologically defined lymphocyte types, such as e.g., hairy cells, prolymphocytes, centrocytes, etc.

3. LGL cells are counted quantitatively.
The number of these lymphocytes should be counted and their proportion included in the differentiation. If they account for less than 10 % of all white blood cells, this is considered normal and will not be mentioned in the findings.

4. LGL cells accounting for more than 10 % of all white blood cells are considered atypical.
If the determined proportion of these lymphocytes accounts for more than 10 % of all white blood cells, they are classified as atypical lymphocytes. It is desired to have a further classification into 'suspect reactive' and 'suspect neoplastic'.

5. The category 'diverse' refers to cells for which an immediate cytological determination is not possible.
These are unclear lymphoid ('atypical lymphocytes, uncertain nature') or non-lymphoid cells, the malignancy/benignancy of which cannot be determined at once by the examiner. This category may, for example, include plasma cells.

6. 'Diverse' cells need to be described.
If the 'diverse' category is used, the description of the cells is mandatory.

7. Shadow nuclei are differentiated as their own group; the name of 'Gumprecht' is no longer applicable.

8. Nucleated red blood cells (NRBC) are counted exclusively (not as a percentage of the WBC count) and reported per 100 white blood cells.

9. In cases of leucopenia, two smears are counted with 50 cells each.
In case of extreme leucopenia, for example, with a value below 1,000/µL, two smears each with 50 cells should be counted in order to avoid double counting of individual cells.

10. In cases of extreme leucocytosis, 200 cells are counted.

If white blood cell counts are extremely high, e.g., >100,000/µL, or if there are many shadow nuclei, it is recommended to differentiate 200 cells in order to reach a more accurate result.

Revised lymphocyte classification after one year of experience – a summary

The most important update 2011 is the two-stage procedure with its simplified classification into typical and atypical lymphocytes that is purely cytological in the first instance. This purely analytical procedure is followed by the second step in which the atypical cells that have been found are interpreted as being suspect reactive or suspect neoplastic. Since in practice, however, this decision must be made directly during differentiation – the DIFF keyboard (Fig. 2) requires the immediate input of an atypical lymphocyte as being either 'suspect reactive' or 'suspect neoplastic' – the steps of detection and interpretation are ultimately interconnected. In practice, this means that the smear must be pre-examined prior to differentiation to gain an impression of the overall distribution of the cells (monomorphous or heterogeneous?). This may also mean that once the individual cells have been initially classified as 'reactive' or 'neoplastic', it may be necessary to revise this in the final assessment of the overall cell image.

Adding the term 'suspect' expresses that there is a justified element of doubt in the accuracy of the classification. This restriction is essential as the cytological options for the classification of atypical lymphocytes are limited and often require further diagnostics, as explained above.

The 'diverse' group is a melting pot of cells, which can be evaluated in diagnostic terms by primary examiners only with the assistance from more experienced colleagues and/or using additional diagnostic methods, such as immunophenotyping, histology, molecular genetics or cytogenetics, meaning that these cells are not interpreted until the second stage. The essential advantage here is that 'compulsory' wrong classifications are avoided and error sources for less experienced examiners are reduced. Furthermore, the obligatory comment prevents pathological forms with conspicuous characteristics falling through the net. The 'diverse' group is likewise useful for rare cells, such as plasma cells or myeloma cells, which do not have any key assigned on the DIFF keyboard. But here as well, a description of the cells including annotation is required.

The revised classification 2011 recommends that the LGL cells should be registered quantitatively. They do not form their own group but are counted either among typical lymphocytes (<10%) or among atypical lymphocytes (>10%). Since it does not have its own category assigned on the DIFF keyboard, this cell type is best registered by simply counting it and pressing the (typical) lymphocyte key. If these cells exceed 10%, they must be subtracted from the typical lymphocytes and categorized as atypical lymphocytes. Proliferation of the LGL cells to over 10% of the white blood cells poses a problem in clinical and diagnostic terms. Such a finding is not very rare, particularly in specialist haematology laboratories, because this phenomenon may, for example, be induced by the therapy used, particularly within the scope of stem cell transplantation. If the appearance is the same, however, neoplasia cannot be excluded.

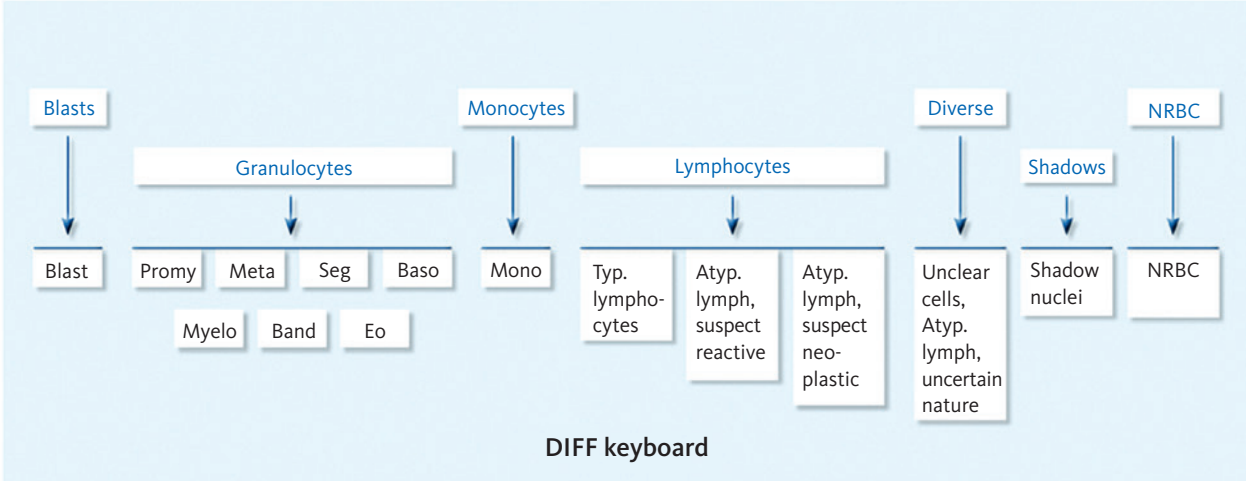

Fig. 2 *Keyboard layout taking the revised new lymphocyte classification into account*

The required classification into 'suspect reactive' and 'suspect neoplastic' can therefore not be made by the examiner in the laboratory because clinical data are often insufficient and/or additional diagnostics are not yet available at the point of assessment. Here a classification into 'diverse' with an additional comment such as 'LGL cell proliferation of unclear nature; further clarification required' can be a helpful option. A (hospital-internal or more far-reaching?) strategy for the clinical approach to LGL proliferations has to be developed to define the further procedure. This could involve an initial follow-up and, if the LGL proliferation persists, further investigation (e.g., at a molecular genetic level). The contentious issue of LGL proliferation calls for further discussion.

The lymphoma cells of chronic lymphocytic leukaemia (CLL) usually appear as typical lymphocytes in the smear although they are pathological cells. In this case, the new classification fails to facilitate characterisation of the lymphoma cells. This is down to the methodical limits of cytology, where functionally atypical cells have to be classified as 'typical' in the differentiation. In this context, it must be pointed out that the valid CLL definition is not restricted to cytology alone. According to the WHO classification 2008, the minimum requirement for a diagnosis of CLL is as follows: >5 x 10^3/µL B lymphocytes with the antigen expression pattern CD19+, CD5+, CD23+ [9].

Other standards for the lymphocyte quantity and morphology apply in paediatrics (preterm infants, neonates and children). The reference range of lymphocytes is higher than in adults and the morphology demonstrates greater variability [8]. The definition of what is normal (typical) in the new lymphocyte differentiation is, however, based exclusively on the situation for adults and does not take the special characteristics of paediatric patients into account. The use of the new DIFF keyboard inevitably produces results with atypical lymphocytes, although these would not be assessed as pathological. The DGHO has not yet clearly defined how to proceed in these cases.

Lymphocyte types in picture and text

An overview of the physiologically and pathologically occurring lymphocytes is shown on the Sysmex lymphocyte poster and the lymphocyte chart, which were developed in cooperation with the Clinic of Oncology/Haematology and Stem Cell Transplantation at the University Hospital of Aachen (Fig. 3). You are welcome to request a copy from your local Sysmex representative.

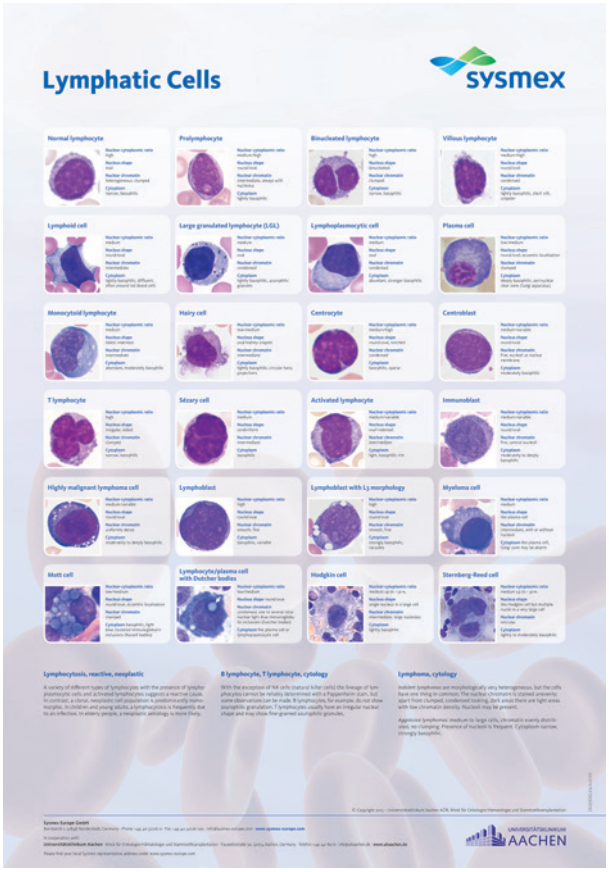

Fig. 3 *Overview of various physiological and pathological lymphocytes available as a wall poster or A4 chart from your Sysmex representative*

Concluding remarks

The new classification of lymphocytes cannot eliminate the difficulties in recognising and classifying pathologically altered lymphocytes. The application in specialist fields where other standard conditions apply (e.g., paediatrics) is also unclear, and leaves many questions unanswered. The standardised nomenclature does, however, lead to a clear definition of what is atypical, and as such is an important step towards standardisation of microscopic differentiation. As a positive side effect, the increased sensitivity to the issue of lymphocyte alterations has also markedly intensified further training activities among clients and users.

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A note by Sysmex Europe:

The above article represents a practical approach to a recently suggested lymphocyte nomenclature in Germany. For a wider European point of view, we would like to add another reference: the ELN Morphology Faculty (EMF), composed of 28 expert morphologists from 17 European countries, started a project with the goal to increase the quality of diagnostics based on cytomorphology and suggested a uniform nomenclature. Amongst others, also for lymphocytes a suggestion has been made.

Zini G, Bain B, Bettelheim P, Cortez J, D'Onofrio G et al. *A European consensus report on blood cell identification: terminology utilized and morphological diagnosis concordance among 28 experts from 17 countries within the European LeukemiaNet network WP10, on behalf of the ELN Morphology Faculty. British Journal of Haematology, Wiley, 2010, 151 (4), pp.359. <10.1111/j.1365-2141.2010.08366.x>. <hal-00573094>.*

The article is freely accessible under: https://hal.archives-ouvertes.fr/hal-00573094/document

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