Counting of highly fluorescent lymphocytes (HFLC) as antibody-producing plasma cells on XE-5000

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Introduction

Fluorescence flow cytometry enhances analytical possibilities beyond the normal 5-part differential by providing excellent cluster resolution and separation of abnormal blood cells. A nucleated red blood cell count (NRBC) and an immature granulocyte count (IG) are part of an extended differential on the XE systems with the potential to improve the diagnostic usefulness of the routine 5-part differential, which can be reported directly for all analysed samples. Besides the IG there is a distinct separation of an abnormal cell population with high fluorescence intensity above the monocyte and lymphocyte region. Highly fluorescent lymphocyte cells (HFLC) are observed in the DIFF channel of the Sysmex XE series haematology analysers in an area of high fluorescence which is used for flagging of 'Atypical Lymphocytes'. They are detected by their characteristically high fluorescence intensity reflecting a high RNA content. The recently introduced XE-5000 analyser with improved gating and flagging excludes those samples in which the analyser cannot discriminate a distinct HFLC population (mostly in cases of systemic diseases or malaria infections).

Figure 1 demonstrates three DIFF scattergrams: one from a normal sample, one showing immature granulocytes (IG) and one demonstrating the presence of immature granulocytes (IG) and highly fluorescent lymphocytes (HFL).

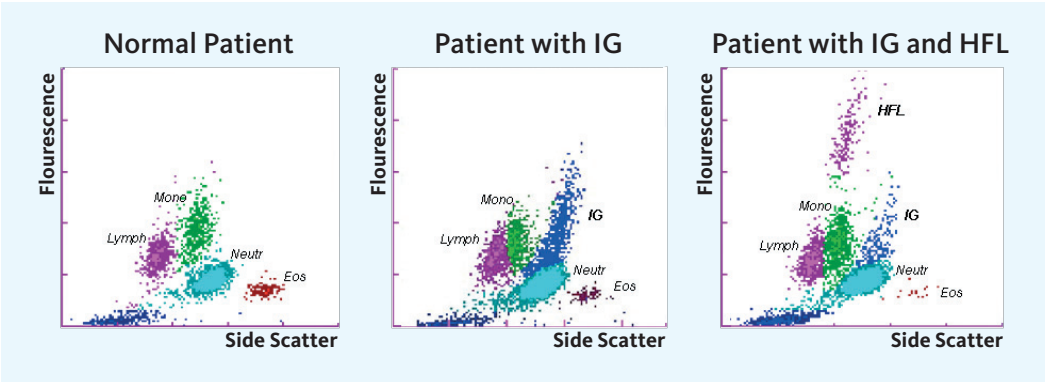

Fig. 1 *Three DIFF scattergrams, the first one coming from a normal patient sample, the second one from a patient sample containing IG, and the third one from a patient sample containing both IG and HFL.*

Cell Biology

Plasma cells or lymphoplasmocytoid cells are the terminally differentiated, non-proliferating effector cells of the B-cell lineage and sole producers of antibodies. Hence they represent both components of the adaptive human immune system being crucial to effective immune response in reactive diseases. They decisively support adequate immune response to microbial pathogens with the needed specificity and rapidity. Plasma cells can be found in bone marrow as so-called 'long-life plasma cells', but they are rarely seen in the peripheral blood of normal healthy individuals. For this reason, the presence of plasma cells in a patient blood sample indicates an immune response as a result of infections.

Monocytes or dendritic cells have receptors to bind many different antigens. Once antigens are bound, the cells circulate to the lymph nodes and present these antigens to the T-cells. The T-cells then stimulate the B-cells to proliferate and differentiate into plasma cells and produce IgM immunoglobulin. This occurs within 1 week after encountering the antigen. Some active B-cells supported by activated T-cells form a germinal centre and proliferate and differentiate (between day 10 and day 14) to class-switch plasma cells (IgG, IgA, or IgE) or memory cells (MC). This is the T-cell-dependent activation of B-cells. In most cases this occurs exclusively within the lymph nodes and the plasma cells do not circulate in peripheral blood; therefore, these are not the plasma cells that are measured as HFLC in peripheral blood.

The majority of peripherally circulating plasma cells and thus candidates for representing the HFL cells in infectious diseases are the splenic marginal-zone B-cells. The first B-cell activation in response to antigens is the differentiation of splenic marginal-zone B-cells into IgM-producing plasma cells without T-cell interaction. These B-cells recognise antigens independently within a few hours of immunisation. With the T-cell-independent antigen they move to the red pulp of the spleen, proliferate from plasma blasts to IgM-producing cells without class-switch possibilities, and populate through the efferent lymph to the blood distant sites (see Figure 2).

Plasma cell enumeration is conventionally done by means of peripheral blood film morphology using light microscopy. However, this manual method is laborious as well as imprecise due to the low number of cells counted, and inter-observer variability. Small percentage numbers of plasma cells, i.e. in neutrophilic acute phase response, are often missed in a standard 100-cell differential count. Flow cytometry with monoclonal antibodies is unsuitable as a screening test. The procedure is not automated, and it is expensive and time consuming.

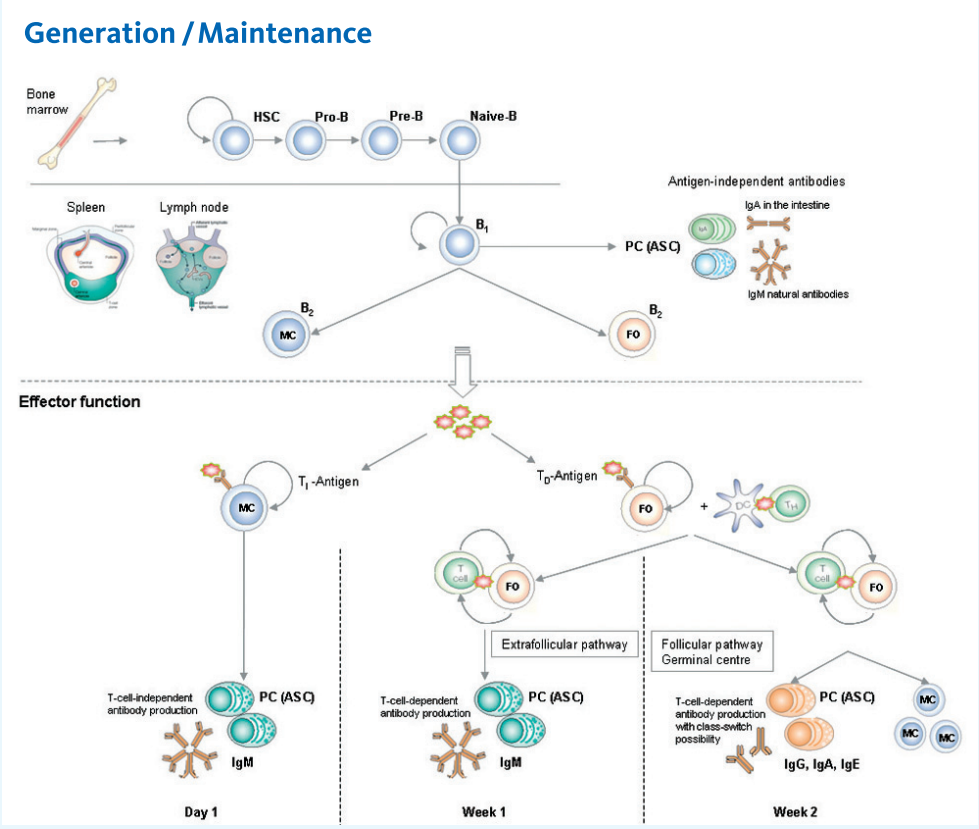

Fig. 2 *Model summarising development, generation, maintenance and function of ASC cells (plasma cells)*

In the previously published study it was to prove that the RNA fluorescence stain method of the XE system can classify and quantify this 'atypical lymphocyte' region as T-independently activated B-lymphocytes synthesizing clone-specific antibodies, plasma cells, or lymphoplasmocytoid cells. In a total of 85 patients the HFLC counts of the XE system were compared with the immunophenotyping flow cytometry method (BD FACSCaliburTM) and a digital 400-cell differential performed on an automated image analysis system with pre-classification (CellaVision® DM96). Basic performance in terms of reproducibility and linearity was also carried out for the HFLC count of the XE system.

Findings of the previously published study to identify the cell type in the HFL area

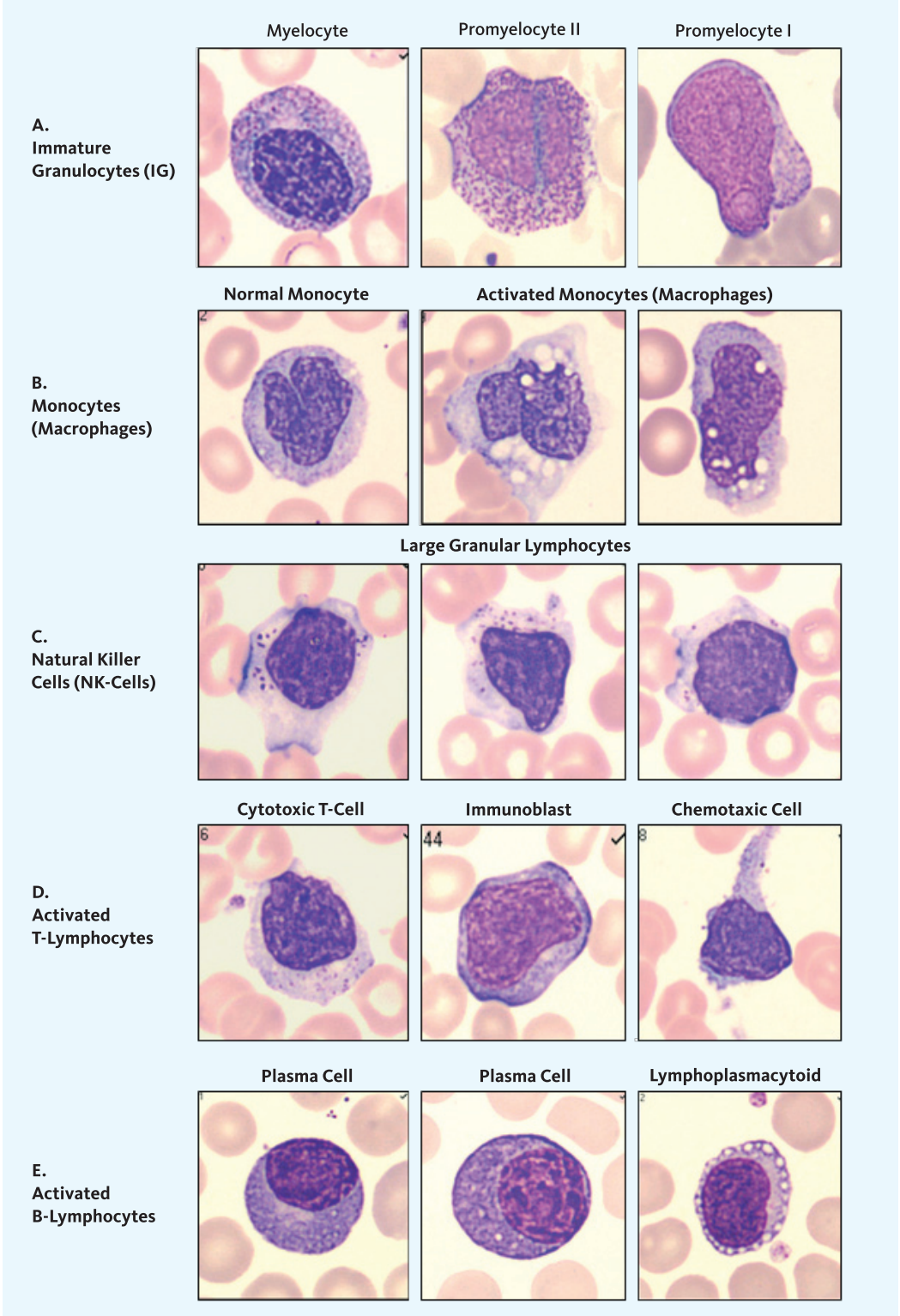

Fig. 3 *The cell classes considered to be possible candidates for the HFL area*

An initial panel of monoclonal antibodies was used to immunophenotype total leukocytes, neutrophils, monocytes, lymphocytes, eosinophils and basophils. Three other antibody panels were used to determine total T-cell lymphocytes and their subgroups T-helper, T-suppressor and cytotoxic T-lymphocytes, natural killer (NK) cells, as well as total B-lymphocytes and subgroups, plasma cells, or cytoplasmic IgM cells. All of these cell types were candidates for the cells appearing in the HFL area. Figure 3 shows the morphology of the cell classes that were being considered as present in the HFL area.

Results of the phenotypic identification of the HFL

The HFLC count on the XE demonstrated excellent reproducibility in low-count and high-count samples and the linearity data showed good correlation between expected and measured HFLC.

There was no correlation found between HFLC and activated monocytes or very immature granulocytes, natural killer cells, activated T-lymphocytes, or cytotoxic T-cells. Activated B-lymphocytes– defined in the peripheral blood smear as plasma cells plus lymphoplasmocytoid cells – and antibody-synthesizing cells (ASC) – defined as $CD19^+$, SSChigh, increase of cytoplasmic IgM and $CD138^+$ – resulted in an excellent correlation with HFLC each. Correlation coefficients were $R^2 = 0.809$ (with $P<0.001$) and $R^2 = 0.804$ (with $P<0.001$), respectively. Correlation of HFLC with plasma cells only (the morphologically defined type) is good with $R^2 = 0.796$.

	Regression	R	R^2	P
Natural Killer Lymphocytes (NK-Cells)				
XE-2100 versus DM96				
HFL # versus LGL # (DM96)	HFL # = 0.0964 + (0.0447 * LGL #)	0.044	0.002	0.700
XE-2100 versus FACSCalibur				
HFL # versus NK-cells # (FACS)	HFL # = 0.0897 + (0.0590 * NK #)	0.072	0.005	0.526
FACSCalibur versus DM96				
NK (FACS) # versus LGL # (DM96)	NK # = 0.126 + (0.688 * LGL #)	0.560	0.314	<0.001
T-Lymphocytes				
XE-2100 versus DM96				
HFL # versus activated T-Lymph # (DM96)	HFL # = 0.0750 + (0.0723 * activated T #)	0.215	0.046	0.056
HFL # versus CTL # (DM96)	HFL # = 0.0888 + (0.187 * CTL #)	0.178	0.032	0.113
HFL # versus chemotaxis # (DM96)	HFL # = 0.0686 + (0.144 * chemotaxis #)	0.259	0.067	0.021
HFL # versus large lymph # (DM96)	HFL # = 0.101 + (0.000664 * large lym #)	0.001	0.000	0.994
XE-2100 versus FACSCalibur				
HFL # versus total T-cells # (FACS)	HFL # = 0.0572 + (0.0370 * total T-cells #)	0.214	0.046	0.056
HFL # versus CD4+ cells # (FACS)	HFL # = 0.0538 + (0.0637 * CD4+ #)	0.229	0.053	0.041
HFL # versus CD8+ cells # (FACS)	HFL # = 0.0901 + (0.0252 * CD8+ #)	0.103	0.011	0.363
HFL # versus CTL cells # (FACS)	HFL # = 0.0865 + (0.131 * CTL #)	0.160	0.026	0.157
FACSCalibur versus DM96				
CD8+ cells # (FACS) versus activated T-lymph # (DM96)	CD8+ # = 0.0387 + (1.134 * activated T #)	0.902	0.814	<0.001
CTL (FACS) # versus CTL # (DM96)	CTL # = 0.0524 + (0.899 * CTL #)	0.703	0.494	<0.001
CD4+ cell # (FACS) versus activated T-lymph # (DM96)	CD4+ # = 8.242 + (2.224 * activated T #)	0.213	0.046	0.056
B-Lymphocytes				
B-cells: XE-2100 versus DM96				
HFL # versus activated B-lymphocytes # (DM96)	HFL # = -0.00499 + (1.127 * activated B #)	0.900	0.809	<0.001
HFL # versus plasma cells # (DM96)	HFL # = 0.0364 + (1.247 * plasma cells #)	0.834	0.796	<0.001
B-cells: XE-2100 versus FACSCalibur				
HFL # versus total B-cells (CD19+) # (FACS)	HFL # = 0.0224 + (0.338 * total B-cells #)	0.534	0.286	<0.001
HFL # versus ASC # (FACS)	HFL # = 0.00192 + (1.518 * ASC #)	0.897	0.804	<0.001
B-cells: FACSCalibur versus DM96				
ASC # (FACS) versus activated B-cells # (DM96)	ASC # = 0.00798 + (0.610 * activated B #)	0.810	0.655	<0.001
Total B-cells # (FACS) versus activated B-cells # (DM96)	Total B # = 0.133 + (1.046 * activated B #)	0.525	0.275	<0.001

Tab. 1 *Linear regression between XE-2100 HFLC and lymphocyte subpopulations analysed from peripheral blood smears and immunophenotyping with the BD FACSCalibur™ flow cytometer (# = absolute cell count / µL blood)*

Figure 4 shows as an example a patient in intensive care seven days after cardiac surgery. There is an increase of the HFLC population from 60 cells to 270 cells. On examination of the flow cytometry scatterplots CD19 positive B-cells with high light scatter are clearly present, as is an increased presence of cytoplasmic IgM and cells positive for CD138 (increase from 80 cells to 280 cells). This correlates with the HFLC counts from the XE system. The numbers of activated B-cells seen in the blood film by the cellavision® DM96 were also very similar to the flow cytometric and XE counts. There were also some cells visible with the characteristics of immunoglobulin production or secretion.

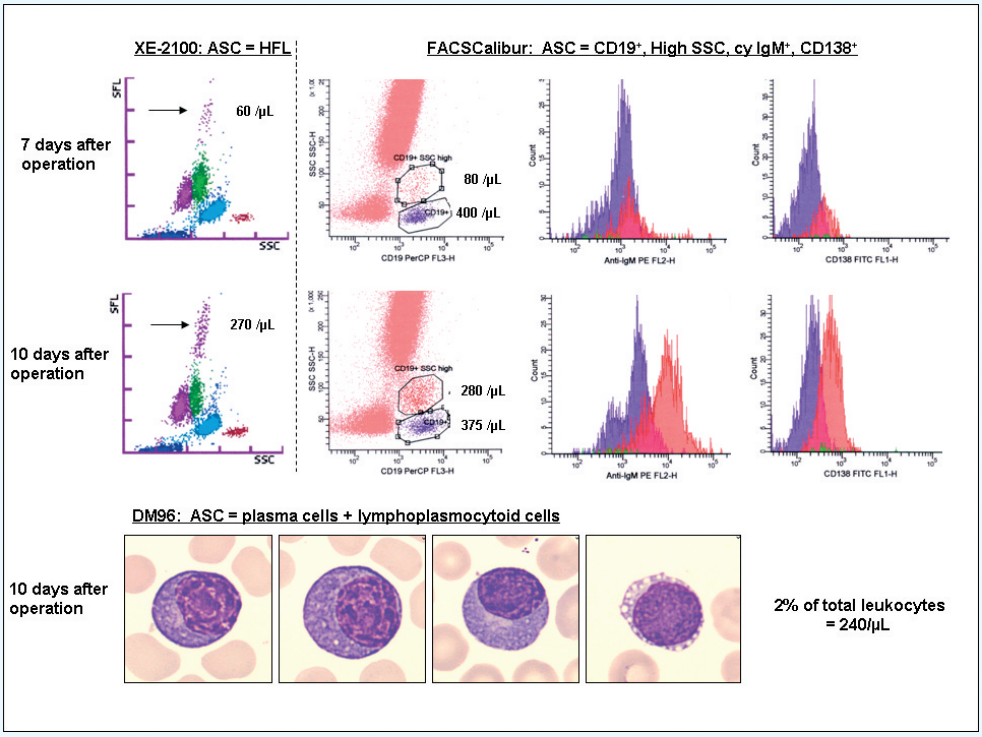

Fig. 4 *Correlation of cell types by the three different methods in a patient ten days post cardiac surgery. Plasma cells and plasmocytoid cells were seen on the blood film; the fourth cell in the figure gives the characteristic appearance of a lymphocyte producing and secreting immunoglobulin.*

Conclusions for the identification of the HFLC

In conclusion, the results deliver evidence that the high-fluorescence lymphocytes count (HFLC) from the automated routine haematology analyser XE-5000 is identified and quantified as antibody-synthesizing or secreting cells (ASC). An elevated count is an indication for an immune response to infectious disease. Lymphoproliferative disorders or other systemic diseases that would also show signals in the HFL area have to be excluded since in such clinical settings the XE-5000 analyser will not discriminate a distinct HFLC population. Such obvious samples will be not flagged as 'atypical lymph' and therefore be exempted in this study. During infectious diseases the peripheral blood plasma cell concentration is not always elevated – despite of high IG values. This is due to antigen presentation by antigen-presenting cells (i.e. dendritic cells) happening predominantly in local lymph nodes. Consequently, the presence of plasma cells in peripheral blood is mirroring the early response of the innate immune system to T-independent antigen by marginal-zone lymphocytes being activated and differentiated to plasma cells in the spleen.

This provides new possibilities for a fast and reliable screening and monitoring of intensive care SIRS (systemic inflammatory response syndrome) patients with suspicion of a local infection or even sepsis.

Further clinical studies are necessary to evaluate the entire usefulness of automated ASC measurement as a screening assay for infectious diseases in clinical settings. Once the automated ASC count has proven its value and is established in clinical medicine as well as accepted by physicians in clinical routine, reevaluation of such results by visual microscopy will not be necessary for blood samples from reactive diseases containing ASC. Automated ASC counting from routine haematology systems without sample preparation and in less than 1 minute will further reduce the workload in haematology laboratories and will provide time for more rewarding tasks other than routine microscopy.

This work was done in collaboration with the University of Cologne.

References

[1] Linssen J, Jennissen V, Hildmann J, Reisinger E, Schindler J, Malchau G, Nierhaus A and Wielckens K. (2007) Identification and quantification of high fluorescence-stained lymphocytes as antibody synthesizing/secreting cells using the automated routine hematology analyzer XE-2100. Clinical Cytometry, Vol 72B, 3, 157-166.

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