Identification of High Fluorescence Lymphocytes (HFL) Count on the XE-5000 with Efficient Multi-Channel Messaging (eMM) as Antibody Synthesizing Cells, c.q. Plasma Cells

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Objectives: *The aim of this study was to classify and quantify the high fluorescence lymphocytes (HFL) area detected with eMM from the SYSMEX XE-5000 (Sysmex Corporation, Kobe, Japan) leucocyte differential channel as antibody-synthesizing cells (ASC, plasma cells or lymphoplasmacytoid cells) in reactive diseases. To unequivocally identify the HFL cells, all possibly eligible cell populations have been investigated: activated B-lymphocytes, activated T-lymphocytes, large granular lymphocytes (LGLs), activated monocytes, and immature granulocytes.*

Methods: *In total, 85 patients were re-analysed on the XE-5000 with eMM and compared with the automated image analysis system CellaVision Diffmaster 96 (Cellavison AB, Lund, Sweden) based on artificial neural network and immunophenotyping method with the BD FACSCalibur™ (Becton, Dickinson and Company, NJ, USA).*

Results: *The comparison with possibly eligible cell populations showed no significant correlation between activated monocytes and immature granulocytes (IG), with most immature granulocytes (promyelocyte I or II), natural killer cells or LGLs, activated T-lymphocytes, and sub-T-lymphocytes populations. However, for activated B-lymphocytes an excellent significant correlation with the peripheral blood smear, and the immunophenotyping method has been found with $R^2 = 0.949$, $P < 0.001$ and $R^2 = 0.913$, $P < 0.001$, respectively.*

Conclusion: *The fully automated SYSMEX XE-5000 HFL count with eMM identifies and quantifies the ASC (activated B-lymphocytes) with high precision and reliability, thus providing a potential screening and monitoring tool for any patient with suspected infection. Additional studies are required to comprehend in more detail the full clinical utility of an HFL (ASC) count as a potential diagnostic indicator of inflammation, infection, or sepsis.*

Key Words High Fluorescence Lymphocytes (HFL) Count, Efficient Multi-channel Messaging (eMM), Antibody Synthesizing Cells (ASC), Activated B-lymphocytes, SYSMEX XE-5000, BD FACSCalibur™, CellaVision Diffmaster 96 (DM96)

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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 immature granulocyte (IG) count is 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. Beside the IG there is a distinct separation of an abnormal cell population with high fluorescence intensity above the monocyte and lymphocyte region. The HFLC are observed in the DIFF channel on the SYSMEX XE-series of haematology analysers in an area of high fluorescence and is used for flagging of atypical lymphocytes. They are detected by their characteristically high fluorescence intensity reflecting a high RNA content. The recently introduce XE-5000 analyser with efficient multi-channel messaging (eMM) exclude those samples where the analyser can not discriminate a distinct HFL population (mostly systemic diseases or malaria infection).

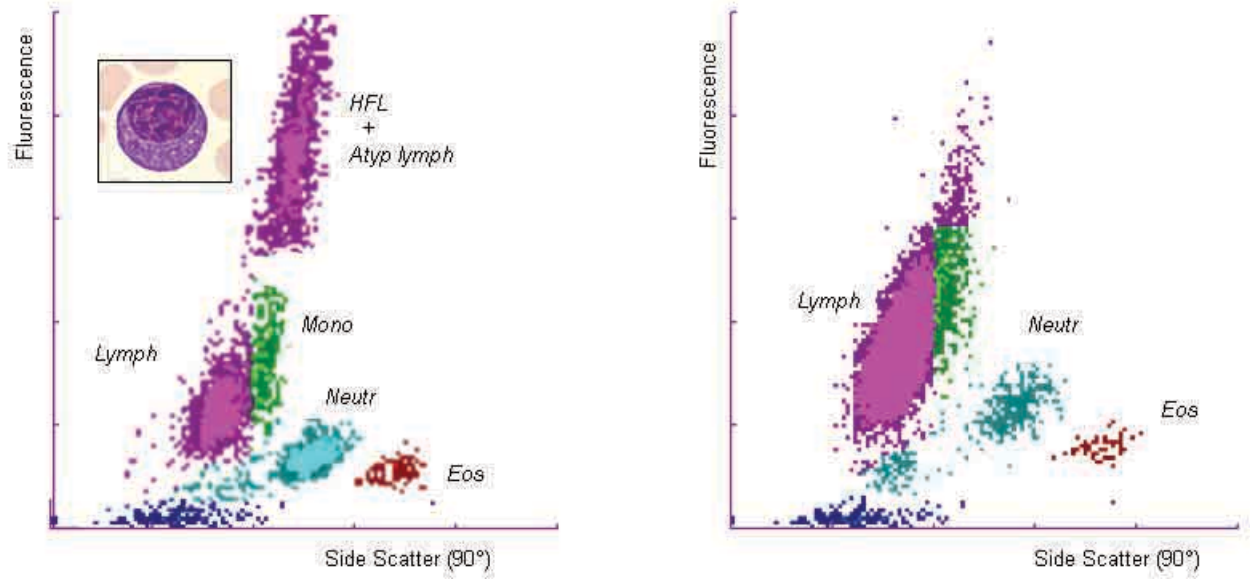

Fig. 1 *HFL are displayed as purple dot plots (as are lymphocytes). Atyp. lymph stands atypical lymphocyte flag; Mono indicates monocytes; Lymph stands for lymphocytes; Neutr for neutrophils; Eos for eosinophils.*

Fig. 1 demonstrates to the left a sample (reactive lymphocytosis c.q. plasma cells) with a distinct HFL population and atypical lymphocyte flag and to the right a sample (Non Hodgkin lymphoma) without a distinct HFL population and no atypical lymphocyte flag.

CELL BIOLOGY[1-8)]

Plasma cells or lymphoplasmacytoid 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 for 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 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 antigen is bound the cells circulate to the lymph nodes and present these antigens to the T-cells. The T-cell then stimulates the B-cells to proliferate and differentiate into plasma cells and produce IgM immunoglobulins this occurs within 1 week after encountering 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 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 the peripheral blood. Therefore these are not the plasma cells that are measured as HFL in the peripheral blood. The most peripheral circulating plasma cells and candidates for representing the HFL in infectious diseases are the spleen marginal-zone B-cells. The first B-cell activation in response to antigens is the differentiation of the spleen marginal-zone B-cells into IgM producing plasma cells without T-cell interaction. These B-cells recognise antigens independently within a few hours of immunization. With the T-cell independent antigen they move to the red pulp of the spleen proliferating from plasma-blasts to IgM producing cells, with no class-switch possibilities en populate through the efferent lymph to the blood distant sites. (see ***Fig. 2***)

Plasma cell enumeration is conventionally done by means of peripheral blood film morphology with 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 plasma cell percentage numbers, 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. It is expensive and time consuming.

This study compared the reanalysed HFL count[9)] on the XE-5000 with eMM flagging with the immuno-phenotyping flow cytometry method and the digital 400 cell automated image analysis system with pre-classification, and to prove that the fluorescence RNA-stain method on XE-5000 with eMM can classify and quantify these atypical lymphocytes region in T-independent activated B-lymphocytes synthesizing clone-specific antibodies, the plasma cells or the lymphoplasmocytoid cells. Basic performance in terms of reproducibility and linearity has also been carried out for the XE-5000 HFL count.

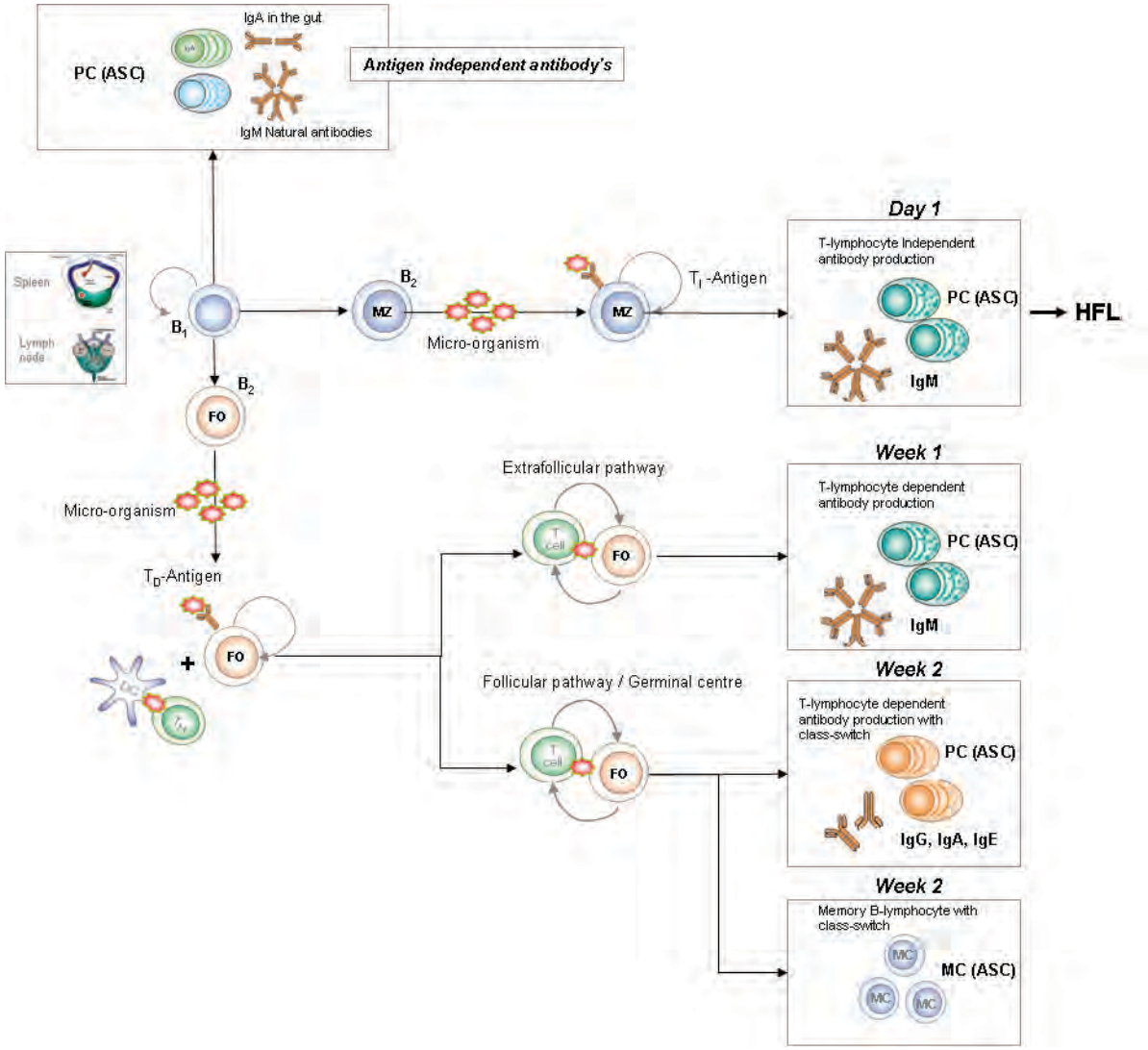

Fig. 2 *Model summarizing development, generation, and function of ASC (plasma cells). Naive B cells that exit the bone marrow continue their development in the spleen. A small proportion home to the marginal zone to become marginal-zone B-cells (MZ), and a higher extent form the long-lived circulated naive follicular B cells (FO). The spleen is also required for the generation and maintenance of B1 cells. They are responsible for the production of natural IgM and for secretion of IgA in the gut. The first B cells to respond to a foreign antigen without help of T-cell (T_I = T-cell independent antigen) by differentiating into plasma cells are marginal-zone B cells (day 1) supposed as HFL. After antigen presentation (T_D =T cell dependent antigen) from active DC's to T helper cells (T_H), some active follicular B cells also differentiate supported by activated T_H cells into extra follicular plasma cells (within week 1). Both are IgM antibody-producers with no possibility to class-switch. After encountering antigen and receiving T-cell help, some follicular B 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).*

MATERIALS AND METHODS

Blood Samples

All blood samples were routine patient samples anticoagulated with K_3EDTA. In total, 97 patient samples were reprocessed on the XE-5000 without any preparation and, immediately after sampling, two separate blood films were prepared by the Sysmex automated slide preparation unit SP-100 , using the May Grünwald Giemsa staining. A manual 2 × 200 cell leukocyte differential count was then performed by the automated image analysis system CellaVision Diffmaster 96 (DM96) (Cellavison AB, Lund, Sweden)[11]. The same samples were further analysed with the BD FACSCalibur™ (Becton, Dickinson and Company, NJ, USA) after whole blood staining by application of monoclonal antibodies. Twenty-nine patients had a negative HFL count (HFL = 0 / mL blood) and 68 patients showed a positive HFL count of up to 750 cells/mL (0.2-14% of total WBC count). Specimens from patients with hematological system disease (AML, ALL, NHL) except plasma cell myeloma / plasmacytoma in general are exclude by the efficient multi-channel messaging and suppressing the atypical lymphocyte flag with an indication of the unreliability of the HFL count. Consequently, such obviously marked samples are automatically excluded from the study.

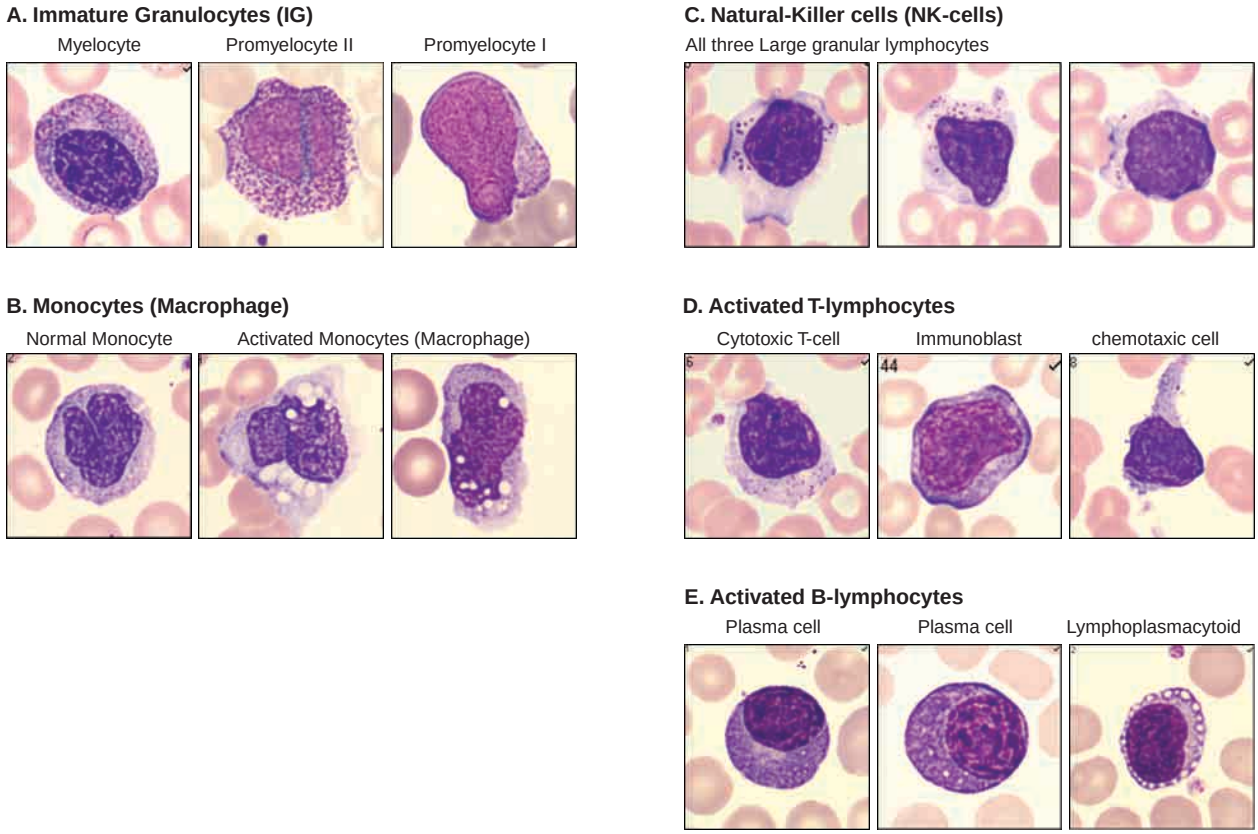

Fig. 3 *Morphologically cell classification in May-Grünwald stain and 1000 × magnification with the CellaVision DM96. Cell classification in five pathological groups: (A) IGs, (B) activated monocytes, (C) LGL, (D) activated T-lymphocytes, and (E) activated B-lymphocytes.*

Principle of HFL count on the Hematology Analyser XE-5000

HFLC and leukocyte differential parameters were measured with the XE-5000. HFLC are differentiated from the lymphocyte population by their high fluorescence and low side scatter intensity in the XE-5000 leukocyte differential channel (***Fig. 1***). The eMM exclude those samples where the analyser can not discriminate a distinct HFL population.
After chemical lysis of erythrocytes and preparation of leukocytes, the fluorescence polymethine dye reacts with the ribonucleic acid of the leukocytes, and the flow cytometry unit detects and classifies the respective cells by side scatter and side fluorescence. They are reported as percentage of the total leukocyte count and as an absolute count.

Manual Differential count with automated image analysis system CellaVision DM96

The 400-cell leukocyte differential count according Clinical and Laboratory Standards Institute (CLSL)[10] was performed with the standardized automated image analysis system DM96 with pre-classification[11]. After automated pre-classification in segmented Neutrophils, Monocytes, Lymphocytes, Eosinophils and Basophils white blood cells were manually classified (see ***Fig. 3***) in immature granulocytes (meta-myelocytes, myelocytes and promyelocytes I and II), activated monocytes (macrophages), natural killer cells (large granular lymphocytes), activated T-lymphocytes (cytotoxic T-lymphocyte [CTL], chemotaxis cells [shape change], large basophilic blast-like cells [immunoblasts]) and activated B-lymphocytes (plasma cells and lymphoplasmacytoid cells).

Flow cytometry immunophenotyping analysis with the BD FACSCalibur™

Flow cytometry analysis was performed on a BD FACSCalibur™ system. FSC, SSC and four-color fluorescence signals were determined for each cell and stored in list-mode data files in FCS 2.0 format. The data files were acquired and analysed by a multi-tube and multi-step gating procedure using BD MultiSET™ 1.1.2, BD CellQuest Pro™ 4.0.2 software and BD DIVA™ 4.1.2 (Becton, Dickinson and Company, NJ, USA).
In a first tube, total leukocytes were differentiated in neutrophils, monocytes, lymphocytes, eosinophils and basophils by using SSC, FSC-H, HLA-DR FITC, CD123 PE, CD45 PerCP-Cy5.5 and CD14 APC.
Monocytes are defined as $CD14^{high}/SSC^{int}$, lymphocytes are defined as $CD45^{high}/SSC^{low}$/NOT monocytes,

basophils are defined as CD123^{high}/anti-HLA DR^{neg}, eosinophils are defined as cells with high side scatter fluorescence and high auto fluorescence appearing as FL1-FL4 intermediate high (SSC^{high}/FL1-FL4^{int}), neutrophils are defined as SSC$^{int-high}$/NOT (lymphocyte OR monocyte OR basophil OR eosinophil).
In a second and a third tube, total lymphocytes were differentiated in B-lymphocytes, T-lymphocytes, and NK-cells by using BD MultiSET™ IMK Kit[11]. One tube was used for the determination of T-lymphocyte subpopulations (T helper-lymphocytes and T-suppressor lymphocytes) with CD3 FITC, CD8 PE, CD45 PerCP and CD4 APC. The second tube was used the for differentiation of B-lymphocytes, T-lymphocytes, cytotoxic T-lymphocytes and NK-cells with CD3 FITC, CD16 + 56 PE, CD45 PerCP and CD19 APC according to manufacturer's preparation and analysis instructions.
In a fourth tube, total lymphocytes were differentiated in total B-Lymphocytes, SSC^{high} and SSC^{low} B-Lymphocytes, cytoplasmic IgM (cyIgM) and CD138 plasma cells[12] by using surface and intracellular staining of CD138 FITC, anti-IgM PE, CD45 PerCP Cy5.5 and CD14 APC (to exclude contamination of monocytes in the lymphocyte gate).
The percentage of all cell types was calculated as a proportion of all leukocyte events. The absolute count of all cell types per µL blood was calculated from the total leukocyte count of the XE-5000.

Statistical Analysis

Linear regression analysis and Pearson product moment correlation coefficient were carried out with MedCalc® Software, Version 9.2.0.1 (MedCalc, Ghent, Belgium). Statistically significant correlations were defined at a P-level of < 0.05.

RESULTS

Identification and quantification of the HFL area

To unequivocally identify the HFLC a total of 97 patients were investigated for all possibly eligible cell populations by comparing the HFL count with the immunophenotyping flow cytometry method on the BD FACSCalibur™ and, the digital 400 cell automated image analysis system DM96. The analysed cell populations were the Non-lymphocyte cell population; IG (metamyelocytes - myelocytes - promyelocytes I and II) and the activated monocytes (macrophages) and the Lymphocyte subpopulation; the natural killer cells (large granular lymphocytes), activated T-lymphocytes and activated B-lymphocytes.

Comparison of XE-5000 HFL count with activated monocytes and IG from automated image analysis system DM96.

The peripheral blood samples were analysed on the XE-5000 instrument and compared with the peripheral blood smear, analysed on the DM96 with pre-classification (5-part differential). Monocytes were manually differentiated further in non-active and activated monocytes, characterised as monocytes with vacuolisation and shape change - the so-called macrophage. IG were manually classified in metamyelocytes, myelocytes and promyelocytes. The linear regression shows no statistically significant ($P > 0.05$) correlation ($R^2 = 0.002$) between HFLC and activated monocytes. Also the total IG or isolated promyelocytes show no correlation with the HFL count ($R^2 = 0.029$, $P > 0.05$ resp. $R^2 = 0.012$, $P > 0.05$).

Comparison of XE-5000 HFL count with different lymphocyte subpopulations analysed with the automated image analysis system DM96 and the immunophenotyping flow cytometry method on the BD FACSCalibur™.

The peripheral blood samples have been analysed on the XE-5000 instrument and the HFL counts were compared to the different lymphocyte subpopulations analysed on the peripheral blood smear and the immunophenotyping flow cytometry method on the BD FACSCalibur™: NK-cells, T-lymphocytes and B-Lymphocytes (see ***Table 1***).
NK-cells were classified microscopically as LGLs and immunophenotypically as CD16$^+$ + CD56$^+$ and CD3$^-$ cells.
T-lymphocytes were differentiated microscopically in activated T-lymphocytes - defined as large lymphocytes (except plasma cells) with ample grey-blue cytoplasm or with azurophilic granules (cytotoxic T-lymphocytes = CTL) or with increased basophilic staining of the cytoplasmic periphery with loose chromatin and nucleoli (Immunoblasts) - and, lymphocytes with chemotaxis (shape change). Immunophenotypically T-lymphocytes are classified in CD4$^+$ (helper cells) and CD8$^+$ (suppressor cells) and, cytotoxic T-lymphocytes as CD16$^+$, CD56$^+$ and CD3$^+$ cells.
B-lymphocytes are classified microscopically in activated B-lymphocytes - defined as plasma cells or lymphoplasmacytoid cells, both being ASC. Immunophenotypically the B-lymphocytes are classified as activated B-lymphocytes, with CD19$^+$, SSC^{high}, increase of cytoplasmic IgM and CD138$^+$.
The linear regressions between HFLC, DM96 and BD FACSCalibur™ for the lymphocyte subpopulations NK-cells, T-lymphocytes and B-lymphocytes are displayed in ***Table 1***.
Fig. 4 shows as an example a patient in intensive care 7 days after cardiac surgery. There is an increase of the HFL population from 60 cells to 270 cells. On examination of the flow cytometry scatterplots CD19 positive B-lymphocytes with high light scatter are clearly present, as is an increased presence of cytoplasmic IgM and cells positive for CD138 (from 80 cells to 280 cells). This correlates with the HFL counts from the XE-system. The numbers of activated B-lymphocytes seen in the blood film by the DM96 were also very similar to the flow cytometric and XE counts.

Table 1 *Linear regression between XE-5000 HFLC and leucocyte subpopulations analysed from peripheral blood smears and immunophenotyping with the BD FACSCalibur™ flow cytometer. (# = absolute cell count / µL blood)*

Leucocyte subpopulation	R value	P value
XE-5000 versus DM 96		
HFLC # versus activated Monocytes # (DM96)	0.018	0.44
HFLC # versus total IG # (DM96)	0.029	0.11
HFLC # versus Promyelocytes # (DM96)	0.012	0.09
T-lymphocytes		
XE-5000 versus DM 96		
HFLC # versus activated T-lymphocytes # (DM96)	0.255	0.07
HFLC # versus CTL cells # (DM96)	0.088	0.12
HFLC # versus Chemotaxis # (DM96)	0.288	0.08
HFLC # versus Large lymphocytes # (DM96)	0.101	0.54
XE-5000 versus FACSCalibur		
HFLC # versus Total T-lymphocytes # (FACS)	0.218	0.06
HFLC # versus $CD4^+$ cells # (FACS)	0.278	0.09
HFLC # versus $CD8^+$ cells # (FACS)	0.089	0.09
HFLC # versus CTL cells # (FACS)	0.242	0.16
FACSCalibur versus DM 96		
$CD8^+$ cells # (FACS) versus activated T-lymphocytes # (DM96)	0.902	<0.001
CTL # (FACS) versus CTL cells # (DM96)	0.703	<0.001
$CD4^+$ cells # (FACS) versus activated T-lymphocytes # (DM96)	0.213	0.056
Natural Killer Lymphocytes (NK-Cells)		
XE-5000 versus FACSCalibur		
HFLC # versus NK cells # (FACS)	0.078	0.42
XE-5000 versus DM 96		
HFLC # versus LGL # (DM96)	0.025	0.07
DM 96 versus FACSCalibur		
LGL # (DM96) versus NK cells # (FACS)	0.560	<0.001
B-lymphocytes		
B-cells: XE-5000 versus DM 96		
HFLC # versus activated B-lymphocytes # (DM96)	0.949	<0.001
HFLC # versus Plasma cells # (DM96)	0.889	<0.001
B-cells: XE-5000 versus FACSCalibur		
HFLC # versus Total B-lymphocytes ($CD19^+$) # (FACS)	0.549	<0.001
HFLC # versus ASC # (FACS)	0.913	<0.001
B-cells: FACSCalibur versus DM 96		
ASC # (FACS) versus activated B-lymphocytes # (DM96)	0.810	<0.001
Total B-Lymphocytes ($CD19^+$) # (FACS) versus activated B-lymphocytes # (DM96)	0.525	<0.001

CONCLUSIONS

In conclusion, the results deliver evidence that the HFL count 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 or lymphoproliferative disorders such as plasma cell myeloma/ plasmacytoma with a distinct HFL population. The XE-5000 eMM software automatically included the samples with a distinct HFL population. Such obvious samples will be flagged as atypical lymph. During infectious diseases the peripheral blood plasma cell concentration is not always elevated - despite 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 or a later reaction through both the T-independent or the T-dependent pathway, to circulated antigens as observed in sepsis. This provides new possibilities for a fast and reliable screening and monitoring of intensive care systemic inflammatory response syndrome (SIRS)

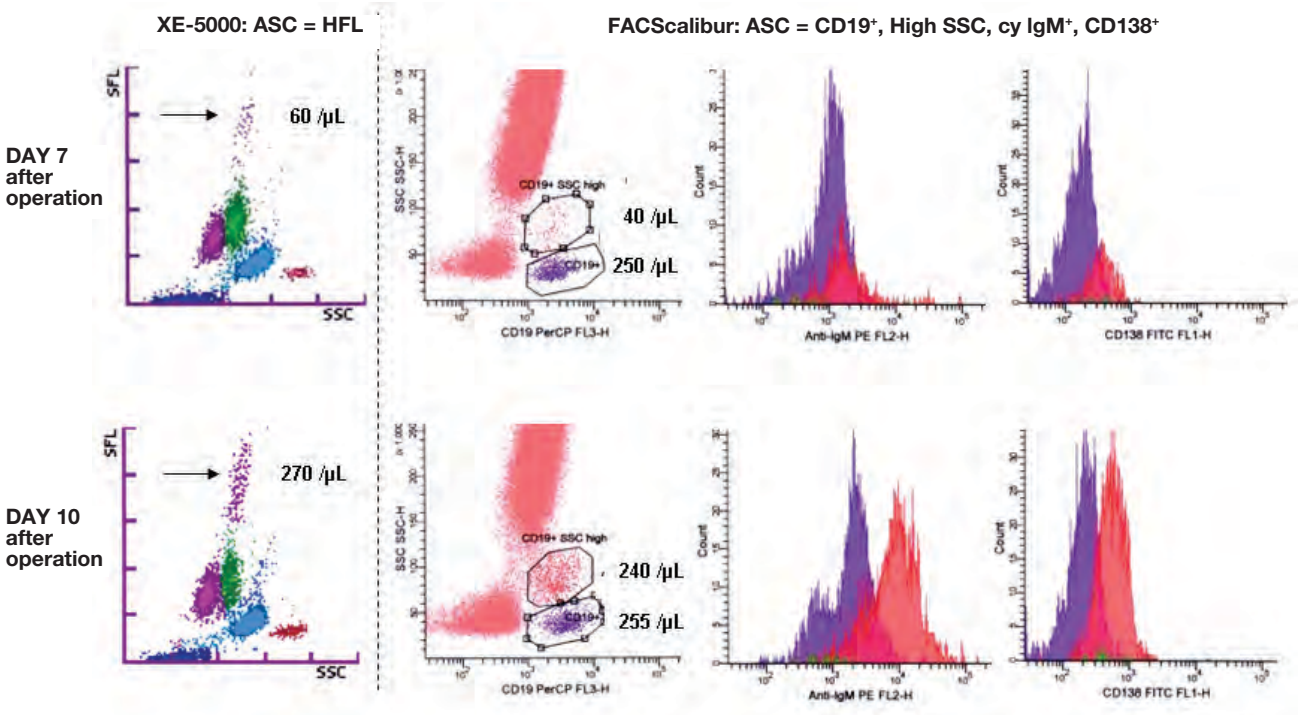

Fig. 4 *Example of correlation of cell types by the two different methods in a patient seven and ten days post cardiac surgery.*

patients with suspect of a local infection or even sepsis. A distinct differentiation between T-independent antigen response and T-lymphocytes supported immune response is obvious by measuring the HFLC concentration in peripheral blood of patients concerned.

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

References

1) *Janeway CA et al. Immunobiology: The immune System in Health and Disease. 5th ed. Heidelberg, Berlin: Taylor & Francis; 2001. 600p.*
2) *Shapiro-Shelef M and Calame K. Regulation of Plasma-cell development. Nat Rev Immunol. March 2005; 5(3): 230-242.*
3) *Flavius Martin and John F. Kearney. Marginal-zone B cells. Nat Rev Immunol. May 2002; 2(5): 323-335.*
4) *Thery C and Amigorena S. The cell biology of antigen presentation in dendritic cells. Curr Opin Immunol. 2001; 13(1): 45-51.*
5) *Liu YJ et al. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. Eur J Immunol. 1991; 21(12): 2951-2962.*
6) *Germain RN. MHC-dependent antigen processing and peptide presentation: Providing ligands for T-Lymphocyte activation. Cell. 1994; 76(2): 287-299.*
7) *Vinuesa CG et al. Follicular B helper T cells in antibody responses and autoimmunity. Nat Rev Immunol. Nov 2005; 5(11): 853-865.*
8) *Kunkel EJ and Butcher EC. Plasma-cell Homing. Nat Rev Immunol. Oct 2003; 3(10): 822-829.*
9) *Linssen J et al. Identification and quantification of high fluorescence-stained lymphocytes as antibody synthesizing/secreting cells using the automated routine hematology analyzer XE-2100. Cytometry PartB: Clinical Cytometry. 2007; 72B(3): 157-166.*
10) *John AK et al. Reference Leucocyte Differential Count (proportional) and Evaluation of Instrumental Methods. Approved Standard-Second Edition. Clinical and Laboratory Standards Institute(CLSI); 2006; 27(4): H20-A2. 63p.*
11) *Swolin B et al. Differential counting of blood leukocytes using automated microscopy and a decision support system based on artificial neural networks -evaluation of DiffMasterTM Octavia. Clin Lab Haematol. 2003; 25(3): 139-147.*
12) *Fred Hilerio, H. Mike Neisler. 4-Color MultiTEST™/ TruCOUNT™ Technology. Becton-Dickinson (BD) FACS® MultiSET™ System.*
13) *Becton-Dickinson (BD). New Specificity in Flow Cytometry CD138. http://www.bdbiosciences.com/pdfs/npas/SJ-0064-00.pdf*