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SEED Haematology

Looking deeper into inflammatory conditions from a laboratory and clinical perspective

Inflammation and infection are two different things, although they very often occur together. 'Inflammation' describes exclusively the body's immunovascular response, irrespective of the cause. It is our body's natural defence against damaged cells, physical or chemical agents, allergens or pathogenic organisms and aims to remove these harmful or foreign invaders and recover.

Generally there are two different types of inflammation. One is acute inflammation, the other is chronic. Acute inflammation starts quickly, presents with redness, swelling, pain, heat, and maybe even loss of function of the inflamed area, and generally disappears in a few days as the tissue heals. Chronic inflammation can last for months or years as a result of a permanent failure to eliminate the cause and/or minor, repeated exposure to one or more proinflammatory agents. A poor diet, stress, minor food allergies, a sedentary lifestyle and more can contribute to chronic inflammation.

As a matter of fact, inflammation isn't always a helpful process. In some cases it has a great destructive potential, which is demonstrated when the immune system mistakenly targets the body's own tissues in autoimmune diseases such as type 1 diabetes, rheumatoid arthritis and lupus.

Inflammation is the body's immunologic response to tissue damage caused by the invasion of foreign bodies, microorganisms or harmful chemicals.

Infection is the invasion of the body by pathogenic microorganisms, including their multiplication and the body's response.

The term 'infection' is used when pathogenic organisms invade the tissue of a host organism, multiply, and the affected tissue reacts to these organisms and the toxins they produce. Infections are caused by microorganisms such as viruses, bacteria and viroids, but also larger organisms such as parasites and fungi. Also prions that are entirely composed of protein material are considered infectious agents. Hosts can fight infection using their immune system and react with an innate response, often involving inflammation, followed by an adaptive response.

Inflammation is therefore not a synonym for infection, not even in cases where inflammation is caused by infection.

Global health challenges – pandemics and epidemics

Infectious diseases are caused by pathogenic microorganisms that infiltrate the body's natural barriers and multiply to induce illnesses, which severity can range from mild to deadly. They can pose a serious threat to public health, not least because epidemics can be difficult to predict. For example, as the 2009/2010 H1N1 flu pandemic illustrated, mutations in the influenza virus can turn annual flu outbreaks into global health threats. Or, taking another example, the Ebola outbreak 2014/2015 created a common awareness that it is no longer a regional problem. According to data of the World Health Organisation (WHO), more than 8,000 healthcare professionals in over 80 countries were trained over the duration of the epidemic [1] since the virus that causes this infection can travel beyond borders – and across oceans – emphasising that major outbreaks of communicable diseases are a shared public health challenge.

Antimicrobial resistance on the advance

Another related topic is the growing problem of antimicrobial resistance. Antibiotics, antivirals, and other antimicrobials have saved millions of lives worldwide, but these drugs are losing their effectiveness because of antimicrobial resistance. Antimicrobial resistance refers to microbes' natural ability to evolve genetically to counter the drugs. Some of this is inevitable, but over-prescription and improper use of antimicrobials play a big role, too. In the EU, about 25,000 patients die each year from an infection caused by these drug-resistant bacteria. Consequences for hospital patients include delayed administration of appropriate antibiotic therapy, longer length of stay, higher healthcare costs and poor patient outcomes [2].

Differentiation is essential

It is useful to differentiate inflammation and infection as there are also many pathological situations where inflammation is not driven by microbial invasion – for example trauma, ischaemia or autoimmune diseases. There are also pathological situations where microbial invasion does not result in a classic inflammatory response, for example parasitosis or eosinophilia.

Fast and efficient differentiation between various inflammatory conditions is clinically very important because the treating physician needs to decide on appropriate therapy for the patient – ideally without delay. A correct differential diagnosis of suspected infections by clinical examination, biochemical markers and microbiological blood cultures is both costly and time-consuming. However, if the laboratory has a fast initial indication, unnecessary follow-up tests, such as smear reviews or flow cytometry analyses, can be avoided. This means the physician can start – or, with treatment already begun, adapt or discontinue – treatment faster.

How blood cell analysis can contribute

The immune system is a mechanism that protects our bodies from harmful substances, foreign microorganisms and even cancer. White blood cells (WBC), as part of the immune system, help to fight infection and defend the body against other foreign substances. Different types of WBC are involved in recognising intruders, killing harmful bacteria, and synthesizing antibodies to protect the body against future exposure to these bacteria and viruses.

The two basic types of immunity are *innate* and *adaptive* – the latter also referred to as *acquired* – immunity. Some of our white blood cells play a role in innate immunity, others in adaptive immunity, while some are involved in both. Innate immune response is activated mainly at the site of infection, whereas adaptive immune response is activated in peripheral lymphoid organs. The two types of responses work together to eliminate invading pathogens.

Both types of immunity include *humoral* and *cell-mediated* immunity components. *Humoral immunity* is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins and certain antimicrobial peptides. The name 'humoral' derives from the idea that the involved substances are found in the humors, or body fluids. *Cell-mediated immunity* follows principal mechanisms by which cells help to defend the body against infection by

- killing the microbes directly,
- killing the cells that harbour microbes,
- preventing access to or expelling the microbes from the body, and
- providing defence against larger parasites such as worms in the gut.

The adaptive immune system can remember previous experiences. This is why we develop lifelong immunity to many common infectious diseases after our initial exposure to the pathogen.

Inflammation is initiated and controlled by phagocytic WBC such as neutrophils, monocytes and macrophages. But also natural killer cells as innate lymphoid cells are involved. Neutrophils migrate from the blood vessels into tissue during an inflammatory response and attack microorganisms such as bacteria and fungi. Basophils initiate an inflammatory response to environmental antigens, whereas eosinophils defend the body against parasites and/or allergens. Natural killer cells use potent chemicals to kill infected cells upon contact. Macrophages act as scavenger cells in tissue. Once a macrophage phagocytoses a foreign invader, it presents typical features of that organism to T lymphocytes, produces inflammatory mediators and initiates progression to the adaptive immune response.

Neutrophil activation

With normal adults, neutrophils account for more than half of the circulating white blood cells [3]. Together with monocytes and macrophages they are commonly known as 'professional' phagocytic cells of the innate immune system. However, neutrophils use at least two different strategies to fight pathogens: phagocytosis and secretion. Once activated, they secrete a variety of proinflammatory cytokines and antibacterial substances and also act as antigen-presenting cells, which are able to activate the adaptive immune response [4].

Alterations in neutrophil morphology (size, shape and composition), mechanics (deformability) and motility (chemotaxis and migration) have been observed during infection [5]. Activated neutrophils can be distinguished morphologically from resting neutrophils by different features (Fig. 1a – d). 'Toxic granulation' is the term used to describe an increase in the staining density and number of granules that occurs regularly with bacterial infection and often with other causes of inflammation. The presence of cytoplasmic vacuoles is indicative of an increased phagocytic activity of the neutrophils in response to bacterial infection. Rarely, in the presence of overwhelming numbers of bacteria or fungi, microorganisms are seen within vacuoles or freely in the cytoplasm of the neutrophils. Many neutrophils in the bloodstream containing Döhle bodies are also a sign of activation after inflammatory stimulation.

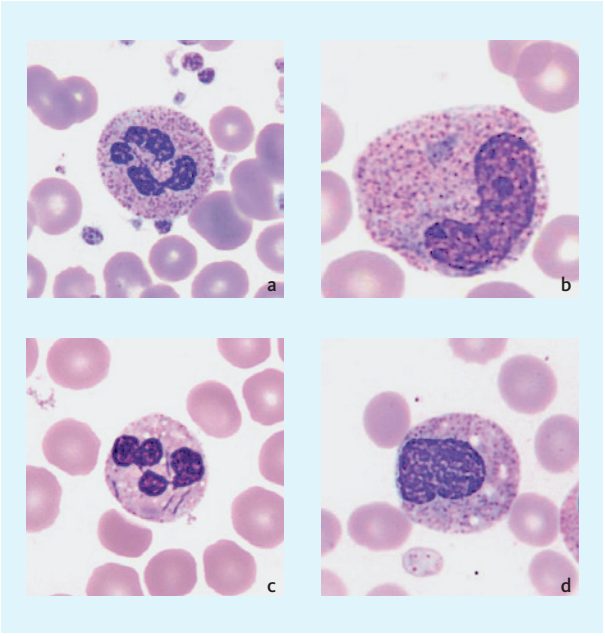

Fig. 1 *Different examples of neutrophils a) toxic granulation b) Döhle bodies c) intracellular gram-negative rods d) vacuolisation*

The above being intracellular activities, neutrophils also entrap and kill bacteria extracellularly by forming neutrophil extracellular traps (NETs) [6], which are networks of extracellular fibres, primarily composed of DNA. NETs provide for a high local concentration of antimicrobial components and bind, disarm and kill microbes extracellularly, independent of phagocytic uptake.

Lymphocyte activation

The primary cells that control the adaptive immune response are the lymphocytes, in particular the T and B cells. Mature T cells become activated by recognising processed foreign antigens on antigen-presenting cells and begin to divide rapidly so that first memory T cells and subsequently effector T cells are generated. In the humoral response, B cells are activated to secrete antibodies, which bind specifically to the foreign antigen that stimulated their production. In certain cases in the early phase of infection also unspecific (T cell-independent) antibodies can be secreted by B cells.

T and B cells become morphologically distinguishable from each other only after they have been activated by antigens [7]. In both bacterial and viral infections, transformed lymphocytes may be seen in the peripheral blood smear. Usually these cells present with heterogeneous morphological features including a larger size, a round nucleus often with a large nucleolus, and abundant, deeply basophilic cytoplasm (Fig. 2 a – c). In their most mature form, which is called 'plasma cell', effector B cells are filled with an extensive rough endoplasmic reticulum (Fig. 2d). In contrast to that, effector T cells contain very little endoplasmic reticulum and do not secrete antibodies. From a morphological point of view, it is sometimes fairly difficult to distinguish reactive lymphocytes from neoplastic ones.

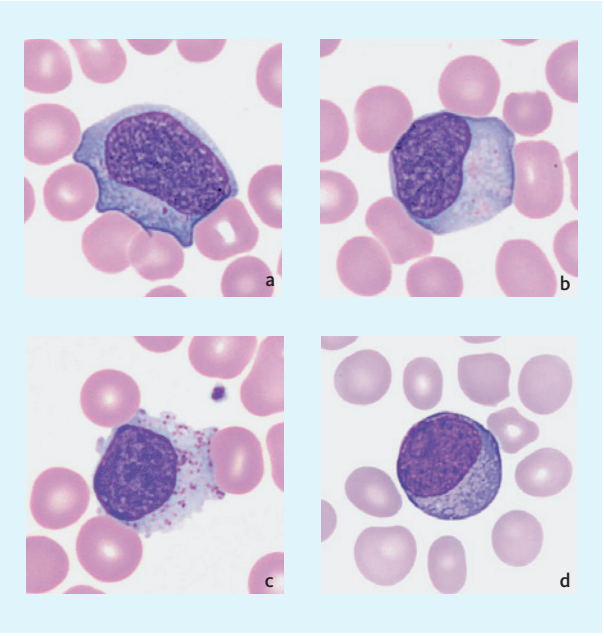

Fig. 2 *Examples of activated lymphocytes as found in infectious mononucleosis*

Activated monocytes

Monocytes and macrophages are the key players in the innate immune system. Their heterogeneity in size, morphology, phagocytic function and cell adhesion had been described, and subsequently three different monocyte subsets – classical, intermediate and non-classical monocytes – were defined based on differences in the expression of the surface markers CD14 and CD16 [8]. Classical monocytes are the cells that had been known to haematologists for a century as monocytes on the basis of structure, whereas the somewhat smaller, non-classical monocytes, which account for only 10 % of all monocytes, were described only 20 years ago. There appears to be a developmental relationship between these cells (from classical via intermediate to non-classical) during the course of an infection or with macrophage colony-stimulating factor (M-CSF) treatment so that there is first an increase of the intermediate cells followed by an increase of the non-classical monocytes [8].

Alterations in the distribution of monocyte subsets are associated with clinical outcomes, e. g. of cardiovascular diseases. When activated (see Fig. 3), monocytes can eliminate pathogens by phagocytosis, release reactive oxygen species (ROS), produce proinflammatory cytokines and modulate the T cell immune response.

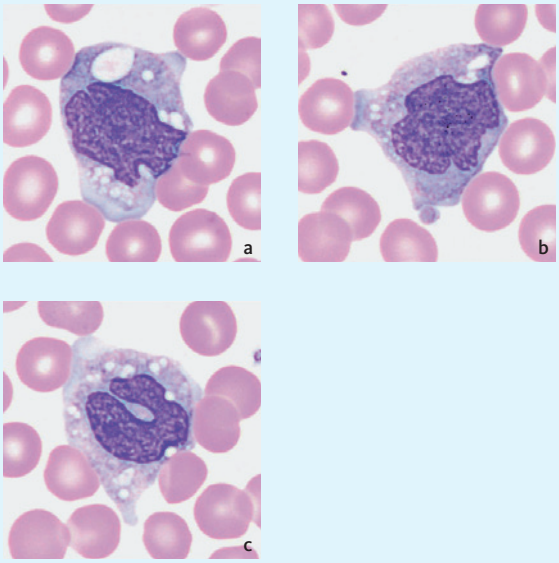

Fig. 3 *Activated monocytes as found in infectious mononucleosis*

Quantification and characterisation of reactive cells beyond a classical 5-part differential

The XN-Series analysers, which use fluorescence flow cytometry as the measurement principle for the WBC differential, contribute to the detection of activated neutrophils and lymphocytes as these cells' measured signals differ significantly from those of resting cells. Using fluorescence flow cytometry allows the measurement of cell functionality – within the scope of a routine blood test.

1. Assessment of neutrophil activation

For each WBC that passes the laser beam, the forward scattered light (FSC), side scattered light (SSC) and fluorescence intensity (SFL) signals are recorded and graphically displayed in a scattergram. The positioning of the neutrophil population in the WDF scattergram (generated in the course of an XN-DIFF analysis) allows an assessment of the neutrophils' activation (Fig. 4 a – c).

When running an XN-DIFF analysis, a unique combination of reagents (lysis and labelling) together with the incubation time permits to separate the different white blood cell populations. First, the lysis reagent perforates cell membranes, whereby the extent of membrane damage depends on the lipid composition, which in turn depends on the cell type (maturity level) and the state of the cell (activation status). Activated cells do not only have a different membrane lipid composition but also show greater activity in the cytoplasm as they actively produce e.g. cytokines. Consequently, the intensity of the fluorescence signal of activated cells is greater than that of resting cells. The parameter NEUT-RI reflects this neutrophil reactivity intensity, expressed in the unit FI (Fluorescence Intensity).

The 90-degree side scattered light of the WBC differential channel provides information about cell density or complexity, which represents the granularity of the cells. Therefore, if the complexity of neutrophils increases upon a change in functionality, e. g. by toxic granulation or vacuolisation, the position of the neutrophil cluster in the scattergram will also be affected. The parameter NEUT-GI, expressed in the unit SI (Scatter Intensity), changes accordingly.

The NEUT-RI and NEUT-GI parameters do not reflect a specific cell count but the intensity of the fluorescence and side scatter signals, respectively, measured at the centroid position of the NEUT population. The following paragraphs explain what this actually signifies.

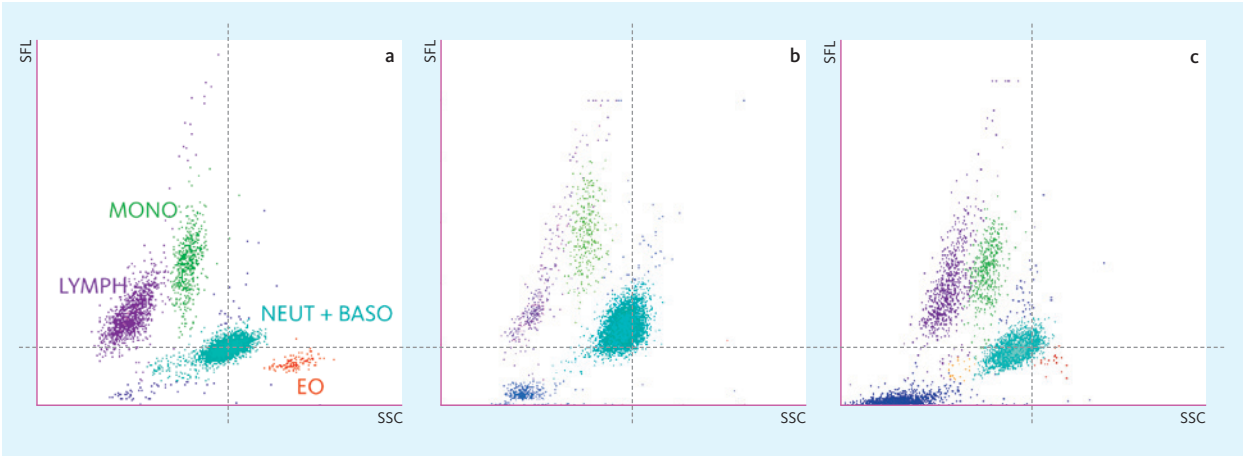

Fig. 4 *The WDF scattergram plots intracellular structure (SSC) on the x-axis and the content of RNA/DNA (SFL) on the y-axis. Each dot represents one analysed cell. a) healthy person b) patient with sepsis c) patient with tuberculosis.*
The dotted lines were added for illustration purposes.

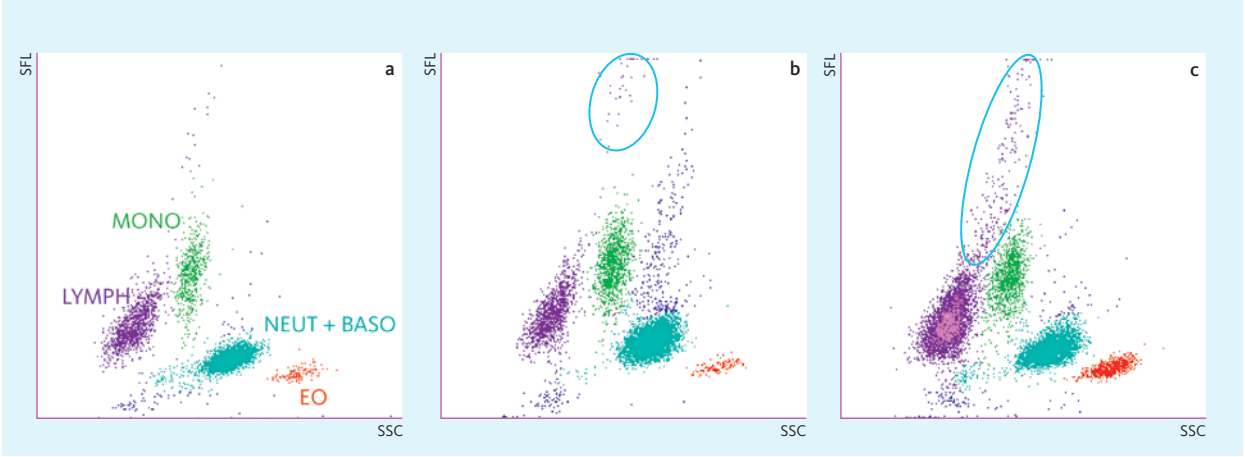

Fig. 5 *WDF scattergrams showing the position of reactive lymphocyte clusters (◯). a) healthy person b) location of AS-LYMP as an independent population at the top of the scattergram c) RE-LYMP with an increased fluorescence signal*

2. Counting activated lymphocytes

The XN-Series also supports the detection of reactive lymphocytes as their activation goes along with an increased cellular activity in the cytoplasm. Reactive lymphocytes are detected by an increased fluorescence signal compared to non-activated lymphocytes (Fig. 5 a – c). Activated B lymphocytes are quantified by the parameter AS-LYMP (antibody-synthesizing lymphocytes) whereas all of the activated lymphocytes (including antibody-synthesizing lymphocytes) are quantified by the parameter RE-LYMP (reactive lymphocytes).

Extended Inflammation Parameters

The Sysmex XN-Series analysers are able to accurately determine counts of reactive mature lymphocytes (RE-LYMP and AS-LYMP) and quantify the activation status of neutrophils (NEUT-GI and NEUT-RI). These new diagnostic parameters are available as 'Extended Inflammation Parameters' (EIP) from XN IPU software version 21.12 onwards. With the EIP the laboratory is able to describe inflammatory conditions in a quantitative manner, and therefore reliably and faster.

The Extended Inflammation Parameters are haematological parameters derived from a routine laboratory test and can be used to characterise reactive samples.

The idea is to use these parameters with reactive samples, meaning a malignant condition has to be ruled out first, since the activated lymphocytes and neutrophils can only be confidently quantified if they are truly reactive and not malignant. In order to exclude a potential malignancy of a sample, analysis in the white precursor and pathological cell (WPC) channel is needed. By running XN-DIFF and WPC analysis and so combining these two flow cytometry channels, malignant and reactive cells can be confidently differentiated due to their differences in cell functionality.

An overview of the Extended Inflammation Parameters describing cell population, immunological interpretation and reference interval is listed in Table 1.

Clinical interpretation of the Extended Inflammation Parameters

The new parameters support the differentiation between inflammation and infection, different pathogenic causes of infection and the different types of immune response: early innate, cellular or humoral immune response [9, 11 – 14]. Using the Extended Inflammation Parameters can help clinicians with the diagnosis, treatment and monitoring of patients with inflammatory disorders by

- supporting the differentiation between inflammation and infection.
- supporting the differentiation between different pathogenic causes of infection (e. g. bacterial, viral).
- supporting the differentiation between different types of immune response: early innate, cellular or humoral immune response. This information may help to identify the stage of infection.
- permitting a detailed monitoring of inflammatory conditions.

Table 1 *Overview of the Extended Inflammation Parameters*

Parameter	Cell population	Detailed description	Immunological interpretation	Reference interval [1]
RE-LYMP	Total reactive lymphocytes	■ RE-LYMP#	Innate and adaptive cell-mediated immune response	0 – 0.5 x 10^9 / L
		■ RE-LYMP% [ii]		0 – 5 %
AS-LYMP [i]	Antibody-synthesizing lymphocytes	■ AS-LYMP#	Innate and adaptive humoral immune response	0 cells / L
		■ AS-LYMP% [ii]		0 %
NEUT-GI	Cytoplasmic granulation of neutrophils	Neutrophil Granularity Intensity	Early innate immune response	142.8 – 159.3 SI [9]
NEUT-RI	Reactivity of neutrophils (metabolic activity)	Neutrophil Reactivity Intensity	Early innate immune response	39.8 – 51.0 FI [9]

1 Reference ranges should be always examined for suitability in a given patient population according to the method recommended by the International Federation of Clinical Chemistry and Laboratory Medicine [10].
i When antibody-synthesizing lymphocytes (AS-LYMP) are present, they are also included in the total reactive lymphocytes (RE-LYMP).
ii As a percentage of all WBC.

For supplementary information on the clinical interpretation of the Extended Inflammation Parameters you can download our freely available white paper: *'Novel haematological parameters for rapidly monitoring the immune system response'*.
www.sysmex-europe.com/whitepapers

Conclusion

The Extended Inflammation Parameters are available from a routine blood laboratory test, together with the complete blood count. They allow a quantification of the activation status of neutrophils (NEUT-GI, NEUT-RI) and activated lymphocytes (RE-LYMP, AS-LYMP). The Extended Inflammation Parameters provide extra information about the activation of the immune response. One should bear in mind, however, that all this information can only be reliably applied if a malignant condition has been confidently ruled out and the sample is confirmed as reactive.

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