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Immunodeficiency and Immunomonitoring

Abstract: Immunological diagnostics is a rapidly developing area in laboratory medicine. Most recently, major developments in the area of immunodeficiency and the monitoring of chronic inflammatory diseases have been observed. Regarding immuno-monitoring, recently a consensus panel for basic flow cytometry has been published together with age-related reference values. In the USA, the search for severe inherited immunodeficiency diseases (such as severe combined immunodeficiency disease, SCID) is part of neonatal screening procedures. Recently, several US states published first results which are based on the measurement of T-cell receptor excision circles (TRECs). Furthermore, age-dependent reference values for the measurement of IgG subclasses and subclass-specific vaccination antibodies have been published. Monitoring of chronic inflammatory disease focuses on asthma. A novel classification based on the cellular distribution of neutrophils versus eosinophils in induced sputum has been developed. Furthermore, novel biomarkers, such as periostin, are currently under evaluation. Such novel approaches of phenotyping are now the basis of individualized therapeutic approaches in patients with (severe) asthma, who respond to certain biologicals.

Keywords: antibody vaccination titers; asthma; eosinophils; flow cytometry; IgG subclasses; induced sputum; periostin; T-cell receptor excision circles (TRECs).

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Immunodeficiencies

Immune system disorders are more common than is generally appreciated. Besides physiological situations in which the immune system cannot yet or can no longer meet optimal functions (neonatal period, age), congenital, acquired and iatrogenic immunodeficiencies can be distinguished (Table 1). The first indication of the presence of an immunodeficiency comes from a clinical examination and a thorough case history.

What responsibilities does the immune system have? It is essentially to protect against the invasion and spread of pathogens that are ultimately no longer compatible with life or survival for the host, or lead to irreversible damage. On the other hand, it is necessary to maintain an immune balance ("homeostasis"), so that the immune system does not attack endogenous molecules (autoantigens) or harmless foreign antigens (allergens).

Therefore, an increased susceptibility to infections is observed in the case of a faulty immune response (Figure 1). The immune system has developed different strategies to deal with the various classes of pathogens that can be distinguished as a matter of principle:

- Defense against most bacterial pathogens occurs at the level of the innate immune system through the use of complement and neutrophils. This initial phase of defending against an infection is followed by the production of pathogen-specific antibodies, which ultimately achieve an optimal elimination of pathogens in concert with complement and neutrophils.
- Viruses are obligate intracellular pathogens. Here, the key strategy of the immune system aims at the destruction of the already infected host cell and/or the prevention of the infection from spreading to host cells not yet infected. This involves a whole series of cytotoxic mechanisms, such as the induction of CD8 T-cells, the use of NK cells, etc.
- The third group of pathogens comprises facultative intracellular microbes, which include mycobacteria, *Pneumocystis carinii*, and a number of fungi, such as *Aspergillus*, *Candida*, and others. The crucial defense reaction is the formation of granulomatous structures consisting mainly of macrophages and their subtypes, as well as the activation of CD4 positive T-cells.

In patients with suspected immunodeficiency, therefore, a detailed pathogen history plays a central role. The

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Table 1 Situations in which an acquired immune deficiency can be observed as a concomitant.

Acquired immunodeficiencies
Possible in the case of:
– HIV infection
– EBV, CMV infection
– Chronic infections (e.g., TB, parasitic diseases)
– Malignant solid tumors
– Leukemias and lymphomas
– Monoclonal gammopathies
– Metabolic disorders, especially: Diabetes mellitus
– Chronic kidney disease
– Autoimmune diseases (e.g., SLE)
– Iatrogenic immunosuppression (e.g. plasmapheresis, immunosuppressants, anti-CD20 therapy)

lab-based diagnostic strategy can then be designed specifically on the basis of such history.

The following focuses on new findings regarding the diagnostics of the T-cell and B-cell systems; diagnostic strategies related to the complement system and neutrophils are merely touched upon.

T-cell defects

T- and B-cells are part of the so-called **adaptive immune system.** The cells or their functions are characterized by some special features that have evolved or specialized within the vertebrate development:

– Antigen specificity
  This is accomplished by the recombination of the T- and/or B-cell receptor. Genetic recombination produces receptor diversity that is capable of recognizing all the antigen specificities (i.e., epitopes) in the environment and in "self". The counterpart of T-cell receptors, for B-cells, is the synthesis of immunoglobulin. The immune response against antigens must be acquired. This requires antigen contact. By presentation of appropriate epitopes, which are presented to the cells of the adaptive immune system by antigen presenters (e.g., monocytes, macrophages, the critical cells), a specific targeted effector response is set in motion at the level of the adaptive immune system.

– Memory function
  Lymphocytes mature in the bone marrow from hematopoietic stem cells. T-cell-receptor-negative lymphocytes then migrate into the thymus. Here, the formation of the T-cell repertoire occurs by means of positive and negative selection. CD4+ or CD8+ T-cells then leave the thymus as mature lymphocytes and colonize the lymphoid organs. After antigen presentation, activation and further functional differentiation in T-cell subpopulations take place, such as Th1, Th2, Th17, etc. B-cells initially remain in the bone marrow where they develop their T-cell receptor repertoire by

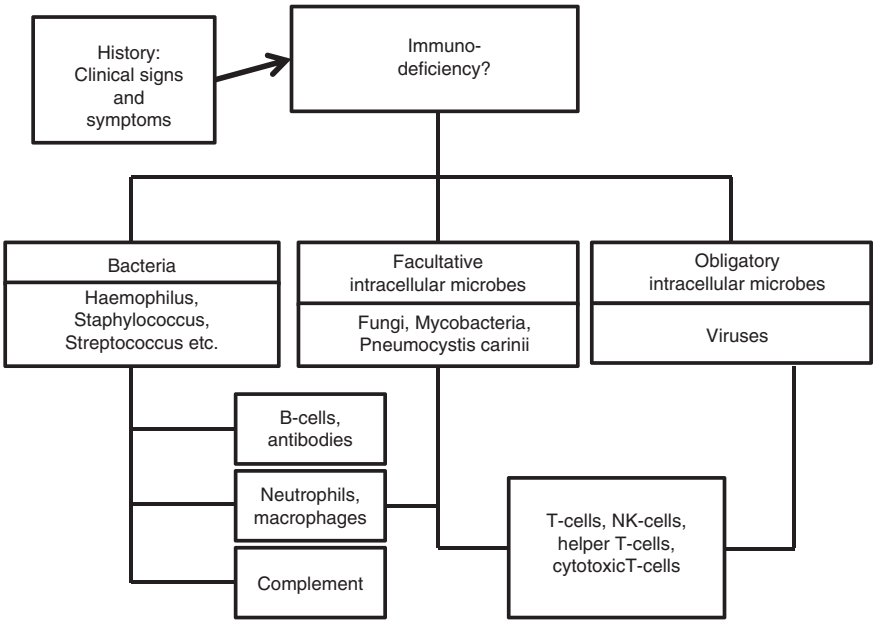

Figure 1 Laboratory diagnostic strategy based on predominant spectrum of pathogens.

expression of a specific antibody (initially IgG and IgM) on the surface of the B-cells. These cells are then able to produce this specific antibody if they have been activated in accordance with the specific antigen. Under the influence of T-cell signals, then, there is a class shift to other immunoglobulin isotypes, such as IgG subclasses, IgE and IgA subclasses. The corresponding signals for the class change are transmitted by (antigen-specific) T-cells.

In this way, errors, usually genetic in origin, can generally occur anywhere.

The focus of lymphocytes diagnostics is the phenotyping of lymphocyte subpopulations in qualitative and quantitative terms. Here, flow cytometry plays a crucial role today.

Age-related standard values must be considered in the interpretation (Table 2). While neutrophilic granulocytes are more numerous than lymphocytes (T+B+NK cells) in adulthood (rule of thumb, by two to one), this ratio is reversed in the infant and toddler years. Reference values for the neonatal and early childhood age groups have recently been published again. They are essentially based on the reference values already published in 2003, taken from the Pediatric AIDS P1009 study [2].

One of the most severe forms of congenital T-cell defects is the severe combined immunodeficiency syndrome (SCID). Children with SCID are healthy at birth, but die from severe infections if they do not receive hematopoietic stem cell transplants, enzyme replacement therapy or gene therapy. SCID is characterized by severe defects in the T- and B-cell system; in some cases, the NK cells are affected as well. It is a genetic disease for which mutations in more than 13 different genes have been described so far [3]. The most important criterion in the diagnosis of SCID has been the absolute lymphocyte or T-cell count. This has been clarified yet again in a recent paper [4].

Table 2 Reference values from the Pediatric AIDS P1009 study published in 2003 [1].

Lymphocyte markers	%	Absolute, µL
$CD3^+$	49–84	2500–5600
$CD3^+/CD4^+$	35–64	1600–4000
$CD3^+/CD8^+$	12–28	500–1700
$CD3^-/CD16^+$ or $CD56^+$	4–18	160–1100
$CD19^+$	6–32	300–2000

Reference values in neonates and early childhood.

Since this is a life-threatening disease that forms on a genetic basis, the question arises whether a newborn screening can be established. For many years efforts in this context have been underway in the US, and have now led to the establishment of such a program in several states. The method that took hold is the quantitative detection of so-called T-cell receptor excision circles (TREC) by means of quantitative PCR [5]. Such TRECs form during the recombination of the T-cell receptor α-chain. During this process, DNA fragments are cut out from the T-cell receptor α gene locus that are no longer needed for the mature T-cell receptor α-chain. The excised DNA material forms a ring that is stable and does not replicate further during the cell cycle. Therefore, the number of these TRECs thins out over the course of the T-cell proliferation. TRECs are then present in cells as byproducts. If one finds TRECs, this is a sign of a normally functioning program of T-cell development. Thus, they are biomarkers for newly produced T-cells.

This methodology has meanwhile been evaluated with a number of technical variations in some U.S. States on more than one million newborns. It has been shown that this test system meets all the requirements for a population-based screening, i.e., false-negative results (that is, when actually ill patients are overlooked) is reduced to an absolute minimum. In return, there are of course a smaller number of false-positive test results that require further follow-up.

The biomaterial used comes from the filter card containing heel blood. As an alternative to PCR, a mass spectrometric method has been developed for certain forms of SCID. This is appropriate for adenosine deaminase deficiency (ADA), which involves the accumulation of toxic metabolites from the purine metabolism. The method has recently been studied in Italy in a pilot project to test its suitability for a population-based screening – with convincing results [6, 7].

B-cell defects

B-lymphocytes, too, can be affected by disorders in all sections and phases of B-cell development. These range from a complete absence of antibodies (e.g., agammaglobulinemia) to impaired production of specific antibodies for antigen contact. Clinically speaking, antibody defects are commonly associated with bacterial infections.

The most common symptomatic primary immunodeficiency is the **common variable immunodeficiency syndrome (CVID).** This disease often occurs in adults

between 20 and 40 years. There is still, despite improved diagnostics, a delay of six to eight years before the diagnosis is established. CVID is associated with a number of chronic complications, including autoimmune diseases, chronic lung disease, chronic inflammatory bowel disease, systemic granulomatosis and malignant lymphoid hyperplasia. Although the genetic defect remains unknown in most CVID cases, some polymorphisms have been described in recent years. These are genes that are involved in T-B-cell communications, B-cell activation and/or the class change.

The diagnosis is crucially determined by a significant reduction in the total IgG, IgA and/or IgM levels. Total IgG is generally lower than 400 mg/dL. In addition, it is required that a deficiency must be present in specific antibody production. If patients have total IgG levels of under 200 mg/dL, this will be seen as a criterion for starting immunoglobulin replacement therapy [8].

IgG subclass defects are also quite common. An appropriate diagnosis can, however, be made only in the simultaneous presence of recurrent infections, plus a proven defect in antibody production. In this context, the age-dependent standard values – particularly of the IgG subclasses – must be taken into account. These were established some time ago with the help of the certified reference material 470 [9].

The clinical relevance of IgG subclass defects varies. While IgG-1 and IgG-2 defects are often associated with recurrent respiratory infections, which can be more or less pronounced, IgG-3 defects are found in patients who develop CVID; IgG-4 defects usually do not have any clinical importance.

Another important step in the diagnostic work-up of antibody-induced immune defects is the **measurement of specific antibody titers.** In this context, the diagnostic vaccination and subsequent determination of specific vaccine antibodies against such standardized antigens play an important role. A recently published document of the American Academy of Allergy, Asthma and Immunology (AAAAI) provides a *state-of-the-art* overview of the procedures and the interpretation of the results [10]. Despite considerable progress in the preparation of vaccine antigens and their frequent use, including for diagnostic purposes, the authors come to the conclusion that further studies are needed in order to definitively describe reference ranges of antibody titers in response to vaccinations. Until further notice, however, the age-dependent reference values can be used, which were published a few years ago for vaccinations following tetanus toxoid, Hemophilus influenzae B and unconjugated pneumococcal polysaccharides [11].

Defects in the complement system and neutrophils

Although defects in the complement system and neutrophils are not the focus of this paper, the authors still want to give a brief introduction to the basic diagnostic workflow program here for the sake of completeness.

Immunomonitoring in connection with chronic inflammation

Allergies and asthma

Allergies are on the rise worldwide. These are chronic inflammatory processes that manifest themselves especially on mucosal surfaces and the skin. The immune system develops a defense reaction against otherwise harmless environmental antigens, such as pollen, animal hair, dust mites, food and other things.

The main allergic diseases are atopic dermatitis (eczema/neurodermatitis), allergic rhinoconjunctivitis and asthma, as well as food allergies in the gastrointestinal tract.

Asthma is the most common chronic disease in pediatrics and the sixth most common chronic disease in adult medicine.

Despite all the advances in understanding the pathogenesis of asthma, there is still a great need for new drugs and/or improved therapeutic strategies. Although the conventional anti-inflammatory asthma therapy works in the majority of patients, about 30% are not well controlled and 5–10% of patients suffer from severe asthma, which responds particularly poorly to conventional therapy.

There is also a growing understanding that asthma involves different, including clinical, phenotypes. This refers not only to the severity (mild, moderate and severe), but also to the clinical representation. Probably the best-studied phenotypes are

- Allergic and
- Non-allergic asthma

In addition, there are also known clinical phenotypes, such as nocturnal asthma, exercise-induced asthma, and others.

From this, the following key questions are derived:

- Can these clinical phenotypes be represented by immunological patterns?
- Does a therapy approach based on the selection of patients on the basis of immunological patterns lead to better clinical results?

New biomarkers of allergic and non-allergic asthma

Significant progress has been made in recent years in this highly exciting and rapidly evolving field of research. These are summarized in an excellent review article by Bhakta and Woodruff [12].

The induced sputum represents an interesting biomaterial for immunological studies. It is well established that not all asthmatics have eosinophils in the sputum. Non-eosinophilic asthma occurs in about 25% of patients without treatment, and about half of the patients under treatment (especially steroids) exhibit this cellular phenotype [13].

This concept has now produced a cellular classification, which can be divided into four categories, based on the cellular composition in induced sputum (Table 3):

- Eosinophilic type
- Neutrophilic type
- Mixed type
- Absence of granulocytes (=paucigranulocytic type)

Is such an immunologically cellular-based classification only of theoretical interest, or does this also have practical implications?

In this context, two important clinical studies should be highlighted that address this very question for the first time:

Asthma patients were treated with a monoclonal anti-IL5 antibody in both studies. These studies were preceded by clinical tests in which a collective (without any specific selection) of asthmatics were treated and in which the study outcome was negative. The scientific rationale for the use of anti-IL5 antibodies is obvious, due to the central role of this mediator in the biology of eosinophils. In fact, it had already been shown earlier – also confirmed in these clinical studies – that patients treated with this antibody can experience even a rapid decrease in eosinophils in the blood (and sputum).

What is new to the studies now carried out is the stratification of patients on the basis of the cellular composition in the induced sputum.

In the study by Nair [14] patients were selected who receive oral steroids (i.e., particularly severe asthmatics) and who still have eosinophils in the sputum (>3%).

As Figure 1 clearly shows, a stable clinical picture of symptoms emerges with respect to asthma exacerbations (the primary endpoint).

In the second study by Haldar and colleagues [15] patients were included who suffered from refractory asthma, required high doses of inhaled steroids, received oral steroids at least twice in the last 12 months, and still exhibited sputum eosinophilia.

As shown graphically in Figure 1, it is also here in the treated group where significantly fewer exacerbations occur (in this case, the cumulative number of exacerbations) than in the placebo group.

These studies can be regarded as a kind of "proof of concept" that a highly specific therapeutic approach for certain, specially selected patients may in fact lead to clinical success. This is a first contribution towards stratified or personalized medicine.

Mediators and immune cells detected in induced sputum are ultimately very predominantly the result of secretion processes of the respiratory epithelium and/or require migration through the respiratory epithelium. This finding raises the question whether the respiratory

Table 3 Cell-biological phenotypes of asthma (according to [13]).

	Non-eosinophilic (2%)	**Eosinophilic (2%)**
Normal neutrophils (60%)	Paucigranulocytic – Controlled or intermittently – If necessary, other diagnosis	Eosinophilic – Allergic asthma – Inadequate ICS therapy
Increased neutrophils (60%)	Neutrophilic – Acute infection (bacterial or viral) – Chronic infection (chlamydia, adenovirus) – Smokers – Ozone (NO_2) – Endotoxins – Occupational – Obesity	Mixed granulocytes – (Severe) asthma exacerbation – Resistant to treatment

Classification of cellular inflammation in induced sputum.

epithelium of patients suffering from certain asthma phenotypes has different signatures and whether new biomarkers can be defined on the basis of such differences.

Woodruff and colleagues intensively investigated this question in recent years. The findings, laid out in a whole series of studies [16–22], led to the identification of three potential biomarkers:

- CLCA1 (calcium-activated chloride channel family member 1)
- Serpin B2 (synonym: plasminogen activator inhibitor 2; PAI2)
- Periostin

These three molecules were used to define the TH2-high phenotype. The clinical relevance of this phenotype was then tested in a study as part of which asthma patients received inhaled steroids over a period of eight weeks. The results show that the TH2-high phenotype is the one that responds to inhaled fluticasone (with improved lung function).

Periostin as a new biomarker?

Can the TH2 phenotype also be used to stratify for a corresponding selective therapy that addresses exactly this TH2 phenotype?

In this context, a recent study was published that deserves special attention. It is a randomized placebo-controlled study with a monoclonal antibody against IL-13, i.e., a traditional TH2 cytokine [23]. A total of 219 asthmatics were included who had been controlled only inadequately despite treatment with inhaled corticosteroids. The primary endpoint was the forced expiratory volume in one second (FEV1).

Patients were stratified according to the TH2 phenotype, using total IgE, blood eosinophils and the blood periostin concentration. In the TH2-high and periostin-high groups, this produced a significant improvement in the primary endpoint.

Periostin is a biomolecule that has gained a great deal of attention in recent times. It has been shown that periostin under the influence of Th2 cytokines supports eosinophilic inflammation (in the respiratory tract and other mucosal organs) [24].

However, this up-regulation of Th2 inflammation by periostin is not limited to this type of inflammation, but may be considered in principle as a general tissue response to injury and stress. Periostin is expressed in the epithelium of the respiratory and intestinal tracts. It is increased in certain tumor types, pulmonary fibrosis and hypertension as well as in dermatitis. Furthermore, it was already described in the context of glomerular injury, renal fibrosis and other inflammations [25–28]. In a recent study, various biomarkers have been studied in patients with asthma who remain symptomatic despite maximal inhaled corticosteroid therapy [29]. The study compared FeNO, eosinophilia and IgE concentrations together with periostin levels in the blood. Compared with each other, periostin reached the highest sensitivity and specificity in the ROC analysis with an AUC of 84%. In the logistic regression model, too, periostin reached the highest level of significance of p=0.007. However, further studies are needed to establish periostin as a systemic marker of airway eosinophilia in asthmatic patients and to emphasize the benefits in relation to the response to particular stratified treatment approaches.

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