UvA-DARE (Digital Academic Repository)

Flow cytometric immunophenotyping in the diagnosis and follow-up of immunodeficient children

de Vries, E.; Noordzij, J.G.; Kuijpers, T.W.; van Dongen, J.J.M.

DOI

[10.1007/s004310100797](https://doi.org/10.1007/s004310100797)

Publication date

2001

Published in

European Journal of Pediatrics

[Link to publication](#)

Citation for published version (APA):

de Vries, E., Noordzij, J. G., Kuijpers, T. W., & van Dongen, J. J. M. (2001). Flow cytometric immunophenotyping in the diagnosis and follow-up of immunodeficient children. *European Journal of Pediatrics*, 160(10), 583-591. <https://doi.org/10.1007/s004310100797>

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

Esther de Vries · Jeroen G. Noordzij · Taco W. Kuijpers
Jacques J.M. van Dongen

Flow cytometric immunophenotyping in the diagnosis and follow-up of immunodeficient children

Received: 29 March 2001 / Accepted: 15 May 2001 / Published online: 19 July 2001
© Springer-Verlag 2001

Abstract From time to time, paediatricians are confronted with children who might suffer from a primary immunodeficiency disease. For practical purposes, these children can be divided into four main clinical categories: (1) a relatively large group of children with recurrent ear-nose and throat and lower respiratory tract infections, in some cases caused by deficiencies of antibodies or complement; (2) children with failure to thrive, intractable diarrhoea or an opportunistic infection which can be caused by a T-lymphocyte or combined immunodeficiency; (3) children with infections with pyogenic bacteria or fungi as seen in case of granulocyte/monocyte function deficiency; and (4) a small heterogeneous group of children with recurrence of particular infections. Also, acquired immunodeficiency becomes a more common problem in paediatric practice. Flow cytometric immunophenotyping of leucocytes appears to be an efficient and rapid tool in the diagnosis and follow-up of immunodeficient patients, supporting early recognition, before serious infections have compromised the child's general condition. This technique can now be performed in many hospitals. In this review, we give directions for the use of flow cytometric immunophenotyping of leucocytes in the diagnosis and follow-up of

immunodeficient children according to the four main clinical categories.

Keywords Flow cytometry · Immunodeficiency · Immunophenotyping

Abbreviations *AID* activation-induced cytidine deaminase · *BTK* Bruton tyrosine kinase · *CD40L* CD40 ligand · *ENT* ear-nose and throat · *FSC* forward scatter · *HIV* human immunodeficiency virus · *IFN* interferon · *IL* interleukin · *LWB* lysed whole blood · *McAb* monoclonal antibody · *SCID* severe combined immunodeficiency · *SSC* side scatter · *TCR* T-cell receptor · *ICF* immunodeficiency-centromeric instability-facial dysmorphism

Introduction

From time to time, paediatricians are confronted with children who might suffer from a primary immunodeficiency disease [21]. For practical purposes, these children can be divided into four main clinical categories [7, 26, 28]. The first clinical category comprises the relatively large group of children with recurrent ear-nose and throat (ENT) and lower respiratory tract infections, only in some cases caused by an underlying immunodeficiency, especially if encapsulated extracellular bacteria are found as pathogens. In patients with hypo- or agammaglobulinaemia, deficient opsonisation with antibodies leads to impaired phagocytosis of these micro-organisms. Complement deficiencies are rare and in most types of complement deficiency affected children present with collagen-vascular disease [15]. However, some complement deficiencies lead to severely impaired opsonisation with recurrent serious bacterial infections resembling agammaglobulinaemia (C3 deficiency, factor D deficiency and factor I deficiency; to a lesser extent C2 deficiency). Most children with recurrent, often viral, ENT and lower respiratory tract infections do not have an antibody or complement

E. de Vries (✉) · J.G. Noordzij · J.J.M. van Dongen
Department of Immunology, Erasmus University Rotterdam,
PO Box 1738, Rotterdam, The Netherlands,
Tel.: +31-10-4088094
Fax: +31-10-4089456

E. de Vries
Department of Paediatrics, Bosch Medicentrum,
's-Hertogenbosch, The Netherlands

T.W. Kuijpers
Department of Paediatrics, Academic Medical Centre,
Amsterdam, The Netherlands

T.W. Kuijpers
Department of Experimental Immunohaematology,
Central Laboratory of the Netherlands Red Cross Blood
Transfusion Service, Amsterdam, The Netherlands

deficiency, but are immunocompetent. Their problems are due to other more common causes such as bronchial hyperreactivity, adenoidal hypertrophy, or an allergic constitution, or they may suffer from rare non-immunological diseases like cystic fibrosis or immotile cilia syndrome.

The second clinical category comprises children with failure to thrive, intractable diarrhoea or an opportunistic infection, which can be caused by a T-lymphocyte or combined immunodeficiency with absent or functionally deficient T-lymphocytes. Their defence against intracellular micro-organisms is inadequate. The third clinical category comprises children with superficial and systemic infections with pyogenic bacteria or fungi, who apparently have problems with their first line of defence against invading micro-organisms as in cases of granulocyte/monocyte function deficiency. Finally, the fourth clinical category comprises a small heterogeneous group of children with recurrence of particular infections such as recurrent neisserial infections in cases of late complement component deficiency, or recurrent mycobacterial infections in cases with a defect in the interferon (IFN)- γ receptor, interleukin (IL)-12 or the IL-12 receptor [19]. These last three clinical categories are only rarely encountered in general paediatric practice.

Most cases of acquired immunodeficiency are iatrogenic and can therefore be anticipated: as more and more children are being treated with immunosuppressive drugs, aggressive chemotherapy protocols, and stem cell transplantation, acquired immunodeficiency becomes a more common problem in paediatric practice [4]. Also, the prevalence of paediatric human immunodeficiency virus (HIV) infection in Europe is increasing, especially in children born to parents who originate from HIV-endemic areas [8].

Laboratory studies for identification and clinical follow-up of immunodeficient children can now be performed in many hospitals. In this review, we focus our discussion on the increasingly important role of flow cytometric immunophenotyping of leucocytes in the diagnosis and follow-up of immunocompromised children in paediatric practice [12]. Application of flow cytometric techniques helps to speed up the diagnostic process, thereby supporting early recognition of primary immunodeficiencies before serious infections have compromised the child's general condition, reducing the chances of survival [21].

Flow cytometric immunophenotyping

Flow cytometric immunophenotyping allows precise assessment of relative frequencies of leucocyte subpopulations by detection of cell surface and intracellular markers with fluorochrome-labelled monoclonal antibodies (McAb). Immunophenotyping can be focussed on lymphocytes, monocytes, or granulocytes, because these leucocyte populations can be defined on the basis

of their light scatter characteristics, such as forward scatter (FSC) as measure for size, and side scatter (SSC) as measure for cellular irregularity (Fig. 1) [12]. Leucocyte count and differential cell count can then be used to calculate absolute counts of the blood lymphocyte subpopulations, which should be compared with age-matched reference values [6].

Blood sample handling, cell separation methodology and labelling techniques influence the reliability of the results obtained by flow cytometric immunophenotyping of leucocytes [12]. Dead cells show increased autofluorescence, potentially leading to incorrect interpretation of staining results. This can be minimised by using freshly collected samples which are fully analysed within 24 h. The immunophenotyping procedures take 4–5 h, implying that the sample should arrive in the laboratory in the morning. Cell separation via density gradients may result in differential loss of specific lymphocyte subpopulations. This is avoided when the lysed whole blood (LWB) technique is used. The currently available LWB techniques require fewer preparation steps and less sample handling via direct incubation of the anticoagulated blood with McAbs, in combination with red blood cell lysis. These LWB techniques also reduce non-specific binding of McAbs, especially if the applied McAbs are directly conjugated with fluorochromes.

Fig. 1 Flow cytometric immunophenotyping of lymphocytes in the blood of a BTK-deficient X-linked agammaglobulinaemia patient as compared to a healthy control. (A) Analysis of peripheral blood of a healthy control. Dot plot with FSC and SSC showing "lympho-gate", "mono-gate", and "granulo-gate" (left). The B-lymphocytes in the left upper quadrant express CD19 on their cell surface (middle). The T-lymphocytes in the right lower quadrant express CD3 on their cell surface (middle and right). The NK-cells in the left upper quadrant express CD16-negative and CD16- and/or CD56-positive (right). (B) Analysis of peripheral blood of a BTK-deficient X-linked agammaglobulinaemia patient. This 3-year-old boy presented at the age of 2 years with skin infections. Dot plot with FSC and SSC showing "lympho-gate" (left). Complete absence of CD19⁺ B-lymphocytes (middle), with CD3⁺ T-lymphocytes and CD16⁺ and/or CD56⁺ CD3⁻ NK-cells normally present (right)

Diagnosing children with primary immunodeficiency

Children with recurrent ENT and lower respiratory tract infections

In some children with recurrent ENT and lower respiratory tract infections, a relatively mild antibody deficiency like IgA deficiency, IgG subclass deficiency, or anti-polysaccharide antibody deficiency is found [21]. Lymphocyte number and immunophenotype are completely normal in these children (Table 1).

It is useful to perform immunophenotyping of lymphocytes according to the "limited" protocol (Table 2), when a rare severe antibody deficiency with hypo- or agammaglobulinaemia is found. Determination of T-lymphocyte counts generally rules out severe combined immunodeficiency (SCID) and determination of B-lymphocyte counts supports the discrimination between the various causes of severe antibody deficiency (Table 1) [21]. If immunophenotyping of lymphocytes shows that B-lymphocytes are virtually absent in the peripheral blood compartment (Fig. 1), this strongly suggests an early stop in B-lymphocyte development. This occurs in X-linked agammaglobulinaemia with Bruton tyrosine kinase (BTK) deficiency as well as in rare autosomal recessive forms of agammaglobulinaemia, such as Ig μ heavy chain deficiency. Using a McAb directed against BTK, it is now possible to detect BTK deficiency in most patients by analysing the monocytes with flow cytometry; normal monocytes also express BTK [14]. Furthermore, the type of B-cell differentiation arrest in bone marrow can be assessed with a special research protocol (Fig. 2) and can direct the diagnostic analysis at the molecular level (Fig. 3).

If B-lymphocytes are present in the peripheral blood compartment of patients with a severe antibody deficiency, common variable immunodeficiency or hyper-IgM syndrome are more likely diagnoses. In common variable immunodeficiency, progressive hypogammaglobulinaemia with combinations of IgA deficiency, IgG subclass deficiency, and/or anti-polysaccharide antibody deficiency can be found. Immunophenotyping of blood lymphocytes can show accompanying T-lymphocytopenia [25]. In X-linked hyper-IgM syndrome with a defect in CD40 ligand (CD40L), severe hypogammaglobulinaemia with normal or increased IgM is found. Immunophenotyping of activated blood T-lymphocytes can show absence or decreased expression of CD40L (Fig. 4) (Table 2) [16]. In some patients CD40L is expressed but not functional; tests for CD40 binding can identify these patients. The defect in CD40L disrupts the CD40L-CD40 mediated T-B cell communication, which is indispensable for isotype switching from IgM to IgG, IgA or IgE. Consequently, X-linked hyper-IgM syndrome is not a pure antibody deficiency but should be considered as a T-lymphocyte disorder, which probably explains why affected children often present with an opportunistic infection. The definitive diagnosis of X-linked

hyper-IgM syndrome can be made via mutation analysis of the *CD40L* gene. The autosomal recessive form of hyper-IgM syndrome is caused by mutations in the activation-induced cytidine deaminase (*AID*) gene. *AID* proteins are specifically expressed in germinal centre B-cells in secondary lymphoid organs and are required for immunoglobulin isotype switching as well [23].

Children with failure to thrive, intractable diarrhoea, or with an opportunistic infection

Many children with T-lymphocyte or combined immunodeficiency do not present with recurrent infections but with failure to thrive or intractable diarrhoea [26]. The differential diagnosis is extensive in these cases. The combination of lymphopenia and agammaglobulinaemia can direct the search towards an immunodeficiency, but these are not invariably present. Opportunistic infections almost always indicate an immunodeficiency, either acquired (see below) or congenital.

The "limited" immunophenotyping protocol as shown in Table 2 can easily detect severe combined immunodeficiency disease. Generally, T-lymphocytes will be absent or very low in number [21]. However, transplacental transfer of maternal T-lymphocytes in SCID patients or remaining (oligoclonal) T-lymphocyte production in Omenn syndrome (Table 1) might coincidentally lead to seemingly normal T-lymphocyte counts. The presence or absence of B-lymphocytes differentiates between B⁺ SCID and B⁻ SCID, as found in X-linked common γ -chain deficiency or autosomal recessive Janus-associated kinase-3 (JAK3) deficiency and in recombination activating genes (RAG)-1 or -2 deficiency, respectively (Fig. 5). Major changes in either the CD4⁺/CD3⁺ helper T-lymphocyte count or the CD8⁺/CD3⁺ suppressor/cytotoxic T-lymphocyte count can be suggestive for the diagnosis of MHC class II deficiency with low CD4⁺/CD3⁺ counts [5] or a diagnosis of 70 kD zeta-associated protein (ZAP70) tyrosine kinase deficiency with low CD8⁺/CD3⁺ counts, respectively (Fig. 3) (Table 1). However, in many cases of combined immunodeficiency, immunophenotyping results are less aberrant with lymphopenia developing over time. Additional tests are then required to obtain a diagnosis. Lymphopenia develops within months after birth in adenosine deaminase or purine nucleotide phosphorylase deficiency due to metabolic poisoning of the cells. Lymphopenia also gradually develops in the first years after birth in Wiskott-Aldrich syndrome and towards adolescence or even adulthood in ataxia teleangiectasia (Table 1) [10].

If immunodeficiency is highly suspected but the "limited" immunophenotyping protocol shows no abnormalities, an "extended" immunophenotyping protocol as shown in Table 2 can identify additional children with specific subtypes of T-lymphocyte or combined immunodeficiency that are not accompanied by major changes in the main lymphocyte populations (Table 1).

Table 1 Primary immunodeficiency diseases

Diseases	Pathogenesis	Immunophenotyping
<i>Mild Antibody Deficiencies (recurrent ENT and lower respiratory tract infections, lamblia, sometimes asymptomatic)</i>		
IgA deficiency	Pathogenesis unknown; in	Generally not useful
IgG-subclass deficiency	anti-polysaccharide antibody	Normal immunophenotyping
Anti-polysaccharide antibody deficiency	deficiency, B-lymphocytes are unable to produce antibodies against polysaccharide antigens, despite adequate responses against protein antigens	results. Focus on serum Ig (sub) classes and antibody reactivity
<i>Severe Antibody Deficiencies (recurrent respiratory tract and ENT infections, life-threatening bacterial infections, enteroviral CNS infections, lamblia)</i>		
BTK enzyme deficiency (X-linked agammaglobulinaemia)	Early arrest in B-cell development	“Limited” protocol (bone marrow protocol)
Ig μ heavy chain deficiency (autosomal recessive)		Absent blood B-lymphocytes. Differentiation arrest reflected in precursor B-cell population in bone marrow (useful for directing DNA diagnostics in non-X-linked agammaglobulinaemia patients)
Several other rare forms (autosomal recessive)		
X-linked hyper IgM syndrome with CD40L deficiency	CD40L deficiency leads to absent isotype switch of B-lymphocytes and associated T-lymphocyte defect due to absent CD40 stimulation	“Extended” protocol (CD40L)
		Absent or decreased CD40L expression on activated T-lymphocytes in X-linked form; blood B-lymphocytes present
Autosomal recessive hyper IgM syndrome	Mutations in the <i>AID</i> gene disrupt isotype switching and somatic hypermutation in germinal centre B-lymphocytes	“Limited” protocol
		Blood B-lymphocytes present
Common variable immunodeficiency	Probably defects in B-cell maturation and helper T-lymphocyte function	“Limited” protocol
		Blood B-lymphocytes present; sometimes T-lymphocytopenia
<i>Combined Immunodeficiency Diseases (failure to thrive, intractable diarrhoea, eczema, and other chronic problems, or opportunistic infections)</i>		
Adenosine deaminase deficiency (autosomal recessive)	Accumulation of toxic purine metabolites due to enzyme deficiency	“Limited” protocol
Purine nucleotide phosphorylase deficiency (autosomal recessive)		
MHC class I expression deficiency (autosomal recessive)	Impaired antigen presentation	“Extended” protocol (β_2 microglobulin)
MHC class II expression deficiency (autosomal recessive)	Impaired antigen presentation	“Extended” protocol (HLA-DR)
		Deficient MHC class II expression; low numbers of CD4 ⁺ T-lymphocytes
Wiskott Aldrich syndrome (X-linked) (patients also suffer from eczema, thrombocytopenia, and malignancy)	Impaired cytoskeletal reorganisation upon activation of platelets and T-lymphocytes	“Limited” protocol
		Increasing T-lymphocytopenia with time; (impaired CD43 expression)
<i>Severe Combined Immunodeficiencies (failure to thrive, intractable diarrhoea, eczema, and other chronic problems, or opportunistic infections)</i>		
X-linked SCID (common γ -chain deficiency)	Defect in T-lymphocyte and NK cell development	“Limited” protocol
		T-lymphocytes absent, NK cells low, and B-lymphocytes present
JAK3 deficiency (autosomal recessive)		
RAG1 or RAG2 (autosomal recessive)	Early arrest in common lymphocyte development	“Limited” protocol
Omenn syndrome (autosomal recessive; partial RAG deficiency) (patients present with severe eczema)	Defective T-lymphocyte development with B-lymphocyte deficiency	“Limited” protocol
		T- and B-lymphocytes absent, NK cells present
ZAP70 protein tyrosine kinase deficiency (autosomal recessive)	Defect in T-lymphocyte development	“Limited” protocol
		Oligoclonal T-lymphocytes, very low or absent B-lymphocytes
CD3 γ - or ϵ -chain deficiency (autosomal recessive)	Defect in T-lymphocyte function	“Limited” protocol
		T-lymphocytes, especially CD8 ⁺ decreased; B-lymphocytes present
IL-7 receptor deficiency	Defect in the α -chain of the IL-7 receptor, leading to a defect in T-lymphocyte development	“Limited” protocol
		T-lymphocytes decreased, CD3 expression decreased; B-lymphocytes present
Reticular dysgenesis (patients present with pancytopenia)	Stem cell defect leading to defective maturation of T- and B-cells and myeloid cells	“Limited” protocol
		T-lymphocytes absent, NK cells and B-lymphocytes present
		Pancytopenia

Table 1 (Contd.)

Diseases	Pathogenesis	Immunophenotyping	
<i>Milder Combined Immunodeficiencies</i>			
DiGeorge (CATCH-22) syndrome (cardiac malformations, hypoparathyroidism, thymic insufficiency)	Disturbance of embryonic development; 22q11 deletion	“Extended” protocol (TCR $\alpha\beta$, TCR $\gamma\delta$)	Sometimes TCR $\alpha\beta$ ⁺ T-lymphocytopenia with normal TCR $\gamma\delta$ ⁺ T-lymphocyte counts
Ataxia teleangiectasia (autosomal recessive)	Mutation in the <i>ATM</i> gene; exact pathogenesis unknown	“Limited” protocol	Sometimes increasing T-lymphocytopenia with time
Chromosomal breakage syndromes (Nijmegen breakage syndrome, Blooms syndrome, ICF syndrome) (mental retardation, photosensitivity, abnormal physionomy)	Mutation in respectively the <i>NBS</i> , <i>BLM</i> , and <i>ICF</i> genes; exact pathogenesis unknown	“Limited” protocol	Immunophenotyping mostly normal, but disturbances may be present
Auto-immune lymphoproliferative syndrome	Defect in apoptosis	“Extended” protocol (CD95)	Absence of CD95 (FAS) on blood leucocytes in some patients, presence of CD3 ⁺ /CD4 ⁺ /CD8 ⁺ T-lymphocytes [24]
Deficiency of IL12, IFN γ receptor, or IL12 receptor (recurrent mycobacterial infections, salmonellosis)	Disturbed communication between T-lymphocytes/NK cells and monocytes; failure of monocyte activation by T-lymphocytes/NK cells	“Extended” protocol (IL12, IFN γ R, IL12R)	Absence of IL-12 receptor on T-lymphocytes and NK cells, or absence of IFN- γ receptor or intracellular IL-12 in monocytes
<i>Granulocyte/monocyte Deficiencies (superficial and systemic infections with pyogenic bacteria and fungi)</i>			
Chronic granulomatous disease (X-linked or autosomal recessive)	Defective microbial killing due to a defect in one of the five NADPH oxidase components	Generally not used; “extended” protocol (b558)	Normal immunophenotyping results; focus on defective microbial killing in granulocyte function tests; (in patients with X-linked chronic granulomatous disease the expression of cytochrome b558 may be defective)
Leucocyte adhesion deficiency type 1 (autosomal recessive)	Defective leucocyte adhesion due to a defect in CD18, the integrin β_2 chain	“Extended” protocol (CD18)	Absence of CD18 expression, otherwise normal
Leucocyte adhesion deficiency type 2 (autosomal recessive)	Defective leucocyte adhesion due to a defect in CD15s, sialyl-Lewis-X	“Extended” protocol (CD15s)	Absence of CD15s expression, otherwise normal
<i>Complement Deficiencies (neisserial infections, serious bacterial infections, collagen-vascular diseases, sometimes asymptomatic)</i>			
C2 deficiency (autosomal recessive)	Impaired opsonisation due to classical pathway defect sometimes leading to serious bacteraemic infections	Not useful	Normal immunophenotyping results; focus on determination of serum complement activity
C3 deficiency, factor I deficiency (autosomal recessive), factor D deficiency	Seriously impaired opsonisation leading to a phenotype similar to that seen in agammaglobulinaemia	Not useful	Normal immunophenotyping results; focus on determination of serum complement activity
C6, C7, C8, C9 deficiency (autosomal recessive), properdin deficiency (X-linked)	Impaired terminal complement pathway leading to predisposition to neisserial infections; exact pathogenesis not clear	Not useful	Normal immunophenotyping results; focus on determination of serum complement activity

For example, in MHC class II deficiency the absence of HLA-DR surface expression gives the clue to the diagnosis [5]; CATCH-22 (DiGeorge) syndrome is sometimes accompanied by severe T-lymphocytopenia with low to absent (TCR)- $\alpha\beta$ ⁺ T-lymphocytes and normal TCR $\gamma\delta$ ⁺ T-lymphocyte counts (J.J.M. van Dongen, unpublished observation). In unclassifiable cases, extensive specialised immunophenotyping protocols may help to unravel the immunodeficiency [12, 13, 21]. These protocols may include markers of maturation, such as CD45RA (“naive”) and CD45RO (“memory”) on T-lymphocytes, markers of activation and/or proliferation, co-stimulatory and/or signalling molecules, and intra-

cellular cytokines, such as IL2 and IFN- γ (T-helper 1 profile), and IL4, IL5, and IL10 (T-helper 2 profile). These tests are generally not performed in routine laboratories and will not be further discussed here.

Children with superficial and systemic infections with pyogenic bacteria or fungi

Most children with superficial and systemic infections with pyogenic bacteria or fungi suffer from neutropenia due to haematological disorders or iatrogenic causes (see below). Flow cytometric immunophenotyping of leuco-

Table 2 Flow cytometric immunophenotyping protocol for the diagnosis of primary immunodeficiency diseases in paediatric practice

“Limited” protocol (performed in many hospitals; use absolute counts and age-matched reference values) [6]		
T-lymphocytes	CD3 ⁺ CD4 ⁺ /CD3 ⁺ CD8 ⁺ /CD3 ⁺	Total T-lymphocytes Helper subset Suppressor/cytotoxic subset
B-lymphocytes	CD19 ⁺ or CD20 ⁺	Total B-lymphocytes
NK cells	Defined as CD3 ⁺ as well as CD16 ⁺ and/or CD56 ⁺ population	
Quality control	The “lymphosum” should equal 92%–98% (= % T + % B + % NK)	
“Extended” protocol (performed in specialised laboratories only; select relevant items based on the information presented in Table 1; always perform the “limited” protocol as well; compare with normal age-matched controls run in parallel, if no reference values are available)		
Analysis of the arrest in B-cell development in the bone marrow and of the intracellular expression of BTK in monocytes of patients with agammaglobulinaemia with absent B-lymphocytes		
Common γ -chain expression (CD132) on lymphocytes in SCID patients with B-lymphocytes		
CD40L expression (CD154) on activated T-lymphocytes in hypogammaglobulinaemia with normal or high IgM; CD40L function is assessed by CD40 binding assays		
HLA-DR (class-II) and β_2 microglobulin (class I) expression on leucocytes in children suspected of severe combined immunodeficiency with normal lymphocyte numbers and phenotype on initial screening		
TCR $\alpha\beta$ - and TCR $\gamma\delta$ -expression on T-lymphocytes in children with neonatal hypocalcaemia and congenital heart disease		
Extensive specialised protocols in unclassifiable cases of combined immunodeficiency with stainings for ‘naive’ (CD45RA) and ‘memory’ (CD45RO) T-lymphocytes, for markers of activation and proliferation, for co-stimulatory and signalling molecules, and for intracellular cytokines (T-helper 1 and T-helper 2 profiles)		
Intracellular cytochrome b558 expression in phagocytes if chronic granulomatous disease is suspected in children with phagocyte function deficiency		
CD18 and CD15s expression on leucocytes if leucocyte adhesion deficiency type 1 or type 2 is suspected in children with phagocyte function deficiency and extreme leucocytosis		
Analysis of the IL-12 receptor β 1 chain on T-lymphocytes/NK cells and of intracellular IL-12 and the IFN- γ receptor chains on monocytes in children with recurrent mycobacterial infections		

cytes plays only a minor role in the diagnostic process in these children. A defect in granulocyte/monocyte function, such as in chronic granulomatous disease or leucocyte adhesion deficiency, is rare. In chronic granulomatous disease, granulocytes show deficient microbial killing due to a defect in one of the five NADPH oxidase complex components [21]. This can be investigated by function tests of granulocytes. Flow cytometric immunophenotyping with McAb directed against cytochrome b558 can show absence of this compound in many but not all patients with the most frequent (65%) X-linked form of chronic granulomatous disease (“extended” protocol in Table 2) [3]. In leucocyte adhesion deficiency type 1 (CD18 deficiency) and type 2 (CD15s deficiency), leucocyte adhesion is impaired. This leads to leucocytosis, which is even more pronounced during infections [21]. CD18 and CD15s expression can be analysed with immunophenotyping (Table 2) (Fig. 6) [1, 11].

Children with recurrent particular infections

Deficiencies in one of the components of the terminal complement pathway result in recurrent neisserial, mainly meningococcal, infections (C6 deficiency, C7 deficiency, C8 deficiency, and C9 deficiency; also properdin deficiency) [15]. Lymphocyte number and immunophenotype are normal in these children (Table 1). Deficiencies in the IFN- γ receptor, IL-12 or the IL-12 receptor lead to recurrent mycobacterial infections [19]. The IL-12 receptor can be detected on T-lymphocytes or NK-cells and the IFN- γ receptor can be detected on

Fig. 2 Flow cytometric immunophenotyping of lymphocytes in the bone marrow of a BTK-deficient X-linked agammaglobulinaemia patient as compared to a healthy control. (A) Analysis of bone marrow of a healthy child (bone marrow donor). Dot plot with FSC and SSC showing “lympho-gate” (left). CD3⁺ T-lymphocytes from contaminating peripheral blood are present in the right lower quadrant (middle). CD19⁺ B-lineage cells are present in the left upper quadrant (middle). CD19 is present on all B-lineage cells from the pre-B-I cell stage onwards. CD10 is expressed on immature B-lineage cells and CD20 is expressed on mature B-lymphocytes. Immature CD10⁺/CD20⁻/CD19⁺ precursor-B-cells as well as mature CD10⁻/CD20⁺/CD19⁺ B-lymphocytes are present in the right lower and left upper regions, respectively (right). (B) Analysis of bone marrow of a BTK-deficient X-linked agammaglobulinaemia patient (same patient as in Fig. 1). Dot plot with FSC and SSC showing “lympho-gate” (left). Few CD19⁺ B-lineage cells are present (middle), which are mostly immature CD10⁺/CD20⁻/CD19⁺ precursor-B-cells (right).

monocytes by flow cytometry. Information about the expression patterns of these molecules can direct the diagnostic analysis at the molecular level, but it is im-

Fig. 5 Flow cytometric immunophenotyping of blood lymphocytes in SCID patients. **(A)** Analysis of peripheral blood of a B-SCID patient with a mutation in the RAG2 gene. This girl presented at the age of 12 months with recurrent otitis media and respiratory infections, and chronic diarrhoea. Dot plot with FSC and SSC shows the "lympho-gate" (*left*). Within the "lympho-gate", virtually no CD19⁺ B-lymphocytes were detected (*middle*), and strongly reduced CD3⁺ T-lymphocyte counts were found (*right*), whereas the NK-cell counts (CD3⁻/CD56⁺/CD16⁺) were within the normal range for age (*right*). **(B)** Analysis of peripheral blood of a B⁺ X-linked SCID patient with a mutation in the γ_c gene. This boy presented at the age of 4 months with pneumonia, diarrhoea and sepsis. Dot plot with FSC and SSC shows the "lympho-gate" (*left*). Within the "lympho-gate" CD19⁺ B-lymphocytes were detected (*middle*), but CD3⁺ T-lymphocytes were absent (*right*), and the NK-cell counts (CD3⁻/CD56⁺/CD16⁺) were decreased (*right*)

of age, <500/ μ l from 1–5 years of age, and <200/ μ l from 6–12 years of age indicate severe suppression [27]. During antiretroviral therapy, monitoring of CD4⁺ helper T-lymphocyte counts is useful for evaluating the treatment effectiveness [20] in addition to HIV RNA quantification [27]. For further guidelines, the reader is referred to the literature [27].

More elaborate immunophenotyping protocols can be reserved for research purposes, e.g. for comparison of immune reconstitution after bone marrow transplantation with normal ontogeny. Such studies are important for understanding the regeneration processes, but have no direct clinical relevance as yet.

Conclusion

Immunophenotyping of leucocytes forms an important part of the diagnostic work-up in children with suspected immunodeficiency. A "limited" protocol (Table 2) can be performed in many hospitals. This enables the general paediatrician to determine the presence and number of the main lymphocyte populations to identify (severe) combined immunodeficiency patients at an early stage and to direct the diagnostic work-up in agammaglobulinaemic patients by determining the presence or absence of B-lymphocytes. In selected cases, a set of extra measurements can be performed in specialised

Fig. 6 Flow cytometric detection of CD18 molecules in the diagnosis of leucocyte adhesion deficiency type 1. In the healthy control (*left panel*), virtually all leucocytes express the CD18 molecule whereas those of the leucocyte adhesion deficiency type 1 patient are deficient for CD18 expression (*right panel*)

laboratories ("extended" protocol in Table 2). This identifies children with immunodeficiencies that are not accompanied by major changes in the main lymphocyte populations. However, it should be noted that not all children with an immunodeficiency show alterations in immunophenotyping results (Table 1). Also, genetic mutations do not always lead to absence of protein expression, even if protein function is severely hampered.

More extensive immunophenotyping protocols used in specialised laboratories or research settings help to recognise new immunodeficiency diseases by identifying as yet undescribed staining patterns. Also, pathogenesis of known defects can be further elucidated: the absence of specific cell surface markers [16, 21], the loss of specific epitopes defined by McAb staining patterns [9, 17], or the determination of the B-cell differentiation arrest in bone marrow can support the precise characterisation of the defect. Therefore, it is important to use these more extensive immunophenotyping protocols in patients with an established immunodeficiency disease, as well as in patients with probable immunodeficiency disease that has not been proven with other methods.

Due to the rapid developments in the field of flow cytometry and antibody production on the one hand and the recognition of new immunodeficiency diseases on the other, it is likely that the "limited" and "extended" immunophenotyping protocols defined here will need to be updated in a few years time.

References

1. Anderson DC, Springer TA (1987) Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annu Rev Med* 38: 175–194
2. Atkinson K (1990) Reconstitution of haematopoietic and immune systems after marrow transplantation. *Bone Marrow Transplant* 5: 209–226
3. Batot G, Martel C, Capdeville N, Wientjes F, Morel F (1995) Characterization of neutrophil NADPH oxidase activity reconstituted in a cell-free assay using specific monoclonal antibodies raised against cytochrome b558. *Eur J Biochem* 234: 208–215

4. Cherry JD, Feigin RD (1996) Infection in the compromised host. In: Stiehm ER (ed.) *Immunologic disorders in infants and children*. Saunders, Philadelphia, pp 975–1013
5. Clement LT, Giorgi JV, Ploeger-Marshall S, Haas A, Stiehm ER, Martin AM (1988) Abnormal differentiation of immunoregulatory T-lymphocyte subpopulations in the major histocompatibility complex (MHC) class II antigen deficiency syndrome. *J Clin Immunol* 8: 503–512
6. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WCJ, Groeneveld K, Hooijkaas H, van Dongen JJM (1997) Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* 130: 389–393
7. Conley ME, Stiehm ER (1996) Immunodeficiency disorders: general considerations. In: Stiehm ER (ed.) *Immunologic disorders in infants and children*. Saunders, Philadelphia, pp 201–252
8. De Kleer IM, Uiterwaal CS, Nauta N, Hirasings RA, Prakken AB, De Graeff-Meeder ER (1999) Toename van gemelde HIV-1-infectie bij kinderen in Nederland, 1982–1997: meer verticale transmissie en groter aandeel van allochtone kinderen (Increase of reported HIV-1 infections in children in the Netherlands 1982–1997: more vertical transmission and a greater proportion of other than Dutch children). *Ned Tijdschr Geneesk* 143: 1696–1700
9. De Vries E, Koene HR, Vossen JM, Gratama J-W, von dem Borne AEGK, Waaijer JLM, Haraldsson A, de Haas M, van Tol MJD (1996) Identification of an unusual Fc γ receptor IIIa (CD16) on natural killer cells in a patient with recurrent infections. *Blood* 88: 3022–3027
10. Elder ME (2000) T-cell immunodeficiencies. *Pediatr Clin North Am* 47: 1253–1274
11. Etzioni A, Harlan JM, Pollack S, Phillips LM, Gershoni-Baruch R, Paulson JC (1993) Leukocyte adhesion deficiency (LAD) II: a new adhesion defect due to absence of sialyl Lewis x, the ligand for selectins. *Immunodeficiency* 4: 307–308
12. Fleisher TA (1994) Immunophenotyping of lymphocytes by flowcytometry. *Immunol Allergy Clin* 14: 225–240
13. Fleisher TA, Tomar RH (1997) Introduction to diagnostic laboratory immunology. *JAMA* 278: 1823–1834
14. Futatani T, Miyawaki T, Tsukada S, Hashimoto S, Kunikata T, Arai S, Kurimoto M, Niida Y, Matsuoka H, Sakiyama Y, Iwata T, Tsuchiya S, Tatsuzawa O, Yoshizaki K, Kishimoto T (1998) Deficient expression of Bruton's tyrosine kinase in monocytes from X-linked agammaglobulinemia as evaluated by a flow cytometric analysis and its clinical application to carrier detection. *Blood* 91: 595–602
15. Johnston RB Jr (1993) The complement system in host defense and inflammation: the cutting edges of a double-edged sword. *Pediatr Infect Dis J* 12: 933–941
16. Kroczeck RA, Graf D, Brugnani D, Giliani S, Korthäuer U, Ugazio A, Senger G, Mages HW, Villa A, Notarangelo LD (1994) Defective expression of CD40 ligand on T cells causes "X-linked immunodeficiency with hyper-IgM (HIGM1)". *Immunol Rev* 138: 39–59
17. Kuijpers TW, van Lier RA, Hamann D, de Boer M, Thung LY, Weening RS, Verhoeven AJ, Roos D (1997) Leukocyte adhesion deficiency type 1 (LAD-1)/variant. A novel immunodeficiency syndrome characterized by dysfunctional beta2 integrins. *J Clin Invest* 100: 1725–1733
18. Mofenson L, Korelitz J, Meyer WA, Bethel J, Rich K, Pahwa S, Moye J, Nugent R, Read J (1997) The relationship between serum human immunodeficiency virus type 1 (HIV-1) RNA level, CD4 lymphocyte percent, and long-term mortality risk in HIV-1 infected children. National Institute of Health and Human Development intravenous immunoglobulin clinical trial study group. *J Infect Dis* 175: 1029–1038
19. Ottenhof TH, Kumararatne D, Casanova JL (1998) Novel human immunodeficiencies reveal the essential role of type-1 cytokines in immunity to intracellular bacteria. *Immunol Today* 19: 491–494
20. Powderly WG, Landay A, Lederman MM (1998) Recovery of the immune system with antiretroviral therapy: the end of opportunism? *JAMA* 280: 72–77
21. Primary immunodeficiency diseases (1999) Report of an IUIS Scientific Committee. International Union of Immunological Societies. *Clin Exp Immunol* 118[Suppl 1]: 1–28
22. Raaphorst FM (1999) Reconstitution of the B cell repertoire after bone marrow transplantation does not recapitulate human fetal development. *Bone Marrow Transplant* 24: 1267–1272
23. Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O et al (2000) activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the hyper-IgM syndrome. *Cell* 102: 565–575
24. Rieux-Laucat F, Blachere S, Danielan S, De Villartay JP, Oleastro M, Solary E, Bader-Meunier B, Arkwright P, Pondare C, Bernaudin F, Chapel H, Nielsen S, Berrah M, Fischer A, Le Deist F (1999) Lymphoproliferative syndrome with autoimmunity: a possible genetic basis for dominant expression of the clinical manifestations. *Blood* 94: 2575–2582
25. Spickett GP, Farrant J, North ME, Zhang J, Morgan L, Webster DB (1997) Common variable immunodeficiency: how many diseases? *Immunol Today* 18: 325–328
26. Stiehm ER, Chin TW, Haas A, Peerless AG (1986) Infectious complications of the primary immunodeficiencies. *Clin Immunol Immunopathol* 40: 69–86
27. Working group on antiretroviral therapy and medical management of infants, children and adolescents with HIV infection (1998) Antiretroviral therapy and medical management of pediatric HIV infection. *Pediatrics* 102[Suppl 4]: 1005–1062
28. Woroniecka M, Ballou M (2000) Office evaluation of children with recurrent infection. *Pediatr Clin North Am* 47: 1211–1224