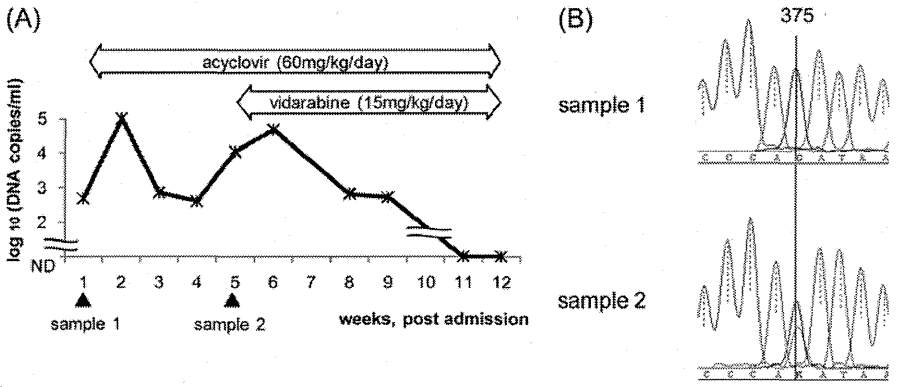

FIG 1 (A) Sequential changes in the HSV-1 DNA level in the CSF determined by quantitative real-time PCR. Arrows below the chart represent the time points of the CSF sample collection for sequencing analysis. ND, not detected. (B) Chromatogram of the *vTK* genes amplified from sample 1 and sample 2. A nucleotide substitution (G375T) was found in sample 2 (lower panel).

and designated *vTK*-375G. Subsequently, a G375T-mutant *vTK* expression plasmid was constructed by site-directed mutagenesis using the following primers, reagents, and PCR conditions and designated *vTK*-375T. Primers 5′-ATATAACAATGGGCATGCC TTATGCCand 5′-GGGCGCTTGTCATTACCAC were designed for the inverse PCR (the underlined T is the targeted nucleotide), and *vTK*-375G was used as the template. The reaction was performed using a PrimeSTAR GXL DNA polymerase kit (TaKaRa Bio, Otsu, Japan), and the amplification conditions included an initial denaturation step of 2 min at 94°C, followed by 10 cycles of 10 s at 98°C, 15 s at 55°C, and 7 min at 68°C. Digestion and self-ligation were performed with a KOD mutagenesis kit (Toyobo, Osaka, Japan). G375T substitution without other nucleotide changes was confirmed by sequencing analysis. The expression plasmid for the ACV-sensitive HSV-1 *vTK* TAS strain (5) was constructed as a positive control and designated *vTK*-TAS. Empty pTARGET served as a negative control. In the final stage of the assay, the titers of the replicated TAR were determined by the standard plaque assays. Then, $\Delta\log_{10}$ PFU values were calculated as follows: $\Delta\log_{10}$ PFU = $\log_{10}$ (PFU per milliliter of the replicated TAR at each ACV concentration) − $\log_{10}$ (PFU per milliliter of the replicated TAR at the ACV concentration of 0 μg/ml). This value represents the inhibitory effect of ACV on TAR replication, which is brought about by the transfection. Thus, the higher the value is, the lower the activity of the expressed *vTK*. In this way, the *vTK*-related resistance of HSV-1 can be judged from the $\Delta\log_{10}$ PFU values. The sensitivities to ganciclovir (GCV; Sigma-Aldrich Chemical Company, St. Louis, MO), penciclovir (PCV; Wako), and brivudine (BVDU; Sigma-Aldrich) were also tested in the same way.

TAR replication in 293T cells transfected with a negative control was not affected by any concentrations of any antiviral compounds (Fig. 2). When ACV was used, $\Delta\log_{10}$ PFU values elicited by *vTK*-375G transfection were at almost the same level as those elicited by *vTK*-TAS transfection, indicating that HSV-1 in sample 1 and TAS had nearly equal levels of sensitivity to ACV. However, $\Delta\log_{10}$ PFU values elicited by *vTK*-375T transfection were significantly higher than those elicited by *vTK*-375G transfection (Welch's *t* test; $P = 0.004$, <0.001, and $= 0.045$, at ACV concentrations of 0.4, 4, and 40 μg/ml, respectively), indicating that the HSV-1 with the G375T mutation in the *vTK* gene had acquired ACV resistance (Fig. 2A). When GCV, PCV, and BVDU were used, transfection of *vTK*-375G, *vTK*-375T, and *vTK*-TAS resulted in almost the same level of $\Delta\log_{10}$ PFU values (Fig. 2B to D). The HSV-1 G375T mutant was therefore considered to be sensitive to these drugs.

To our knowledge, this is the first report of a patient with ACV-resistant neonatal HSV-1 disease. Neonatal HSV infection is estimated to occur in 1 in every 3,500 to 5,000 deliveries (6). Approximately 30% of the patients are diagnosed as having NHE (7). Although the introduction of ACV has significantly improved the prognosis, NHE is still a severe disease with a mortality rate of 6%, and 70% of the survivors suffer from moderate-to-severe neurological abnormalities (7, 8). ACV-resistant HSV mainly threatens immunocompromised patients, and the prevalence among them is reported to range from 3.5% to 10%. In immunocompetent individuals, the prevalence of ACV-resistant HSV is far lower, ranging from 0.1% to 0.7% (2). Neonatal ACV-resistant HSV infections are quite rare, and all the cases previously described have been caused by ACV-resistant HSV-2 (9–11).

The present study also showed for the first time that a Q125H amino acid substitution in the *vTK* polypeptide induces ACV resistance. Using a method previously described (4), it was confirmed that the Q125H mutation was not a part of natural polymorphism. Q125 of HSV-1 TK has been shown to be located above the nucleotide binding pocket in the three-dimensional (3D) structure of the *vTK* protein (12). Several studies have shown that substitution of Q125 to other amino acids changes *vTK* activity; Q125E and Q125L are associated with resistance to ACV, and Q125N leads to hypersensitivity to ACV (13, 14). Interestingly, the Q125H mutation did not induce cross-resistance to GCV, PCV, and BVDU, suggesting that these drugs may be effective with respect to this specific mutant.

This study showed also for the first time a central nervous system infection caused by a virologically confirmed ACV-resistant HSV-1 strain. There is one report of a possibly ACV-resistant HSV-1 encephalitis adult patient (15). In that report, virus isolation from the CSF failed, but an amino acid substitution of R41H found in the *vTK* polypeptide was suspected to be responsible for the ACV resistance, although it has not been virologically confirmed whether the mutation confers ACV resistance. The method

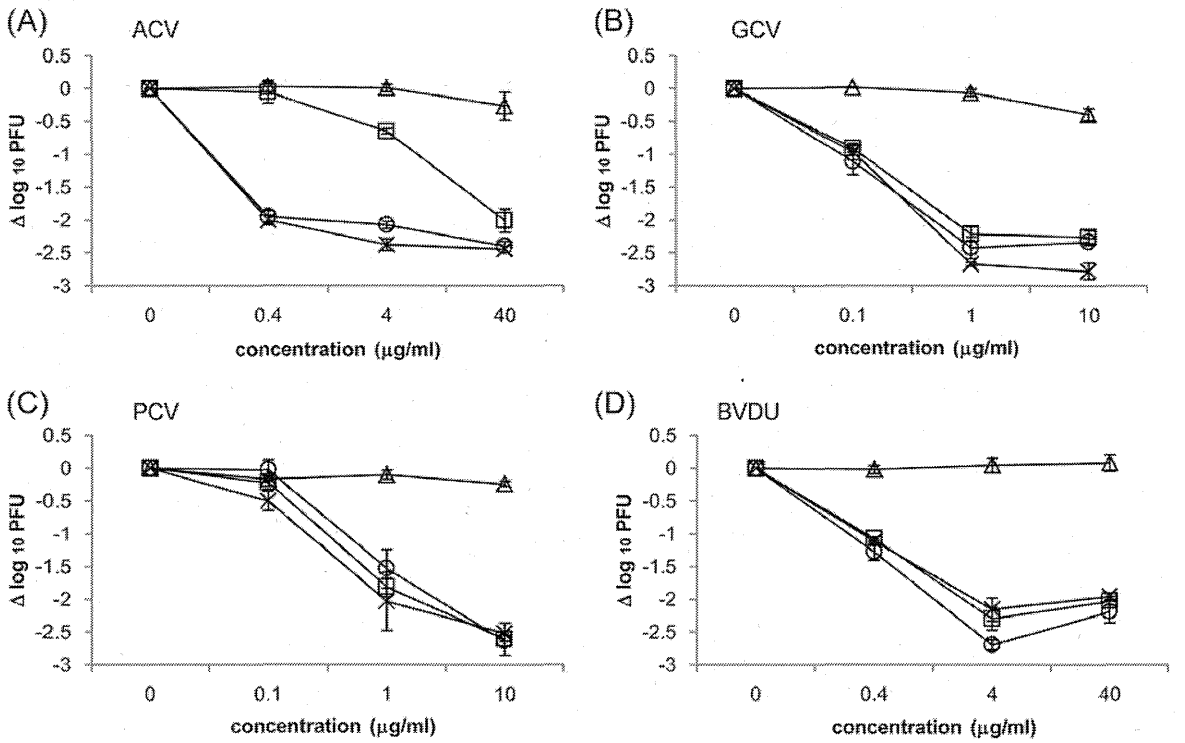

FIG 2 The inhibitory effects of antiviral compounds on replication of TAR in 293T cells transfected with each of the plasmids *vTK*-375G (○), *vTK*-375T (□), *vTK*-TAS (×), and empty pTARGET (Δ). Each experiment was performed in triplicate, and the error bars indicate standard deviations.

used in the present study may be suitable for such a condition. However, it should be kept in mind that the method can be applied only for *vTK*-related ACV resistance and not for DNA polymerase-related resistance.

ACV-resistant HSV is usually seen in patients with a history of ACV treatment (16). In the present case, administration of ACV may possibly have induced the appearance of the ACV-resistant HSV-1 strain. Although a recent study showed a benefit of oral ACV suppressive therapy for survivors of NHE (17), emergence of ACV-resistant HSV during the suppressive therapy was also reported (18). Thus, sensitivity of the causative HSV to antiviral drugs should be carefully monitored. This patient did not receive the suppressive therapy because he suffered from NHE before the beneficial effect of the therapy was reported.

It is recommended to repeat the lumbar puncture after 21 days from the initiation of ACV administration in the treatment of NHE (19). On the other hand, persistence of CSF HSV DNA is reported to be associated with poor neurodevelopmental outcomes of NHE patients (20). CSF HSV DNA quantification was conducted weekly in this patient to monitor the HSV-1 genome level in a real-time manner. We considered that the practice was beneficial, although further discussion is needed. In fact, the frequent monitoring enabled us to treat NHE with an appropriate choice of antiviral drugs.

In conclusion, ACV-resistant HSV-1 was virologically confirmed for the first time in a NHE patient. A nucleotide mutation, G375T in the HSV-1 TK gene, leading to a Q125H amino acid substitution, conferred ACV resistance.

Nucleotide sequence accession numbers. The vTK DNA sequence data have been deposited in the DNA Data Bank of Japan (DDBJ) under accession no. AB713519 (CSF sample 1) and AB713520 (CSF sample 2).

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We have no conflicts of interest.

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ORIGINAL RESEARCH

A Phenotypic Approach for IUIS PID Classification and Diagnosis: Guidelines for Clinicians at the Bedside

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Abstract The number of genetically defined Primary Immunodeficiency Diseases (PID) has increased exponentially, especially in the past decade. The biennial classification published by the IUIS PID expert committee is therefore quickly expanding, providing valuable information regarding the disease-causing genotypes, the immunological anomalies, and the associated clinical features of PIDs. These are grouped in eight, somewhat overlapping, categories of immune dysfunction. However, based on this immunological classification, the diagnosis of a specific PID from the clinician's observation of an individual clinical and/or immunological phenotype remains difficult, especially for non-PID specialists. The purpose of this work is to suggest a phenotypic classification that forms the basis for diagnostic trees, leading the physician to particular groups of PIDs, starting from clinical features and combining routine immunological investigations along the way. We present 8 colored diagnostic figures that correspond to the 8 PID groups in the IUIS Classification, including all the PIDs cited in the 2011 update of the IUIS classification and most of those reported since.

Keywords Primary immunodeficiency · classification · IUIS · diagnosis tool

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Abbreviations

αFP	Alpha- fetoprotein
Ab	Antibody
AD	Autosomal dominant inheritance
ADA	Adenosine deaminase
Adp	Adenopathy
AIHA	Auto-immune hemolytic anemia
AML	Acute myeloid leukemia
Anti PSS	Anti- pneumococcus polysaccharide antibodies
AR	Autosomal recessive inheritance
BL	B lymphocyte
CAPS	Cryopyrin-associated periodic syndromes
CBC	Complete blood count
CD	Cluster of differentiation
CGD	Chronic granulomatous disease
CID	Combined immunodeficiency
CINCA	Chronic infantile neurologic cutaneous and articular syndrome
FCM*	Flow cytometry available
CMML	Chronic myelo-monocytic leukemia
CNS	Central nervous system
CVID	Common variable immunodeficiency disorders
CT	Computed tomography
CTL	Cytotoxic T-lymphocyte
DA	Duration of attacks
Def	Deficiency
DHR	DiHydroRhodamine
Dip	Diphtheria
EBV	Epstein-barr virus
EDA	Anhidrotic ectodermal dysplasia
EDA-ID	Anhidrotic ectodermal dysplasia with immunodeficiency
EO	Eosinophils
FA	Frequency of attacks
FCAS	Familial cold autoinflammatory syndrome
FISH	Fluorescence in situ hybridization
GI	Gastrointestinal
Hib	*Haemophilus influenzae* serotype b
HIDS	Hyper IgD syndrome
HIES	Hyper IgE syndrome
HIGM	Hyper Ig M syndrome
HLA	Human leukocyte antigen
HSM	Hepatosplenomegaly
Hx	Medical history
Ig	Immunoglobulin
IL	Interleukin
LAD	Leukocyte adhesion deficiency
MKD	Mevalonate kinase deficiency
MSMD	Mendelian susceptibility to mycobacteria disease
MWS	Muckle-Wells syndrome
N	Normal, not low
NK	Natural killer
NKT	Natural killer T cell
NN	Neonate
NOMID	Neonatal onset multisystem inflammatory disease
NP	Neutropenia
PAPA	Pyogenic sterile arthritis pyoderma gangrenosum, Acne syndrome
PMN	Neutrophils
PT	Platelet
SCID	Severe combined immune deficiencies

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Sd	Syndrome
SLE	Systemic lupus erythematosus
SPM	Splenomegaly
Subcl	IgG subclass
TCR	T-cell receptor
Tet	Tetanus
TL	T lymphocyte
TNF	Tumor necrosis factor
TRAPS	TNF receptor-associated periodic syndrome
WBC	White blood cells
XL	X-linked

Introduction

Primary Immunodeficiency Diseases (PID) comprise at least 200 genetically-defined inborn errors of immunity [1–3]. The International Union of Immunological Societies (IUIS) PID expert committee has proposed a PID classification [1], which facilitates clinical care and clinical research studies world-wide; it is updated every other year to include new information. The PIDs are grouped into eight categories based on the principal mechanism in each disease, though if more than one mechanism is involved, there are diseases that could appear in more than one category. For each individual PID, the genotype, immunological and clinical phenotypes are briefly described. Since the number of disorders is quickly increasing every year [4–6], at an even faster pace since the advent of next-generation sequencing, the classification and these tables are therefore cumbersome. They offer limited assistance to most physicians at the bedside, especially those outside the field of PIDs and those in training; clinicians in regions

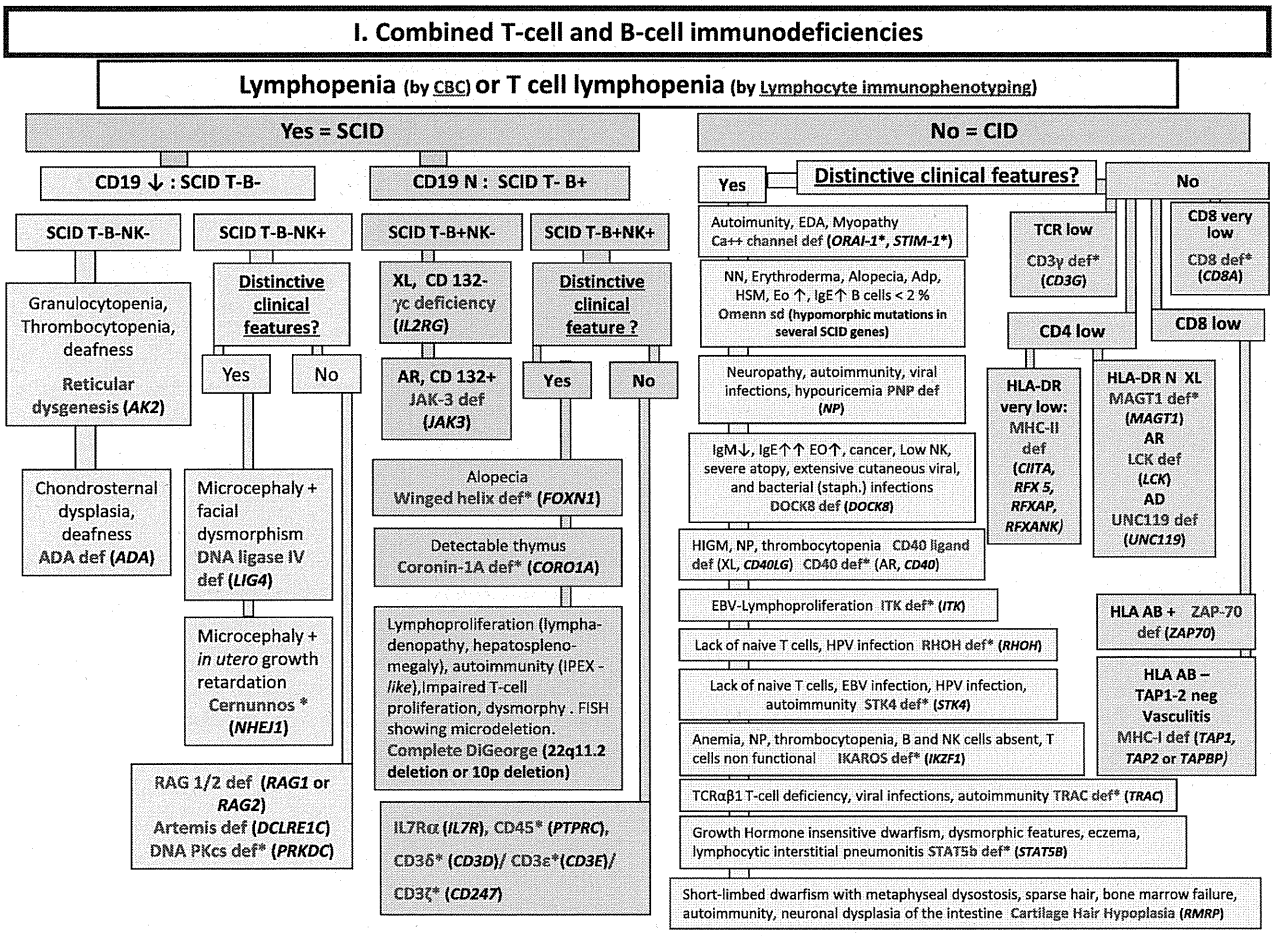

Fig. 1 Combined T- and B- cell immunodeficiencies. ADA: Adenosine Deaminase; Adp: adenopathy; AIHA: Auto-Immune Hemolytic Anemia; AR: Autosomal Recessive inheritance; CBC: Complete Blood Count; CD: Cluster of Differentiation; CID: Combined Immunodeficiency; EBV: Epstein-Barr Virus; EDA: Anhidrotic ectodermal dysplasia; EO: Eosinophils; **FISH:** Fluorescence in situ Hybridization; HIGM: Hyper IgM syndrome; HLA: Human Leukocyte Antigen; HSM: Hepatosplenomegaly; Ig: Immunoglobulin; N: Normal, not low; NK: Natural Killer; NN: Neonate; NP: Neutropenia; PT: Platelet; SCID: Severe Combined ImmunoDeficiency; TCR: T-Cell Receptor; XL: X-Linked

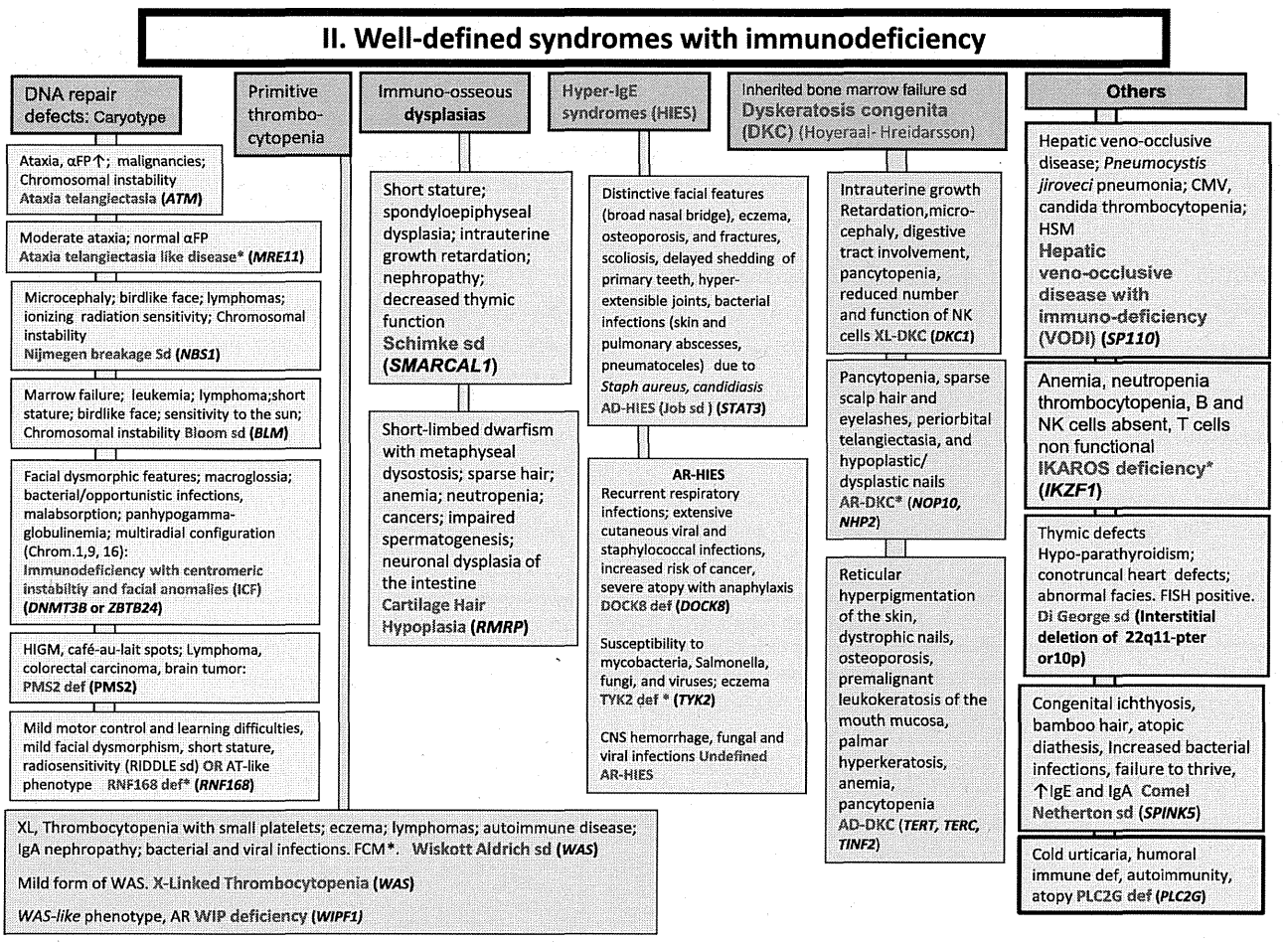

Fig. 2 Well-defined syndromes with immunodeficiencies. These syndromes are generally associated with T-cell immunodeficiency. αFP: alpha- fetoprotein; AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; CNS: Central Nervous System; FCM*: Flow cytometry available; FISH: Fluorescence in situ Hybridization; HSM: Hepatosplenomegaly; Ig: Immunoglobulin; NK: Natural Killer; XL: X-Linked inheritance

of the world where awareness for PIDs is limited may also find the tables tricky.

Patients with a PID may first present to many types of medical and surgical disciplines and this is likely to be increasingly common given the growing number of patients with known or suspected PIDs [7]. Such physicians, who may lack familiarity with PIDs, need a classification that is based on a clinical and/or biological phenotype that they observe. This prompted IUIS PID experts to work on a simplified classification, based on simple clinical and immunological phenotypes, in order to provide some easy-to-follow algorithms to diagnose a particular PID or group of PIDs. This will optimize collaboration between primary centers and specialized centers, particularly for genetic studies, and will lead to faster and more precise molecular diagnosis and genetic counseling, paving the way to more appropriate management of affected patients and families. This work presents a user-friendly classification of PIDs, providing a tree-based decision-making process based on the observation of clinical and biological phenotypes.

Methodology

We included all diseases from the 2011 update of IUIS PID classification [1]. To stay up-to-date, we also included new diseases described in the last 2 years [2]. However, there may be other genes associated with

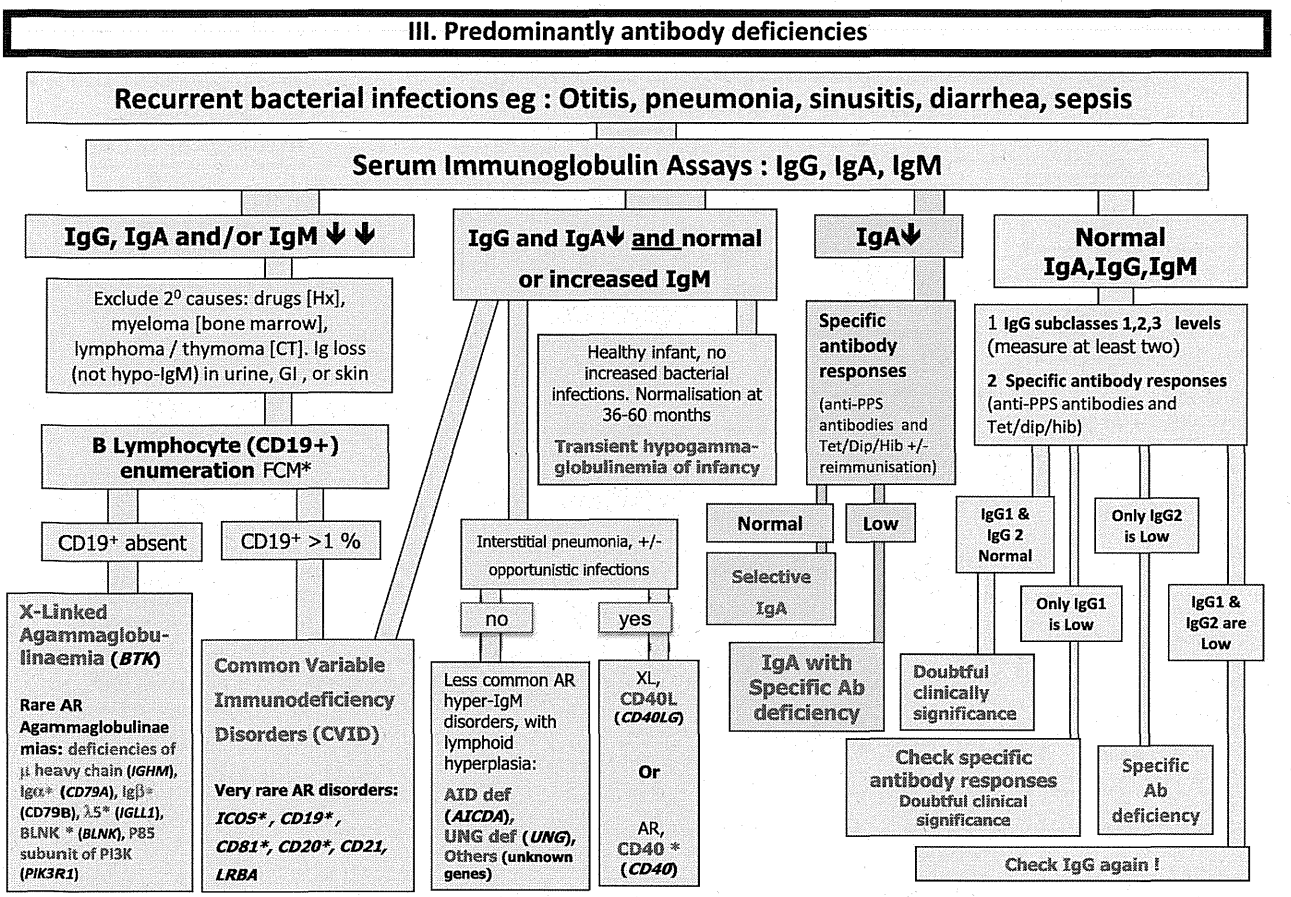

Fig. 3 Predominantly antibody deficiencies. Ab: Antibody; Anti PPS: Anti- pneumococcal polysaccharide antibodies: AR: Autosomal Recessive inheritance; CD: Cluster of Differentiation; CVID: Common Variable Immunodeficiency Disorders; CT: Computed Tomography; Dip: Diphtheria; FCM*: Flow cytometry available; GI: Gastrointestinal; Hib: *Haemophilus influenzae* serotype b; Hx: medical history; Ig: Immunoglobulin; subcl: IgG subclass; Tet; Tetanus; XL: X-Linked inheritance

PIDs that are not included here to be faithful to our inclusion criteria. An algorithm was assigned to each of the eight main groups of the classification. We used the same color for each group of similar conditions. Disease names are written in red. As in the IUIS Classification, an asterisk is added to highlight extremely rare disorders (less than 10 cases reported in the medical literature). These algorithms were first established by a small committee; then validated by one or two experts for each figure.

Results

A classification validated by the IUIS PID expert committee is presented in Figs. 1, 2, 3, 4, 5, 6, 7 and 8.

Discussion

These figures are diagnostic tools that represent a modified and simplified version of the 2011 IUIS classification [1]. They are based on patients' clinical and biological phenotypes and are mostly presented as decision trees for diagnostic orientation. These figures serve as diagnostic orientation tools for the typical forms of PID; the more atypical presentations of PIDs are not covered in these figures. These figures do not therefore aim to replace decisional trees or diagnostic protocols proposed by other teams or scientific societies [8–11]. Rather they aim at being a user-friendly first approach to the IUIS classification [1]. These figures enable non-PID specialists to select the most appropriate diagnostic tree and to undertake some preliminary

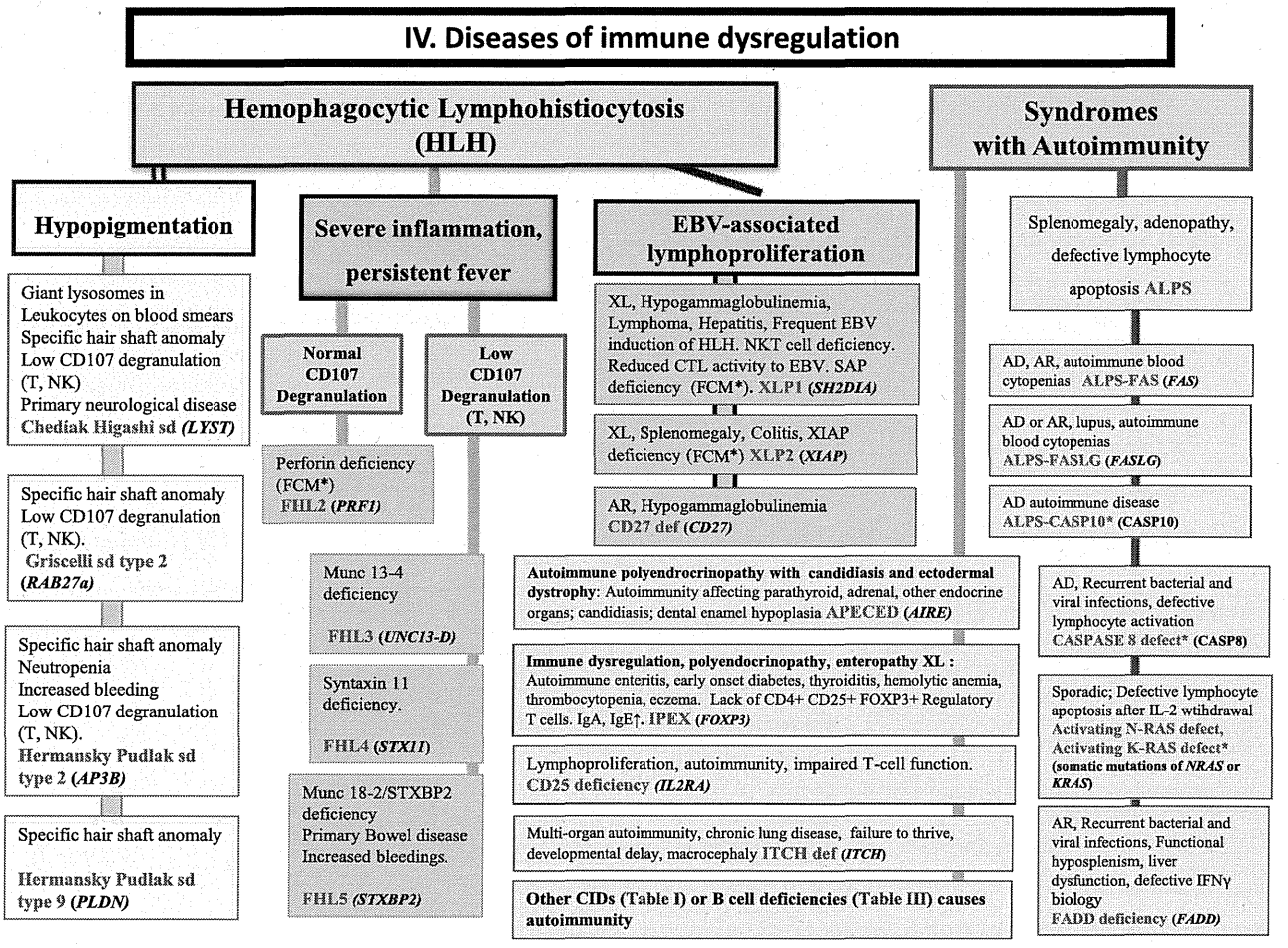

Fig. 4 Diseases of immune dysregulation. AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; CD: Cluster of Differentiation; CTL: Cytotoxic T-Lymphocyte; EBV: Epstein-Barr Virus; FCM*: Flow cytometry available; HSM: Hepatosplenomegaly; Ig: Immunoglobulin; IL: interleukin; NK: Natural Killer; NKT: Natural Killer T cell; TL: T lymphocyte; XL: X-Linked inheritance

investigations, whilst contacting an expert in PIDs. They may also help in the selection of the center or expert to whom the patient should be referred, given the patient's particular phenotype. In all cases, whether a tentative diagnosis can be made based on these figures or not, we recommend that the practitioner outside the field who sees a patient with a possible PID seeks specialist advice.

To simplify our figures, we did not systematically include all data from the IUIS classification (OMIM number, presumed pathogenesis, affected cells or function...) [1]. In order to present the 24 pages from the IUIS classification in only 8 figures, we used common abbreviations familiar to most physicians (explained in footnotes). The use of a color code makes these figures easy to follow, so that they could be hung, in larger format, in clinical wards. This is also suitable for informing young clinicians and students.

To make these figures easier to use by clinicians and biologists, we highlighted the clinical and biological features, adding to the data from the IUIS classification some other features typical of the PID in question. This allows an initial orientation towards a particular disease or group of diseases. Whenever it was possible, we have focused on clinical or routine laboratory features that distinguish disorders that are closely related. Example: A female infant with an opportunistic infection in whom lymphocyte subpopulation investigation reveals profound CD3 and CD16/56 lymphopenia without CD19/20 lymphopenia

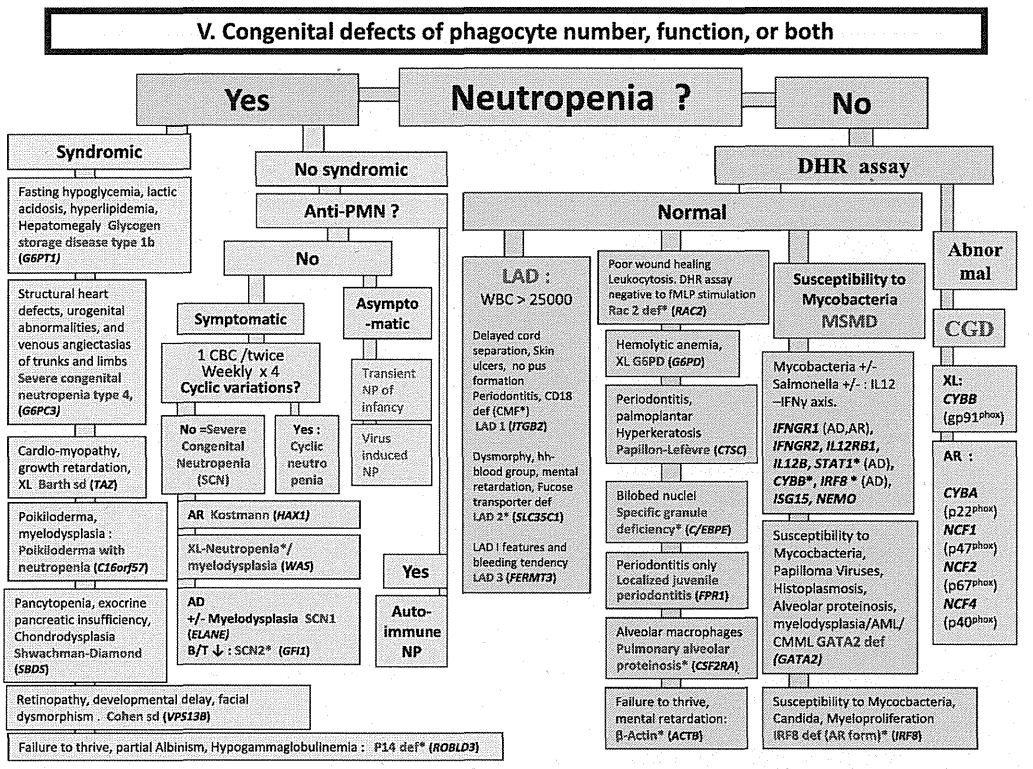

Fig. 5 Congenital defects of phagocyte number, function, or both. For DHR assay, the results can distinguish XL-CGD from AR-CGD, and gp40phox defect from others AR forms. AD: Autosomal Dominant inheritance; AML: Acute Myeloid Leukemia; AR: Autosomal Recessive inheritance; CBC: Complete Blood Count; CD: Cluster of Differentiation; CGD: Chronic Granulomatous Disease; CMML: Chronic Myelo-monocytic Leukemia; DHR: DiHydroRhodamine; LAD: Leukocyte Adhesion Deficiency; MSMD: Mendelian Susceptibility to Mycobacteria Disease; NP: Neutropenia; PNN: Neutrophils; WBC: White Blood Cells; XL: X-Linked inheritance

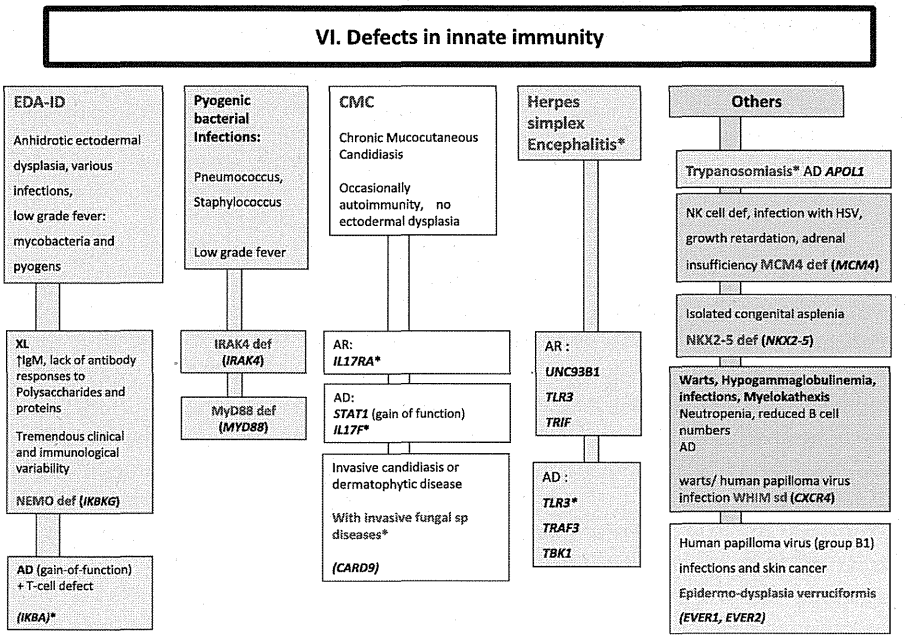

Fig. 6 Defects in innate immunity. AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; BL: B lymphocyte; EDA-ID: Anhidrotic Ectodermal Dysplasia with Immunodeficiency; Ig: Immunoglobulin; PNN: Neutrophils; XL: X-Linked inheritance

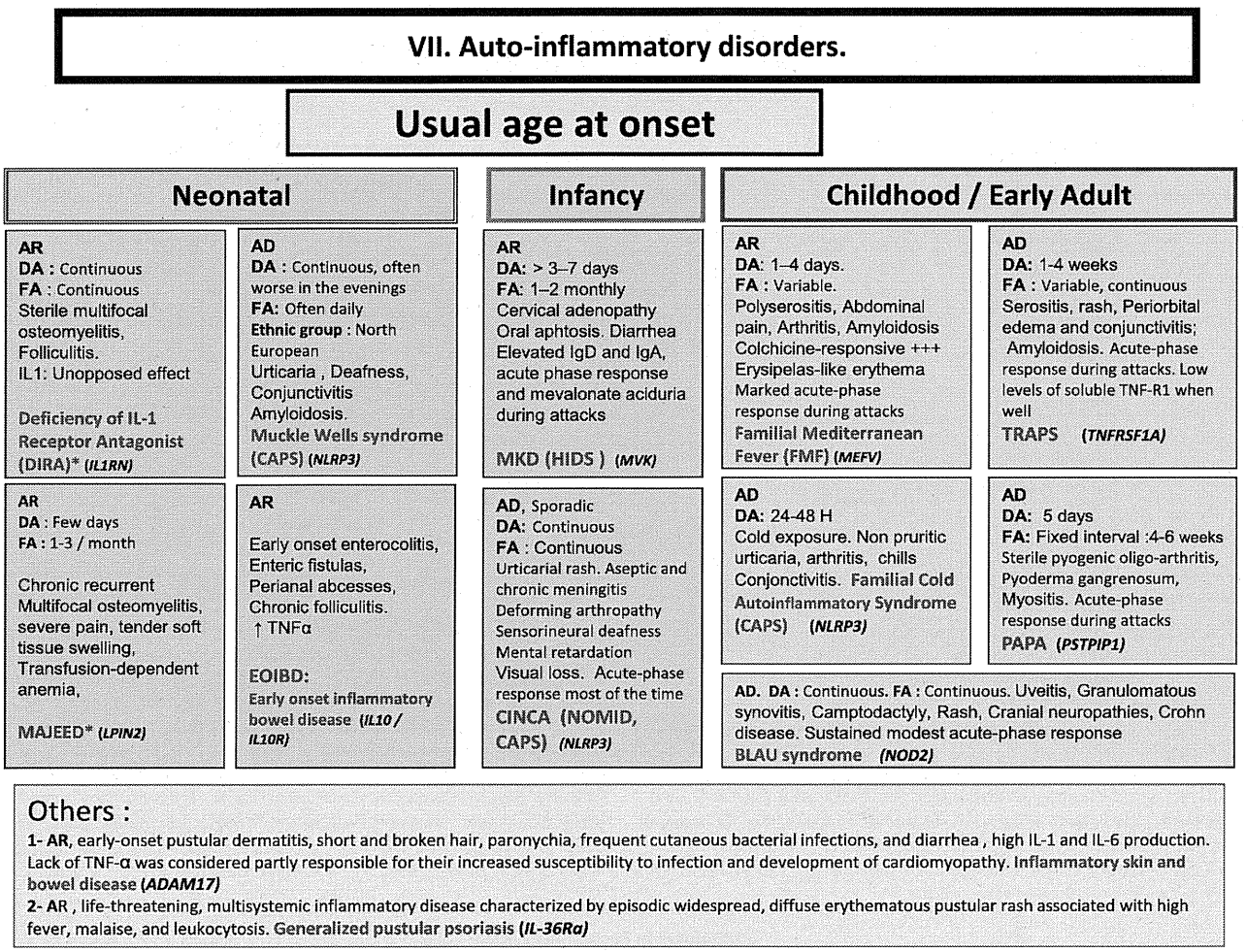

Fig. 7 Autoinflammatory disorders. AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; CAPS: Cryopyrin-Associated Periodic syndromes; CINCA: Chronic Infantile Neurologic Cutaneous and Articular syndrome; DA: Duration of Attacks; FA: Frequency of Attacks; FCAS: Familial Cold Autoinflammatory Syndrome; HIDS: Hyper IgD syndrome; Ig: Immunoglobulin; IL: interleukin; MKD: Mevalonate Kinase deficiency; MWS: Muckle-Wells syndrome; NOMID: Neonatal Onset Multisystem Inflammatory Disease; PAPA: Pyogenic sterile Arthritis, Pyoderma gangrenosum, Acne syndrome; SPM: Splenomegaly; TNF: Tumor Necrosis Factor; TRAPS: TNF Receptor-Associated Periodic Syndrome

has a SCID T-B+NK- phenotype, which strongly suggests Jak3 deficiency (Fig. 1). After discussion with a team specialized in the diagnosis and treatment of SCID patients, an analysis of the *JAK3* gene will be arranged as a priority, while expert advice will be given on the appropriate management for the infant.

Though atypical forms of PID are increasingly reported in the literature [12–15], typical presentations of these conditions remain predominant, permitting this classification to be useful in most of cases. Moreover, the genetic heterogeneity of most PIDs is high and patients with almost any PID may lack coding mutations in known disease-causing genes. This manuscript will therefore be up-dated every other year along with the IUIS classification. Meanwhile, we hope that this phenotypic approach to diagnosis of PID can constitute a useful tool for physicians or biologists from various related specialties, especially in the setting of pediatric and adult medicine (internal medicine, pulmonology, hematology, oncology, immunology, infectious diseases, etc...) who may encounter the first presentation of PID patients.

Conclusion

The strengths of this algorithmic approach to the diagnosis of PID are its simplified format, reliance on phenotypic

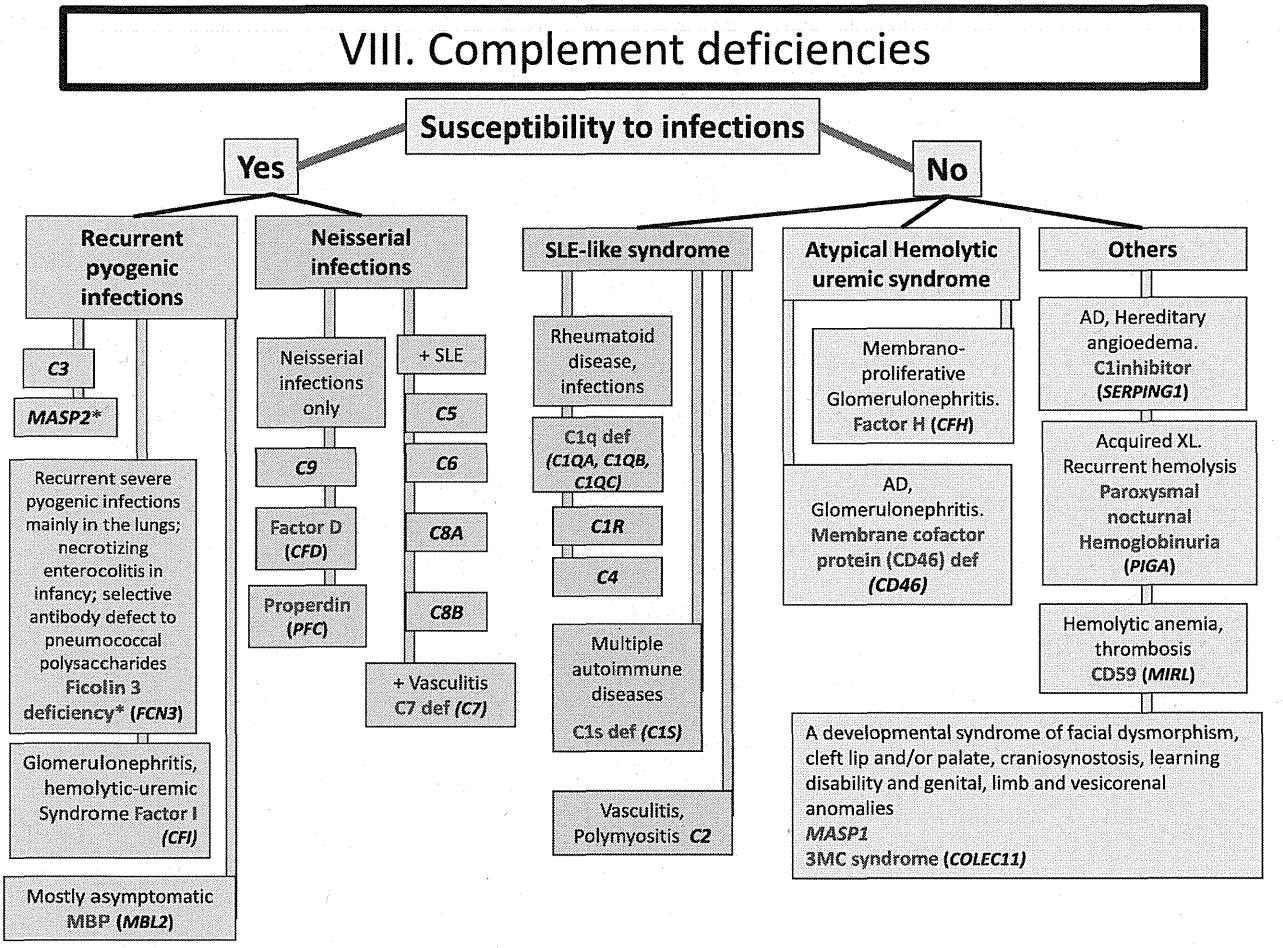

Fig. 8 Complement deficiencies. Def: deficiency; SLE: Systemic Lupus Erythematosus

features, presentation in user-friendly pathways, and validation by a group of PID experts. We hope they will be useful to physicians at the bedside in several areas of pediatrics, internal medicine, and surgery. While these algorithms cannot be comprehensive, due to the tremendous genetic and phenotypic heterogeneity of PIDs, they will be improved over time with progress in the field as well as by feed-back from users. They will also be expanded with the discovery of new PIDs and the refined description of known PIDs.

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Article

Fetal liver stromal cells support blast growth in transient abnormal myelopoiesis in Down syndrome through GM-CSF†

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Abstract

Transient abnormal myelopoiesis (TAM) in neonates with Down syndrome, which spontaneously resolves within several weeks or months after birth, may represent a very special form of leukemia arising in the fetal liver (FL). To explore the role of the fetal hematopoietic microenvironment in the pathogenesis of TAM, we examined the in vitro influences of stromal cells of human FL and fetal bone marrow (FBM) on the growth of TAM blasts. Both FL and FBM stromal cells expressed mesenchymal cell antigens (vimentin, α-smooth muscle actin, CD146 and nestin), being consistent with perivascular cells/mesenchymal stem cells that support hematopoietic stem cells. In addition, a small fraction of the FL stromal cells expressed an epithelial marker, cytokeratin 8, indicating that they could be cells in epithelial-mesenchymal transition (EMT). In the coculture system, stromal cells of the FL, but not FBM, potently supported the growth of TAM blast progenitors, mainly through humoral factors. High concentrations of hematopoietic growth factors were detected in culture supernatants of the FL stromal cells and a neutralizing antibody against granulocyte-macrophage colony-stimulating factor (GM-CSF) almost completely inhibited the growth-supportive activity of the culture supernatants. These results indicate that FL stromal cells with unique characteristics of EMT cells provide a pivotal hematopoietic microenvironment for TAM blasts and that GM-CSF produced by FL stromal cells may play an important role in the pathogenesis of TAM.

INTRODUCTION

In mammals as well as non-mammalian vertebrates, the first wave of hematopoiesis ("primitive erythropoiesis") begins in the extra-embryonic yolk sac, originating from a common mesodermal precursor for hematopoietic and endothelial cell lineages [Dzierzak and Speck, 2008]. Various types of hematopoietic precursors produced later in the yolk sac, allantois and aorta-gonad-mesonephros region migrate to the liver, where the second wave of hematopoiesis (adult-type or "definitive hematopoiesis") begins and continues until birth. The major site of adult-type hematopoiesis finally shifts to the bone marrow afterwards, where it continues throughout life. Leukemia generally arises in the bone marrow, but certain types of leukemia have been shown to arise in utero. These include infantile and childhood acute lymphoblastic leukemia (ALL) with *MLL* gene rearrangements [Ford et al., 1993] or *TEL-AML1* rearrangements [Ford et al., 1998; Wiemels et al., 1999] and transient abnormal myelopoiesis (TAM) in neonates with Down syndrome (DS) [Ahmed et al., 2004]. In such cases, the microenvironment of prenatal hematopoietic organs, not bone marrow, might play an important role in the leukemogenesis.

Children with DS have a higher risk of developing leukemia [Hitzler and Zipursky, 2005; Roy et al., 2009]. ALL is the major form of leukemia in patients with DS at the age of 3 years or older, as in the case of non-DS patients, while acute myeloid leukemia (AML) is as commonly seen as ALL under the age of 3 years, and acute megakaryoblastic leukemia (AMKL), a very rare subtype of AML in non-DS children, comprises the majority of AML cases. In 4-10% of neonates with DS, abnormal blasts indistinguishable from those of AMKL in DS (AMKL-DS) appear in the blood, but usually spontaneously disappear within several weeks or months. A variety of terms have been given to this special disorder, including TAM, transient myeloproliferative disorder

(TMD) and transient leukemia (TL) [Hitzler and Zipursky, 2005; Roy et al., 2009; Roy et al., 2012; Zipursky, 2003]. AMKL arises in 20-30% of patients with TAM after spontaneous remission by the age of 4 years. Somatic mutations affecting the *GATA1* gene, which encodes one of the GATA family of zinc-finger transcription factors, have been detected exclusively in AMKL-DS and TAM in nearly all cases [Hitzler et al., 2003; Mundschau et al., 2003; Wechsler et al., 2002]. A variety of *GATA1* mutations have been reported, but almost all of them result in the lack of the 50-kD full-length GATA1 protein and the generation of a 40-kD short isoform of GATA1, called GATA1s, lacking the N-terminal activation domain [Gurbuxani et al., 2004]. The precise role of GATA1s in the pathogenesis of TAM and AMKL-DS remains largely unknown.

Myelofibrosis is a common complication of AMKL and is thought to be caused by cytokines, including transforming growth factor β (TGF-β), that are produced by leukemic megakaryoblasts and stimulate fibroblasts to induce fibrosis in the bone marrow [Terui et al., 1990]. Although TAM blasts have the features of megakaryoblasts similar to those of AMKL-DS blasts, myelofibrosis is absent in most cases but, instead, hepatic fibrosis is often found in fatal cases of TAM [Miyauchi et al., 1992; Schwab et al., 1998]. On the basis of these findings and considering that TAM is a disorder of neonates, we hypothesized that TAM may be a very special form of leukemia arising in the fetal liver (FL) but not in the bone marrow and that cytokines produced by TAM blasts in the FL cause hepatic fibrosis in a similar manner to myelofibrosis in AMKL [Miyauchi et al., 1992]. Consistent with this hypothesis, the expression of TGF-β has been immunohistochemically demonstrated in megakaryocytic cells in hepatic sinusoids of patients with TAM [Arai et al., 1999]. Furthermore, using a *GATA1* knock-in allele, the dominant action of

N-terminus-truncated GATA1 protein, leading to the hyperproliferation of hematopoietic progenitor cells in the yolk sac and FL, but not the bone marrow, has been shown [Li et al., 2005], indicating that the source of TAM is hematopoietic progenitor cells of embryonic/fetal origin. If the above hypothesis is the case and the growth of blast progenitors in TAM is dependent on the microenvironment of FL, cessation of fetal hematopoiesis in the liver after birth might cause spontaneous resolution of TAM [Miyauchi et al., 1992]. To explore the role of the fetal hematopoietic microenvironment in the pathogenesis of TAM directly, we investigated the in vitro influence of stromal cells derived from human FL and fetal bone marrow (FBM), the major hematopoietic organs in fetal stage, on the growth of blasts in TAM. The obtained findings indicate that blast progenitors in TAM are dependent on the microenvironment of the FL and that granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by FL stromal cells is particularly important for the growth of TAM blasts.

MATERIALS AND METHODS

Cells

TAM blasts were obtained from the peripheral blood of 4 patients with TAM (TAM-1 through TAM-4) after obtaining the informed consent of their parents. The clinical profile of these patients and *GATA1* mutations of the blasts have been published (TAM-1 through TAM-4 correspond to patient 1 through 4 in the reference, respectively) [Miyauchi et al., 2010]. AML blasts from 2 adult patients (AML-1 and AML-2; FAB subtypes, M5a and M1, respectively) were also obtained from the peripheral blood after obtaining informed consent. Blast cells were enriched by depleting

monocytes and lymphocytes by plastic adherence and immuno-magnetic bead methods, respectively, as previously described [Miyauchi et al., 2010], and these patient samples were confirmed to contain more than 95% blasts morphologically and to possess *GATA1* mutations.

Stromal cells of human FL and FBM were obtained from two fetuses (FL1/FBM1 from fetus 1; FL2/FBM2 from fetus 2) that had been artificially aborted during the 16th and 13th weeks of gestation, respectively, because of maternal health problems, after informed consent for the use of fetal tissues for research purposes had been obtained. Single-cell suspensions of FL and FBM stromal cells were prepared as previously described [Campagnoli et al., 2001]. Briefly, FBM cells were flushed out of the femur using a syringe with a 23-gauge needle into the growth medium [α-minimum essential medium (αMEM) supplemented with 10% fetal calf serum (FCS)], whereas FL was minced with scalpels, incubated in 0.25% trypsin solution containing 1 mM EDTA, filtered through a 70-μm nylon mesh and suspended in the growth medium. These cells were incubated at 37°C in 5% CO_2. Nonadherent cells were removed after 48 h and adherent cells were expanded in fresh growth medium for 2-3 weeks with 3-4 passages. The confluent adherent cell layers in primary cultures of the FL and FBM, consisting of morphologically homogeneous populations of fibroblast-like spindle cells, were trypsinized and cryopreserved.

A stromal cell line, KM101, which is derived from human adult bone marrow and known to support the growth of hematopoietic progenitors [Harigaya and Handa, 1985], was provided by Prof. K. Harigaya at Chiba University, Japan, and stem-cell-factor (SCF)-dependent leukemic cell line KPAM1 [Toki et al., 2009], derived from AMKL-DS, was provided by Dr. T. Toki and Prof. E. Ito at Hirosaki University, Japan. The experimental procedures using human cells described