J Clin Immunol (2013) 33:1078–1087
DOI 10.1007/s10875-013-9901-6

ORIGINAL RESEARCH

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

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Received: 11 December 2012 / Accepted: 9 April 2013 / Published online: 9 May 2013

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

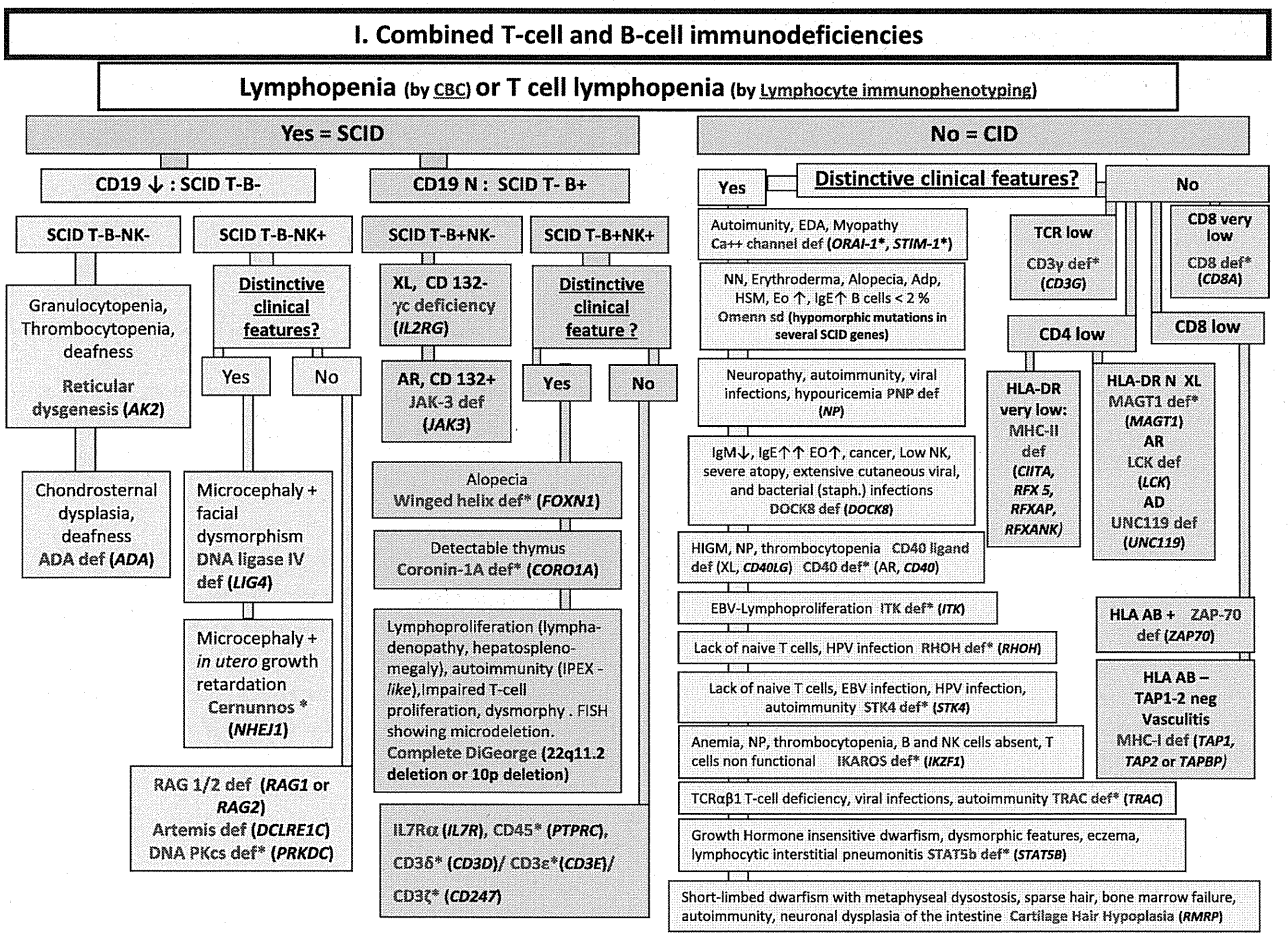

Fig. 1 Combined T- and B- cell immunodeficiencies. ADA: Adenosine Deaminase; Adp: adenopathy; AIHA: Auto-Immune Hemolytic Anemia; AR: Autosomal Recessive inheritance; CBC: Complete Blood Count; CD: Cluster of Differentiation; CID: Combined Immunodeficiency; EBV: Epstein-Barr Virus; EDA: Anhidrotic ectodermal 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

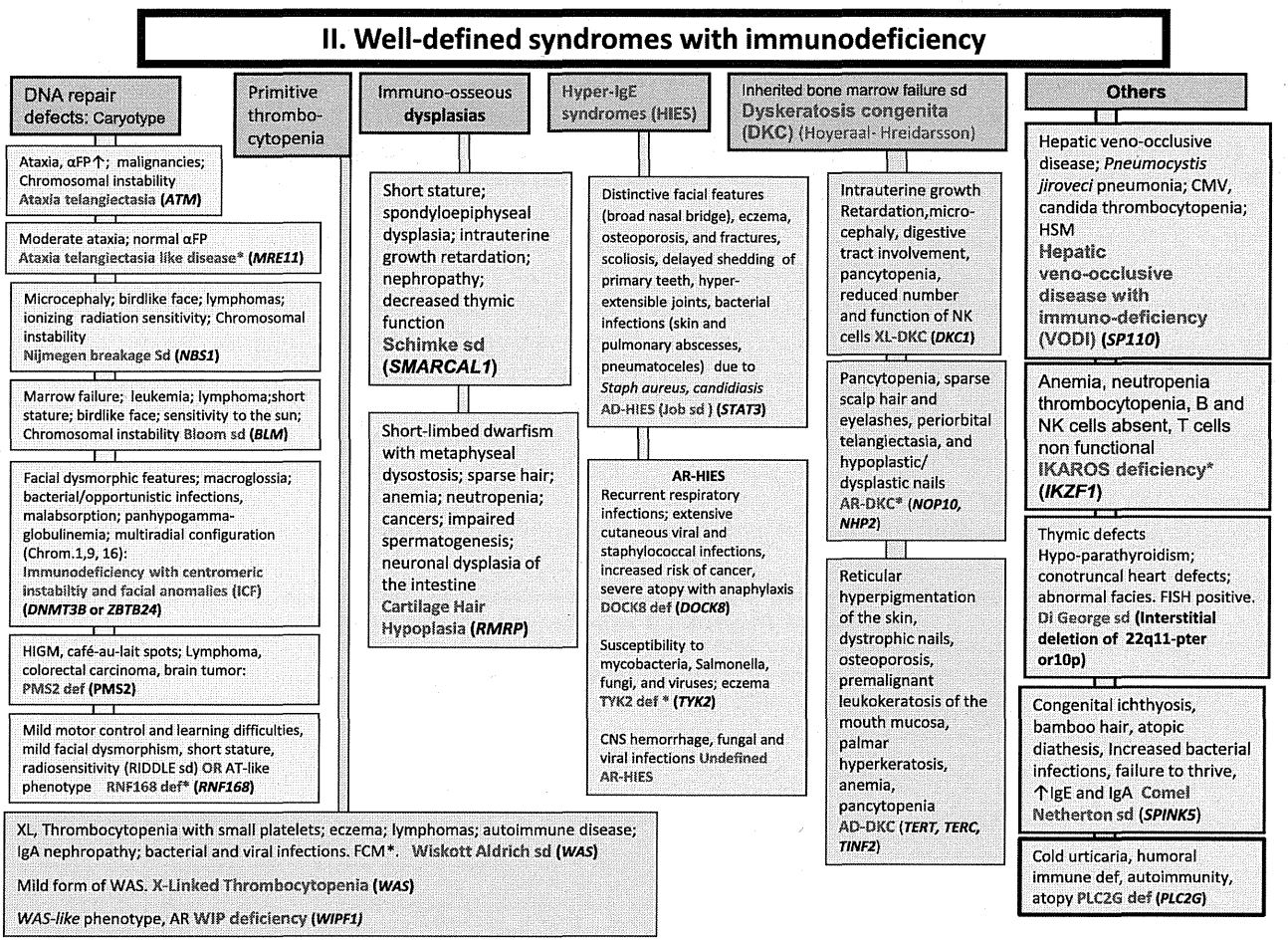

Fig. 2 Well-defined syndromes with immunodeficiencies. These syndromes are generally associated with T-cell immunodeficiency. αFP: alpha- fetoprotein; AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; CNS: Central Nervous System; FCM*: Flow cytometry available; FISH: Fluorescence 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

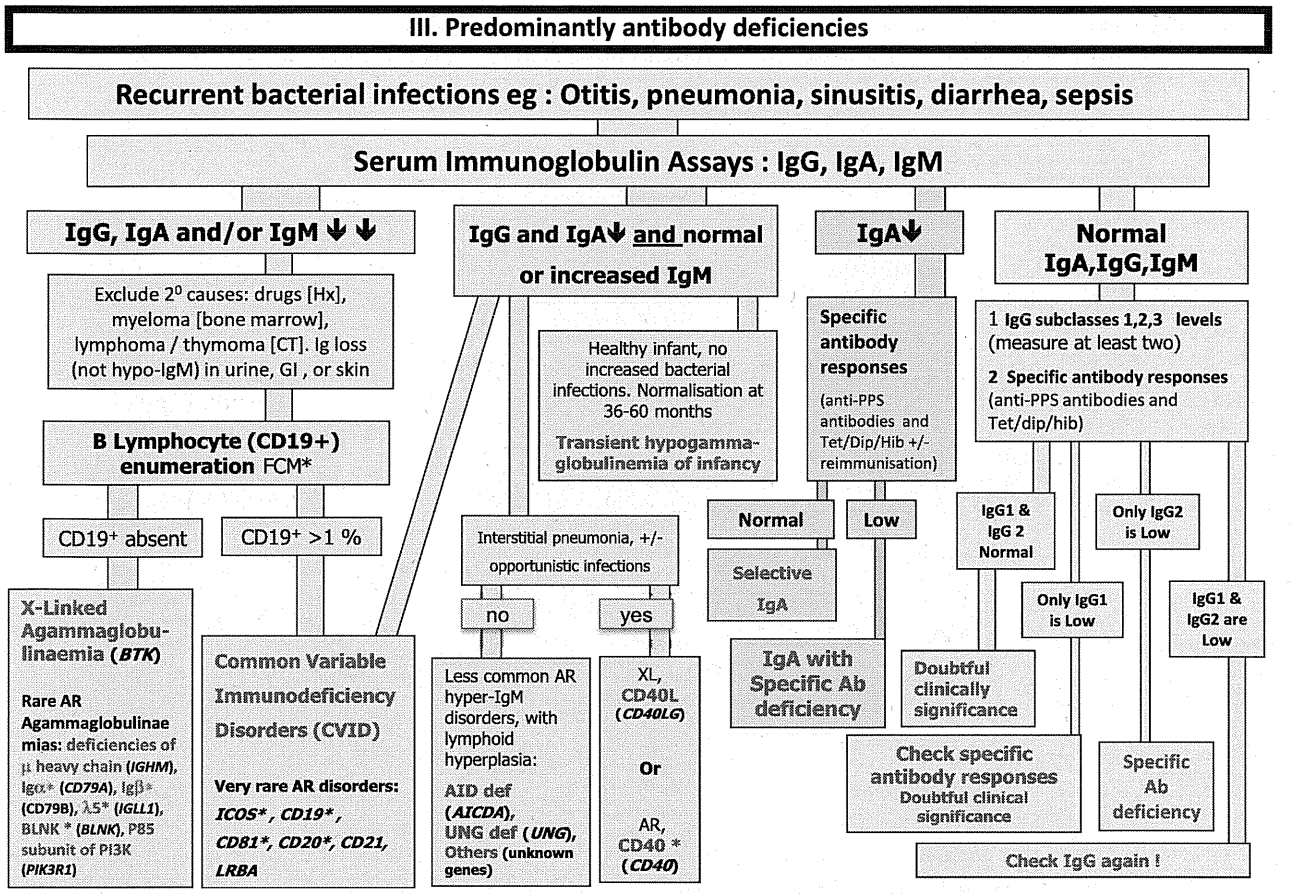

Fig. 3 Predominantly antibody deficiencies. Ab: Antibody; Anti PPS: Anti- pneumococcal polysaccharide antibodies: AR: Autosomal Recessive inheritance; CD: Cluster of Differentiation; CVID: Common Variable Immunodeficiency Disorders; CT: Computed Tomography; Dip: Diphtheria; FCM*: Flow cytometry available; 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

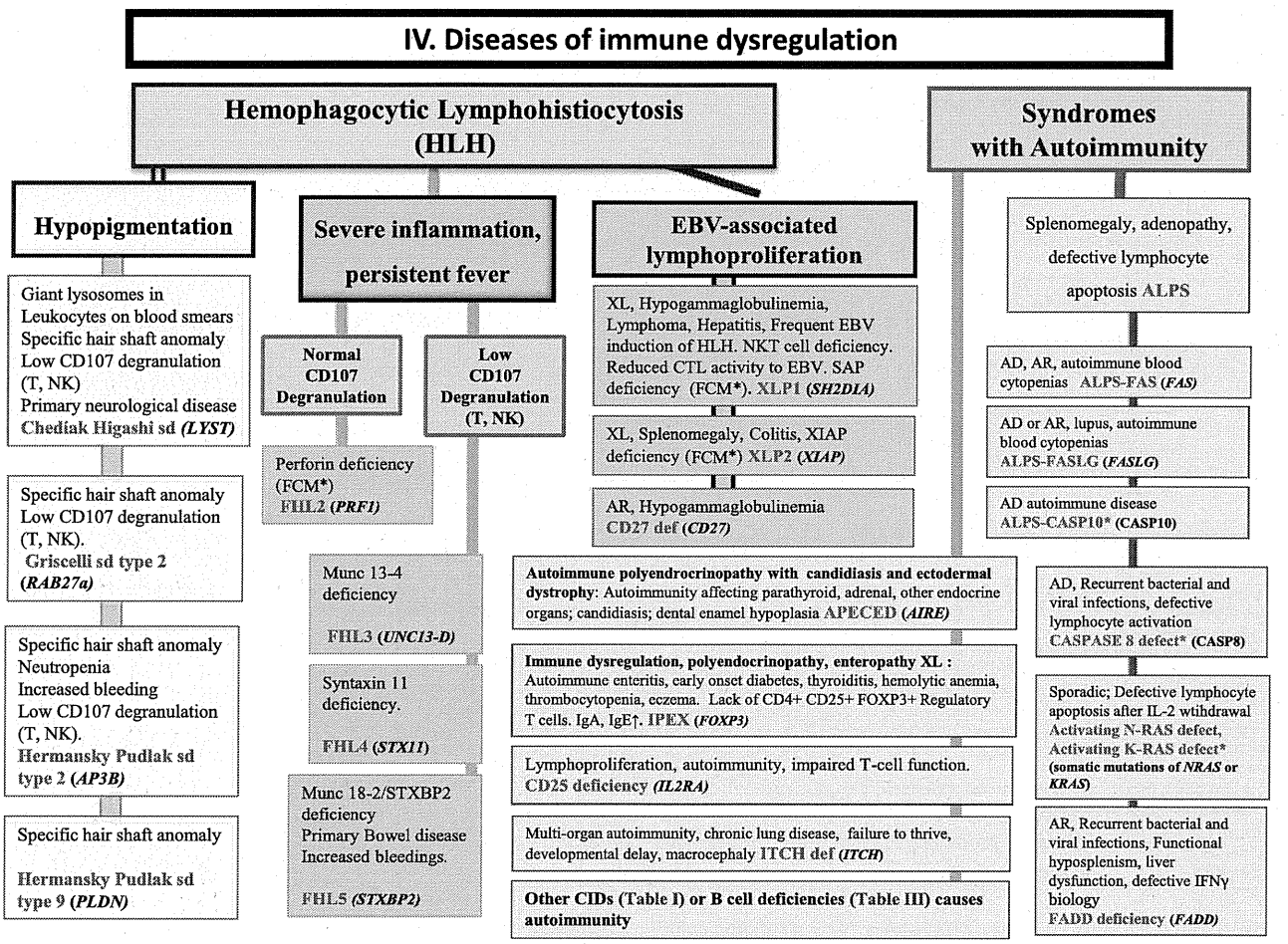

Fig. 4 Diseases of immune dysregulation. AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; CD: Cluster of Differentiation; CTL: Cytotoxic T-Lymphocyte; EBV: Epstein-Barr Virus; FCM*: Flow cytometry available; HSM: Hepatosplenomegaly; Ig: Immunoglobulin; IL: interleukin; NK: Natural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

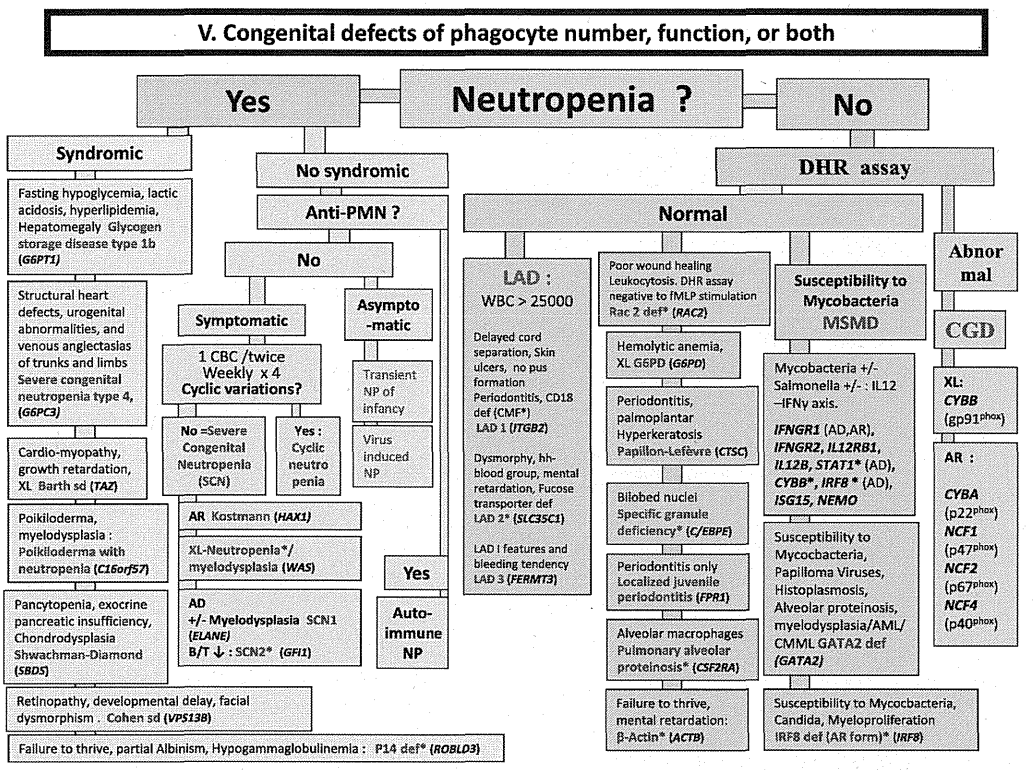

Fig. 5 Congenital defects of phagocyte number, function, or both. For DHR assay, the results can distinguish XL-CGD from AR-CGD, and gp40phox defect from others AR forms. AD: Autosomal Dominant inheritance; AML: Acute Myeloid Leukemia; AR: Autosomal Recessive inheritance; CBC: Complete Blood Count; CD: 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

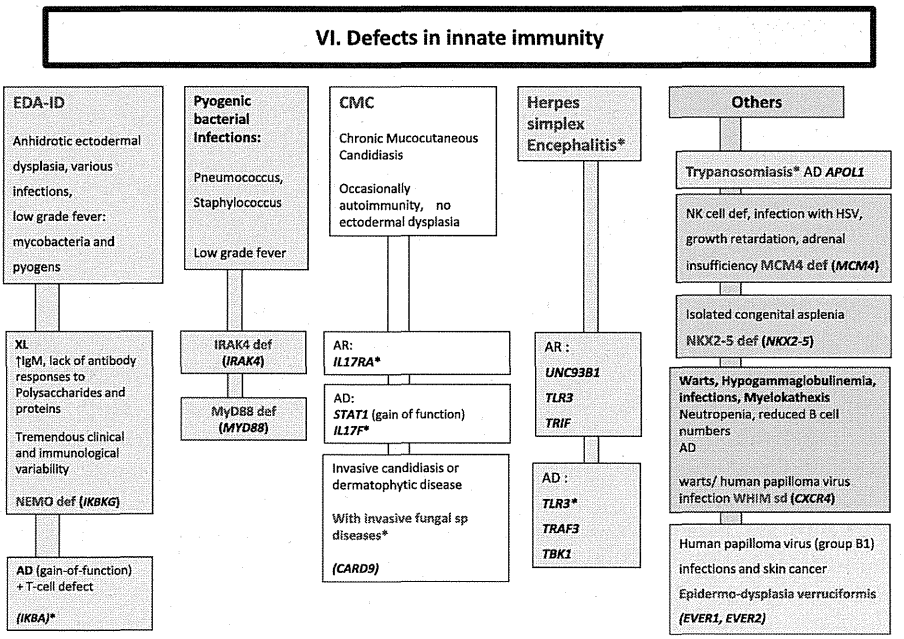

Fig. 6 Defects in innate immunity. AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; BL: B lymphocyte; EDA-ID: Anhidrotic Ectodermal Dysplasia with Immunodeficiency; Ig: Immunoglobulin; PNN: Neutrophils; XL: X-Linked inheritance

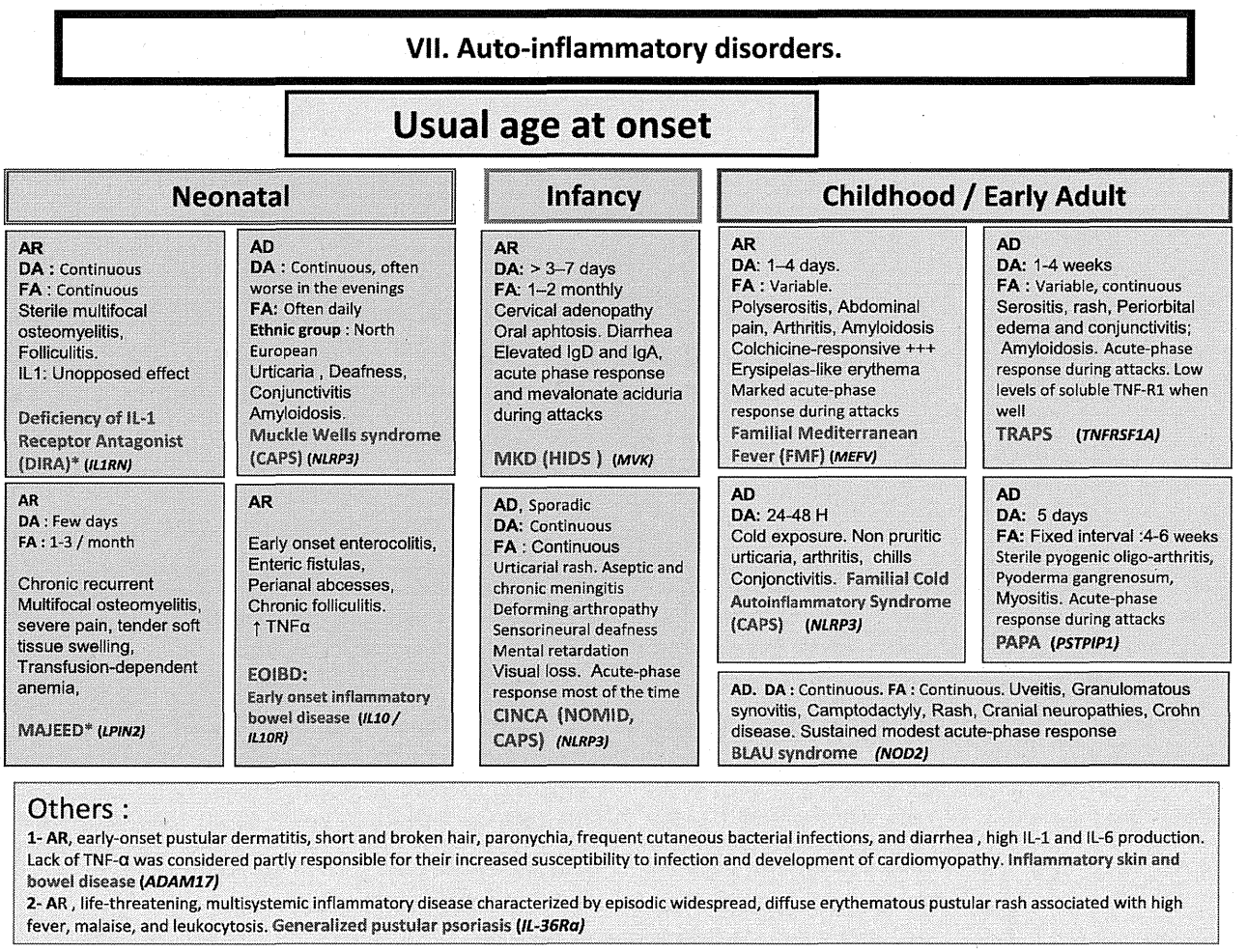

Fig. 7 Autoinflammatory disorders. AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; CAPS: Cryopyrin-Associated Periodic syndromes; CINCA: Chronic Infantile Neurologic Cutaneous and Articular syndrome; DA: Duration of Attacks; FA: Frequency of Attacks; FCAS: Familial Cold Autoinflammatory 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

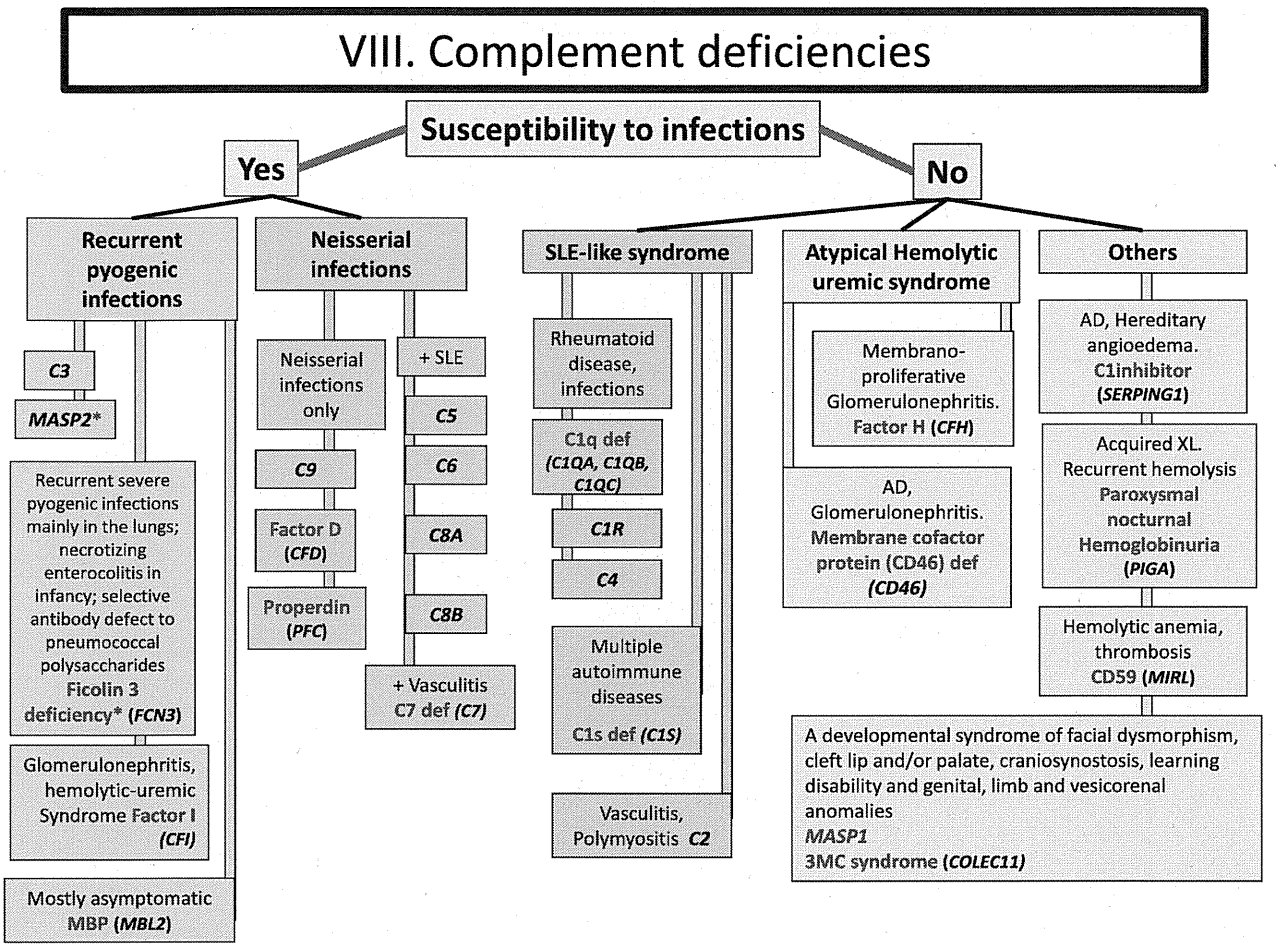

Fig. 8 Complement deficiencies. Def: deficiency; SLE: Systemic Lupus Erythematosus

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.

Acknowledgments We thank Dr Capucine Picard and Dr Claire Fieschi for their contribution to this work.

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Journal of Clinical Microbiology

Neonatal Herpes Encephalitis Caused by a Virologically Confirmed Acyclovir-Resistant Herpes Simplex Virus 1 Strain

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J. Clin. Microbiol. **2013, 51(1):356. DOI: 10.1128/JCM.02247-12.**
Published Ahead of Print 24 October 2012.

JCM
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Neonatal Herpes Encephalitis Caused by a Virologically Confirmed Acyclovir-Resistant Herpes Simplex Virus 1 Strain

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A neonate with herpes simplex virus 1 encephalitis was treated with intravenous acyclovir. During the course of therapy, the infection became intractable to the treatment and a mutation in the viral thymidine kinase gene (nucleotide G375T, amino acid Q125H) developed. This mutation was demonstrated *in vitro* to confer acyclovir resistance.

CASE REPORT

A 13-day-old boy was admitted to National Defense Medical College Hospital due to lethargy and failure to thrive. He was born at 39 weeks 5 days of gestation and 2,558 g birth weight to a healthy 35-year-old mother (gravida 2, para 2). Group B streptococcus (GBS) was detected from the mother's vagina in the third trimester, but the baby's bacterial culture tests performed at birth, including throat, skin, and blood analyses, were negative for GBS. The mother did not have a history of genital herpes. Her herpes simplex virus 1 (HSV-1) and HSV-2 serostatus was not examined, and her history of acyclovir (ACV) use was not clear. Furthermore, the genital swab culture examination for HSV was not performed. On admission, physical examination of the boy revealed skin blisters on the forehead and upper lip. A swab from the blister showed positive and negative reactions for the specific antigens of HSV-1 and HSV-2, respectively, in a direct immunofluorescent antibody assay (Denka Seiken Co. Ltd., Tokyo, Japan) performed by a qualified clinical laboratory (SRL Inc., Tokyo, Japan). A serum sample collected on admission showed positive and negative reactions in the enzyme-linked immunosorbent assay for detection of anti-HSV IgM and IgG antibody (SRL Inc.), respectively. A lumbar puncture revealed pleocytosis (547 cells/μl) and an elevated protein level (168 mg/dl) in the cerebrospinal fluid (CSF). The CSF was also positive for HSV-1 DNA, which was determined by a previously reported method (1) in PCR testing (SRL Inc.). The boy was diagnosed as having neonatal herpes encephalitis (NHE), and intravenous high-dose ACV (60 mg/kg/day) treatment was initiated. His general status improved with resolution of the skin lesions within a few days of the beginning of treatment. However, the viral load in the CSF determined by TaqMan-based quantitative real-time PCR (SRL Inc.), which dropped temporarily, increased again after 4 weeks from the initiation of ACV treatment (Fig. 1A) without obvious deterioration in clinical symptoms. Because the standard dose of ACV was given and drugs which have antagonistic effects for ACV were not used, we assumed that an ACV-resistant HSV-1 strain had developed. The ACV concentration in the CSF was not measured. Foscarnet, an antiviral drug recommended for treatment of ACV-resistant HSV infections (2), was not immediately available. Therefore, vidarabine (15 mg/kg/day) was added to the therapeutic regimen from the fifth week of the treatment course. Subsequently, HSV-DNA in the CSF decreased to a level that was finally undetectable; hence, the antiviral drug treatment was terminated. Because virus isolation from the CSF of the patient was unsuccessful, as is common in herpes encephalitis cases (3), we could not perform a plaque reduction assay to test the *in vitro* inhibition concentration of ACV. Neuroimaging showed residual necrotic changes of the bilateral temporal lobes. Unfortunately, neurodevelopmental sequelae remained in this patient.

To reveal the mechanism of the clinical ACV resistance, sequencing analysis of the viral thymidine kinase (*vTK*) gene was conducted using the CSF samples collected at two different time points. As denoted by the arrows in Fig. 1A, sample 1 and sample 2 were collected before the initiation of and at the 5th week of ACV treatment, respectively. Full-length *vTK* genes were successfully amplified from both samples by a previously reported nested PCR method (4). By direct sequencing, one nucleotide substitution, G375T, leading to a Q125H amino acid substitution was detected. CSF sample 2 contained a mixture of *vTK* genes with and without this mutation (Fig. 1B). To examine whether or not this mutation induced HSV-1 ACV resistance, further analysis was conducted.

The analysis was performed according to a method developed by our group (4). The concept for the novel assay system is to assess the sensitivity of the HSV-1 to ACV and other *vTK*-associated drugs by measuring the replication capacity of the *vTK*-deficient and highly ACV-resistant HSV-1 TAR strain (5) in 293T cells expressed with the recombinant *vTK* protein of the HSV-1 strain of interest. In this study, *vTK* expression plasmid vectors were constructed using pTARGET (Promega, Madison, WI). A *vTK* expression plasmid without the G375T mutation, which was inserted with the *vTK* PCR product from sample 1, was constructed

Received 27 August 2012 Returned for modification 18 September 2012
Accepted 17 October 2012
Published ahead of print 24 October 2012
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doi:10.1128/JCM.02247-12

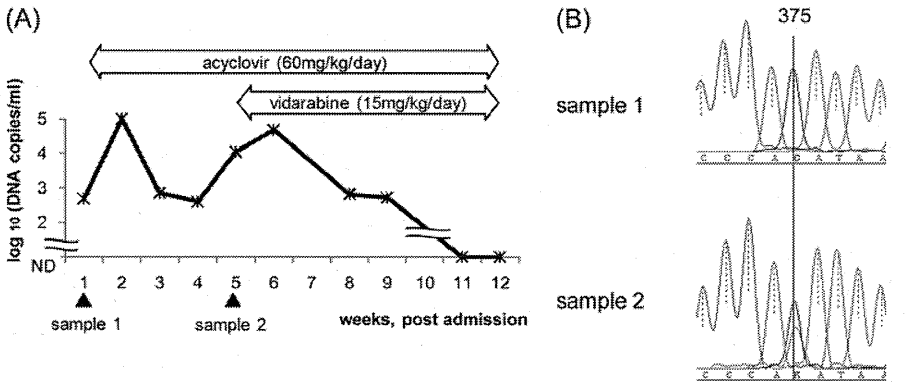

FIG 1 (A) Sequential changes in the HSV-1 DNA level in the CSF determined by quantitative real-time PCR. Arrows below the chart represent the time points of the CSF sample collection for sequencing analysis. ND, not detected. (B) Chromatogram of the *vTK* genes amplified from sample 1 and sample 2. A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

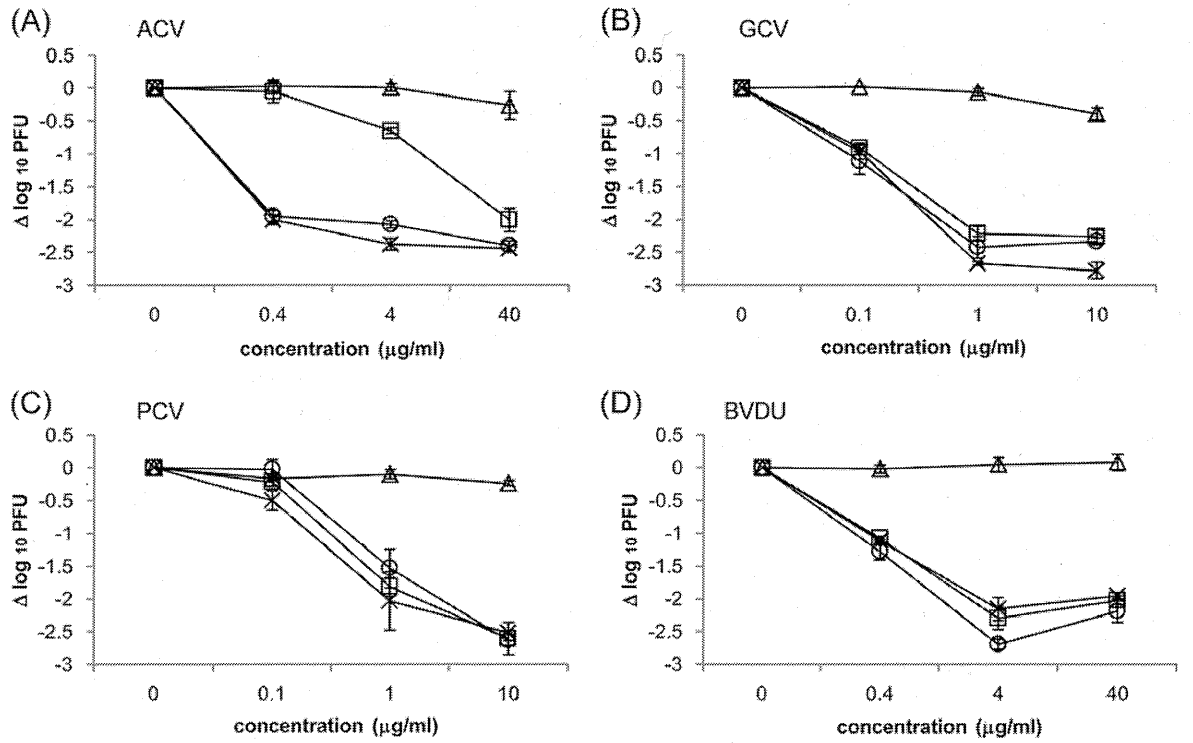

FIG 2 The inhibitory effects of antiviral compounds on replication of TAR in 293T cells transfected with each of the plasmids *vTK*-375G (○), *vTK*-375T (□), *vTK*-TAS (×), and empty pTARGET (Δ). Each experiment was performed in triplicate, and the error bars indicate standard deviations.

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).

ACKNOWLEDGMENT

We have no conflicts of interest.

This study was financially supported by grants-in-aid from the Japan Society for the Promotion of Science (no. 21591402 and no. 24591591).

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In the United States the regulation of nonstandardized AEs presented some similarities with our approach. AEs were classified into 4 categories according to scientific data supporting their use in diagnosis and treatment, and the extracts were regularly evaluated by the regulatory agencies. The last update was conducted between 2003 and 2011, and the process was recently reviewed by Slater et al.[1] It was shown that for nearly half of nonstandardized AEs there were, in fact, little or no data to support their effectiveness. We had similar results: 66 of 84 AEs were validated for diagnosis, but only for 29 of 66 was there at least 1 published piece of data to support their effectiveness for immunotherapy (Table I). Among those 66 authorized AEs, approximately one third are standardized. There is no consensus about the standardization methods, and the European approaches present some differences compared with the US approach (see Table E1 in this article's Online Repository at www.jacionline.org). Briefly, in-house reference preparation (IHRP) AEs are standardized *in vivo* and *in vitro*. Each manufacturer has its own IHRP, and there is no national standard. Batch-to-batch standardization is performed *in vitro* through a comparison of the AEs with the IHRP.[9]

In the future, the NPP list will be updated every 5 years, and requests for MA will be made and processed for standardized AEs produced industrially and frequently used for immunotherapy.

In conclusion, for the first time in Europe, this work guarantees that available AEs are clinically relevant and safe. Moreover, it guarantees that all AEs comply with recent European guidelines on APs, including rare allergens for which it is not possible to obtain large clinical studies requested for MA. The process involved all the representatives of allergists and manufacturers and is still ongoing.

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Disclosure of potential conflict of interest: F. de Blay and A. de Laval have received research support from Stallergènes and ALK-Abelló. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online January 30, 2013.
http://dx.doi.org/10.1016/j.jaci.2012.11.003

Common variable immunodeficiency classification by quantifying T-cell receptor and immunoglobulin κ-deleting recombination excision circles

To the Editor:

Common variable immunodeficiency (CVID) is the most frequent primary immunodeficiency associated with hypogammaglobulinemia and other various clinical manifestations. CVID was originally reported to be a disease primarily caused by defective B-cell function, with defective terminal B-cell differentiation rendering B cells unable to produce immunoglobulin. However, combined immunodeficiency (CID) involving both defective B and T cells is often misdiagnosed as CVID.[1] Indeed, one study reported that $CD4^+$ T-cell numbers were decreased in 29% of 473 patients with CVID[2]; similarly, another study found that naive T-cell numbers were markedly reduced in 44% (11/25) of patients with CVID.[3] These observations indicated that a subgroup of patients with clinically diagnosed CVID is T-cell deficient. Consistently, some patients with CVID have complications that might be related to T-cell deficiency, including opportunistic infections, autoimmune diseases, and malignancies, which is similar to that observed in patients with CID.[1,4] Therefore identifying novel markers to better classify CVID and distinguish CID from CVID will be required to best manage medical treatment for CVID.

We recently performed real-time PCR-based quantification of T-cell receptor excision circles (TREC) and signal joint immunoglobulin κ-deleting recombination excision circles (KREC) for mass screening of severe combined immunodeficiency (SCID)[5] and B-lymphocyte deficiency[6] in neonates. TREC and KREC are associated with T-cell and B-cell neogenesis, respectively.[7] Here we retrospectively report that TREC and KREC are useful for classifying patients with clinically diagnosed CVID.

Hypogammaglobulinemic patients (n = 113) were referred to our hospital for immunodeficiency from 2005-2011, and the following patients were excluded from the CVID pool by estimating their SCID genes based on clinical manifestations and lymphocyte subset analysis: 18 patients with SCID diagnoses; 14 patients less than 2 years of age (transient infantile hypogammaglobulinemia); 10 patients with IgM levels of greater than 100 mg/dL (hyper-IgM syndrome); 26 patients with diseases other than CVID caused by known gene alterations (10 with X-linked agammaglobulinemia and 11 with hyper-IgM syndrome

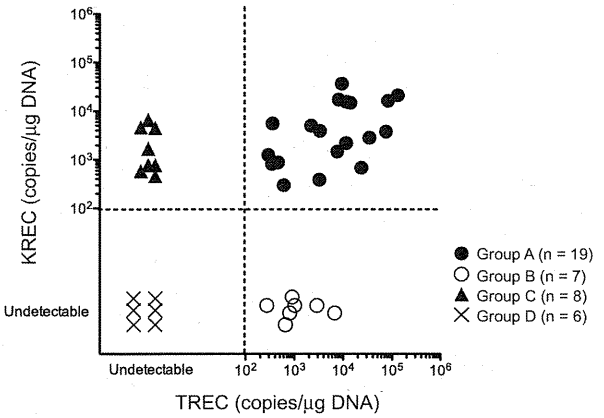

FIG 1. Quantifying TREC and KREC classifies patients with CVID into 4 groups. Patients with CVID were classified as follows: TREC(+)/KREC(+), group A (19 patients); TREC(+)/KREC(−), group B (7 patients); TREC(−)/KREC(+), group C (8 patients); and TREC(−)/KREC(−), group D (6 patients). *Undetectable,* Less than 100 copies/μg DNA.

[*CD40L* or *AICDA* mutated]), (2 with DiGeorge syndrome, and 3 with *FOXP3*, *IKBKG*, or *6p* deletions); and 5 patients with drug-induced hypogammaglobulinemia. The remaining 40 patients with decreased IgG (≥2 SDs below the mean for age), IgM, and/or IgA levels, as well as absent isohemagglutinins, poor response to vaccines, or both were included in this study as patients with CVID and analyzed for TREC/KREC levels, retrospectively.

Ages of patients with CVID ranged from 2 to 52 years (median age, 15.5 years). The sex ratio of the patients was 21 male/19 female patients. Serum IgG, IgA, and IgM levels were 370 ± 33 mg/dL (0-716 mg/dL), 30 ± 7 mg/dL (1-196 mg/dL), and 40 ± 6 mg/dL (2-213 mg/dL), respectively. TREC and KREC quantification was performed by using DNA samples extracted from peripheral blood, as reported previously.[5,6] Clinical symptoms were then assessed retrospectively. The study protocol was approved by the National Defense Medical College Institutional Review Board, and written informed consent was obtained from adult patients or parents of minor patients in accordance with the Declaration of Helsinki.

Based on TREC and KREC copy numbers, the 40 patients with CVID were classified into 4 groups (groups A, B, C, and D; Fig 1). Comparing lymphocyte subsets, $CD3^+$ T-cell numbers were similar among groups A, B, and D but were significantly lower in group C ($P < .05$; group A, 1806 ± 204 cells/μL; group B, 1665 ± 430 cells/μL; group C, 517 ± 124 cells/μL; and group D, 1425 ± 724 cells/μL; $P = .0019$, Tukey multiple comparison test based on 1-way ANOVA). $CD3^+CD4^+CD45RO^+$ memory T-lymphocyte percentages in groups B, C, and D were significantly higher than those in group A ($P < .0001$; group A, 37% ± 16%; group B, 67% ± 13% [$P = .0006$]; group C, 92% ± 8.2% [$P < .0001$]; and group D: 83% ± 14% [$P < .0001$]; see Fig E1 in this article's Online Repository at www.jacionline.org); additionally, the percentages of these cells in groups C and D were higher than in group B ($P = .0115$). These results indicate that group C and D patients have markedly decreased $CD4^+CD45RA^+$ naive T-cell counts than group A patients and that counts in group B are also significantly decreased, although less so than in groups C or D, which is consistent with a report showing lower TREC copy numbers in $CD4^+CD45RO^+$ cells. Some patients in groups B, C, and D exhibited normal $CD4^+CD45RO^+$ percentages, although TREC levels, KREC levels, or both decreased. This discrepancy indicates that TREC/KREC levels could be independent markers to determine the patient's immunologic status in addition to $CD4^+CD45RA^+$; the reasons underlying the discrepancy between $CD4^+CD45RA^+$ and TREC/KREC levels remain unsolved.

$CD19^+$ B-cell numbers in group A were significantly higher ($P < .05$) than those in groups B and D (group A, 269 ± 65 cells/μL; group B, 35 ± 16 cells/μL; group C, 60 ± 11 cells/μL; and group D, 29 ± 16 cells/μL; $P = .0001$). However, B-cell subpopulations, including $CD27^-$, IgD^+CD27^+, and IgD^-CD27^+ cells, were not significantly different among the groups. Standardizing KREC copy numbers for each patient by dividing their $CD19^+$ by their $CD27^+$ percentages revealed the same patient classification as that shown in Fig 1 (data not shown), indicating that the original classification was independent of $CD19^+$ B-cell or $CD27^+$ memory B-cell percentages.

Because TREC and KREC levels decrease with age (see Fig E2 in this article's Online Repository at www.jacionline.org)[5,6] and age distribution was wide in this study, we compared patients' ages among groups at the time of analysis to determine whether classification was associated with age. TREC/KREC-based classification was independent of both age and sex because age distribution was not significantly different among groups ($P > .05$; group A, 12.7 ± 2.3 years [2-30 years]; group B, 23.4 ± 4.2 years [6-39 years]; group C, 21.5 ± 6.1 years [4-52 years]; and group D, 25.5 ± 4.4 years [15-46 years]; data not shown) nor was male/female sex ratio (overall, 21/19; group A, 10/9; group B, 2/5; group C, 5/3; and group D, 4/2; $P = .4916$, χ^2 test; data not shown).

We next evaluated whether any correlation existed between TREC/KREC-based classification and clinical symptoms in each patient group. All patients in the study had been treated with intravenous immunoglobulin (IVIG) substitution at the time of analysis. We found that the cumulative events of complications (opportunistic infections, autoimmune diseases, and malignancies) per 10 patient-years were highest in group D (0.98 events/10 patient-years), followed by group C (0.63 events/10 patient-years), group B (0.30 events/10 patient-years), and group A (0.04 events/10 patient-years), where events in groups D and C were significantly higher than group A (group A vs group D, $P = .0022$; group A vs group C, $P = .0092$; group A vs group B, $P = .0692$; Fig 2). Furthermore, we found similar results when evaluating only patients 19 years old or older for group D (1.01 events/10 patient-years), group C (0.56 events/10 patient-years), group B (0.32 events/10 patient-years), and group A (0.06 events/10 patient-years; group A vs group D, $P = .0074$; group A vs group C, $P = .0407$; group A vs group B, $P = .1492$; data not shown). Categorizing patients by using several different previously reported CVID classifications (focused primarily on separating patients based on levels of circulating B-cell subsets), we found that no classification scheme showed any significant event increases in any particular group (see Fig E3 in this article's Online Repository at www.jacionline.org). Assessing longitudinal cumulative opportunistic infection incidence among the groups, group D and C values were significantly higher than in group A (see Fig E4, A, in this article's Online Repository at www.jacionline.org; $P = .0059$). Autoimmune and malignant diseases ($P = .5168$ and $P = .6900$, respectively) were observed in groups B and D but not in group A (see Fig E4, *B* and *C*). Cumulative events were significantly different between groups ($P = .0313$, log-rank test; group A, 5.3% and 5.3%; group B, 14.3% and

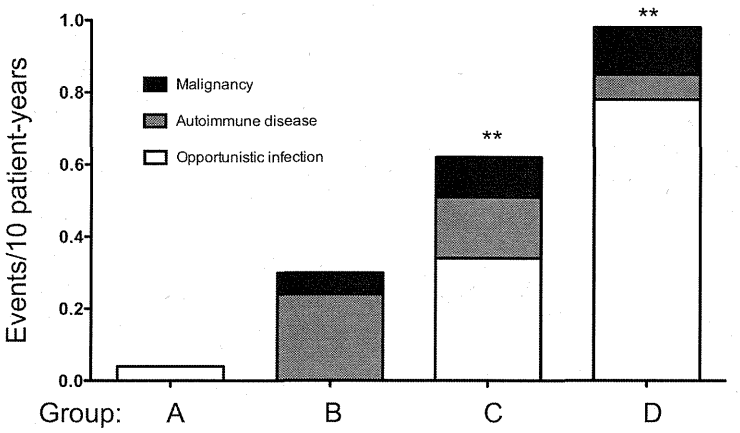

FIG 2. Cumulative incidence of complication events per 10 patient-years differs among groups. Opportunistic infections, autoimmune diseases, and malignancies were evaluated for each patient group. Complication incidences in group D (0.98 events/10 patient-years), group C (0.63 events/10 patient-years), and group B (0.30 events/10 patient-years) were higher than in group A (0.04 events/10 patient-years). Group A versus group D: $^{**}P = .0022$; group A versus C: $^{**}P = .0092$; group A vs group B: $P = .0692$.

57.1%; group C, 27.1% and 63.5%; and group D, 33.3% and 83.3% at 10 and 30 years of age, respectively; see Fig E4, *D*). One patient in group D died of *Pneumocystis jirovecii* pneumonia, and 2 other patients in the same group received hematopoietic stem cell transplantation after complications caused by EBV-related lymphoproliferative disorder.

Assessing these data, TREC/KREC-based classification matches clinical outcomes. Because group D patients exhibited the most frequent complications (opportunistic infections, autoimmune diseases, and malignancies), they could receive a diagnosis of CID based on these symptoms. If they are indeed determined to have CID, then TREC/KREC analysis is helpful to distinguish between CID and CVID. Their TREC(−)/KREC(−) phenotype might relate to defective V(D)J recombination in T- and B-cell development[8] because patients with B-negative SCID (*RAG1*, *RAG2*, *Artemis*, and *LIG4*), as well as patients with ataxia-telangiectasia (AT) and Nijmegen breakage syndrome (NBS; see Fig E5 in this article's Online Repository at www.jacionline.org),[5,6] were also negative for both TREC and KREC; it is intriguing to speculate that an unknown V(D)J recombination gene or genes is responsible. As for treatment, hematopoietic stem cell transplantation should be considered the preferred treatment to "cure" group D patients, as reported in patients with severe CVID/CID, because event-free survival is poor.[9]

In contrast to group D patients, TREC(+)/KREC(+) group A patients treated with IVIG substitution therapy remained healthy. One possible explanation is that these patients harbor defects only in terminal B-cell differentiation, but not in T cells, and represent typical patients with CVID, as originally reported.

Group C patients had a high frequency of both opportunistic infections and malignancies, suggesting that these TREC(−) patients have T-cell defects. Although group C patients had a similar TREC/KREC pattern to patients with SCID with B cells (*IL2RG* and *JAK3*; see Fig E5, *A*), they do not fulfill the European Society for Immunodeficiencies criteria for SCID, and no mutation was identified in the SCID genes estimated from clinical manifestation and lymphocyte subset analysis. However, from our data, they would likely benefit from undergoing similar treatment to patients with SCID or CID to prevent these complications.

Although opportunistic infections were rare in group B patients, autoimmune diseases were often observed. This is consistent with this group being TREC(+)/KREC(−) and the idea that balance between T and B cells is important to prevent autoimmune diseases in patients with CVID.[1] Intriguingly, a group of patients with AT and NBS were also TREC(+)/KREC(−) (see Fig E4, *B*), which is similar to group B patients. Additionally, $CD45RA^{+}CD4^{+}$ naive T-cell numbers were reduced in most group B patients, which is similar to the phenotype exhibited by patients with AT and NBS. This finding raises the possibility that although some group B patients are also T-cell deficient, as well as B-cell deficient, and should be treated similarly to patients with CID, other patients have only B-cell deficiency and are effectively treated with IVIG substitution therapy.

By analyzing a large CVID patient cohort, the overall survival rate of patients with more than 1 complication was worse than that for patients without other complications.[4] Our findings indicate that low TREC levels, KREC levels, or both are useful markers that correlate well with the overall survival rate in patients with CVID. Therefore we conclude that TREC and KREC are useful markers to assess the clinical severity and pathogenesis of each patient with CVID and to distinguish CID from CVID. Thus patient classification based on TREC/KREC levels would provide a helpful tool for deciding on an effective treatment plan for each patient with CVID.

We thank the following doctors who contributed patient data to this study: Satoshi Okada, Kazuhiro Nakamura, Masao Kobayashi, Tomoyuki Mizukami, Yoshitora Kin, Hironobu Yamaga, Shinsuke Yamada, Kazuhide Suyama, Chihiro Kawakami, Yuko Yoto, Kensuke Oryoji, Ayumu Itoh, Takao Tsuji, Daisuke Imanishi, Yutaka Tomishima, Minako Tomiita, Kaori Sasaki, Akira Ohara, Hanako Jimi, Mayumi Ono, Daisuke Hori, Yuichi Nakamura, Yoshitoshi Otsuka, Toshiyuki Kitoh, Toshio Miyawaki, Akihiko Maeda, Terumasa Nagase, Takahiro Endo, Yoshiaki Shikama, Mikiya Endo, Satoru Kumaki, Lennart Hammarström, Janine Reichenbach, and Reinhard Seger. We also thank Professor Junichi Yata for critical reading and Ms Kaori Tomita, Ms Kimiko Gasa, and Ms Atsuko Kudo for their skillful technical assistance.

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Supported in part by grants from the Ministry of Defense; the Ministry of Health, Labour, and Welfare; and the Ministry of Education, Culture, Sports, Science, and Technology.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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Available online December 28, 2012.
http://dx.doi.org/10.1016/j.jaci.2012.10.059

Homing frequency of human T cells inferred from peripheral blood depletion kinetics after sphingosine-1-phosphate receptor blockade

To the Editor:

Naive and central memory (CM) T cells home through lymph nodes (LNs), whereas T cells with an effector memory (EM) phenotype preferentially screen peripheral tissues in search of cognate antigen.[1] LN entry and egress are distinct and highly regulated processes mediated by an orchestrated interplay of chemokines/chemokine receptors and adhesion molecules.[2] Interaction of peripheral node addressins with L-selectin on T cells allows tethering/rolling along high endothelial venules (HEVs).[2] Interaction of the chemokine receptor CCR7 with its ligands CCL19/CCL21 and CXCR4 with CXCL12 then mediates firm adhesion to HEVs through high-affinity interactions of lymphocyte function–associated antigen 1 and intercellular adhesion molecule 1, permitting transmigration of T cells across the HEV cell layer.[2] Within the LNs, T-cell migration is directed through T-cell zones toward the cortical sinuses.[3] A sphingosine-1-phosphate (S1P) gradient established across the endothelial cells of the cortical sinuses is directing LN egress of T cells through efferent lymph back to the peripheral blood circulation.[4] Acting as a functional antagonist on the S1P receptor, the pharmacologic compound fingolimod, which has shown efficacy in the treatment of multiple sclerosis (MS), blocks this egress.[4,5] As a consequence, in fingolimod-treated subjects naive and CM T cells are trapped in LNs and reduced in the blood circulation.[6]

Here, by studying depletion kinetics of T cells in the blood of *de novo* fingolimod-exposed subjects in combination with *in vitro* migration experiments, homing frequencies and LN access hierarchy between T-cell subsets were derived indirectly. First, we defined the effect of *de novo* fingolimod exposure on the number of circulating $CD4^+$ and $CD8^+$ phenotypic T-cell subsets in patients with MS during a 6-hour observation period (hourly measurements, 1 time before and 6 times after drug exposure) by using flow cytometry (detailed information on patients and methods is provided in the Methods section and Table E1 in this article's Online Repository at www.jacionline.org). In fingolimod-treated subjects, 6 hours after the first drug dose, numbers of $CD4^+$ T-cell subsets with an LN homing phenotype (ie, naive and CM T cells) were significantly reduced (Fig 1, *A* [representative example; absolute cell counts], and Fig 1, *B* [pooled data; proportional change]). Intriguingly, the kinetics of reduction differed between phenotypic naive (CD62 ligand [CD62L]–positive $CD45RA^+$) and CM ($CD62L^+CD45RA^-$) $CD4^+$ T cells. Specifically, compared with baseline measurements, naive $CD4^+$ T-cell counts started to decrease earlier than CM $CD4^+$ T-cell counts (2 vs 5 hours after fingolimod exposure; Fig 1, *B*). In $CD8^+$ T cells, contrasting $CD4^+$ T cells, only naive ($CD62L^+CD45RA^+$) $CD8^+$ T-cell counts decreased significantly (after 3 vs 2 hours in naive $CD4^+$ T cells) after the first dose of fingolimod (Fig 1, *C* [representative example; absolute cell counts], and Fig 1, *D* [pooled data; proportional change]).

On the basis of these *ex vivo* depletion kinetics, *in vitro* chemotaxis experiments were performed, as described in the Methods section in this article's Online Repository. In a transwell system spontaneous migration of bulk $CD4^+$ and $CD8^+$ T cells was comparably low in healthy control subjects and untreated patients with MS (and was further decreased in the presence of fingolimod; see Fig E1 in this article's Online Repository at www.jacionline.org). Gradients of CXCL12, CCL19, and CCL21 mediated a clear increase in migration of bulk $CD4^+$ and $CD8^+$ T cells from healthy control subjects and untreated patients with MS, which was not significantly influenced by fingolimod (see Fig E1). Dot plot distribution (as a percentage) of migrated versus nonmigrated, phenotypic naive, CM, EM, and (for $CD8^+$ T cells) CD45RA re-expressing EM cells (EMRA) was then compared between control cells (spontaneous migration) and cells that migrated toward CXCL12, CCL19, or CCL21. An example of CXCL12-mediated changes in the

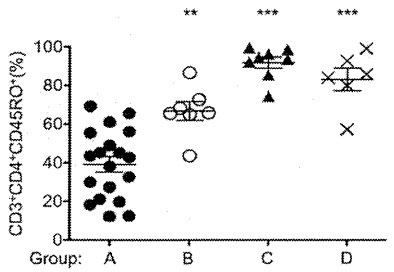

FIG E1. $CD45RO^+CD3^+CD4^+$ T-cell frequency within $CD4^+CD3^+$ lymphocytes was analyzed among groups. $CD45RO^+CD3^+CD4^+$ lymphocyte counts were significantly higher in groups B, C, and D compared with those in group A ($P < .0001$). Group A: 37% ± 16%; group B: 67% ± 13% (**$P < .01$); group C: 92% ± 8.2% (***$P < .001$); and group D: 83% ± 14% (***$P < .001$).