the HPA axis under stress may be one explanation of stress-induced exacerbation of AD symptoms. Similar endocrine irregularities of the HPA axis have also been reported after pharmacologic challenge in patients with AD (5,6). It may be argued that hyporesponsiveness of the HPA axis in patients with AD is simply a side effect of topical corticosteroid (TCS) therapy, which may lead to steroid phobia (fear of TCS use) in patients with AD and even in health care professionals (7). Previous reports have investigated the influence of TCS on the HPA axis in children with AD, with contradictory results (8–10). Other studies have suggested that AD disease activity, rather than the use of TCS, is responsible for changes in the HPA axis in patients with severe AD (5,11). Young children are believed to be at special risk for systemic effects of TCS because of high percutaneous absorption of TCS due to their relatively large body surface area per weight. Therefore, it is important to investigate whether HPA dysfunction is observed in young children with AD and is related to TCS therapy.

Blood cortisol concentration increases in response to unpredictable, uncontrollable, and novel situations (12,13). Salivary cortisol levels correlate with plasma unbound cortisol levels (14). Moreover, because saliva samples can be obtained without stress, salivary cortisol assessment is a reliable tool for investigating HPA axis function, especially in young children (13). Previous studies reported a significantly attenuated cortisol response to standardized laboratory psychological stress, which consisted mainly of public speaking and mental arithmetic tasks in front of an audience, in children and adults with AD (3,4), but those procedures are difficult to implement in children younger than 6 years of age. Moreover, venipuncture is an unpredictable, uncontrollable, novel situation for young children, so it itself might be a significant stressor resulting in activation of the HPA axis in young children, detected as high cortisol levels. We used venipuncture as the acute stressor to investigate changes in the level of salivary cortisol.

We evaluated whether impairment of the salivary cortisol response to the stress of venipuncture in young children with AD was related to the severity of the AD symptoms and whether TCS use affected the response.

METHODS

Subjects

We studied 38 young children (24 boys and 14 girls) with AD (median age 16.5 months, range 3–66 months) being treated at our outpatient unit. Patients who had received regular TCS therapy were included, but those who had received inhaled or oral corticosteroids in the preceding 6 months were excluded. All patients were Japanese whose parents were of middle socioeconomic status. An allergist made the diagnosis of AD in accordance with the clinical criteria defined by the Japanese Dermatological Association (15). The severity of AD in each patient was assessed using the scoring of atopic dermatitis (SCORAD) index (16). The severity of AD was defined according to the SCORAD index (mild <25, moderate 25–50, severe ≥ 50).

Previous use of TCS was assessed by a modification of the previously defined score, the treatment score (Table 1) (10), which was based on the potency of the preparation used (TCS preparations were grouped according to clinical potency, as described in the Japanese Therapeutic Guideline for AD (17)), percentage of body surface area to which it was applied, the duration of treatment in the last month. The hospital ethics committee of the National Center for Child Health and Development approved this study. Informed consent was obtained from all caregivers.

Procedures

To evaluate the responsiveness of the HPA axis to stress, salivary cortisol concentrations were determined before and after venipuncture. Venipuncture for clinical purposes and saliva sampling were performed between 10:00 A.M. and 3:00 P.M. to avoid any possible inhibitory effect of a high morning basal cortisol level on further cortisol release (18). To determine free cortisol concentration, saliva samples were collected 5 minutes before and 15 to 20 minutes after the venipuncture was completed, because that is when salivary cortisol level peaks (13).

Cortisol Measurement

Saliva was obtained using a Sorbette sampling device (Salimetrics, State College, PA), which consists of a

TABLE 1. *Score for Topical Corticosteroid Treatment (Ref. 10)*

Score (total 10)	0	1	2	3	4
Potency of preparation (0 to 4)	None	Mild	Strong	Very strong	Strongest
Body surface area treated, % (0 to 3)		<9		>36	
Treatment duration* (0 to 3)		<7	≥ 7	Continuous	

*Days in the past month.

TABLE 2. *Clinical Characteristics*

Characteristic	Mild n = 12	Moderate n = 14	Severe n = 12	p-value*
Sex, male/female, n	6/6	9/5	9/3	0.44
Age, months, median (range)	19 (4–66)	21 (3–60)	10.5 (3–57)	0.41
SCORAD, median (range)	16 (8–25)	40 (26–48)	64.5 (51–86)	<0.001
Score for TCS treatment, median (range)	4.5 (4–7)	5 (0–7)	0 (0–7)	0.38
Non-TCS users, n	0	2	7	0.002
Number of venipunctures, median (range)	2 (1–3)	1 (1–4)	1 (1–3)	0.10
Total immunoglobulin E, IU/mL, median (range)	163 (5.2–4,562)	212 (12.9–9,500)	1,093.5 (55.6–4,161)	0.12

TCS, topical corticosteroid.
*Kruskal–Wallis test or chi-square test.

microsponge with a short plastic shaft as a handle. After the microsponge had been put under the tongue for 1 minute, the Sorbette was placed in a plastic tube and centrifuged for 15 minutes at 1,800 g, resulting in a clear, watery supernatant. The samples were stored at −30°C until analysis. For cortisol determination, 50 μL of saliva was used for duplicate analysis with a salivary cortisol enzyme-linked immunosorbent assay kit (Salimetrics), according to the manufacturer's protocol.

Statistical Analysis

Salivary cortisol concentrations did not show a normal distribution and were therefore log-transformed for analysis. Group variables were compared using the Mann–Whitney U test, the Kruskal–Wallis test, or the chi-square test. The paired t test was used to compare salivary cortisol response to the stressor (venipuncture). Spearman rank correlation was used to investigate the relationship between the parameters of the salivary cortisol response to the stressor and the variables (disease severity and TCS treatment). Data were analyzed using STATA software (Windows version 8.0, Stata Corp., College Station, TX). $P < 0.05$ was considered to indicate statistical significance in all comparisons.

RESULTS

Clinical Characteristics

Clinical characteristics of the patients are summarized in Table 2. The patients were grouped according to severity of AD (mild [12 patients], moderate [14 patients], and severe [12 patients]). There were no significant differences between the AD severity groups in terms of the age, sex, score for TCS treatment, number of venipunctures, or total immunoglobulin (Ig)E level (IU/mL), but there was a statistically significant difference in the number of patients not treated with TCS ($p = 0.002$).

Salivary Cortisol Response to Venipuncture

First, we evaluated whether venipuncture could be a stressor that induced significantly higher salivary cortisol levels in young children with AD. The number of venipunctures and the change in salivary cortisol level were investigated for possible association in patients in whom—for technical reasons—several venipunctures were needed to achieve blood sampling. The change in salivary cortisol level was calculated by dividing the postvenipuncture cortisol concentration by the prevenipuncture cortisol concentration, yielding a ratio. When subjects were stratified according to number of venipunctures, the increase in salivary cortisol level depended on the number of venipunctures ($p = 0.04$; Kruskal–Wallis test, Fig. 1). This result suggested that venipuncture was a sufficient stressor to induce a change in cortisol level in children younger than 6 years of age.

There were no significant differences in prevenipuncture and postvenipuncture salivary cortisol levels between the three groups, but the salivary cortisol level increased significantly after venipuncture in all groups ($p < 0.05$; paired t test, Fig. 2).

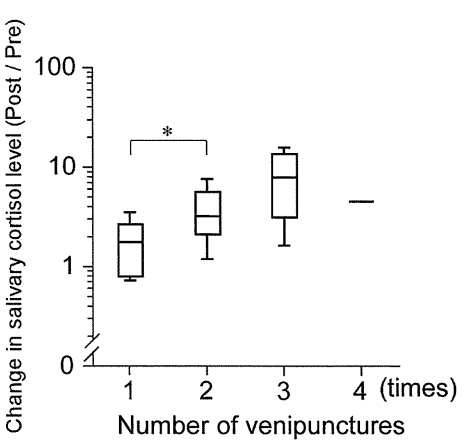

Figure 1. Change in salivary cortisol level (post/pre) versus number of venipunctures (*p = 0.02; Mann–Whitney test).

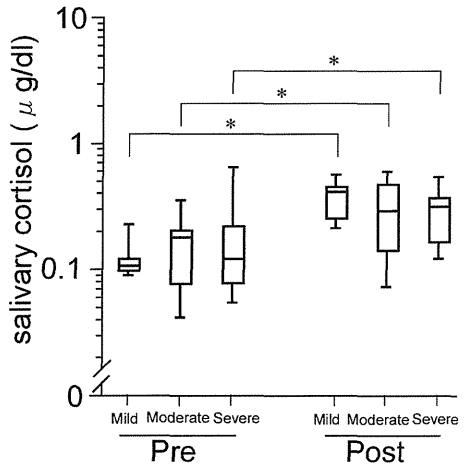

Figure 2. Salivary cortisol level in response to venipuncture in individuals with atopic dermatitis (*p < 0.05; Paired *t* test).

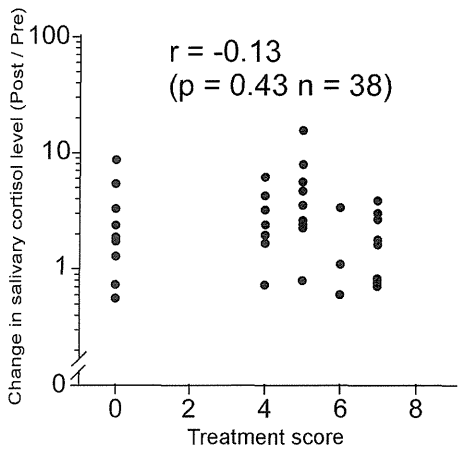

Figure 4. Correlation between change in salivary cortisol level (post/pre) and treatment score ($n = 38$, $r = -0.13$, p = 0.43).

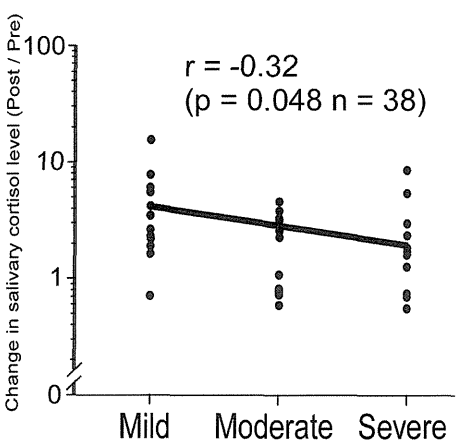

Figure 3. Correlation between change in salivary cortisol level (post/pre) and severity of atopic dermatitis ($n = 38$, $r = -0.32$, p = 0.048).

Correlation Between Salivary Cortisol Response and Severity of AD or TCS Treatment

The change in salivary cortisol level correlated significantly with the pre- and postvenipuncture salivary cortisol levels ($r = -0.50$, p = 0.01; $r = 0.61$, p < 0.001, respectively; data not shown). Conversely, it correlated negatively with severity of AD ($r = -0.32$, p = 0.048; Fig. 3). No significant correlation was found between change in salivary cortisol level and score for TCS treatment (p = 0.43; Fig. 4), age, sex, or total IgE (data not shown).

DISCUSSION

In previous studies, children and adults with AD showed attenuated salivary cortisol responses to psychosocial stressors, suggesting hyporesponsiveness of the HPA axis, which may be one explanation for stress-induced exacerbation of AD symptoms (3,4), but few studies have been performed in young children, who are vulnerable to systemic side effects of TCS therapy. In this study, we found that salivary cortisol response to the stress of venipuncture in young children with AD correlated negatively with the severity of symptoms.

We first evaluated whether venipuncture could be a stressor in children younger than 6 years of age. Although previous studies reported that changes in cortisol level after venipuncture were not significant in children aged 6 and older (19,20), we found that venipuncture is a stressor in children younger than 6 years of age. This difference might be because children have more occasions to undergo venipuncture and become used to it as they get older. Although venipuncture is difficult to standardize, and the individual characteristics of the children would influence its role as a stressor, salivary cortisol level rose dependent on the number of venipunctures. These results suggest that venipuncture is a sufficient stressor to induce a change in cortisol level in children younger than 6 years of age. The number of venipunctures did not differ significantly between the three groups of patients with different AD severity (mild 1.75 ± 0.72, moderate 1.29 ± 0.80, severe 1.42 ± 0.64, mean ± SD; p = 0.10), suggesting that patients in all three groups received virtually the same level of stress.

The salivary cortisol response to venipuncture as a stressor correlated negatively with the severity of AD. This result is in line with previous studies that found a weaker cortisol response to stressors in adults and children with AD (3,4). Meanwhile, there were no significant

differences in prevenipuncture cortisol levels between the groups with various AD severity, which is also in line with previous studies (3,4). Dysfunction of the HPA axis in individuals with AD may become apparent only when a stress stimulus is present.

According to a previous study (21), the normal salivary cortisol level in young children was approximately 0.16 to 0.36 μg/dL at baseline. The cortisol level in each group of subjects before venipuncture was compatible with that range, and there were no significant differences between the groups. Therefore, we think that our time points for performing venipuncture (10:00 A.M. and 3:00 P.M.) were sufficient for detecting any elevation in cortisol level, regardless of whether severe pruritus might have somewhat altered the circadian rhythm of cortisol release.

The underlying psychobiologic mechanisms of hyporeactivity of the HPA axis in patients with AD are not fully understood. Historically, these changes have been interpreted as a consequence of an ongoing chronic allergic inflammatory process, which releases pro-inflammatory cytokines (22). Some studies reported that an atopic disposition in neonates is associated with greater responsiveness of the HPA axis to stressors, which may promote the development of AD in later life (21,23). It remains to be determined whether these changes in HPA function precede or follow the onset of AD.

Most individuals with AD have high serum concentrations of total and allergen-specific IgE, and the severity of AD is known to be weakly associated with serum IgE levels and degree of Th2-type immune predisposition (24). In the present study, we also found that serum IgE levels were higher in those with more severe AD, but the differences were not statistically significant, presumably because of limited power or the fact that we studied very young infants, but further study is needed to elucidate whether hyporeactivity of the HPA axis is simply a consequence of the chronic inflammatory process or is specific to Th2-type immune responses.

The effects of TCS on HPA function in children with AD have been studied using various methods but with contradictory results (8–10). Our present study found no significant correlation between salivary cortisol level and previous TCS treatment, suggesting that there are other factors related to the disease. Our results are in line with previous findings showing that patients with AD and not treated with TCS had a weaker cortisol response than control subjects (3,4). Moreover, some studies showed that a significant decrease in the disease activity of AD after intensive treatment with large amounts of a potent TCS during hospitalization was associated with normalization of the basal serum cortisol level compared with levels at admission (5,11). These results suggest that the disease activity of AD, rather than TCS use, is responsible for dysfunction of the HPA axis in patients with severe AD.

In children with AD, percutaneous absorption of TCS was proven to be significantly lower in the convalescent phase of the disease than in the acute phase, probably because of the restoration of the skin barrier (25). Although percutaneous absorption of a potent TCS is likely to occur, especially during the acute phase of severe AD, the positive effect of adequate disease control seems to clinically outweigh the suppressive effect on adrenal gland function. Therefore, early restoration of the skin barrier by appropriate TCS therapy might contribute to reducing any undesirable effect of TCS on the HPA axis in individuals with AD in the long term. Good control of AD would improve HPA axis function, which might reduce stress-induced exacerbation of AD symptoms.

In addition, to clarify the effect of TCS on the HPA axis in patients with AD, we would like to compare the cortisol responses of patients with similar severity of AD but different TCS usage. We believe that appropriate use of TCS may improve the skin condition and quality of life of patients and in turn improve the HPA axis response. Suppression of the HPA axis can be seen only in patients with extremely severe AD or inappropriate administration of TCS. Further large-scale, longitudinal studies should be undertaken to elucidate the underlying mechanisms of HPA axis hyporeactivity in patients with AD. Limitations of this study are that the number of subjects was not sufficiently large and the sleep patterns of the patients were not closely determined, but a strength of the study is that we can evaluate HPA axis function to stress non-invasively in young children with AD.

Salivary cortisol responsiveness to the stress of venipuncture correlated negatively with the severity of AD but showed no correlation with previous use of TCS in young children with AD. These findings have major implications for daily practice when treating young patients with moderate to severe AD and steroid phobia.

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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

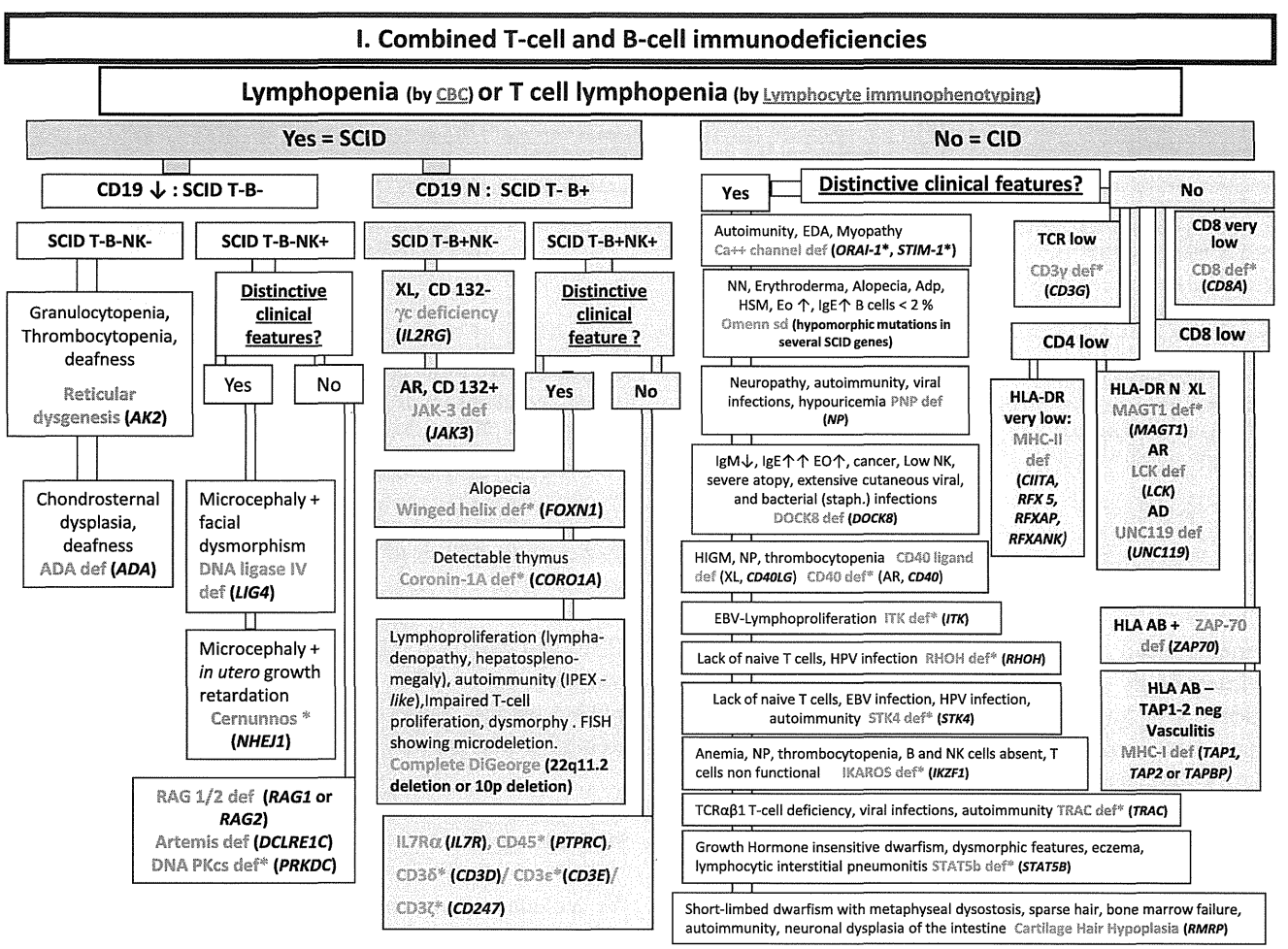

Fig. 1 Combined T- and B- cell immunodeficiencies. ADA: Adenosine Deaminase; Adp: adenopathy; AIHA: Auto-Immune Hemolytic Anemia; AR: Autosomal Recessive inheritance; CBC: Complete Blood Count; CD: Cluster of Differentiation; CID: Combined Immunodeficiency; EBV: Epstein-Barr Virus; EDA: Anhidrotic ectodermal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

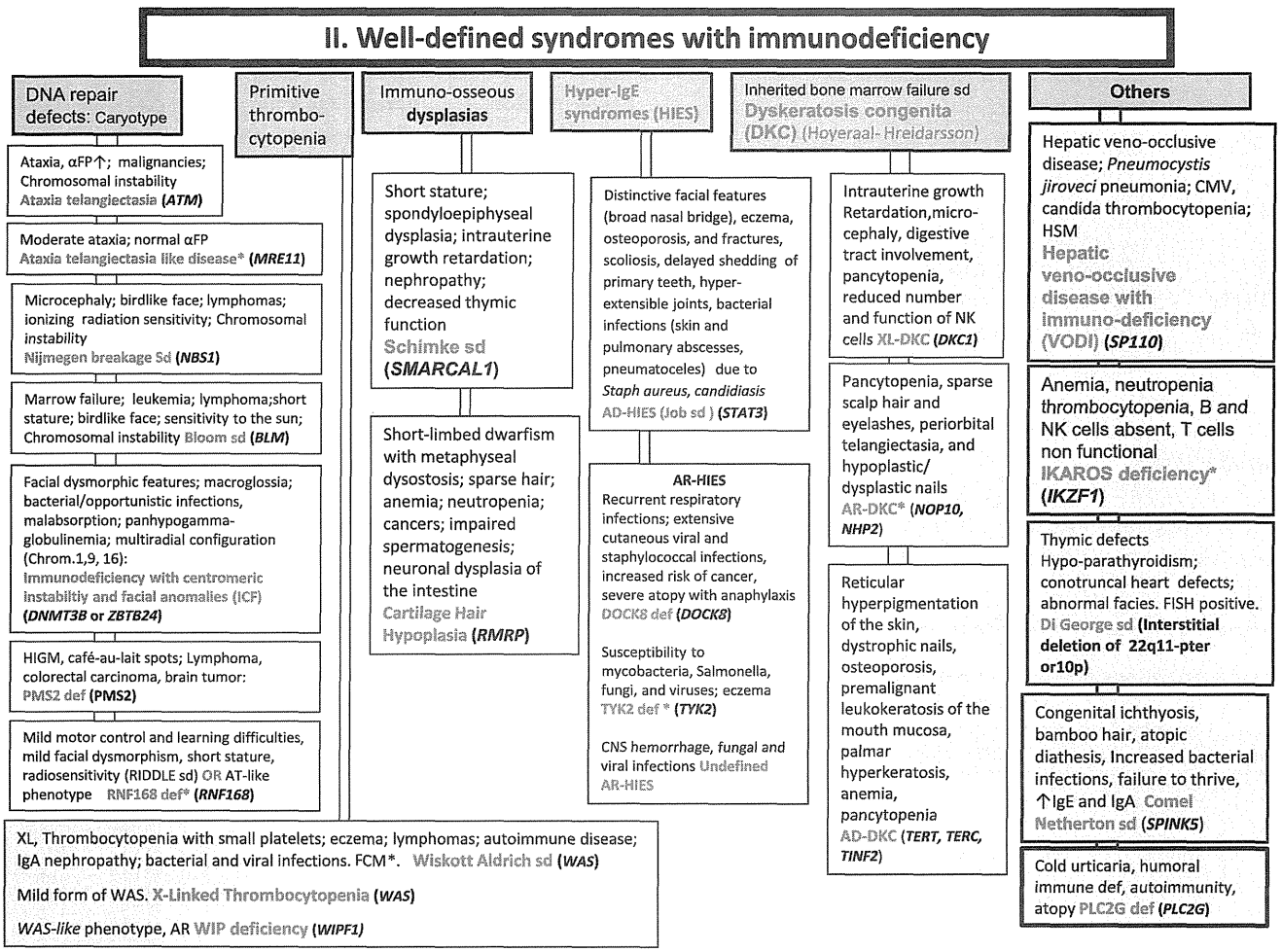

Fig. 2 Well-defined syndromes with immunodeficiencies. These syndromes are generally associated with T-cell immunodeficiency. αFP: alpha- fetoprotein; AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; CNS: Central Nervous System; FCM*: Flow cytometry available; FISH: Fluorescence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

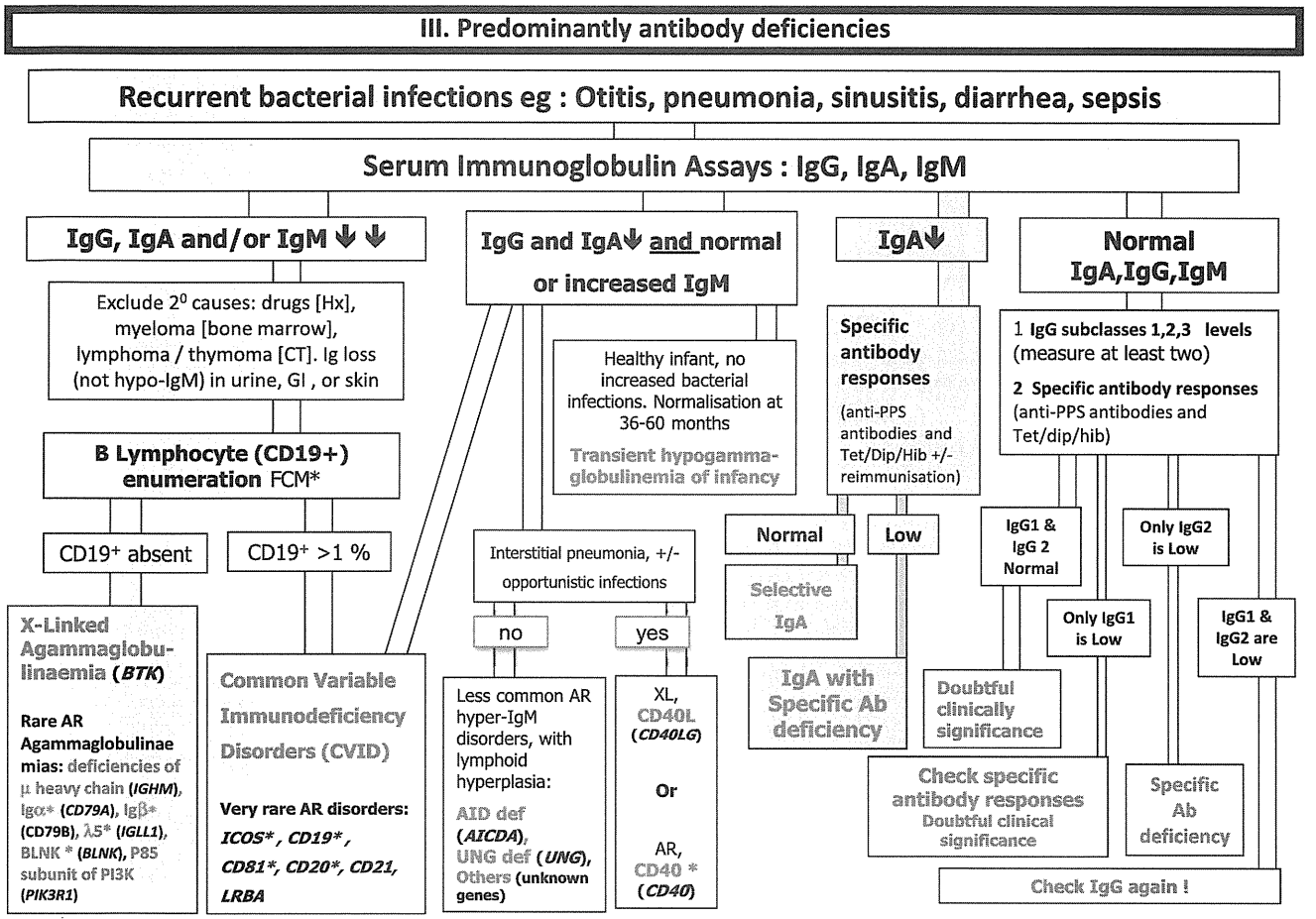

Fig. 3 Predominantly antibody deficiencies. Ab: Antibody; Anti PPS: Anti- pneumococcal polysaccharide antibodies: AR: Autosomal Recessive inheritance; CD: Cluster of Differentiation; CVID: Common Variable Immunodeficiency Disorders; CT: Computed Tomography; Dip: Diphtheria; FCM*: Flow cytometry available;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

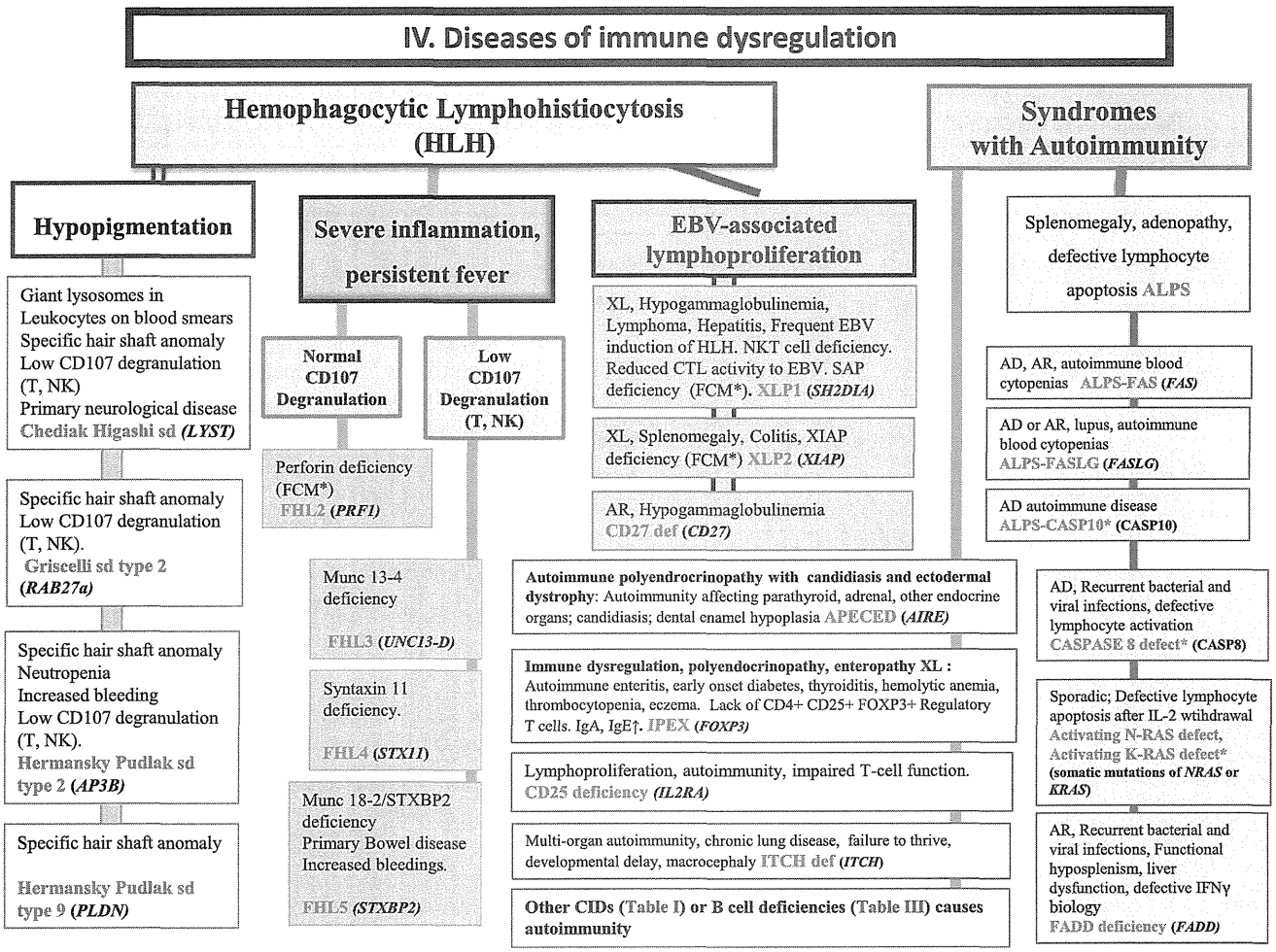

Fig. 4 Diseases of immune dysregulation. AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; CD: Cluster of Differentiation; CTL: Cytotoxic T-Lymphocyte; EBV: Epstein-Barr Virus; FCM*: Flow cytometry available; HSM: Hepatosplenomegaly; Ig: Immunoglobulin; IL: interleukin; NK: Natural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

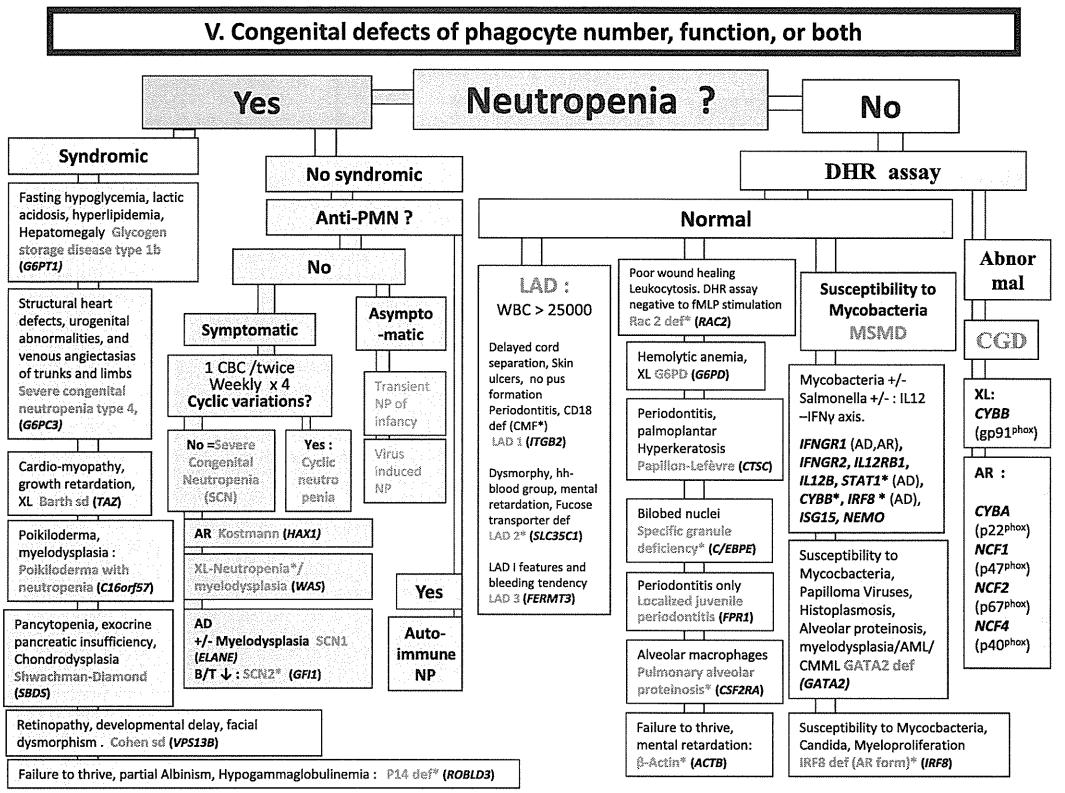

Fig. 5 Congenital defects of phagocyte number, function, or both. For DHR assay, the results can distinguish XL-CGD from AR-CGD, and gp40phox defect from others AR forms. AD: Autosomal Dominant inheritance; AML: Acute Myeloid Leukemia; AR: Autosomal Recessive inheritance; CBC: Complete Blood Count; CD: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

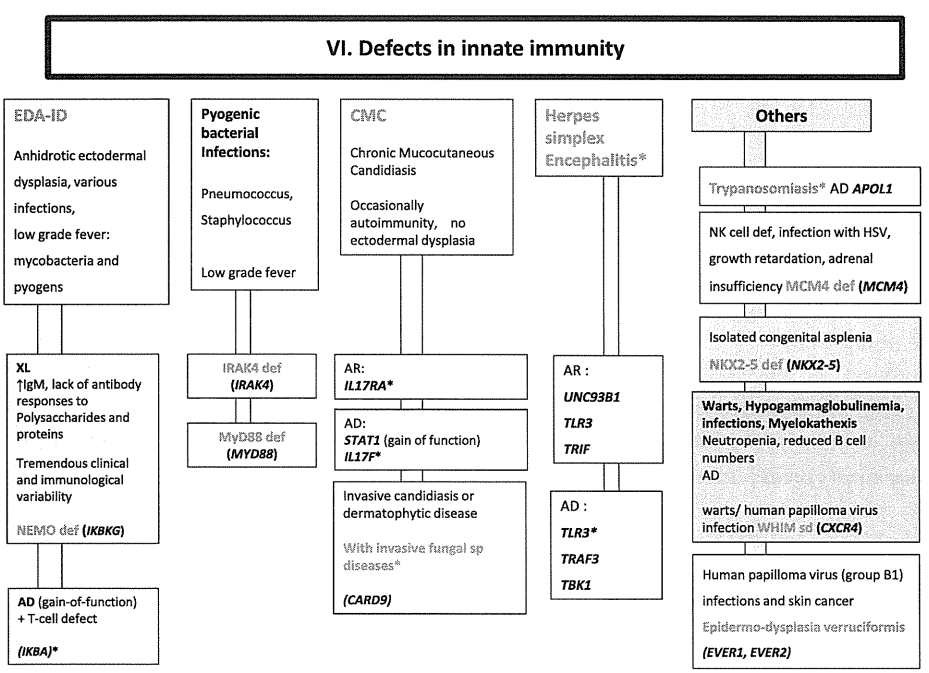

Fig. 6 Defects in innate immunity. AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; BL: B lymphocyte; EDA-ID: Anhidrotic Ectodermal Dysplasia with Immunodeficiency; Ig: Immunoglobulin; PNN: Neutrophils; XL: X-Linked inheritance

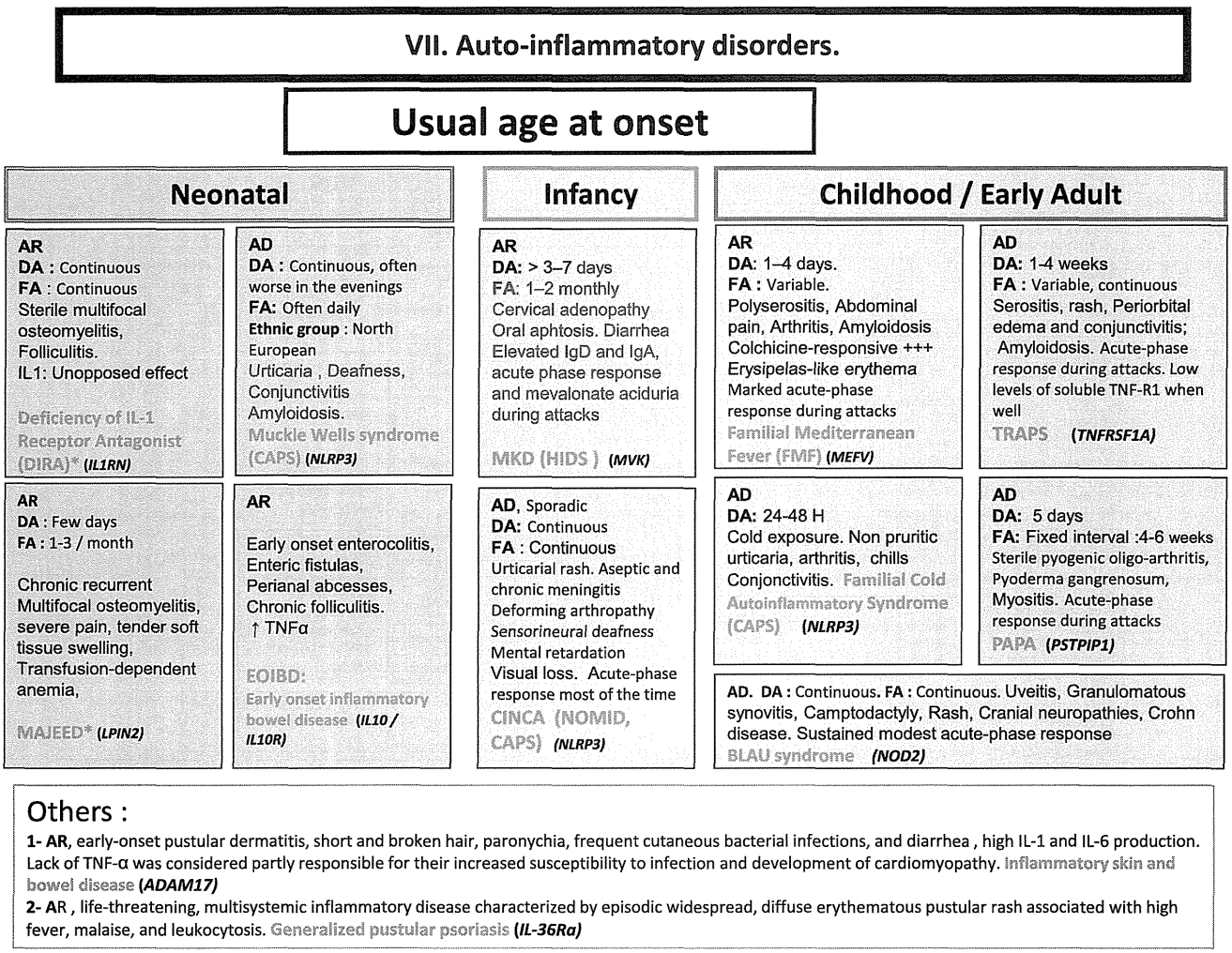

Fig. 7 Autoinflammatory disorders. AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; CAPS: Cryopyrin-Associated Periodic syndromes; CINCA: Chronic Infantile Neurologic Cutaneous and Articular syndrome; DA: Duration of Attacks; FA: Frequency of Attacks; FCAS: Familial Cold Autoinflammatory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

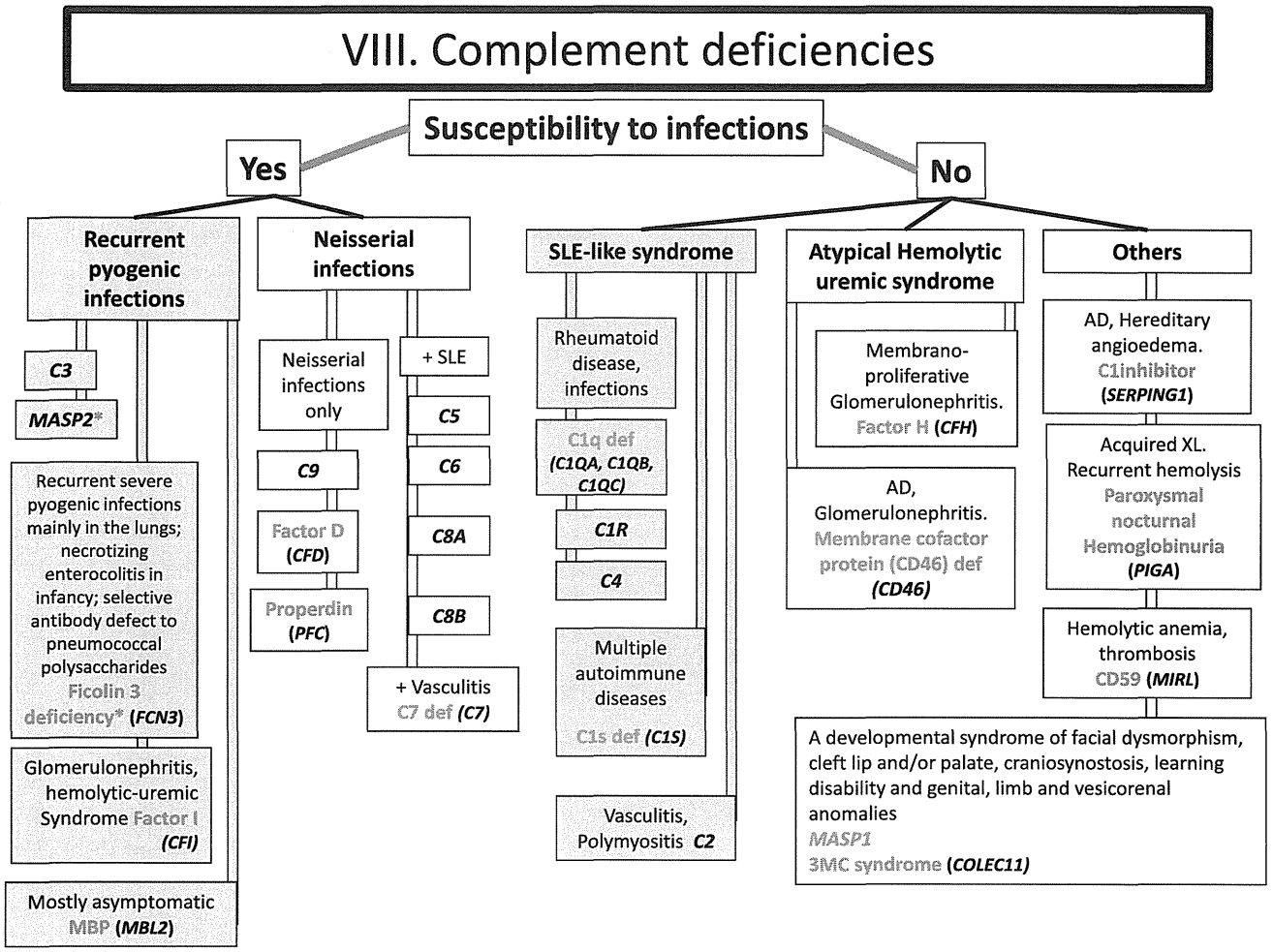

Fig. 8 Complement deficiencies. Def: deficiency; SLE: Systemic Lupus Erythematosus

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.

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

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Galectin-9 Enhances Cytokine Secretion, but Suppresses Survival and Degranulation, in Human Mast Cell Line

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Abstract

Galectin-9 (Gal-9), a lectin having a β-galactoside-binding domain, can induce apoptosis of Th1 cells by binding to TIM-3. In addition, Gal-9 inhibits IgE/Ag-mediated degranulation of mast cell/basophilic cell lines by binding to IgE, thus blocking IgE/Ag complex formation. However, the role of Gal-9 in mast cell function in the absence of IgE is not fully understood. Here, we found that recombinant Gal-9 directly induced phosphorylation of Erk1/2 but not p38 MAPK in a human mast cell line, HMC-1, which does not express FcεRI. Gal-9 induced apoptosis and inhibited PMA/ionomycin-mediated degranulation of HMC-1 cells. On the other hand, Gal-9 induced cytokine and/or chemokine production by HMC-1 cells, dependent on activation of ERK1/2 but not p38 MAPK. In addition, the lectin activity of Gal-9 was required for Gal-9-mediated cytokine secretion by HMC-1 cells. These observations suggest that Gal-9 has dual properties as both a regulator and an activator of mast cells.

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Competing Interests: Toshiro Niki and Mitsuomi Hirashima are board members of GalPharma Co., Ltd. The authors have the following patents related to material pertinent to this article: "Novel modified galectin 9 proteins and use thereof" which is applied by GalPharma and issued in Japan (4792390), the USA (8,268,324), EPC (1736541), Canada (2,561,696), India (239130), and Korea ((10-1222281) as of 2013.12.2). The authors have the following products related to material pertinent to this article: stable-form Gal-9. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Galectin-9 (Gal-9) was first identified as a chemoattractant and activating factor for eosinophils. [1–3] It is abundantly expressed in various tissues, especially the epithelium of the gastrointestinal tract, and in a variety of cells such as macrophages, eosinophils, mast cells, fibroblasts and synovial cells. [4–7].

Gal-9 influences various biological functions such as cell aggregation, adhesion, apoptosis, survival, activation and differentiation by binding to T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3). [5,8,9] Like Gal-9, Tim-3 is also expressed on various types of cells, including Th1 cells, [9] Tc1 cells, [10] Th17 cells, [11] NK cells, [12] NKT cells, [13,14] dendritic cells (DC) [15] and mast cells (MCs). [16,17] It is known that Gal-9 has anti-tumor activity by promoting activation of NK cells [18] and cytotoxic T lymphocytes by enhancing DC maturation. [19] Moreover, Gal-9 induces aggregation of melanoma and breast cancer cell lines and suppresses metastasis. [20–22] It was suggested that Gal-9 is a negative regulator of development of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) in mice. Indeed, like anti-TIM-3 mAb, [10] Gal-9 can suppress development of EAE by inducing Th1 cell apoptosis via TIM-3. [9] Gal-9 can also attenuate development of CIA by inhibiting differentiation of Th17 cells while enhancing differentiation of regulatory T cells. [23] Moreover, expression of each of Gal-9 and TIM-3 was shown to be increased in the lungs of rodents during allergic airway inflammation, [24–26] suggesting roles for Gal-9 and TIM-3 in induction of that disease. Indeed, Gal-9 administration to mice suppressed ovalbumin- and house dust mite antigen-induced airway inflammation and hypersensitivity. [27] In the setting, Gal-9 bound to CD44, interfering with binding of hyaluronan, a known ligand for CD44, and resulting in inhibition of Th2 cell recruitment through CD44-hyaluronan

interaction. [27] On the other hand, the role of TIM-3 in development of ovalbumin-induced airway inflammation and hypersensitivity is controversial. That is, the response was attenuated in mice treated with anti-TIM-3 mAb, [24] but normal in TIM-3-deficient mice. [28] Although the reason for that apparent discrepancy is unclear, the report using anti-TIM-3 mAbs did not fully characterize them, i.e., whether they were agonistic, blocking or depletion Abs. These observations suggest that the biological function of Gal-9 may be mediated independently of TIM-3 in certain settings. In support of this, binding of Gal-9 to IgE blocks IgE/Ag complex formation and thus inhibits IgE/Ag-FcεRI crosslinking-induced degranulation of mast cell/basophilic cell lines. [29] In contrast, we showed that anti-TIM-3 agonistic antibody promoted cytokine secretion, but did not influence degranulation, by mouse bone marrow cell-derived cultured mast cells (BMCMCs) after IgE/Ag-FcεRI crosslinking. [16] On the other hand, the role of Gal-9 in mast cell function in the absence of IgE remains unclear. Therefore, in the present study we examined the role of Gal-9 in the functions of a human mast cell line, HMC-1, which does not express FcεRI, in the absence of IgE/Ag stimulation. We found that human Gal-9 enhanced cytokine secretion, but suppressed survival and degranulation, of HMC-1. These observations suggest that Gal-9 has dual properties as a regulator and activator of mast cells.

Materials and Methods

Cell Culture

HMC-1 cells (a human mast cell line) [30] were cultured in α-minimum essential medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin under a humidified atmosphere of 5% CO_2 at 37°C. Half of the medium was replaced twice per week. Normal human bronchial epithelial cells (NHBEs), normal human coronary artery endothelial cells (HCAECs) and normal human lung fibroblasts (NHLF) were obtained from Lonza (Wakersville, MD, USA) and were cultured as described elsewhere. [31].

Quantitative PCR

Total RNA samples were isolated from HMC-1 cells, NHBEs and HCAECs using RNeasy (Qiagen, Valencia, CA, USA) and digested with RNase-free DNase I (Qiagen) in accordance with the manufacturer's instructions. Human universal reference (HUR) RNA (BD Biosciences, Palo Alto, CA, USA) was used as a positive control. Then first-strand cDNA was synthesized from the isolated RNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Primers for TIMs and GAPDH were designed as follows: TIM-1 (sense, 5′-TGT TCC TCC AAT GCC TTT GC-3′; antisense, 5′-TTG CTC CCT GCA GTG TCG TA-3′), TIM-3 (sense, 5′-CAA TGC CAT AGA TCC AAC CAC C-3′; antisense, 5′-GCA GTG GAC AGA ACC TCC AAA A-3′), TIM-4 (sense, 5′-TCC TGC TGA CAT CCA AAG CA-3′; antisense, 5′-TGG GAG ATG GGC ATT TCA TT-3′) and GAPDH (sense, 5′-GAA GGT GAA GGT CGG AGT C-3′; antisense, 5′-GAA GAT GGT GAT GGG ATT TC-3′). To determine the exact copy numbers of the target genes, quantified concentrations of the purified PCR products of TIM-1, TIM-3, TIM-4 and GAPDH were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 5 ng of the total RNA samples were used for each quantitative PCR. The mRNA expression levels were normalized to the GAPDH level in each sample.

Flow Cytometry

Cells (HMC-1 cells and PBMCs) were incubated with human AB serum (Lonza) at 4°C for 5 min, and then stained with PE-conjugated anti-human TIM-1 mAb (Clone Name 1D12, BioLegend, San Diego, CA, USA), PE-conjugated anti-human TIM-3 mAb (Clone Name F38-2E2, BioLegend), PE-conjugated anti-human TIM-4 mAb (Clone Name 9F4, BioLegend) and PE-conjugated mouse IgG1 (Clone Name MOPC-21, BioLegend) at 4°C for 30 min. The expression of TIMs on the cells was determined with a FACS Canto II using Diva Software (BD Biosciences, San Jose, CA, USA).

Western Blotting

HMC-1 cells (5×10^5 cells/well in a 24-well plate) were treated with 1 μM recombinant human galectin-9 (rhGal-9) (GalPhama Co., Ltd., Kagawa, Japan) at 37°C for the indicated time periods. Then the cells were lysed and sonicated in 200 μl of NuPAGE sample buffer (Invitrogen, Carlsbad, CA, USA) containing 5% 2-mercaptoethanol. Proteins in the whole-cell lysates were separated by SDS-PAGE (5–15% Ready Gels J; Bio-Rad) gel electrophoresis and transferred to nitrocellulose membranes (iBlot Gel Transfer Stacks, mini; Invitrogen). Immunoblotting was performed using rabbit anti-phospho-p44/42 MAPK (Erk1/2) mAb (clone D13.14.4E; Cell Signaling Technology, Danvers, MA) and rabbit anti-p44/42 MAPK (Erk1/2) mAb (clone 137F5; Cell Signaling Technology) as the 1st Abs and horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) as the 2nd Ab. The protein bands were visualized by enhanced Pierce Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA).

Cell Survival

HMC-1 cells were pretreated with or without 5 μg/ml mitomycin-C (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) for 2 hours. After washing, the cells (4×10^5 cells/ml) were cultured in the presence and absence of 0.25, 0.5 and 1 μM rhGal-9 at 37°C for 0, 24 or 48 h. Live cells were counted under a microscope after trypan blue staining. The cells were incubated with FITC-conjugated annexin V and propidium iodide (MEBCYTO-Apoptosis Kit; MBL Co., Ltd., Nagoya, Japan), and the percentage of propidium iodide-negative and annexin V-positive apoptotic cells was determined using a FACSCanto II with Diva Software.

Caspase Activity

HMC-1 cells (1×10^5 cells/ml) were cultured in the presence and absence of 0.5 μM rhGal-9 or 0.1 μM staurosporine (Cayman Chemical Company, Ann Arbor, MI, USA) at 37°C for 16 hours. Then the caspase-3/7 activities in the cells were determined by Caspase-Glo 3/7 assay (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. The luminescence (Relative Light Unit [RLU]) of each sample was measured with a fluorescence plate reader (ARVO X5, PerkinElmer, Waltham, MA, USA) at 490/535 nm.

β-hexosaminidase Release Assay

HMC-1 cells (1×10^5 cells/well in a 96-well plate; not treated with mitomycin C) were pre-treated with rhGal-9 (0, 0.25, 0.5 and 1 μM) at 37°C for 30 min, and then stimulated with 0.1 μg/ml PMA (Sigma Chemical Co.) and 1 μg/ml ionomycin (Sigma Chemical Co.) at 37°C for 30 min. The culture supernatants were collected, and the activity of β-hexosaminidase in each was determined as described previously, with minor modification. [32] In brief, 50-μl samples were incubated with 100 μl of 1.3 mg/ml

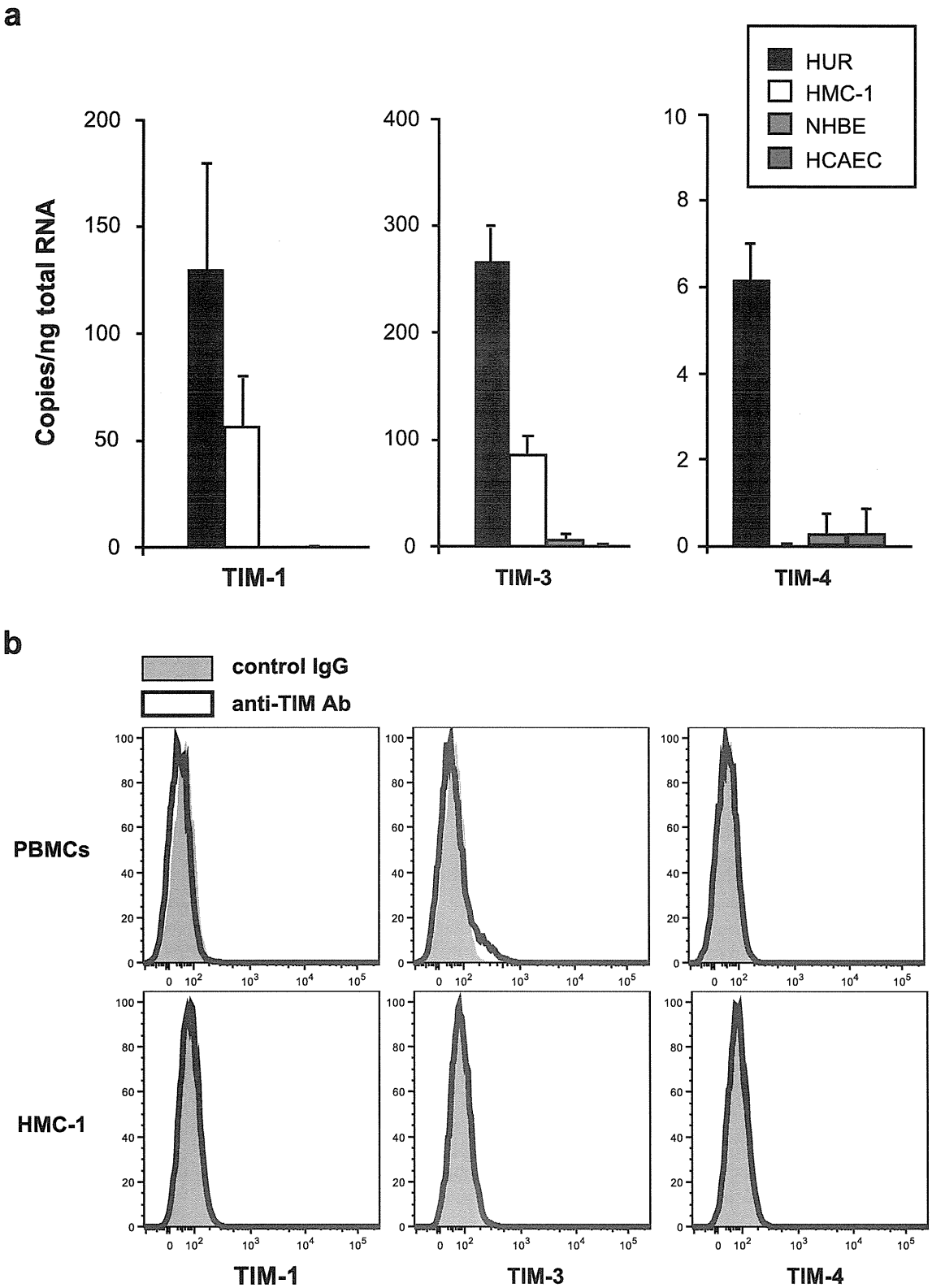

Figure 1. Expression of TIM family members in cultured human mast cell line and primary cells. (a) The mRNA expression for TIM family members (TIM-1, TIM-3 and TIM-4) in a human mast cell line, HMC-1, normal human bronchial epithelial cells (NHBEs) and normal human coronary artery endothelial cells (HCAECs) was determined by quantitative PCR. Human universal reference (HUR) RNA was used as a control. (b) The cell surface expression of TIM family members (TIM-1, TIM-3 and TIM-4) on HMC-1 cells and PBMCs was determined by flow cytometry. Shaded areas = isotype-matched control IgG staining, and bold lines = anti-TIM mAb staining. Data show a representative result of two independent experiments.

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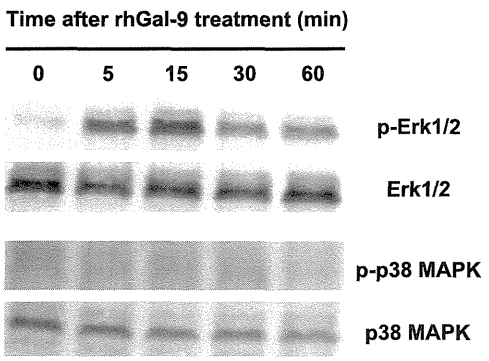

Figure 2. Galectin-9 induces phosphorylation of Erk1/2, but not p38 MAPK, in HMC-1 cells. HMC-1 cells were cultured in the presence of 1 μM recombinant human galectin-9 (rhGal-9) for the indicated times. Then the levels of phosphorylation of Erk1/2 and p38 MAPK in the cells were determined by western blot analysis. Data show a representative result of three independent experiments.
doi:10.1371/journal.pone.0086106.g002

p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Chemical Co.) in 0.1 M sodium citrate (pH 4.5) in a 96-well microtiter plate at 37°C for 1 h. The enzymatic reaction was stopped by addition of 50 μl of 0.4 M glycine (pH 10.7) to each well. Enzymatic activities (OD405) were measured using a plate reader. Data show the percent release of β-hexosaminidase under various conditions of stimulation relative to the total amount of β-hexosaminidase in the cells, as measured in the supernatants of frozen and thawed cells.

ELISA

HMC-1 cells (1×10^5 cells/well in a 96-well plate) were treated with various concentrations of rhGal-9 in the presence and absence of 20 mM lactose (Nacalai Tesque, Kyoto, Japan) or sucrose (Wako, Osaka, Japan) at 37°C for 18 h. In some cases, HMC-1 cells were treated with ERK inhibitor (PD98059; Calbiochem, La Jolla, CA, USA), ERK inhibitor control (SB202474; Calbiochem) or solvent (0.1% (v/v) DMSO) alone at 37°C for 30 min, and with rhTIM-3-Fc (R&D Systems, Minneapolis, MN, USA) or human IgG (Sigma Chemical Co.) at 37°C for 1 hour before exposure to rhGal-9. The levels of IL-6, IL-8 and MCP-1 in the culture supernatants were determined with ELISA kits (R&D Systems) in accordance with the manufacturer's instructions.

Statistics

All data are expressed as means ± SD. The unpaired Student's t-test, two-tailed, or ANOVA, as appropriate, was used for statistical evaluation of the results. $P<0.05$ was considered statistically significant.

Results

Expression of TIM Family Members' mRNA in Human Mast Cell Line

As in our earlier study using mouse mast cells (16), we first examined the expression of mRNA for TIM family members (TIM-1, TIM-3 and TIM-4) in HMC-1 cells and other human primary cells (NHBE and HCAEC, as negative controls) by quantitative PCR. We found constitutive expression of mRNA for both TIM-1 and TIM-3 in HMC-1 cells, but not in NHBE or HCAEC (Figure 1a). On the other hand, expression of TIM-4 mRNA was barely detectable in these cells. Next, we determined the surface protein expression of the TIM family members (TIM-1, TIM-3 and TIM-4) in HMC-1 cells and PBMCs by flow cytometry. In contrast to mRNA expression, TIM-1 and TIM-3 as well as TIM-4 were barely detectable on either HMC-1 cells or PBMCs (Figure 1b).

Gal-9 Induces Phosphorylation of Erk1/2 in HMC-1 Cells

We previously demonstrated that IL-33 can induce cytokine secretion by human mast cells, although surface expression of ST2, a component of IL-33R, is barely detectable on these cells by flow cytometry [33]. Likewise, although surface expression of TIM-3 is barely detectable on HMC-1 cells, Gal-9, which is a ligand for TIM-3, may play some role in activation and/or regulation of HMC-1 cells. Thus, we examined the effect of rhGal-9 on the phosphorylation of signaling molecules in those cells by immunoblot analysis. We found that rhGal-9 induced phosphorylation of Erk1/2, but not p38 MAPK, in HMC-1 cells (Figure 2), suggesting that rhGal-9 may influence the function of HMC-1 cells.

Gal-9 Induces Apoptosis of HMC-1 Cells

We next examined the effects of Gal-9 on HMC-1 cell survival. After treatment with or without mitomycin C, HMC-1 cells were cultured in the presence and absence of 0.25, 0.5 and 1 μM rhGal-9 for 24, 48 and 72 hours. The number of trypan blue-negative viable cells was significantly decreased by 1 μM rhGal-9, while the percentage of propidium iodide-negative and annexin V-positive apoptotic cells was significantly increased irrespective of mitomycin C treatment (Figure 3a and 3b). In association with this, as in the case of staurosporine treatment, the levels of caspase-3/7 activity in HMC-1 cells were also significantly increased after rhGal-9 treatment (Figure 3c). These findings indicate that rhGal-9 induces apoptosis of HMC-1 cells.

Gal-9 Inhibits Degranulation of HMC-1 Cells

We next evaluated the effect of rhGal-9 on degranulation of HMC-1 cells. HMC-1 cells were treated with 0, 0.25, 0.5 and 1 μM of rhGal-9 for 30 min prior to stimulation with PMA+ionomycin. The level of degranulation, assessed by the release of β-hexosaminidase from the cells, was significantly suppressed by pretreatment with the optimal dose (0.5 μM) of rhGal-9 (Figure 4a). In the setting, pre-treatment with 0.5 μM rhGal-9 resulted in inhibition of cell survival (assessed as trypan blue-negative cells, and propidium iodide-negative and annexin V-positive apoptotic cells by flow cytometry) as well as degranulation after stimulation with PMA+ionomycin (Figure 4b–d), suggesting that the reduced degranulation of HMC-1 cells may be due to apoptosis of the cells after rhGal-9 treatment. On the other hand, the relative levels of degranulation per live HMC-1 cells were significantly reduced by pre-treatment with rhGal-9 (Figure 4e), suggesting that Gal-9 also inhibited PMA- and ionomycin-induced degranulation of HMC-1 cells independently of Gal-9-mediated apoptosis. Thus, these observations suggest that Gal-9 can inhibit PMA- and ionomycin-induced degranulation of HMC-1 cells, both directly and indirectly.

Gal-9 Induces Cytokine Production by HMC-1 Cells

In contrast to the inhibitory effect of rhGal-9 on degranulation, we found that IL-6, IL-8 and MCP-1 production by HMC-1 cells was dose-dependently induced by rhGal-9 (Figure 5a). It is known that most biological effects of galectins are mediated by their carbohydrate-binding activities. [5] In support of that, rhGal-9-mediated IL-6 production by HMC-1 cells was strongly suppressed by addition of an excessive amount of lactose but not sucrose (Figure 5b). Moreover, rhGal-9-mediated IL-6 production

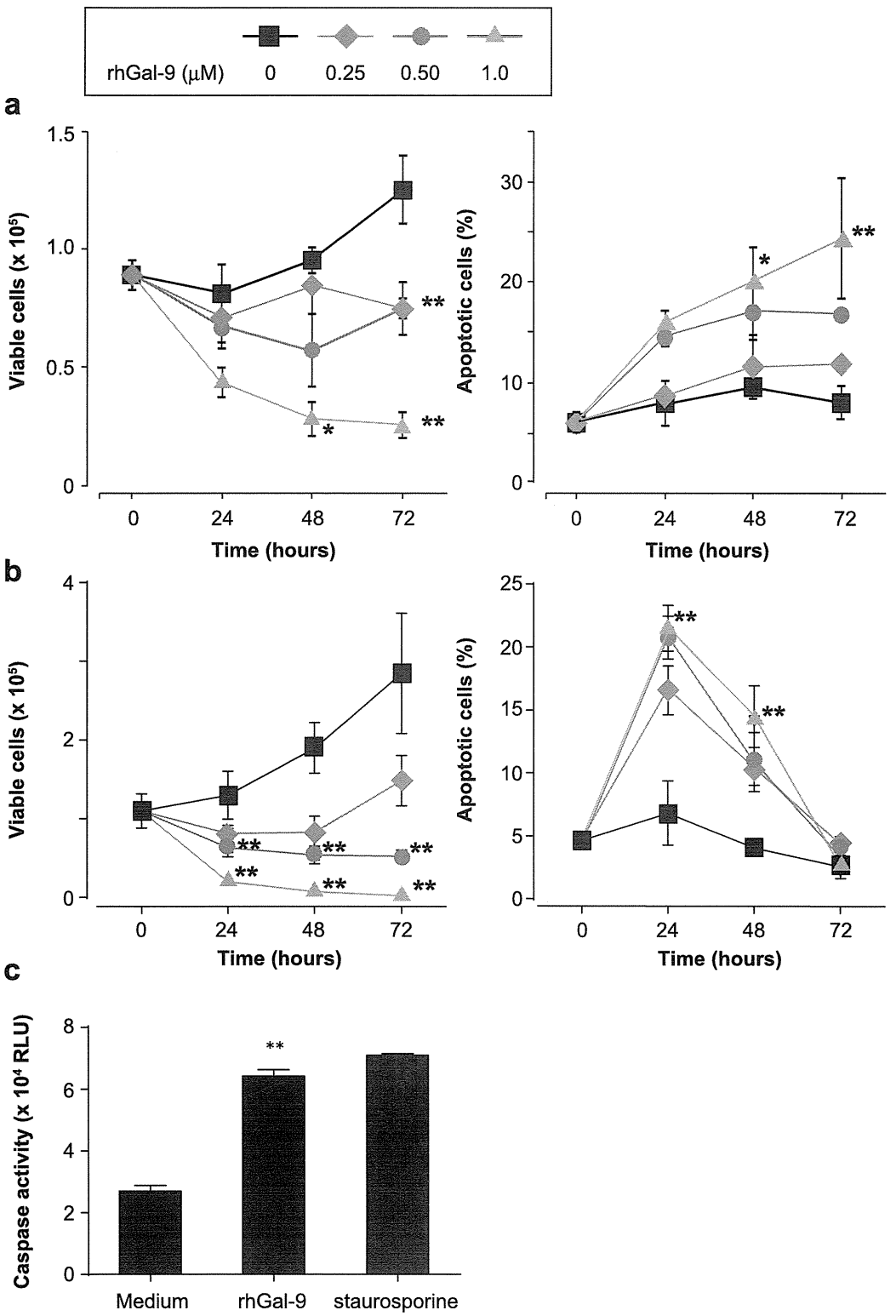

Figure 3. Galectin-9 induces apoptosis of HMC-1 cells. (a, b) HMC-1 cells pre-treated with (a) or without mitomycin C (b) were cultured in the presence of 0, 0.25, 0.5 or 1 μM recombinant human galectin-9 (rhGal-9) for the indicated time periods. The number of viable cells was determined by trypan blue staining. The proportion of propidium iodide-negative and annexin V-positive apoptotic cells was assessed by flow cytometry. (c) HMC-1 cells (no mitomycin C pre-treatment) were cultured in the presence and absence of 0.5 μM rhGal-9 or 0.1 μM staurosporine for 16 hours. Then the levels of caspase activity in the cells were determined. Data show the mean ± SD of triplicate samples and are a representative result of three independent experiments. *$p<0.05$ and **$p<0.01$ versus the corresponding values for the vehicle control.
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