

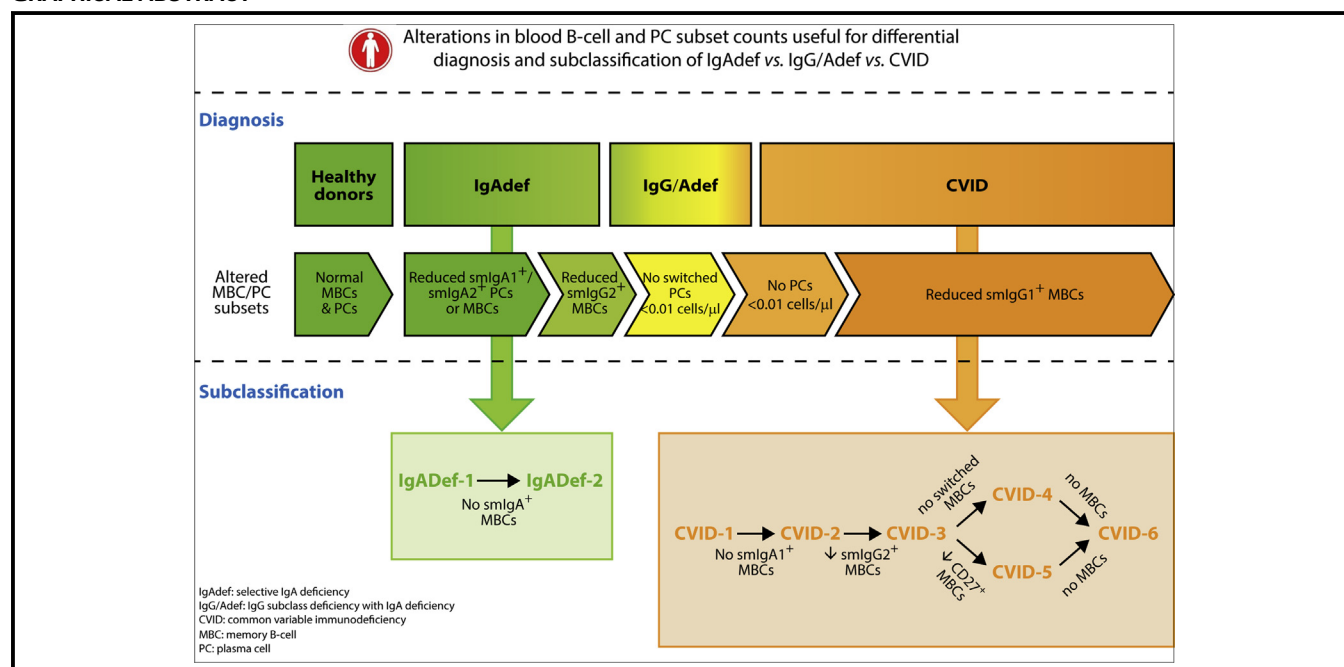
Defects in memory B-cell and plasma cell subsets expressing different immunoglobulin-subclasses in patients with CVID and immunoglobulin subclass deficiencies



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GRAPHICAL ABSTRACT



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Background: Predominantly antibody deficiencies (PADs) are the most prevalent primary immunodeficiencies, but their B-cell defects and underlying genetic alterations remain largely unknown.

Objective: We investigated patients with PADs for the distribution of 41 blood B-cell and plasma cell (PC) subsets, including subsets defined by expression of distinct immunoglobulin heavy chain subclasses.

Methods: Blood samples from 139 patients with PADs, 61 patients with common variable immunodeficiency (CVID), 68 patients with selective IgA deficiency (IgAdef), 10 patients with IgG subclass deficiency with IgA deficiency, and 223 age-matched control subjects were studied by using flow cytometry with EuroFlow immunoglobulin isotype staining. Patients were classified according to their B-cell and PC immune profile, and the obtained patient clusters were correlated with clinical manifestations of PADs.

Results: Decreased counts of blood PCs, memory B cells (MBCs), or both expressing distinct IgA and IgG subclasses were identified in all patients with PADs. In patients with IgAdef, B-cell defects were mainly restricted to surface membrane (sm)IgA⁺ PCs and MBCs, with 2 clear subgroups showing strongly decreased numbers of smIgA⁺ PCs with mild versus severe smIgA⁺ MBC defects and higher frequencies of nonrespiratory tract infections, autoimmunity, and affected family members. Patients with IgG subclass deficiency with IgA deficiency and those with CVID showed defects in both smIgA⁺ and smIgG⁺ MBCs and PCs. Reduced numbers of switched PCs were systematically found in patients with CVID (absent in 98%), with 6 different defective MBC (and clinical) profiles: (1) profound decrease in MBC numbers; (2) defective CD27⁺ MBCs with almost normal IgG₃⁺ MBCs; (3) absence of switched MBCs; and (4) presence of both unswitched and switched MBCs without and; (5) with IgG₂⁺ MBCs; and (6) with IgA₁⁺ MBCs.

Conclusion: Distinct PAD defective B-cell patterns were identified that are associated with unique clinical profiles. (J Allergy Clin Immunol 2019;144:809-24.)

Key words: Immunodeficiency, primary antibody deficiency, selective IgA deficiency, common variable immunodeficiency, immunophenotyping, immunoglobulins, immunoglobulin subclasses, memory B cells, plasma cells, flow cytometry, diagnosis, classification

Abbreviations used

CVID:	Common variable immunodeficiency
ESID:	European Society for Immunodeficiencies
GC:	Germinal center
HD:	Healthy donor
IgAdef:	Selective IgA deficiency
IgG/Adef:	IgG subclass deficiency with IgA deficiency
IgH:	Immunoglobulin heavy chain
IUIS:	International Union of Immunological Societies
LLN:	Lower limit of normal
MBC:	Memory B cell
NPV:	Negative predictive value
PAD:	Predominantly antibody deficiency
PC:	Plasma cell
PPV:	Positive predictive value
sm:	Surface membrane

Predominantly antibody deficiencies (PADs) are the most prevalent primary immunodeficiencies (50% to 70% of all primary immunodeficiencies)^{1,2} and comprise a heterogeneous spectrum of disorders with defective production of 1 or more immunoglobulin isotypes and/or immunoglobulin subclasses; the underlying pathogenic mechanisms remain largely unknown.^{1,2} Current classification of PADs strongly relies on the affected serum immunoglobulin heavy chain (IgH) isotype and subclass levels and includes (1) selective IgA deficiency (IgAdef) characterized by an isolated defect of serum IgA (prevalence, approximately 1:100-1,000 subjects)³⁻⁵; (2) IgG subclass deficiency with IgA deficiency (IgG/Adef) with reduced IgA and 1 or more IgG subclass serum levels (approximately 15% to 20% of IgA deficiencies)⁶; and (3) common variable immunodeficiency (CVID), which is characterized by low (total) IgG serum levels, decreased IgA and/or IgM levels, and a more severe clinical presentation but a lower prevalence (approximately 1:25,000-50,000 subjects).^{4,5} Although recurrent bacterial infections of the respiratory tract are the clinical hallmark of PADs, clinical manifestations vary substantially among patients, from (almost) asymptomatic cases to patients presenting with recurrent severe infections associated with other noninfectious disorders, such as autoimmunity, allergy, lymphoproliferation and organomegalies, enteropathy, and granulomatous disease.^{1,3,7-10}

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Disclosure of potential conflict of interest: E. Blanco, M. Pérez-Andrés, T. Kalina, M. Vlkova, E. López-Granados, M. van der Burg, J. J. M. van Dongen, and A. Orfao each report being one of the inventors on the EuroFlow-owned patent PCT/NL 2015/050762 (Diagnosis of primary immunodeficiencies), which is licensed to Cytognos, a company that pays royalties to the EuroFlow Consortium. J. J. M. van Dongen and A. Orfao

report an Educational Services Agreement from BD Biosciences. The rest of the authors declare that they have no relevant conflicts of interest.

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Despite extensive efforts, genetic (ie, monogenic) alterations responsible for PADs are detected in less than 10% of cases.^{1,2,11} In such settings altered distributions of distinct blood B- and T-cell subpopulations determined by using flow cytometry might provide key (complementary) diagnostic information, particularly for patients with low serum antibody isotype levels and nonspecific clinical features.^{5,12,13} Thus controversial results have been reported in patients with CVID concerning the potential association between specific B-cell alterations, such as decreased (relative) numbers of CD27⁺ (antigen-experienced) switched B cells in blood and relevant clinical manifestations (eg, splenomegaly, granulomatous disease, and autoimmunity),¹⁴⁻¹⁸ whereas preservation of CD27⁺ class-switched memory B cells (MBCs) has been considered a surrogate marker for the ability to respond to vaccination.⁵ Similarly, decreased CD27⁺ (antigen-experienced) switched B-cell counts in blood have been associated with a worse clinical outcomes in patients with IgAdef,¹⁹ whereas decreased percentages of CD21⁺ B cells and increased proportions of immature/transitional B-cells have both been correlated to distinct CVID clinical profiles.^{15,18,20}

Despite all the above associations, the actual clinical relevance of these B-cell defects in patients with PADs still remains elusive. In addition, a significant clinical and functional B-cell heterogeneity is still observed among patients who present with similar patterns of alteration of B cells by using flow cytometry (eg, reduced numbers of switched MBCs).^{18,21} This is probably due to the limited number of B-cell populations investigated, the lack of appropriate age-matched reference ranges, or both in most studies. For example, in many studies focused on antigen-experienced B cells, no distinction is made between (relative long-living) MBCs and (newly generated) circulating plasma cells (PCs),^{14,15,19} and very few reports have investigated the precise relationship between defects in specific immunoglobulin isotypes and the number of blood B cells and PCs that express them.^{22,23} Moreover, thus far, no study has investigated the IgG₁ to IgG₄ and IgA₁ and IgA₂ subclass distribution within the PC and MBC compartments of patients with PADs. Finally, despite the fact that PADs can present at any age^{1,7,9,13} and major age-related differences exist in the distribution of blood B-cell subsets throughout life,²⁴ most reports on B-cell compartments in patients with PADs do not consider (normal) age-associated variations, and only a few studies subdivided healthy donors (HDs) and patients with PADs into a few ($n = 3-4$) age groups.^{17,20,23} Altogether, this reflects the potential relevance of a more in-depth evaluation of the B-cell compartment and its alterations in patients with PADs versus age-matched control subjects for improved diagnosis and classification of PADs.

Here, for the first time, we investigated the distribution of 41 distinct blood B-cell and PC subsets in 139 patients with PADs versus 223 age-matched control subjects. Based on the B-cell and PC defects encountered, distinct defective immune profiles were identified that are associated with both the diagnostic subtype and clinical manifestations of PADs.

METHODS

Patients and control subjects

Overall, 139 patients with PADs⁴ (mean age, 32 ± 19 years; range, 4-87 years) and 223 HDs (mean age, 39 ± 28 years; range, 4-99 years) were studied. Patients with PADs were subclassified by the International Union of Immunological Societies (IUIS)⁴ and European Society for

Immunodeficiencies (ESID)⁵ criteria into 68 and 42 patients with IgAdef (mean age, 24 ± 17 years), respectively; 10 patients with IgG/Adef (mean age, 24 ± 14 years); and 61 patients with CVID (mean age, 41 ± 17 years). Twenty-six asymptomatic patients with IgAdef (mean age, 24 ± 15 years) with serum IgA levels of less than 7 mg/dL did not fulfill the ESID criteria⁵ for IgAdef and are referred to hereafter as ESID⁻ versus ESID⁺ IgAdef cases. EDTA-anticoagulated blood samples were collected at 8 different sites and centrally processed in 2 of them after informed consent was provided by each subject, their legal representatives, or both. The study was approved by local ethics committees.

Flow cytometric identification of blood B cells and their subsets

Total B-cell counts and distribution of 41 distinct B-cell subsets were analyzed by using flow cytometry after staining 10^7 nucleated cells with the EuroFlow 12-color immunoglobulin isotype B-cell tube (see Table E1 in this article's Online Repository at www.jacionline.org) and bulk-lyse standard operating procedure (www.EuroFlow.org), as described elsewhere.^{25,26} Per sample, 5×10^6 or more leukocytes were measured in LSRFortessa X-20 flow cytometers (Becton Dickinson Biosciences, San Jose, Calif). Instrument set-up and calibration were performed according to EuroFlow standard operating procedures (www.EuroFlow.org).²⁷ For data analysis, Infinicyt software (Cytognos S.L., Salamanca, Spain) was used.

CD19⁺ B-cells and PCs were both identified by using low-to-intermediate forward light scatter and sideward light scatter and subsequently subclassified into 41 different subpopulations based on their maturation stage and expression of distinct immunoglobulin isotypes and immunoglobulin subclasses, as previously described,²⁴ by using the gating strategy detailed in the Methods section and Fig E1 in this article's Online Repository at www.jacionline.org. Briefly, the following B-cell subpopulations were defined based on their staining profile for CD19, CD38, CD24, CD21, CD27, CD5, surface membrane (sm)IgM, and smIgD: (1) CD27⁺CD38^{hi}CD24^{hi}CD5⁺smIgM⁺IgD⁺ immature/transitional B-cells; (2) CD27⁺CD38^{lo}CD24^{het}CD5^{het}smIgM⁺IgD⁺ naive B lymphocytes; (3) CD27⁺CD38^{lo}CD5⁻CD24^{het}smIgM⁺IgD⁺ unswitched MBCs; (4) CD27⁺/⁻CD38^{lo}CD5⁻CD24^{het}smIgM⁻IgD⁻ switched MBCs; and (5) CD27⁺CD38^{hi}CD5⁻CD21⁻CD24⁻ PCs. MBCs and PCs were further subclassified according to their immunoglobulin isotypes and immunoglobulin subclasses into (1) smIgM⁺IgD⁺, smIgD⁺-only, smIgA₁⁺, smIgA₂⁺, smIgG₁⁺, smIgG₂⁺, smIgG₃⁺, and smIgG₄⁺ MBCs and (2) smIgM⁻-only, smIgD⁻-only, smIgA₁⁺, smIgA₂⁺, smIgG₁⁺, smIgG₂⁺, smIgG₃⁺, and smIgG₄⁺ PCs, respectively. Finally, the above subpopulations of naive B lymphocytes and MBCs were placed in further subsets based on CD21 (CD21⁺ vs CD21⁻ naive and MBC subsets) and CD27 expression (CD27⁺ and CD27⁻ MBCs, see Fig E1). Absolute counts were calculated by using total B-cell counts based in a double-platform assay²⁸ and used throughout the study. Intralaboratory and interlaboratory variability was assessed at the participating centers based on replicate measurements of the same samples to ensure comparable results at distinct sites (see Fig E2 in this article's Online Repository at www.jacionline.org).

Statistical analyses

Statistical analyses were performed with either the R (version 3.2.3; <https://www.r-project.org/>)²⁹ or SPSS (version 23.0; IBM, Armonk, NY) software packages. Kruskal-Wallis and Mann-Whitney *U* tests (for continuous variables) and χ^2 and Fisher exact tests (for categorical variable) were used, respectively, to investigate the statistical significance (set at $P \leq .05$) of differences observed between groups in B-cell subset counts and clinical features. Unsupervised clustering analysis of patient data based on the K-means learning algorithm³⁰ and Euclidean distances was performed by using blood B-cell subset absolute counts normalized by age group (see Table E2 in this article's Online Repository at www.jacionline.org) based on (previously reported) reference values of 140 age-matched subjects²⁴ and extended here to 223 individual $-\log_{10}$ values (patient value/minimum normal value). Age-normalized B-cell/PC subset values per patient were represented in heat maps by using gplots (R package).³¹

and 5th to 95th percentile values were used to define normal ranges per age group defined by a minimum of 20 subjects (see Table E3 in this article's Online Repository at www.jacionline.org). Those B-cell and PC subsets with absolute counts that were less than the method's limit of detection (undetectable; <0.01 cells/ μ L) in at least 1 subject for more than 1 reference (HD) age group were excluded from the analysis (ie, IgD-only and IgG₄⁺ PCs and MBCs and IgG₁⁺, IgG₂⁺, and IgG₃⁺ PCs).

RESULTS

Blood B-cell and PC subset defects in patients with IgAdef

Once compared with age-matched HDs, most patients with IgAdef displayed normal total B-cell counts (93%), including normal immature/transitional (90%), naive (94%), and MBC (87%) counts (see Table E4 in this article's Online Repository at www.jacionline.org). In contrast, numbers of (total) PCs, although being detected in every case (≥ 0.07 PCs/ μ L), were decreased in 49% of patients (Fig 1 and see Table E4). When MBCs and PCs were dissected according to their pattern of expression of immunoglobulin subclasses, a greater frequency of altered cases was observed. Thus smIgA₁⁺ and/or smIgA₂⁺ PC counts were found to be reduced in blood in 97% of cases, with still detectable residual smIgA⁺ PCs in 38% of the patients (PCs expressing both IgA subclasses were found in 26%, smIgA₁⁺-only subclasses were found in 9%, and smIgA₂⁺-only subclasses were found in 3% of all patients with IgAdef).

In line with these findings, reduced smIgA₁⁺ and/or smIgA₂⁺ MBC counts were also observed in virtually all patients with IgAdef (99%), although still present in half (50%) of them (both smIgA₁⁺ and smIgA₂⁺ MBCs were detected in 40% and smIgA₁⁺-only MBCs were detected in 10% of all patients with IgAdef; Fig 2 and see Table E4). Thus decreased smIgA₁⁺ or smIgA₂⁺ MBC counts showed a sensitivity of 99% with a negative predictive value (NPV) of 100% (see Table E5 in this article's Online Repository at www.jacionline.org), although when combined with decreased smIgA₁⁺ or smIgA₂⁺ PC counts, reached a 100% sensitivity and NPV (see Table E6 in this article's Online Repository at www.jacionline.org). In turn, absence of the above MBC or PC subpopulations showed a specificity of 100% and positive predictive value (PPV) of 98% for identification of patients with IgAdef (see Table E6). In contrast, smIgG⁺ PCs were present in virtually every patient with IgAdef (91%), with normal smIgG⁺ PC counts in 71% of them. Similarly, smIgG₁⁺ to smIgG₃⁺ MBC counts were only decreased in 13% or fewer patients (Fig 1 and see Fig E3 and Table E4).

Blood B-cell and PC subset defects in patients with IgG/Adef

Similarly to patients with IgAdef, total peripheral blood B-cell counts, including immature/transitional, naive and MBC counts, were within the normal range in most patients with IgG/Adef (90%, Fig 2 and see Table E4); in contrast, decreased PC counts were observed in 90% of patients with IgG/Adef, mostly because of a significant decrease in both smIgA⁺ and smIgG⁺ PC counts (100% and 90%, respectively), which were undetectable in 90% and 50% of cases, respectively. Although total blood MBC counts were within the normal range in 70% of patients with IgG/Adef showed decreased smIgA₁⁺ MBC and/or smIgA₂⁺ MBC counts in association with decreased smIgG₂⁺ MBC counts; meanwhile,

smIgG₁⁺ and smIgG₃⁺ MBC counts were normal in 80% and 90% of patients with IgG/Adef, respectively (Fig 1 and see Fig E3 and Table E4).

Based on these results, the observation of undetectable PCs combined with decreased smIgG₂⁺ MBC counts also showed a high sensitivity (90%), specificity (96%), and NPV (100%) for IgG/Adef in addition to those MBC and PC populations that identified IgAdef. In contrast, the PPV was only 50% because of the low number of patients with IgG/Adef analyzed (Fig 1 and see Tables E6 and E7 in this article's Online Repository at www.jacionline.org).

Blood B-cell and PC subset defects in patients with CVID

In contrast to patients with IgAdef, total B-cell and PC counts were decreased in around half (51%) and the majority (98%) of patients with CVID, respectively ($P < .001$ vs patients with IgAdef). In addition, immature/transitional and naive B lymphocytes were decreased in 42% and 43% of patients with CVID, mostly at the expense of CD21⁺ B cells (Fig 2 and see the Results section, Fig E4, and Table E4 in this article's Online Repository at www.jacionline.org), with only 10% and 3% of patients with CVID showing undetectable immature/transitional and naive B cells, respectively (Fig 2 and see Table E4). Reduced smIgA₁⁺ and/or smIgA₂⁺ PC counts were found in all patients with CVID, being undetectable in virtually every (98%) case. In line with these findings, only 2% of patients with CVID showed circulating smIgG⁺ PCs (Fig 1 and see Fig E3 and Table E4). Thus the absence of switched PCs was highly accurate (100% specificity, 100% PPV, and 100% NPV, with a sensitivity of 98%) for identification of CVID (see Table E8 in this article's Online Repository at www.jacionline.org). Of note, no other parameter or combination of parameters showed an improved sensitivity, specificity, PPV, and NPV for identification of CVID than the absence of switched PCs or the lack of smIgA₂⁺ PCs (see Table E6). However, the lack of switched PCs was not specific enough for an accurate differential diagnosis among distinct PAD subgroups because 9% of patients with IgAdef and 50% of patients with IgG/Adef also had undetectable switched PCs. Because of this, for a clear-cut discrimination among distinct PAD diagnostic categories, the lack of switched PCs needs to be combined with the absence or decrease in other B-cell subsets in patients with CVID that are typically normal among patients with IgAdef and those with IgG/Adef (eg, smIgG₁⁺ or smIgG₂⁺ MBCs, or total PCs; Fig 1 and see Table E4). Interestingly, the (small) subgroup of patients with IgAdef who had undetectable switched PCs also had lower serum IgG levels at diagnosis (data not shown).

Finally, despite abnormally low total MBC counts being observed in most patients with CVID (70%) and being undetectable (<0.01 MBCs/ μ L) in only 13% of cases, the degree of involvement of MBCs expressing different immunoglobulin isotypes and immunoglobulin subclasses varied significantly. Thus reduced smIgA₁⁺ and/or smIgA₂⁺ MBC counts were observed in virtually all patients with CVID (98%), being absent in most of them (80%). Regarding MBC subsets expressing distinct IgG subclasses, patients with CVID more frequently showed decreased or absent smIgG₂⁺ (95% and 67% of patients, respectively) than smIgG₁⁺ MBC counts (90% and 33% [$P > .05$]).

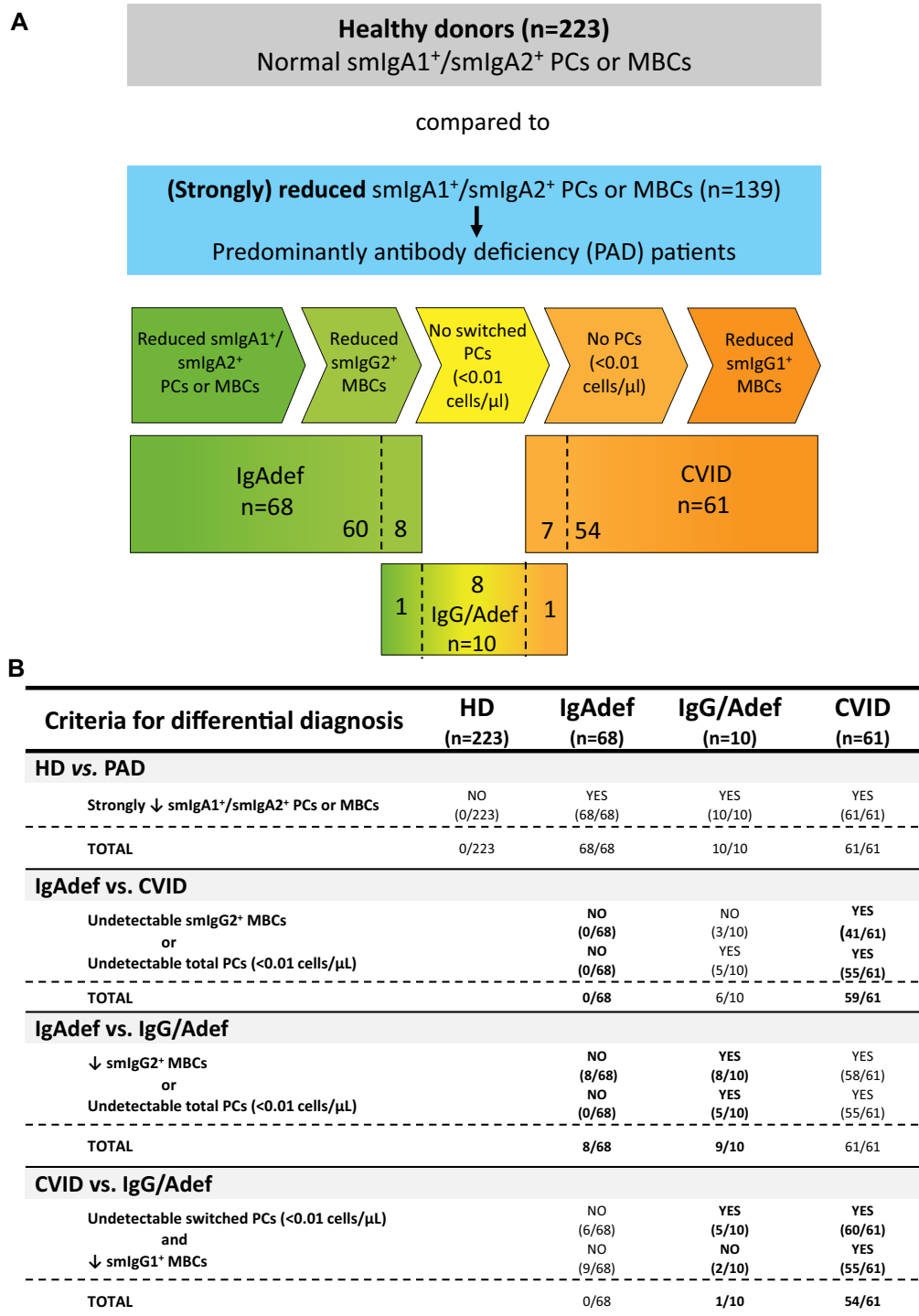


FIG 1. Alterations in blood B-cell and PC subset counts useful for the diagnosis of PADs and for the differential diagnosis of IgAdef versus IgG/Adef versus CVID. **A**, Scheme illustrating the most useful peripheral blood B-cell subset alterations for the diagnosis of PADs (vs HDs; strongly reduced: absolute numbers lower than the minimum value in HDs) and the differential diagnosis of patients with IgAdef versus patients with IgG/Adef versus patients with CVID are shown. As displayed, these criteria showed a 100% and approximately 98% accuracy in the diagnosis of PADs and the discrimination between IgAdef and CVID, respectively, whereas approximately 10% of cases within both diagnostic subgroups overlapped with 10% and 10% of patients with IgG/Adef, respectively. **B**, Most useful peripheral blood B-cell subset criteria for the diagnosis of PAD versus HDs and the distinction between patients with IgAdef versus patients with CVID, patients with IgAdef versus patients with IgG/Adef, and patients with CVID versus patients with IgG/Adef are shown, together with the number of cases within the different diagnostic categories that fulfilled these criteria.

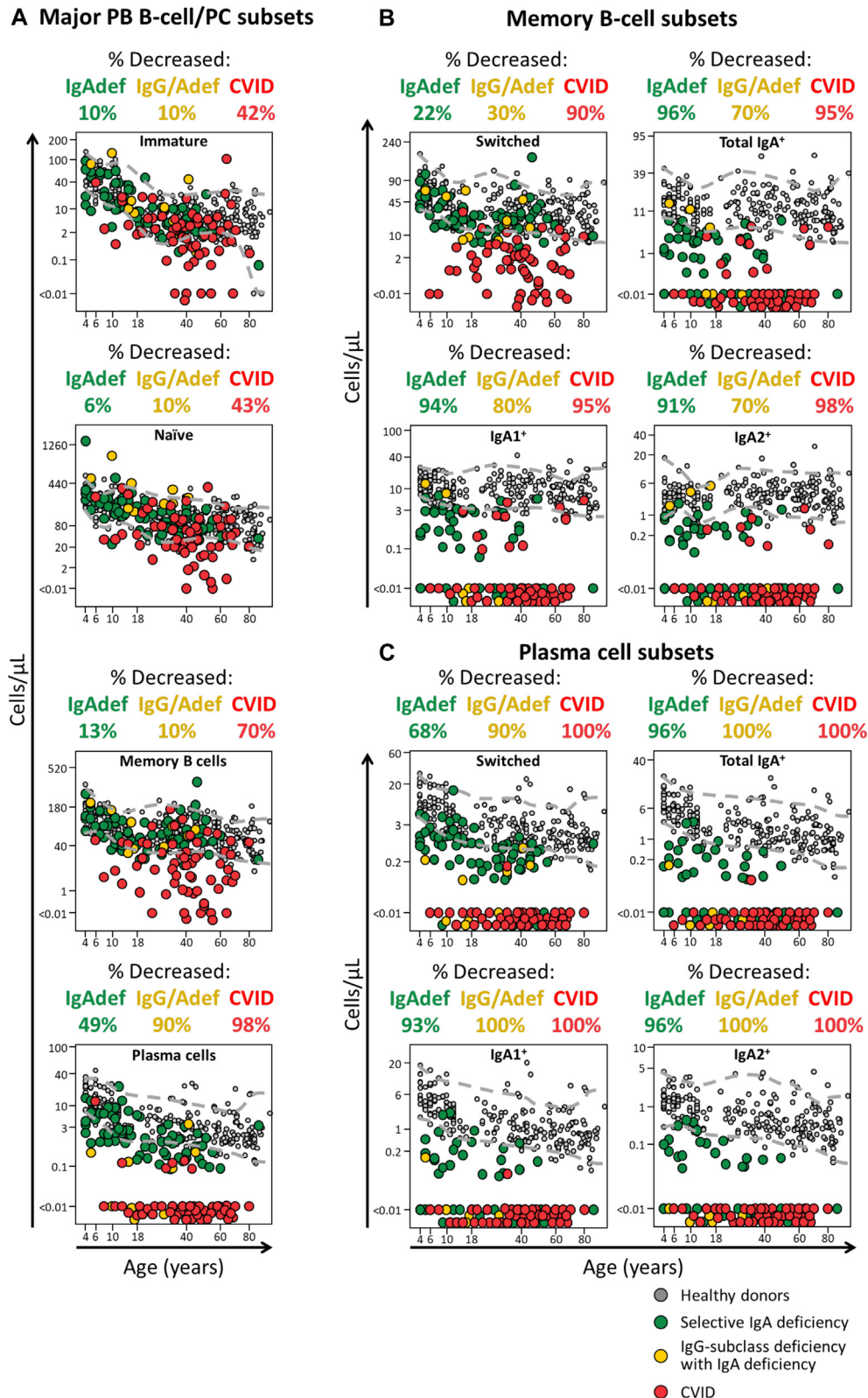


FIG 2. Absolute counts of distinct maturation-associated subpopulations of blood B cells and PCs (**A**) and total switched, total IgA, and IgA subclass subsets of MBCs (**B**) and PCs (**C**) in patients with IgAdef ($n = 68$), patients with IgG/Adef ($n = 10$), and patients with CVID ($n = 61$) versus HDs ($n = 223$) grouped by age. Individual cases are represented as green dots (IgAdef), yellow dots (IgG/IgAdef), red dots (CVID), and gray dots (HDs). Dotted gray lines represent age-associated reference 5th and 95th values. Percentages of patients with reduced numbers compared with reference values per age group are depicted above each plot by using the same color code.

and $P < .001$], respectively) and smIgG₃⁺ MBC counts (61% and 23%, respectively; $P < .001$; Fig 1 and see Fig E3 and Table E4).

Classification of patients with PADs based on blood B-cell and PC subset immune profiles

Unsupervised clustering analysis identified 5 major immune profiles of altered blood B-cell and PC subset counts in patients with PADs (classification criteria are provided in Table I), which were closely related to the IUIS diagnostic categories of PADs and are termed hereafter PAD-1 to PAD-5 (Fig 3, A).⁴ Thus patients with IgAdef were split between the PAD-1 and PAD-2 clusters (with 1 outlier in PAD-3), and patients with IgG/Adef were split between the PAD-1, PAD-2, and PAD-3 groups, whereas most (54/61) patients with CVID fell into the PAD-3, PAD-4, and PAD-5 clusters, with 7 of 61 outliers falling into the PAD-1 and PAD-2 groups (Fig 3, A).

In detail, PAD-1 included 40 patients with reduced but detectable numbers of smIgA⁺ PCs and/or smIgA⁺ MBCs (smIgA⁺ MBCs and smIgA⁺ PCs ranging from <12-fold below the lower limit of normal [LLN] and undetectable to virtually normal counts, respectively). Thirty-two (80%) of 40 PAD-1 cases had been given a previous diagnosis of IgAdef and 2 (5%) of IgG/Adef, and 6 (15%) were patients with CVID with decreased but detectable numbers of IgA₁⁺ or IgA₂⁺ MBCs with limited effect on the overall number of smIgA⁺ MBCs (never decreased >12 times the LLN) and virtually normal IgG₁⁺ to IgG₃⁺ MBC counts.

PAD-2 was characterized by severely decreased numbers of smIgA⁺ MBCs (>40 times below the LLN; absent in 37 of 39 patients) and absence of smIgA⁺ PCs but (similarly to PAD-1) virtually normal smIgG₁⁺ to smIgG₃⁺ MBC counts. This PAD-2 cluster included 35 (90%) patients with IgAdef, 3 (7.5%) patients with IgG/Adef, and 1 (2.5%) patient with CVID who lacked PCs and smIgA⁺ MBCs but had normal smIgG⁺ MBC numbers.

PAD-3 cases consisted of patients with severely decreased switched smIgG⁺ and smIgA⁺ PC counts (absent in 34/35 cases) and smIgA₁⁺/smIgA₂⁺ MBC counts (absent in 30/36 cases) but presenting with a heterogeneous defect on IgG⁺ MBCs, consisting of severely reduced smIgG₂⁺ MBC counts (absent in 19/36), with a milder decrease in smIgG₁⁺ (86% of cases) and particularly smIgG₃⁺ (39% of cases) MBC counts. This subgroup included 30 patients with CVID (83%), 5 patients with IgG/Adef (14%), and 1 patient with IgAdef (3%).

Finally, all PAD-4 and PAD-5 cases had undetectable smIgG₂⁺ MBCs (14/14 cases) with severely reduced smIgG₁⁺ MBC counts (14/14; absent in 9/14 cases; PAD-4) or no MBCs at all (PAD-5), except for 2 PAD-5 cases who showed detectable IgG₃⁺ MBCs at levels of greater than 15-fold below the LLN; all PAD-4 and PAD-5 cases corresponded to CVID.

Blood B-cell and PC immune profiles in patients with IgAdef

Patients with IgAdef were split into 2 subgroups termed hereafter IgAdef-1 and IgAdef-2 (classification criteria are provided in Table I) with different patterns of alteration of smIgA⁺ MBCs (Fig 3, B): smIgA₁⁺ and/or smIgA₂⁺ MBCs were present in the IgAdef-1 group, whereas they were virtually absent in IgAdef-2 cases (Fig 4, B, and Figs E5, A, and E6 in this article's Online Repository at www.jacionline.org). Interestingly, these 2

subgroups did not show a strong association with the ESID diagnostic criteria⁵ for clinical IgAdef, which were met in 53% of IgAdef-1 cases versus 69% of IgAdef-2 cases ($P > .05$; see Table E9, A, in this article's Online Repository at www.jacionline.org). Of note, IgAdef-1 cases were older than IgAdef-2 cases both at the time of analysis (31 ± 19 years vs 17 ± 13 years, respectively; $P = .001$) and at diagnosis (28 ± 19 years vs 14 ± 13 years, respectively; $P = .006$), with a similar male/female distribution. Despite no differences being observed in IgM serum levels at diagnosis, serum IgG levels were slightly lower in IgAdef-1 versus IgAdef-2 cases (1305 ± 290 vs 1467 ± 232 mg/dL, $P = .03$). In turn, although around one third of both IgAdef-1 and IgAdef-2 cases had a past history of recurrent respiratory tract infections at presentation, IgAdef-2 cases showed a greater frequency of other (recurrent) infections (17% vs 0%, respectively; $P = .02$), tissue-specific autoimmunity (31% vs 6%, respectively; $P = .01$), and other family members affected (22% vs 3%, respectively; $P = .03$; Fig 5, A, and see Table E10 in this article's Online Repository at www.jacionline.org).

Blood B-cell and PC immune profiles in patients with CVID

Overall, 6 subgroups of CVID (designated hereafter as CVID-1 to CVID-6) with different patterns of altered B-cell subsets and complete absence of switched PCs in 98% of cases (classification criteria are provided in Table I) were identified (Figs 3, C, and 4, C, and see Figs E5, B, and E6).

The CVID-1 and CVID-2 groups included patients with both detectable smIgMD⁺ MBCs and switched MBCs of all smIgG₁ to smIgG₃ subclasses, with CVID-1 (but not CVID-2) cases also presenting normal or slightly reduced IgA₁⁺ MBC counts. In contrast, CVID-3 cases showed a more severe smIgG₂⁺ MBC defect (>4-fold below the LLN), frequently with undetectable (<0.01 cells/ μ L) smIgG₂⁺ MBCs (17/22 cases). CVID-4 cases had no switched MBCs, whereas CVID-5 cases showed more severe defects involving all CD27⁺ MBC subsets (≥ 6 -fold below the LLN) but almost normal CD27⁻ smIgG₃⁺ MBC counts. Finally, CVID-6 cases had severely decreased switched and unswitched MBC counts, including 0.06 or fewer IgG₃⁺ MBCs/ μ L (>15-fold below the LLN; Figs 3, C, and 4, C).

Overall, a close association between the CVID-1 and CVID-6 clusters and the EUROclass classification (see Table E11 in this article's Online Repository at www.jacionline.org)¹⁸ of CVID was observed. Thus EUROclass smB⁺ patients were subclassified here into the CVID-1 (58%), CVID-2 (17%), and CVID-3 (25%) clusters, depending on their normal versus low smIgA⁺ and smIgG₂⁺ MBC counts. EUROclass B⁻ patients were included in our CVID-6 cluster, except for 2 cases with less than 1% peripheral blood B cells but preserved MBC counts, who were thereby classified as CVID-4 and CVID-5, respectively. In fact, in 8 of 9 patients classified as B⁻, we could identify naive B cells, and in 4 of 9 cases we could also identify MBCs, despite these cells being severely decreased in 2 of them. In contrast, EUROclass smB⁻ patients split across the different CVID-1 to CVID-6 clusters: CVID-1, 2.5%; CVID-2, 10%; CVID-3, 47.5%; CVID-4, 12.5%; CVID-5, 20%; and CVID-6, 7.5% of smB⁻ cases (Fig 3, C, and see Table E9, B). Inclusion of other EUROclass parameters, such as CD21 expression (see Table E9, B) or immature/transitional B-cell counts did not result in significantly

TABLE I. Criteria used for subclassification of patients with PAD, patients with IgAdef, and patients with CVID into the PAD-1 to PAD-5, IgAdef-1 to IgAdef-2, and CVID-1 to CVID-6 clusters, respectively

Clusters	MBCs				
	Total MBCs	CD27 ⁺ MBCs	CD21 ⁺ MBCs	smlgM ⁺⁺ IgD ⁺ MBCs	Switched MBCs
PAD-1	Normal or ↓<2-fold			Normal or ↓<1.4-fold	Normal or ↓<3-fold
PAD-2	Normal or ↓<1.4-fold			Normal or ↓<1.1-fold	Normal or ↓<2-fold
PAD-3	Normal or ↓<10-fold			Normal or ↓<2-fold†	Normal or ↓<100-fold
PAD-4	↓1.4-46 fold			↓≥2-fold or undetectable§	↓>12-fold or undetectable
PAD-5	↓>500-fold or undetectable			Undetectable	↓>190-fold or undetectable
IgAdef-1					
IgAdef-2					Normal or ↓<12-fold ↓>40-fold or undetectable
CVID-1	Normal	Normal or ↓<1.4-fold	Normal or ↓<1.8-fold	Normal or ↓<1.4-fold	Normal or ↓<3-fold
CVID-2	Normal	Normal	Normal	Normal	Normal or ↓<5-fold
CVID-3	Normal or ↓<5-fold#	Normal or ↓<6-fold#	Normal or ↓<8-fold#	Normal or ↓<3-fold	↓>3-100 fold
CVID-4	↓1.4-30 fold	↓1.3-30 fold	↓1.5-35 fold	Normal or ↓<14-fold	Undetectable
CVID-5	↓5-45 fold	↓≥6-fold or undetectable	↓8-1000-fold	↓≥3-fold or undetectable	↓>2-80 fold
CVID-6	↓>500-fold or undetectable	Undetectable	↓>780-fold to undetectable	Undetectable	↓>185-fold or undetectable

B-cell subpopulations that were not required for patient subclassification are plotted as empty cells. *Undetectable* is defined as less than 0.01 cells/μL. The most relevant subsets for discrimination between 2 or more subgroups are highlighted in boldface.

*Less than 15% of cases showed reduced smlgG₂⁺ MBC counts systematically associated with the presence of switched PCs or normal to less than 2-fold reduced smlgA⁺ MBC counts.

†Those cases with smlgMD⁺ MBC counts reduced more than 2-fold systematically had normal smlgG₃⁺ MBC or detectable smlgG₂⁺ MBC counts.

‡One case had detectable switched PCs (55-fold below the LLN) associated with decreased smlgG₁⁺ (>1.5-fold) and smlgG₂⁺ MBC counts and undetectable smlgA⁺ MBCs.

§One case had normal values associated with undetectable switched MBCs.

||Reduced smlgA₁⁺ or smlgA₂⁺ PC counts were observed in all patients with IgAdef except 2 patients who had decreased smlgA⁺ MBC counts.

¶One CVID case showed detectable but reduced switched PC counts.

#When smlgG₂⁺ MBCs were present, these subsets were systematically decreased.

different distributions of smlgB⁺ patients across our CVID-1 to CVID-6 clusters (data not shown).

When considering the 6 CVID clusters, no overall differences were observed among them regarding age (at time of study and at diagnosis) and immunoglobulin serum levels, whereas significant differences were found in the frequency of autoimmunity ($P = .02$), autoimmune cytopenias ($P = .02$), and (a statistical trend) hepatomegaly ($P = .06$). Subsequent pairwise comparisons confirmed a similar age and sex distribution and frequency of recurrent infections (range, 83% to 100%) was observed among the 6 CVID clusters, except for CVID-6 cases, who were significantly older than CVID-2 cases ($P = .04$). In addition, no differences were observed regarding serum immunoglobulin levels at diagnosis and the clinical manifestations of the disease among patients with preserved smlgG₁⁺ MBCs (CVID-1, CVID-2, and CVID-3 cases). Conversely, all CVID-4 cases presented with autoimmunity versus 25% in CVID-1 ($P = .009$), 33% in CVID-2 ($P = .03$), and 50% in CVID-3 ($P = .04$) cases, including a greater frequency of autoimmune cytopenias (67% vs 0% in CVID-1 and CVID-2 cases and 20% in CVID-3 cases, $P \leq .05$) and a tendency ($P > .05$) toward a greater frequency of systemic

autoimmunity (50% vs 25% in CVID-1, 0% in CVID-2, and 10% in CVID-3 cases). Although systemic autoimmunity was not detected among CVID-5 cases ($P = .04$ vs CVID-4 cases), these cases more frequently had other adverse clinical features, such as hepatomegaly (44% vs 5% in CVID-3 cases, $P = .02$), autoimmunity (89% vs 25% in CVID-1 cases [$P = .01$], 33% in CVID-2 cases [$P < .05$], and 50% in CVID-3 cases [$P = .05$]), and cytopenias (44% vs 0% in CVID-1 cases, $P = .05$).

Finally, CVID-6 cases displayed a mixed clinical profile between CVID-4 and CVID-5 cases, with a high frequency of autoimmune cytopenias (50%), as well as hepatomegaly (56%), bronchiectasis (80%), and enteropathy (78%; Fig 5, B). Additionally, CVID-6 cases presented with granulomatous disease more frequently than all other CVID patient groups (30% vs 0% to 15%, $P = .06$; Fig 5, B, and see Table E12 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Current IUIS and ESID guidelines for diagnosis and classification of PADs rely on antibody serum levels, response to

MBCs				PCs	
smlgA ₁ ⁺ MBCs	smlgG ₃ ⁺ MBCs	smlgG ₁ ⁺ MBCs	smlgG ₂ ⁺ MBCs	Switched PCs	smlgA ⁺ PCs
	Normal or ↓<2-fold	Normal or ↓<3-fold	Normal*	Detectable in >80% of cases	↓ or undetectable in >90% of cases
	Normal or ↓<2-fold	Normal or ↓<1.3-fold	Normal*	Detectable in >80% of cases	Undetectable
	Normal or ↓<12-fold	Normal or ↓<65-fold	↓>1.4-fold or undetectable	Undetectable‡	
	↓>1.2-fold or undetectable	↓>13-fold or undetectable	Undetectable	Undetectable	
	↓>15-fold or undetectable	Undetectable	Undetectable	Undetectable	
					↓ or undetectable
					Undetectable
Normal or ↓<2-fold		Normal or ↓<3-fold	Normal or ↓<11-fold	Undetectable¶	
↓>34-fold or undetectable		Normal or ↓<4-fold	Normal or ↓<12-fold	Undetectable	
↓>9-fold or undetectable		↓2-65-fold	↓>4-fold or undetectable	Undetectable	
Undetectable		Undetectable	Undetectable	Undetectable	
↓>20-fold or undetectable		↓>9-fold or undetectable	↓>80-fold or undetectable	Undetectable	
Undetectable		Undetectable	Undetectable	Undetectable	

vaccination, and clinical manifestations of PADs^{4,5,7} in the absence of well-defined genetic markers^{32,33}; in addition, an increased susceptibility to infections and autoimmunity or the existence of affected family members is required for the diagnosis of IgAdef per the ESID⁵ (but not IUIS) criteria. Although the number of affected serum antibody isotypes provides a rough estimation of susceptibility to less (eg, IgAdef) versus more severe (CVID) disease complications in the short term in patients with PADs,^{4,5} it cannot accurately predict the longer-term outcome of individual patients within each PAD subgroup, particularly after immunoglobulin replacement therapy. In these settings B-cell maturation-associated defects identified by using flow cytometry have proved useful for the diagnosis and classification of patients with CVID^{5,18,20} because they more precisely reflect the medium-term B cell-associated protective potential than their corresponding serum antibody isotype levels. However, some of the relationships observed in these studies between B-cell subset defects in blood and clinical manifestations of the disease^{14,15,18} have not been confirmed in other studies.¹⁶ Moreover, patients are usually classified based on relative B-cell subset numbers,^{14,15,18} which might be modified by changes in the other subsets,²⁰ and no

reference values per age group are used,^{14,15,18} which might limit the applicability of these classifications in, for example, children.³⁴ In addition, such B-cell defects have been poorly explored in patients with IgG/Adef and those with IgAdef,^{19,35} whereas the blood distribution of B cells and PCs expressing distinct immunoglobulin subclasses has not been investigated thus far in either patients with CVID or those with IgAdef.

Here, for the first time, we investigated the distribution of MBC and PC subsets that express distinct immunoglobulin isotypes and IgH subclasses in the blood of patients with PADs and correlated the altered immune profiles identified with the diagnostic subgroups and clinical manifestations of the disease. Because the blood B-cell compartment is highly dynamic across a patient's lifetime,^{24,36-39} B-cell defects were defined per age group.

Overall, every patient with CVID, IgG/Adef, or IgAdef studied here showed decreased counts for 1 or more B-cell subsets. This contrasts with previous flow cytometric studies that detected B-cell defects in only 6% to 86% of patients with PADs, namely 77% to 86% in patients with CVID,^{14,15,18,20,23,40,41} 6% to 25% in patients with IgAdef,^{19,35} and 30% in patients with selective IgG subclass deficiency (with or without IgAdef).⁴¹ This high

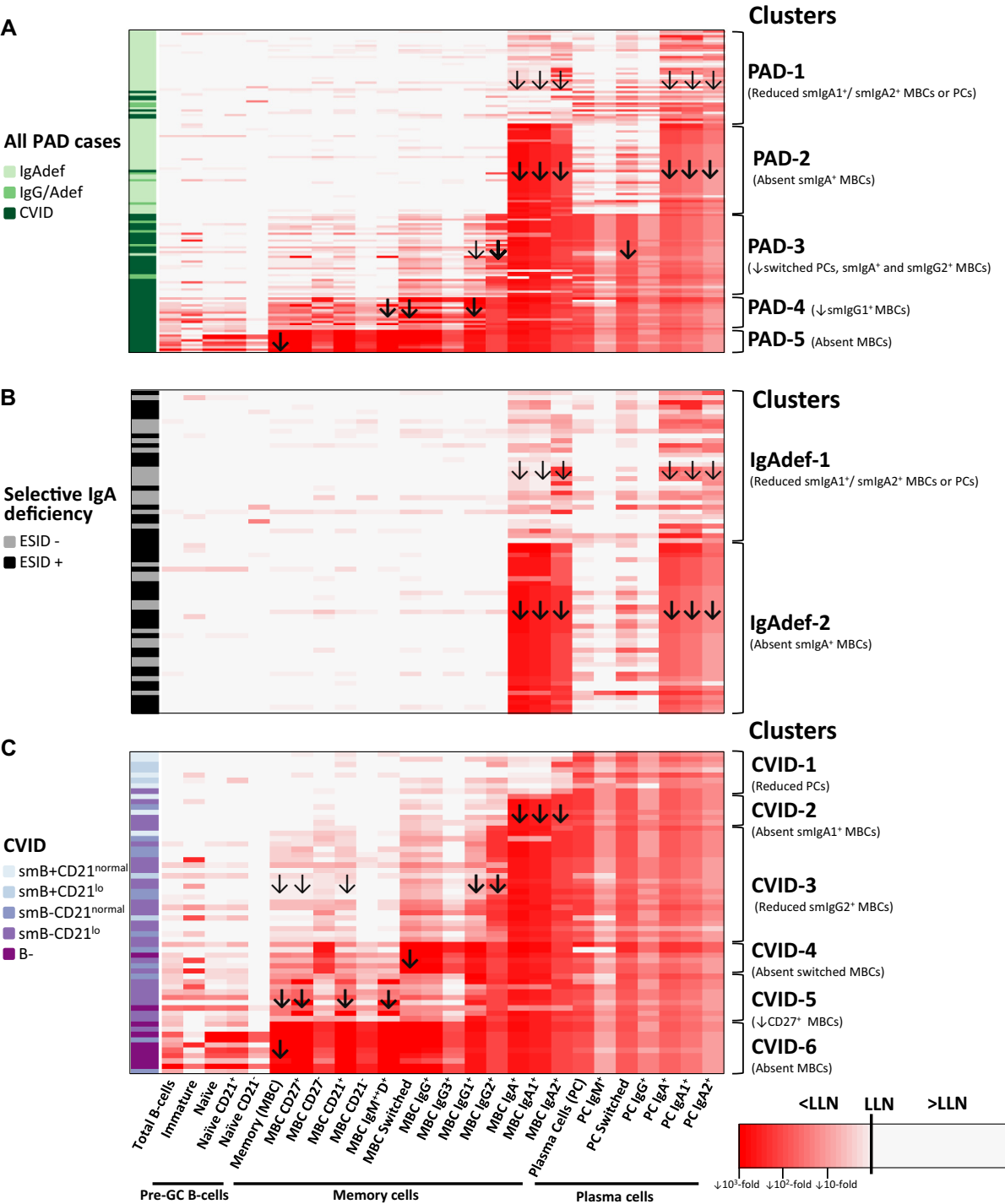
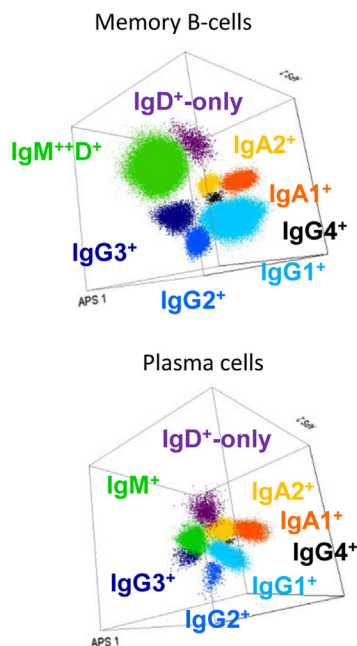


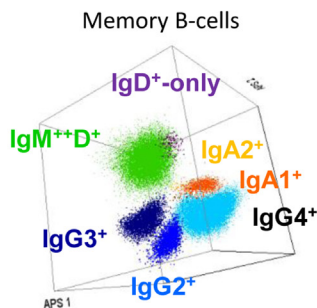
FIG 3. Clustering analysis–based heat map representing all patients with PADs (**A**) and those with IgAdef (**B**) and CVID (**C**) grouped according to their (altered) blood MBC and PC subset immune profiles. Each heat map represents absolute counts of the different B-cell subsets normalized by the LLN in HDs for the corresponding age group (*columns*) versus individual cases (*rows*). Higher red color intensities represent a deeper degree of deficiency in a log₁₀ scale compared with the corresponding age-matched LLN. Individual patients (*rows*) are identified by (1) their IUIS (clinical) diagnosis (Fig 3, A; light green for patients with IgAdef, intermediate green for patients with IgG/Adef, and dark green for patients with CVID); (2) their corresponding ESID IgAdef diagnosis (Fig 3, B), including IgAdef cases that fulfilled (black) or not (gray) the ESID criteria for IgAdef; and (3) CVID EUROclass classification subgroup (Fig 3, C), smB⁺CD21^{normal}, smB⁺CD21^{lo}, smB⁻CD21^{normal}, smB⁻CD21^{lo}, and B⁻ cells from lighter to darker violet. The here-defined PAD-1 to PAD-5 (Fig 3, A), IgAdef-1 and IgAdef-2 (Fig 3, B), and CVID-1 to CVID-6 (Fig 3, C) clusters identified by using the K-means algorithm, as well as the main characteristics of these groups, are depicted at the right side of each heat map. Black arrows indicate those MBC and PC subsets that contributed most to the specific identification of each patient cluster.

A Healthy donor

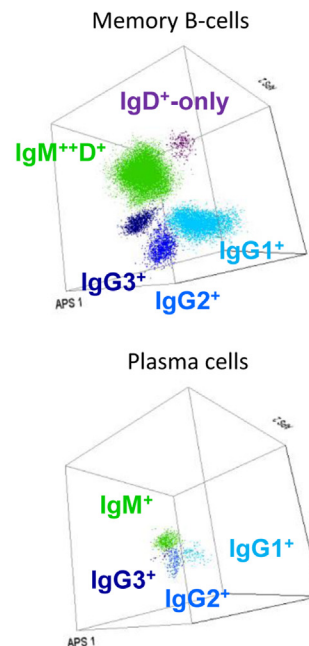


B Selective IgA deficiency

IgAdef-1 cluster

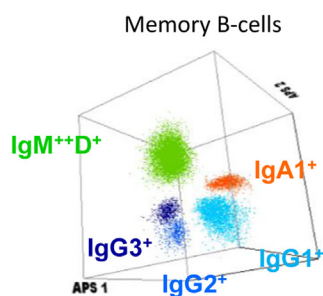


IgAdef-2 cluster

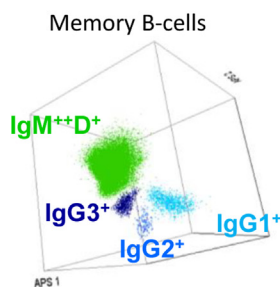


C CVID

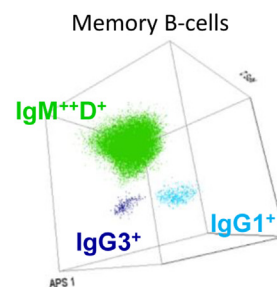
CVID-1 cluster



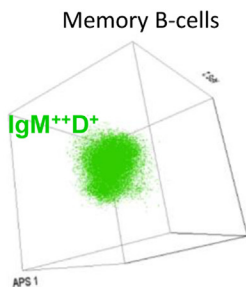
CVID-2 cluster



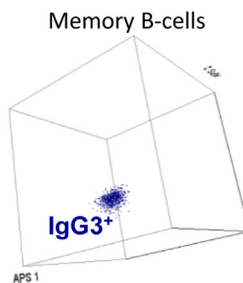
CVID-3 cluster



CVID-4 cluster



CVID-5 cluster



CVID-6 cluster

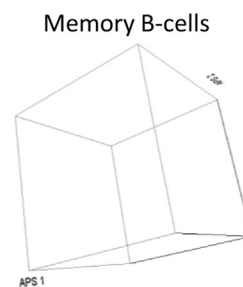


FIG 4. Illustrating dot plot examples of the numeric distribution of blood unswitched and switched MBC and PC subsets expressing different immunoglobulin isotypes and subclasses in a representative HD (**A**) and in representative patients with IgAdef (**B**) and CVID (**C**). Each plot corresponds to 3-dimensional Automated Population Separator (APS) views of principal component 1 (PC1) versus PC2 versus PC3 of the distinct subsets of MBCs and PCs defined by the immunoglobulin isotype and subclass expressed: IgM(D⁺) in green, IgG₁ in light blue, IgG₂ in intermediate blue, IgG₃ in dark blue, IgG₄ in black, IgA₁ in orange, IgA₂ in yellow, and IgD in violet. Additional cases from each group of patients with PADs are displayed in [Figs E5 and E6](#).

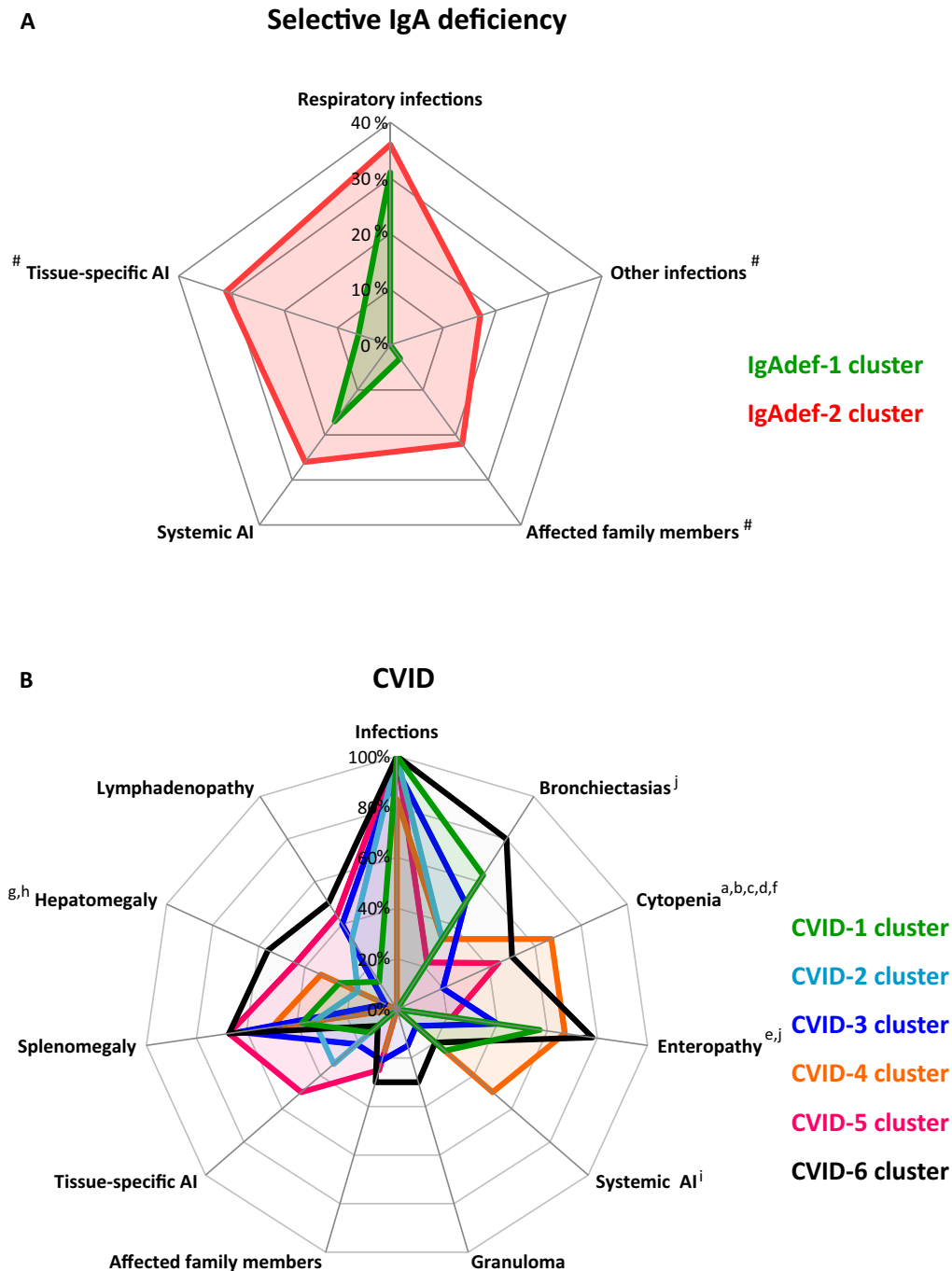


FIG 5. Frequency of distinct clinical manifestations of PADs and the existence (vs absence) of affected family members among the distinct clusters (ie, groups) of patients with IgAdef and those with CVID defined by their distinct patterns of altered blood B-cell and PC subset counts. Radar charts represent the percentage of patients with IgAdef (**A**) and patients with CVID (**B**) presenting with each type of clinical manifestation of the disease and the presence of family members affected by PADs. *Colored lines* indicate the distinct patient groups as defined by clustering analysis based on the B-cell and PC subset defects identified (see also Fig 3). # $P \leq .05$ for patients with IgAdef-1 versus IgAdef-2. ^a $P \leq .05$ for CVID-1 versus CVID-4. ^b $P \leq .05$ for CVID-1 versus CVID-5. ^c $P \leq .05$ for CVID-1 versus CVID-6. ^d $P \leq .05$ for CVID-2 versus CVID-4. ^e $P \leq .05$ for CVID-2 versus CVID-6. ^f $P \leq .05$ for CVID-3 versus CVID-4. ^g $P \leq .05$ for CVID-3 versus CVID-5. ^h $P \leq .05$ for CVID-3 versus CVID-6. ⁱ $P \leq .05$ for CVID-4 versus CVID-5. ^j $P \leq .05$ for CVID-5 versus CVID-6. AI, Autoimmunity.

frequency of B-cell defects most likely reflects the more detailed dissection of the blood B-cell and PC compartments together with the greater sensitivity of our method versus previous methods, use of age-matched reference ranges, or both. However, despite the

high sensitivity of the flow cytometric approach used here (similar to that of minimal residual disease detection by using next-generation flow^{25,42}), several minor B-cell subsets, particularly within the smIgG⁺ PC compartment (ie, smIgG₁⁺ to smIgG₄⁺

PCs), were undetectable (<0.01 cells/ μ L) in 1 or more HDs from 2 or more age groups, limiting their potential diagnostic utility. Consequently, these subsets were not considered in the present study. Acquisition of greater numbers of cells with a greater sensitivity will become feasible soon with the new generation of high-speed cytometers and might overcome this limitation.

Recently produced short-lived blood PCs,⁴³ particularly IgA⁺ PCs, emerged as the most sensitive population for diagnosis of PADs, followed by switched and nonswitched MBCs, with progressively more severe immunologic defects in the spectrum of IgAdef to IgG/Adef and CVID. Thus decreased smIgA₁⁺ and/or smIgA₂⁺ PC counts were found in all patients with PADs, except in 2 patients with IgAdef, who showed reduced smIgA₁⁺ and smIgA₂⁺ MBC counts. In addition, decreased total and/or switched PC counts emerged as a hallmark of CVID, which is in line with previous bone marrow and lymph node findings.^{44,45} Of note, patients with IgAdef showed cellular defects typically restricted to smIgA⁺ PCs and MBCs, despite IgG⁺ MBC and PC counts also being decreased in 15% and 29% of patients with IgAdef versus 30% and 90% patients with IgG/Adef and 90% and 98% patients with CVID. Nevertheless, compared with a previous study²² in which less than 10% of patients with IgAdef had smIgA⁺ MBCs, a greater percentage of our patients with IgAdef showed circulating smIgA⁺ MBCs (50%), PCs (approximately 40%), or both. This discrepancy is probably caused by the greater sensitivity of our EuroFlow strategy and method with 5×10^6 or more (vs 5×10^4) cells analyzed.²²

Our findings are in line with those of previous studies demonstrating S α -switch recombination in blood B cells from 2 of 4 patients with IgAdef.⁴⁶ Interestingly, patients with IgAdef who showed a preserved IgA-switching capacity (IgAdef-1 cases) displayed a milder clinical phenotype, with less risk factors for CVID progression (eg, autoimmunity)⁴⁷ but a similar prevalence of recurrent respiratory tract infections. In addition, they were younger (both at presentation and at time of analysis) than IgAdef-2 cases, which could potentially reflect progressive accumulation of more severe defects in blood IgA⁺ MBCs and PCs in parallel to a greater frequency and severity of clinical manifestations. However, all cases categorized as IgAdef-1 that have been re-evaluated (11/32) after a median follow-up of 25 months (range, 10–52 months) continue to show preserved IgA-switching capacity (data not shown), and none of the 26 IgAdef-2 cases followed since their inclusion in this study have evolved to CVID (median follow-up, 2 years; data not shown). Nonetheless, longer follow-up times are needed to rule out an effect of age at diagnosis on the altered blood B-cell immune profile and clinical manifestations of patients with IgAdef. Altogether, these findings suggest that detailed evaluation of blood B-cell and PC defects might contribute to an improved classification and clinical management of IgAdef patients.

Complete lack of blood switched PCs was the hallmark of CVID. Although reduced switched MBC counts have been extensively reported in patients with CVID,^{14–16,18,20} this is the first time that these cells were dissected at the immunoglobulin subclass level, similar to what is routinely done for serum IgG_{1–4} levels. Progressive deterioration in IgG-switching capacity was observed in MBCs of patients with CVID, which directly correlated with their consecutive location in the *IGHC* gene locus: IgM $<$ IgG₃ $<$ IgG₁ $<$ IgG₂. In line with these results, Piqueras et al¹⁴ showed a similar pattern of reduced mRNA expression for the different immunoglobulin isotypes/immunoglobulin

subclasses: IgM $>$ IgG₃ $\geq$ IgG₁ $>$ IgG₂ $>$ IgA₁ $>$ IgA₂ $>$ IgG₄. At present, it is well established that downstream IgG subclasses are produced, at least in part, by consecutive switching of B-cells during repeated rounds of MBC response,^{48–51} leading to a greater frequency of somatic hypermutation^{48,50,51} and switch regions bearing remnants of indirect class-switching⁵⁰ in cells expressing downstream immunoglobulin isotypes/immunoglobulin subclasses. Interestingly, we recently identified a similar pattern of sequential production of MBC expressing distinct immunoglobulin subclasses during a lifetime.²⁴

These findings, together with recent observations using genome-wide sequencing approaches, suggest that consecutive switching along the *IGHC* locus might deteriorate in patients with PADs, possibly because of combined hypomorphic/deleterious variants,^{52–54} haploinsufficient genes,^{55–58} and epigenetic modifications⁵⁹ involving B-cell response pathways rather than a single genetic defect. Progressive deterioration of sequential class-switching along the *IGHC* locus, along with reduced MBCs and lack of PCs, leads to a progressively more restricted repertoire and decreased functional capacity of MBCs expressing downstream IgG subclasses. Of note, previous flow cytometric approaches typically excluded patients with CVID with less than 1% B cells from further analyses (and subclassification) caused by insufficient B-cell numbers for robust dissection of its major subsets.^{15,18} However, here we were able to identify B cells also in all patients with CVIDs presenting less than 1% B cells, including circulating blood naive B cells in 8 of 9 cases and MBCs in 4 of 9 cases; this is in contrast to *BTK*-deficient patients evaluated with this same highly sensitive approach, who systematically showed undetectable peripheral blood B cells (data not shown).

Among different approaches used to categorize CVID, the EUROclass classification (see Table E11) is the most widely used because of its clinical utility. This classification allows us to relate alterations in the distribution of peripheral blood B-cell subsets with the presence of clinical manifestations, such as a decrease in MBC counts (smB[–] group) and the occurrence of splenomegaly, as also confirmed here (see Table E13 in this article's Online Repository at www.jacionline.org). In this regard our proposed stratification criteria for CVID into CVID-1 to CVID-6 clusters based on MBC immunoglobulin isotype and IgH-subclass subset immune profile in blood also showed association with other disease features (eg, autoimmune cytopenias and hepatomegaly) that have been related to a lower survival in patients with CVID¹⁰ but that did not correlate with the EUROclass classification either in the present or other larger previously reported CVID patient series.¹⁸ In addition, the highly sensitive approach used here allowed detection of low blood MBC and PC counts expressing IgG₁ to IgG₄ and IgA₁ to IgA₂ subclasses, demonstrating that most patients with CVID retain the ability for class-switching, including the great majority ($>70\%$) of smB[–] cases presenting with dramatically reduced numbers of switched MBCs.¹⁸ This is consistent with more laborious functional studies that demonstrated the (residual) capacity of B cells to produce IgG, also among smB[–] patients.²¹ In fact, our EuroFlow strategy for highly sensitive immunoglobulin subclass analysis of blood B cells and PCs identified 6 CVID subgroups with different IgG-switching patterns and clinical profiles, even within smB[–] patients with CVIDs. The 3 clinically milder subgroups included patients capable of producing MBCs of the (first 3) IgM/IgD, IgG₃, and IgG₁ immunoglobulin isotypes/subclasses located upstream in

the *IGHC* locus (independently of smIgG₂⁺ and IgA⁺ MBC counts), who might require less IgG substitution therapy.²¹ In fact, despite patients of all groups having a greater frequency of infection, those within the CVID-1 to CVID-3 groups required less hospital care (data not shown). CVID-4 cases were still capable of CD27⁺ unswitched MBC production and typically presented with cytopenias, such as in patients with hyper-IgM syndromes.⁶⁰⁻⁶² However, they had no PCs (including no IgM⁺ PCs in all but 1 case), and they showed a typical CVID-related serum antibody profile in the absence of *in vitro* functional defects associated with hyper-IgM syndromes (data not shown).⁶³ Interestingly, 3 of 4 patients with rheumatoid arthritis (an immune complex-mediated autoimmune disease⁶⁴) in our series clustered together in the CVID-4 cluster (data not shown), which only has preserved IgM⁺ IgD⁺ MBCs.

The 2 clinically more severe CVID-5 and (particularly) CVID-6 patient subgroups had dramatically decreased CD27⁺ unswitched and switched MBC counts, except for CD27⁺CD21⁺IgG₃⁺ MBC counts, which were found to be almost normal in CVID-5 (but not CVID-6) cases. From the clinical point of view, CVID-5 and CVID-6 cases specifically showed disease symptoms (eg, organomegalies) reflecting an impaired ability to mount germinal center (GC) responses.^{24,50,65} Altogether, these findings suggest that even if the residual CD27⁺CD21⁺smIgG₃⁺ MBCs could offer some immune protection in CVID-5 cases, in CVID-5 and CVID-6 cases the underlying immune dysregulation leads to a polyclonal lymphocytic infiltration of secondary lymphoid tissues previously associated with increased risk for lymphoid malignancy in patients with CVID.⁶⁶ In fact, all patients with hematologic tumors were clustered as CVID-5 and CVID-6 cases (see Table E12). Although it is tempting to hypothesize that such stepwise deterioration of IgG-switching capacity might reflect disease progression, no significant differences in age (or time from diagnosis) were observed among the above CVID patient subgroups (except for CVID-6 cases who were older at the time of analysis than CVID-2 cases and the time from diagnosis, which was greater in CVID-6 vs CVID-2 and CVID-3 cases, data not shown).

The most severe CVID immunologic phenotype, CVID-6, also showed significantly reduced pre-GC B-cell counts, reflecting a markedly defective bone marrow B-cell production.^{18,20,45} Most blood B cells in these patients showed an immature/transitional phenotype, reflecting their premature egress from bone marrow,⁴³ whereas residual naive B cells were enriched in the minor CD21^{lo} naive B-cell subset. Reduced pre-GC B-cell counts, together with the low *in vitro* response of both immature and CD21^{lo} naive B cells,^{43,67} might explain the marked antigen-experienced B-cell defect involving all immunoglobulin isotypes found in CVID-6 cases. In line with previous observations,²⁰ these patients also had decreased naive T CD4⁺ and T CD8⁺ counts versus age-matched HDs and other patients with CVID (data not shown), but they did not fulfill the diagnostic criteria for late-onset combined immunodeficiency.⁵ The potential existence of underlying hypomorphic defects and variants of genes related to the production of lymphocytes (*RAG*, *DCLRE1C*, and *NHEJ1*) previously related to CVID-like clinical phenotypes remains to be more deeply investigated in these CVID-6 cases.⁵²⁻⁵⁴

In summary, detailed dissection of circulating MBCs and PCs in patients with PADs into subsets expressing distinct immunoglobulin subclasses provides complementary information to serum antibody isotype levels and might contribute to a better

understanding of the pathogenesis of PADs and an improved diagnosis, subclassification, and monitoring (particularly in case of immunoglobulin replacement therapy) of the disease. Blood PCs emerged here as the most sensitive diagnostic blood cellular compartment, whereas analysis of blood MBC subsets appeared informative to discriminate patients with different clinical profiles. However, further multicentric studies in large age-matched case-control cohorts are needed to replicate and validate the clinical utility and feasibility of our proposed approach for detailed and sensitive dissection of blood B-cell and PC subsets for the diagnosis and classification of PADs. At the same time, use of EuroFlow databases and tools for automated gating and reporting of flow cytometric data will facilitate its implementation in routine diagnostics.^{68,69}

Key messages

- Evaluation of blood B cells and PCs expressing distinct immunoglobulin subclasses provides a new highly sensitive approach for identification of specific B-cell defects of potential diagnostic relevance in patients with PADs.
- Detailed dissection of blood MBC and PC subsets expressing different immunoglobulin subclasses identifies distinct deficient immune profiles in patients with primary antibody deficiencies, which correlate with both the diagnostic subtype and clinical manifestations of the disease.

REFERENCES

1. Durandy A, Kracker S, Fischer A. Primary antibody deficiencies. *Nat Rev Immunol* 2013;13:519-33.
2. Wood PM. Primary antibody deficiency syndromes. *Curr Opin Hematol* 2010;17:356-61.
3. Wang N, Hammarstrom L. IgA deficiency: what is new? *Curr Opin Allergy Clin Immunol* 2012;12:602-8.
4. Picard C, Bobby Gaspar H, Al-Herz W, Bousfiha A, Casanova J-L, Chatila T, et al. International Union of Immunological Societies: 2017 Primary Immunodeficiency Diseases Committee Report on Inborn Errors of Immunity. *J Clin Immunol* 2018;38:96-128.
5. Abinun M, Albert M, Buckland S.B.C.M., Bustamante J, Cant A, Casanova J-L, et al. ESID registry—working definitions for clinical diagnosis of PID; European Society for Immunodeficiencies. Available at: <https://esid.org/Working-Parties/Registry-Working-Party/Diagnosis-criteria>. Accessed January 10, 2019.
6. Ballou M. Primary immunodeficiency disorders: antibody deficiency. *J Allergy Clin Immunol* 2002;109:581-91.
7. Jolles S. The variable in common variable immunodeficiency: a disease of complex phenotypes. *J Allergy Clin Immunol Pract* 2013;1:545-56.
8. Yazdani R, Azizi G, Abolhassani H, Aghamohammadi A. Selective IgA deficiency: epidemiology, pathogenesis, clinical phenotype, diagnosis, prognosis and management. *Scand J Immunol* 2017;85:3-12.
9. Cunningham-Rundles C. The many faces of common variable immunodeficiency. *Hematology Am Soc Hematol Educ Progr* 2012;2012:301-5.
10. Chapel H, Lucas M, Patel S, Lee M, Cunningham-Rundles C, Resnick E, et al. Confirmation and improvement of criteria for clinical phenotyping in common variable immunodeficiency disorders in replicate cohorts. *J Allergy Clin Immunol* 2012;130:1197-8.
11. Chapel H. Common variable immunodeficiency disorders (CVID)—diagnoses of exclusion, especially combined immune defects. *J Allergy Clin Immunol Pract* 2016;4:1158-9.
12. Bertinchamp R, Gérard L, Boutboul D, Malphettes M, Fieschi C, Oksenhendler E, et al. Exclusion of patients with a severe T-cell defect improves the definition of common variable immunodeficiency. *J Allergy Clin Immunol Pract* 2016;4:1147-57.
13. Bonilla FA, Barlan I, Chapel H, Costa-Carvalho BT, Cunningham-Rundles C, de la Morena MT, et al. International consensus document (ICON): common variable immunodeficiency disorders. *J Allergy Clin Immunol Pract* 2016;4:38-59.

14. Piqueras B, Lavenu-Bombled C, Galicier L, Bergeron-van der Cruyssen F, Mouthon L, Chevret S, et al. Common variable immunodeficiency patient classification based on impaired B cell memory differentiation correlates with clinical aspects. *J Clin Immunol* 2003;23:385-400.
15. Warnatz K, Denz A, Dräger R, Braun M, Groth C, Wolff-Vorbeck G, et al. Severe deficiency of switched memory B cells (CD27(+)IgM(-)IgD(-)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. *Blood* 2002;99:1544-51.
16. Al Kindi M, Mundy J, Sullivan T, Smith W, Kette F, Smith A, et al. Utility of peripheral blood B cell subsets analysis in common variable immunodeficiency. *Clin Exp Immunol* 2012;167:275-81.
17. Piątoś B, Pac M, Siewiera K, Pietrucha B, Klaudel-Dreszler M, Heropolitańska-Pliszka E, et al. Common variable immune deficiency in children—clinical characteristics varies depending on defect in peripheral B cell maturation. *J Clin Immunol* 2013;33:731-41.
18. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. *Blood* 2008;111:77-85.
19. Aghamohammadi A, Abolhassani H, Biglari M, Abolmaali S, Moazzami K, Tabatabaeiyan M, et al. Analysis of switched memory B cells in patients with IgA deficiency. *Int Arch Allergy Immunol* 2011;156:462-8.
20. Driessen GJ, van Zelm MC, van Hagen PM, Hartwig NG, Trip M, Warris A, et al. B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. *Blood* 2011;118:6814-23.
21. Rösel AL, Scheibenbogen C, Schliesser U, Sollwedel A, Hoffmeister B, Hanitsch L, et al. Classification of common variable immunodeficiencies using flow cytometry and a memory B-cell functionality assay. *J Allergy Clin Immunol* 2015;135:198-208.
22. Marasco E, Farroni C, Cascioli S, Marcellini V, Scarsella M, Giorda E, et al. B-cell activation with CD40L or CpG measures the function of B-cell subsets and identifies specific defects in immunodeficient patients. *Eur J Immunol* 2017;47:131-43.
23. Driessen GJ, Dalm VASH, van Hagen PM, Grashoff HA, Hartwig NG, van Rossum AMC, et al. Common variable immunodeficiency and idiopathic primary hypogammaglobulinemia: two different conditions within the same disease spectrum. *Haematologica* 2013;98:1617-23.
24. Blanco E, Pérez-Andrés M, Arriba-Méndez S, Contreras-Sanfeliciano T, Criado I, Pelak O, et al. Age-associated distribution of normal B-cell and plasma cell subsets in peripheral blood. *J Allergy Clin Immunol* 2018;141:2208-19.
25. Flores-Montero J, Sanoja-Flores L, Paiva B, Puig N, García-Sánchez O, Böttcher S, et al. Next Generation Flow for highly sensitive and standardized detection of minimal residual disease in multiple myeloma. *Leukemia* 2017;31:2094-103.
26. Blanco E, Perez-Andres M, Sanoja-Flores L, Wentink M, Pelak O, Martín-Ayuso M, et al. Selection and validation of antibody clones against IgG and IgA subclasses in switched memory B-cells and plasma cells. *J Immunol Methods* 2017 [Epub ahead of print].
27. Kalina T, Flores-Montero J, van der Velden VHJ, Martín-Ayuso M, Böttcher S, Ritgen M, et al. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia* 2012;26:1986-2010.
28. Rudolf-Oliveira RCM, Gonçalves KT, Martignago ML, Mengatto V, Gaspar PC, de Moraes ACR, et al. Determination of lymphocyte subset reference ranges in peripheral blood of healthy adults by a dual-platform flow cytometry method. *Immunol Lett* 2015;163:96-101.
29. R Core Team. R: a language and environment for statistical computing. Vienna (Austria): R Foundation; 2015.
30. MacQueen J. Some methods for classification and analysis of multivariate observations. In: *Proceedings of the Fifth Berkeley Symposium on Mathematical Statistics and Probability, Volume 1: Statistics*. Berkeley (CA): University of California Press; 1967. pp. 281-97.
31. Warnes GR, Bolker B, Bonebakker L, Gentleman R, Liaw WHA, Lumley T, et al. *gplots: various R programming tools for plotting Data*. R Packag. version 2.17.0.2015.
32. Bogaert DJA, Dullaers M, Lambrecht BN, Vermaelen KY, De Baere E, Haerynck F. Genes associated with common variable immunodeficiency: one diagnosis to rule them all? *J Med Genet* 2016;53:575-90.
33. Kienzler A-K, Hargreaves CE, Patel SY. The role of genomics in common variable immunodeficiency disorders. *Clin Exp Immunol* 2017;188:326-32.
34. Schatorjé EJH, Gemen EFA, Driessen GJA, Leuvenink J, van Hout RWNM, van der Burg M, et al. Age-matched reference values for B-lymphocyte subpopulations and CVID classifications in children. *Scand J Immunol* 2011;74:502-10.
35. Nechvatalova J, Pikulova Z, Stikarovska D, Pesak S, Vlkova M, Litzman J. B-lymphocyte subpopulations in patients with selective IgA deficiency. *J Clin Immunol* 2012;32:441-8.
36. Piątoś B, Wolska-Kuśnierz B, Pac M, Siewiera K, Gałkowska E, Bernatowska E. B cell subsets in healthy children: reference values for evaluation of B cell maturation process in peripheral blood. *Cytometry B Clin Cytom* 2010;78:372-81.
37. van Gent R, van Tilburg CM, Nibbelke EE, Otto SA, Gaiser JF, Janssens-Korpela PL, et al. Refined characterization and reference values of the pediatric T- and B-cell compartments. *Clin Immunol* 2009;133:95-107.
38. Morbach H, Eichhorn EM, Liese JG, Girschick HJ. Reference values for B cell subpopulations from infancy to adulthood. *Clin Exp Immunol* 2010;162:271-9.
39. van den Heuvel D, Jansen MAE, Nasserinejad K, Dik WA, van Lochem EG, Bakker-Jonges LE, et al. Effects of nongenetic factors on immune cell dynamics in early childhood: the Generation R Study. *J Allergy Clin Immunol* 2017;139:1923-34.e17.
40. Huck K, Feyen O, Ghosh S, Beltz K, Bellert S, Niehues T. Memory B-cells in healthy and antibody-deficient children. *Clin Immunol* 2009;131:50-9.
41. Bogaert DJA, De Bruyne M, Debacker V, Depuydt P, De Preter K, Bonroy C, et al. The immunophenotypic fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives. *Haematologica* 2017;102:192-202.
42. Theunissen P, Mejstrikova E, Sedek L, van der Sluijs-Gelling AJ, Gaipa G, Bartels M, et al. Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. *Blood* 2017;129:347-57.
43. Perez-Andres M, Paiva B, Nieto WG, Caraux A, Schmitz A, Almeida J, et al. Human peripheral blood B-cell compartments: a crossroad in B-cell traffic. *Cytometry B Clin Cytom* 2010;78(suppl 1):S47-60.
44. Unger S, Seidl M, Schmitt-Graeff A, Böhm J, Schrenk K, Wehr C, et al. Ill-defined germinal centers and severely reduced plasma cells are histological hallmarks of lymphadenopathy in patients with common variable immunodeficiency. *J Clin Immunol* 2014;34:615-26.
45. Ochtrop MLG, Goldacker S, May AM, Rizzi M, Draeger R, Hauschke D, et al. T and B lymphocyte abnormalities in bone marrow biopsies of common variable immunodeficiency. *Blood* 2011;118:309-18.
46. Wang Z, Yunis D, Irigoyen M, Kitchens B, Bottaro A, Alt F. Discordance between IgA switching at the DNA level and IgA expression at the mRNA level in IgA-deficient patients. *Clin Immunol* 1999;91:263-70.
47. Aghamohammadi A, Mohammadi J, Parvaneh N, Rezaei N, Moin M, Espanol T, et al. Progression of selective IgA deficiency to common variable immunodeficiency. *Int Arch Allergy Immunol* 2008;147:87-92.
48. Jackson KJL, Wang Y, Collins AM. Human immunoglobulin classes and subclasses show variability in VDJ gene mutation levels. *Immunol Cell Biol* 2014;92:729-33.
49. Collins AM, Jackson KJL. A temporal model of human IgE and IgG antibody function. *Front Immunol* 2013;4:235.
50. Berkowska MA, Driessen GJA, Bikos V, Grosserichter-Wagener C, Stamatoopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood* 2011;118:2150-8.
51. de Jong BG, IJsspeert H, Marques L, van der Burg M, van Dongen JJ, Loos BG, et al. Human IgG2- and IgG4-expressing memory B cells display enhanced molecular and phenotypic signs of maturity and accumulate with age. *Immunol Cell Biol* 2017;95:744-52.
52. Volk T, Pannicke U, Reisli I, Bulashevskaya A, Ritter J, Björkman A, et al. DCLRE1C (ARTEMIS) mutations causing phenotypes ranging from atypical severe combined immunodeficiency to mere antibody deficiency. *Hum Mol Genet* 2015;24:7361-72.
53. Abolhassani H, Cheraghi T, Rezaei N, Aghamohammadi A, Hammarstrom L. Common variable immunodeficiency or late-onset combined immunodeficiency: a new hypomorphic JAK3 patient and review of the literature. *J Invest Allergol Clin Immunol* 2015;25:218-20.
54. Abolhassani H, Wang N, Aghamohammadi A, Rezaei N, Lee YN, Frugoni F, et al. A hypomorphic recombination-activating gene 1 (RAG1) mutation resulting in a phenotype resembling common variable immunodeficiency. *J Allergy Clin Immunol* 2014;134:1375-80.
55. Kuehn HS, Boisson B, Cunningham-Rundles C, Reichenbach J, Stray-Pedersen A, Gelfand EW, et al. Loss of B cells in patients with heterozygous mutations in IKAROS. *N Engl J Med* 2016;374:1032-43.
56. Schubert D, Bode C, Kenefick R, Hou TZ, Wing JB, Kennedy A, et al. Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. *Nat Med* 2014;20:1410-6.
57. Fliegau M, Bryant VL, Frede N, Slade C, Woon S-T, Lehnert K, et al. Haploinsufficiency of the NF-kappaB1 subunit p50 in common variable immunodeficiency. *Am J Hum Genet* 2015;97:389-403.
58. Tuijnburg P, Lango Allen H, Burns SO, Greene D, Jansen MH, Staples E, et al. Loss-of-function nuclear factor kappaB subunit 1 (NFKB1) variants are the most common monogenic cause of common variable immunodeficiency in Europeans. *J Allergy Clin Immunol* 2018;142:1285-96.

59. Rodríguez-Cortez VC, del Pino-Molina L, Rodríguez-Ubreva J, Ciudad L, Gómez-Cabrero D, Company C, et al. Monozygotic twins discordant for common variable immunodeficiency reveal impaired DNA demethylation during naïve-to-memory B-cell transition. *Nat Commun* 2015;6:7335.
60. Leven EA, Maffucci P, Ochs HD, Scholl PR, Buckley RH, Fuleihan RL, et al. Hyper IgM Syndrome: a Report from the USIDNET Registry. *J Clin Immunol* 2016;36:490-501.
61. Qamar N, Fuleihan RL. The hyper IgM syndromes. *Clin Rev Allergy Immunol* 2014;46:120-30.
62. de la Morena MT. Clinical phenotypes of hyper-IgM syndromes. *J Allergy Clin Immunol Pract* 2016;4:1023-36.
63. O'Gorman MR, Zaas D, Paniagua M, Corrochano V, Scholl PR, Pachman LM. Development of a rapid whole blood flow cytometry procedure for the diagnosis of X-linked hyper-IgM syndrome patients and carriers. *Clin Immunol Immunopathol* 1997;85:172-81.
64. Derksen VFAM, Huizinga TWJ, van der Woude D. The role of autoantibodies in the pathophysiology of rheumatoid arthritis. *Semin Immunopathol* 2017;39:437-46.
65. Fecteau JF, Côté G, Néron S. A new memory CD27-IgG+ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation. *J Immunol* 2006;177:3728-36.
66. Chapel H, Lucas M, Lee M, Bjorkander J, Webster D, Grimbacher B, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. *Blood* 2008;112:277-86.
67. Isnardi I, Ng Y, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement receptor 2/CD21- human naïve B cells contain mostly autoreactive unresponsive clones. *Blood* 2010;115:5026-36.
68. Lhermitte L, Mejstrikova E, van der Sluijs-Gelling AJ, Grigore GE, Sedek L, Bras AE, et al. Automated database-guided expert-supervised orientation for immunophenotypic diagnosis and classification of acute leukemia. *Leukemia* 2018;32:874-81.
69. van der Burg M, Kalina T, Pérez-Andrés M, Vlokova M, López-Granados E, Blanco E, et al. The EuroFlow PID orientation tube for flow cytometric diagnostic screening of primary immunodeficiencies of the lymphoid system. *Front Immunol* 2019, eCollection.