The immunophenotypic fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives

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Haematologica 2017
Volume 102(1):192-202

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Received: May 13, 2016.

Accepted: September 8, 2016.

Pre-published: September 15, 2016.

doi:10.3324/haematol.2016.149112

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/102/1/192

ABSTRACT

The etiology of primary antibody deficiencies is largely unknown. Beside rare monogenic forms, the majority of cases seem to have a more complex genetic basis. Whereas common variable immunodeficiency has been investigated in depth, there are only a few reports on milder primary antibody deficiencies such as idiopathic primary hypogammaglobulinemia and IgG subclass deficiency. We performed flow cytometric immunophenotyping in 33 patients with common variable immunodeficiency, 23 with idiopathic primary hypogammaglobulinemia and 21 with IgG subclass deficiency, as well as in 47 asymptomatic first-degree family members of patients and 101 unrelated healthy controls. All three groups of patients showed decreased memory B- and naïve T-cell subsets and decreased B-cell activating factor receptor expression. In contrast, circulating follicular helper T-cell frequency and expression of inducible T-cell co-stimulator and chemokine receptors were only significantly altered in patients with common variable immunodeficiency. Asymptomatic first-degree family members of patients demonstrated similar, albeit intermediate, alterations in naïve and memory B- and T-cell subsets. About 13% of asymptomatic relatives had an abnormal peripheral B-cell composition. Furthermore, asymptomatic relatives showed decreased levels of $CD4^+$ recent thymic emigrants and increased central memory T cells. Serum IgG and IgM levels were also significantly lower in asymptomatic relatives than in healthy controls. We conclude that, in our cohort, the immunophenotypic landscape of primary antibody deficiencies comprises a spectrum, in which some alterations are shared between all primary antibody deficiencies whereas others are only associated with common variable immunodeficiency. Importantly, asymptomatic first-degree family members of patients were found to have an intermediate phenotype for peripheral B- and T-cell subsets.

Introduction

Primary antibody deficiencies (PAD) are the most prevalent primary immune deficiencies and are characterized by impaired production of one or more immunoglobulin (Ig) isotypes. Since the description of Bruton agammaglobulinemia in 1952,[1] our understanding of PAD has improved substantially.[2] Nonetheless, the etiology of many PAD remains largely unknown.[2] Common variable immunodeficiency (CVID) is one of the most common PAD and is a clinically and immunologically heterogeneous disorder.[2,3] Indeed, the definition of CVID is a topic of ongoing debate. The term CVID was introduced in 1971 to distinguish less well-defined PAD from those with a consistent phenotype and inheritance.[4] In 1999, CVID was redefined by the European Society for Immunodeficiencies (ESID) and the Pan-American Group for Immunodeficiency (PAGID): a marked decrease in serum IgG with a marked decrease in serum IgM and/or IgA, poor antibody response to vaccines and/or absent isohemagglutinins, and exclusion of secondary or other defined causes of hypogammaglobulinemia.[5] About 15 years later, two different revisions of the ESID/PAGID 1999 criteria were made: the Ameratunga 2013 criteria[6] and the revised ESID registry 2014 criteria.[7] Remarkably, both revisions proposed reduced (switched) memory B cells as an alternative criterion for impaired vaccine responses.[7] The revised ESID registry 2014 criteria additionally stated that both IgG and IgA must be decreased to confer a diagnosis of CVID.[7] However, not all practitioners agree on the obligatory decrease in IgA.[3] In 2016, an international consensus statement on CVID proposed less stringent diagnostic criteria, closely resembling the ESID/PAGID 1999 criteria and not including a reduction in memory B cells.[3]

CVID patients have an increased susceptibility to infections, predominantly of the respiratory tract.[3,8] Moreover, they are prone to developing non-infectious complications such as autoimmunity, polyclonal lymphoproliferation, and malignancies.[3,8] Patients with hypogammaglobulinemia showing clinical features reminiscent of CVID but not fulfilling all laboratory criteria are often encountered in daily practice.[2,3] For the latter group of patients, consensus diagnostic criteria, prevalence rates and clinical and immunophenotypic data are scarce.[9] These patients are henceforth referred to as having idiopathic primary hypogammaglobulinemia (IPH),[9] although various other terminologies have also been used such as CVID-like disorders[10] and unclassified hypogammaglobulinemia.[11] Patients with a marked decrease in one or more IgG subclasses but normal total IgG are diagnosed with IgG subclass deficiency (IgGSD).[12] Since IgG1 constitutes 66% of total IgG, IgG1 deficiency typically results in decreased total IgG.[12] IgG4 only forms a minor portion of total IgG (3%), and isolated IgG4 deficiency is usually asymptomatic.[12] Patients with isolated IgG2 and/or IgG3 deficiency can suffer from recurrent infections and some develop non-infectious, especially autoimmune, complications.[12,13] However, subnormal Ig isotype levels and in particular subnormal IgG subclass levels are not always accompanied by a clinical phenotype.[2,13] On the other hand, milder PAD phenotypes can sometimes evolve into a complete CVID phenotype over time.[3]

There is increasing evidence that besides rare monogenic forms, the majority of PAD are complex disorders in which multiple genes and/or environmental factors determine the final phenotype.[3] This has been best documented for CVID.[14] A monogenic cause has only been identified in 2-10% of cases of CVID (e.g. *ICOS*, *CD19*, *CD21*), and in several families the same primary genotype resulted in a large phenotypic variability from asymptomatic over milder PAD forms to complete CVID (e.g. *TACI*, *BAFF-R*, *NFKB1*, *CTLA4*).[14] Furthermore, it has been recently reported that sporadic patients with CVID have variants in multiple CVID-associated genes and an enrichment of variants in pathways important in B-cell function, suggesting a polygenic nature of CVID.[15] In addition, B cells in CVID patients have been shown to have DNA methylation alterations in genes critical for B-cell function, implicating a role for epigenetic factors in the pathogenesis of CVID.[16]

While the immunological phenotype of CVID has been investigated in depth, there are only few reports on IPH and IgGSD and none on cohorts of asymptomatic PAD family members. We, therefore, performed a detailed immunophenotypic analysis of CVID, IPH and IgGSD patients as well as asymptomatic first-degree relatives of the PAD patients included in the study (asymptomatic family members, AFM) and unrelated healthy controls (HC). With this study, we demonstrate for the first time in a relatively large cohort that the spectrum of immunophenotypic alterations ranges from AFM through milder PAD entities, such as IPH and IgGSD, to the most severe entity, CVID, supporting the notion of a multifactorial origin of PAD.

Methods

Study population

The study populations consisted of 33 patients with CVID, 23 with IPH, 21 with IgGSD, 47 AFM, and 101 HC. Patients were recruited between October 2013 and November 2015 at the Departments of Pediatrics, Hematology and Pulmonology at Ghent University Hospital. Clinical data were retrospectively collected from patients' medical records. CVID was defined as decreased [from hereon always meaning: at least 2 standard deviations (SD) below the age-adjusted mean according to the local laboratory reference values, measured at least twice] IgG, decreased IgA and/or IgM, and poor antibody responses to protein and/or polysaccharide vaccines.[3] IPH was defined as decreased IgG, normal or decreased IgA and/or IgM, and good antibody responses to protein and/or polysaccharide vaccines. IgGSD was defined as decreased IgG2 and/or IgG3, normal total IgG and IgM, normal or decreased IgA, and good or poor antibody responses to protein and/or polysaccharide vaccines. Patients with other defined causes of antibody deficiency and/or profound T-cell defects, as determined by the ESID registry criteria for CVID (http://esid.org/Working-Parties/Registry/Diagnosis-criteria), were excluded from the study. At the time of analysis, 32 of 33 CVID patients, 21 of 23 of those with IPH, and 15 of 21 patients with IgGSD were receiving regular immunoglobulin replacement therapy. One of the CVID patients was being given tacrolimus and rapamycin as maintenance immunosuppressive drugs after liver transplantation; the remaining patients were not on immunosuppression.

AFM were defined as first-degree family members of included patients who did not suffer from recurrent infections, autoimmunity or other signs of immune deficiency or dysregulation. HC were recruited from hospital and university staff and their children. Exclusion criteria for HC were pregnancy, medical conditions that affect the immune system, and treatment with immuno-

suppressive or immune-modulating drugs. To obtain medical information regarding AFM and HC, a detailed clinical history was taken at the time of their inclusion in the study.

The study was approved by the ethical committee of Ghent University Hospital (2012/593). All reported subjects (or their parents in the case of pediatric subjects) provided written informed consent to participation in the study, in accordance with the Helsinki Declaration of 1975.

Serum immunoglobulins, flow cytometry, unsupervised computational clustering analysis, and statistics

Details on measurement of serum Ig levels and absolute white blood cell counts, flow cytometric analysis of peripheral blood mononuclear cells, unsupervised computational clustering methods, the statistical analysis and conversion into z-scores are provided in the *Online Supplementary Methods*.

Results

Characteristics of the study population

A comprehensive analysis was performed of the clinical and immunological phenotype of 77 patients with PAD (33 with CVID, 23 with IPH, and 21 with IgGSD), 47 AFM, and 101 HC. The demographics of the study population are shown in Table 1. One IPH patient was born from a consanguineous marriage; both parents were asymptomatic and included as AFM.

Median levels of IgG at diagnosis were significantly lower in CVID than in IPH (Figure 1A). As expected from the clinical definition, median IgG levels were also significantly lower in CVID than in IgGSD, and median IgA and IgM levels were significantly lower in CVID than in either IPH or IgGSD (Figure 1A). None of the HC or AFM had severely decreased Ig levels (i.e. z-score below 2.5), except for one AFM (mother of an IPH patient) who had a total IgG level of 3.5 SD below the age-adjusted mean (Figure 1A). Interestingly, mean IgG and IgM levels were significantly lower in AFM than in HC (Figure 1A).

Details on absolute white blood cell counts from routine laboratory evaluations are provided in the *Online Supplementary Results*, *Online Supplementary Figure S1* and *Online Supplementary Table S2*. Patients' clinical characteristics are summarized in *Online Supplementary Table S3*. At the time of analysis, all patients suffered from recurrent infections, predominantly of the respiratory tract. A higher proportion of IPH patients developed warts or fungal infections (8.7% and 17.4%, respectively) compared to CVID patients (3.0% and 3.0%, respectively) and IgGSD patients (0% and 9.5%, respectively), although these differences were not statistically significant (P=0.312 and P=0.184, respectively). Bronchiectasis was seen in 54.2% of CVID patients, 30.8% of IPH patients and 16.7% of IgGSD patients, which fits with their gradually milder defects in Ig production (*Online Supplementary Table S3*). The overall occurrence of non-infectious disease-related complications was significantly higher in CVID patients than in IPH patients (P=0.031), and also higher in patients with CVID than in those with IgGSD although in this case the difference did not reach statistical significance (P=0.070) (Figure 1B). In particular, autoimmune manifestations (symptoms related to autoimmune disease) and benign lymphadenopathy (cervical, mediastinal and/or abdominal lymph nodes >1 cm diameter, detected at least twice on medical imaging) were significantly greater in CVID than in IPH or IgGSD (Figure 1C).

Abnormalities in peripheral B-cell subsets

An abnormal distribution of peripheral B-cell subsets is one of the best-established features in CVID.[3] We, therefore, examined peripheral B-cell subsets extensively. A representative gating strategy is shown in *Online Supplementary Figure S2*. Mean total B-cell percentages were comparable between all groups of patients (*Online Supplementary Figure S3*), but absolute B-cell numbers were significantly lower in CVID than in IPH or IgGSD (*Online Supplementary Figure S1*). On average, the B-cell phenotype was most deviant in CVID with significantly decreased IgD^+CD27^+ marginal zone-like B cells, IgD^-CD27^+ memory B cells and plasmablasts and significantly increased naïve, transitional and $CD21^{low}$ B cells (Figure 2). Although differences did not always reach statistical significance, IPH and IgGSD patients as well as AFM showed a similar trend towards a decrease in antigen-experienced B-cell subsets and an increase in immature/naïve B-cell subsets (Figure 2). The levels of IgD^-CD27^+ memory B cells, in particular, were significantly decreased in all three groups of patients compared to the levels in HC. $IgD^-CD27^+IgG^+$ memory B cells were reduced in CVID and IPH but not in IgGSD, corresponding with the inclusion criteria. Remarkably, $IgD^-CD27^+IgG^+$ memory B-cell levels were also significantly lower in AFM than in HC (Figure 2). $IgD^-CD27^+IgA^+$ memory B-cell levels were significantly lower in CVID but unexpectedly higher in IgGSD than in HC (Figure 2). As expected, $IgD^-CD27^+IgG^+$ and IgA^+ memory B-cell levels showed strong positive correlations with serum IgG and IgA levels, respectively (both P<0.001, *data not shown*). Mean levels of $IgD^-CD27^+IgM^+$ memory B cells were significantly higher in CVID and IPH, probably reflecting the relative decrease in IgG^+ and IgA^+ memory B cells (*Online Supplementary Figure S3*). No significant differences were found in plasma cells or $IgM^+IgD^+CD27^+$ marginal zone-like B cells (*Online Supplementary Figure S3*).

Based on the composition of peripheral B-cell subsets, Driessen *et al.* distinguished five patterns indicating at what stage (early to late) in peripheral B-cell development a defect may be located, as explained in the legend to Figure 2B.[17] Here, study subjects were categorized using age-adjusted B-cell subset proportions (z-scores) instead of absolute counts. All HC and the majority of AFM, IPH and IgGSD showed a normal peripheral B-cell composition (pattern 5), whereas CVID patients were divided over the five patterns (Figure 2B). B-cell patterns 1 and 2 were only seen in CVID and IgGSD patients, whereas patterns 3 and 4 were observed in CVID, IPH, and IgGSD patients and, remarkably, even in AFM (Figure 2B). The distribution of B-cell patterns was significantly different in patient groups

Table 1. Demographics of the study population.

	Number (male/female)	Age at inclusion in years (mean, range)
HC	101 (44/57)	30.8 (4.5 - 82.2)
AFM	47 (23/24)	45.1 (6.2 - 71.0)
CVID	33 (19/14)	28.0 (7.7 - 83.2)
IPH	23 (9/14)	35.6 (10.3 - 86.5)
IgGSD	21 (3/18)	39.0 (11.9 - 65.0)

AFM: asymptomatic family member; CVID: common variable immunodeficiency; HC: healthy control; IgGSD: IgG subclass deficiency; IPH: idiopathic primary hypogammaglobulinemia.

and AFM compared to HC (all $P \leq 0.001$). In addition, B-cell patterns in CVID differed significantly from those in IPH, IgGSD and AFM (all $P<0.01$). B-cell patterns were not significantly different between IPH, IgGSD and AFM.

Abnormalities in B-cell co-stimulatory molecules and chemokine receptors

Defects in B-cell co-stimulatory molecules and chemokine receptors have been associated with CVID.[3] In this study we examined the expression of various co-stimulatory molecules [B-cell activating factor belonging to the TNF family - receptor (BAFF-R), human leukocyte antigen – antigen D-related (HLA-DR), cluster of differentiation 40 (CD40) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI)] and chemokine receptors [CXC-chemokine receptor 5 (CXCR5) and CC-chemokine receptor 7 (CCR7)].

Average BAFF-R expression on B cells was severely decreased in CVID and moderately decreased in IPH and IgGSD (Figure 2C). Mean HLA-DR expression on B cells

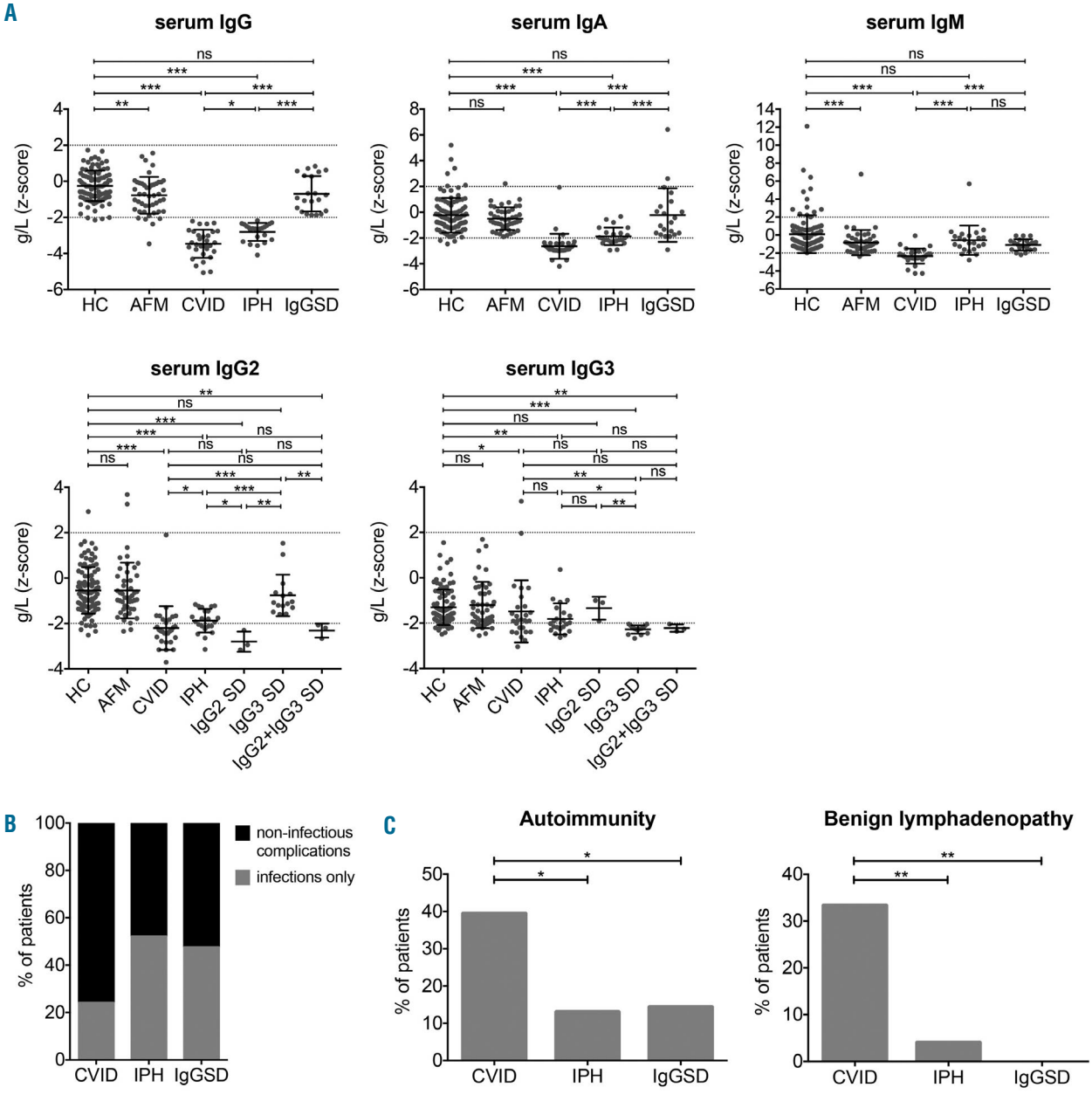

Figure 1. Serum immunoglobulin levels and clinical phenotype. (A) Serum immunoglobulin (Ig) levels at diagnosis (CVID, IPH, IgGSD) or at inclusion in the study (HC, AFM). For IgG2 and IgG3 levels, IgGSD patients were subdivided in accordance with the decreased IgG subclasses. Graphs represent mean ± SD. Ig values were expressed as z-scores to adjust for age. Values normal for age have a z-score between -2 and 2 (dotted lines). (B) The clinical phenotype of CVID, IPH and IgGSD patients. Non-infectious complications included benign lymphadenopathy, lymphocytic interstitial pneumonitis, granulomata, splenomegaly, hepatomegaly, unexplained enteropathy and/or autoimmunity. (C) Prevalence of autoimmune manifestations (symptoms related to autoimmune disease) and benign lymphadenopathy (cervical, mediastinal and/or abdominal lymph nodes >1 cm diameter, detected at least twice on medical imaging) in CVID, IPH, and IgGSD patients. AFM: asymptomatic family member; CVID: common variable immunodeficiency; HC: healthy control; IgGSD: IgG subclass deficiency; IPH: idiopathic primary hypogammaglobulinemia. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons).

was markedly elevated in CVID and to a lesser extent in IgGSD (*Online Supplementary Figure S4*). All groups of patients had similarly increased mean CD40 expression on B cells compared to the mean expression on B cells from HC (*Online Supplementary Figure S4*). Mean TACI expression was lower in IgGSD but was not aberrant in the other groups of patients (*Online Supplementary Figure S4*). On average, AFM did not have deviant expression of the investigated co-stimulatory molecules (Figure 2C, *Online Supplementary Figure S4*).

CXCR5 and CCR7 are implicated in B- and T-cell migration to secondary lymphoid organs and in differential localization of these cells in follicles.[18] In our cohort, CXCR5 and CCR7 expression on B cells was severely reduced in CVID but not in the other groups of patients or in AFM (Figure 3A). Furthermore, CXCR5 and CCR7 expression on B cells was positively correlated with levels of IgD$^-$CD27$^+$ memory B cells, IgD$^-$CD27$^+$IgG$^+$ and IgA$^+$ memory B cells and IgD$^+$CD27$^+$ marginal zone-like B cells, and negatively correlated with CD21^{low} B-cell levels (Figure 3B).

Abnormalities in the T-cell compartment

Alongside B-cell abnormalities, multiple alterations have been described in the T-cell compartment of CVID patients.[3] *Online Supplementary Figure S5* depicts a representative gating strategy of the here-examined T-cell subsets.

CVID patients showed, on average, skewing towards memory T-cell subsets evidenced by significantly increased central memory T (TCM) cells and decreased naïve T cells and CD4$^+$ recent thymic emigrants (RTE) (Figure 4). A similar though less pronounced trend was also observed in AFM and patients with IPH: both groups

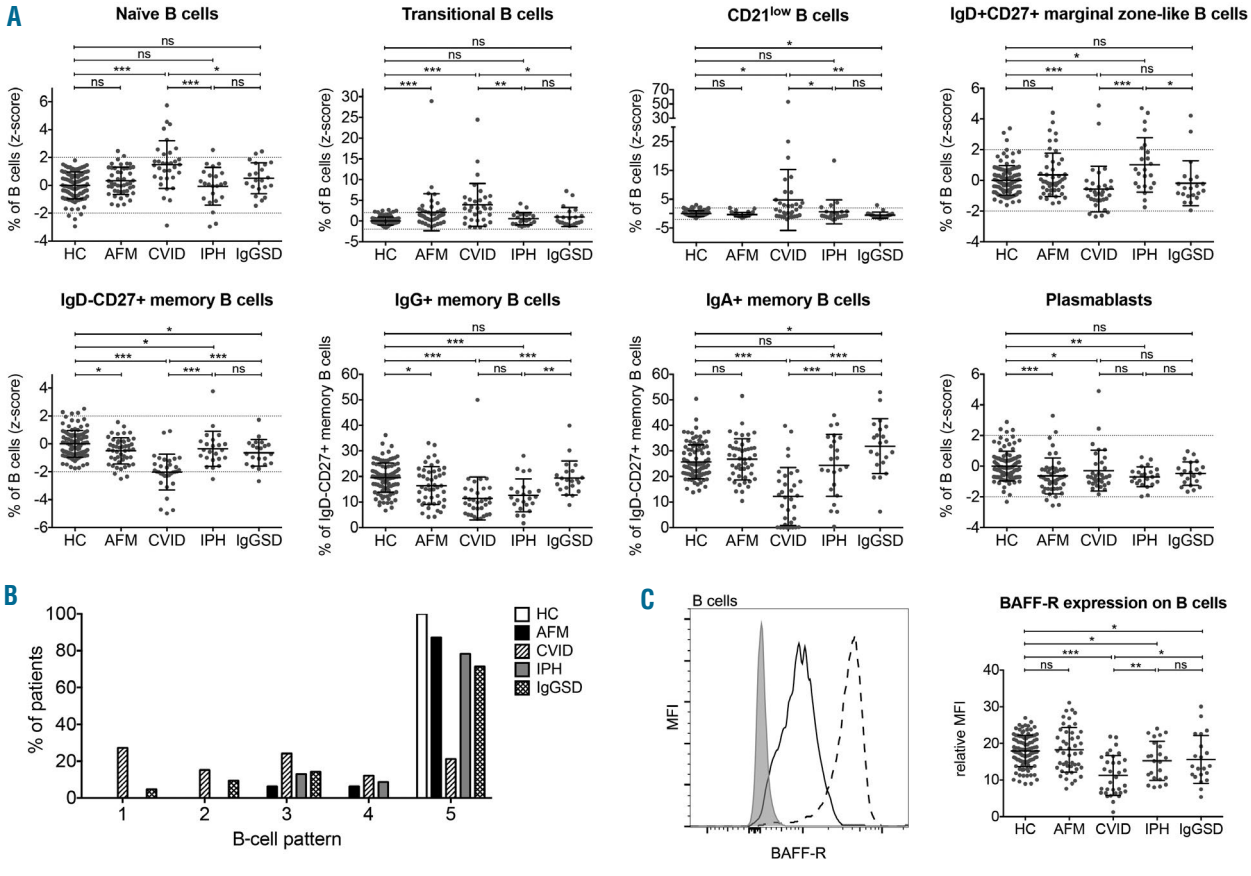

Figure 2. Peripheral B-cell subsets and BAFF-R expression on B cells. (A) Total B cells were gated as CD19$^+$CD20$^+$ in living cells. Within B cells, naïve B cells were gated as IgD$^+$CD27$^-$, transitional B cells as CD24^{high}CD38^{high}, CD21^{low} B cells as CD21^{low}CD38^{low}, marginal zone-like B cells as IgD$^+$CD27$^+$, memory B cells as IgD$^-$CD27$^+$, and plasmablasts as CD38^{high}CD24$^-$. Graphs represent mean ± SD. B-cell subsets were expressed as z-scores to adjust for age if applicable. Values normal for age have a z-score between -2 and 2 (dotted lines). (B) B-cell patterns according to Driessen *et al.*[17] using age-adjusted B-cell subset proportions (z-scores). B-cell pattern 1: reduced transitional and IgD$^-$CD27$^+$ memory B cells, indicative of a defect in B-cell production and germinal center function. B-cell pattern 2: normal transitional B cells and reduced naïve, IgD$^+$CD27$^+$ marginal zone-like and IgD$^-$CD27$^+$ memory B cells, indicative of a defect in early B-cell maturation or survival. B cell pattern 3: reduced IgD$^+$CD27$^+$ marginal zone-like and IgD$^-$CD27$^+$ memory B cells, indicative of a defect in B-cell activation and proliferation. B-cell pattern 4: isolated reduction of IgD$^-$CD27$^+$ memory B cells, indicative of a defect in germinal center function. B-cell pattern 5: normal IgD$^+$CD27$^+$ marginal zone-like and IgD$^-$CD27$^+$ memory B cells; in individuals with decreased serum immunoglobulin levels, this could be indicative of a post-germinal center defect. (C) BAFF-R expression on B cells. The graph represents mean ± SD. Representative flow cytometric analysis is shown on the left. The full black line represents a CVID patient, the dashed black line represents a HC. Relative mean fluorescence intensity (MFI) was calculated by dividing the MFI of the positive population (black line) by the MFI of the "fluorescence minus one" (FMO) population (gray). AFM, asymptomatic family member; CVID, common variable immunodeficiency; HC, healthy control; IgGSD, IgG subclass deficiency; IPH, idiopathic primary hypogammaglobulinemia. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons).

displayed significantly increased CD8⁺ TCM cells, and AFM also had increased CD4⁺ TCM cells and decreased CD4⁺ RTE (Figure 4). In contrast, IgGSD patients showed skewing towards naïve T-cell subsets evidenced by significantly increased levels in naïve T cells and CD4⁺ RTE, without significant differences in memory T-cell subsets (Figure 4).

HLA–DR⁺CD8⁺ T-cell counts were significantly elevated in CVID patients and AFM compared to HC. CVID patients also had higher numbers of HLA–DR⁺CD4⁺ T cells (*Online Supplementary Figure S6*). This is analogous to the increase in mean HLA-DR expression on B cells in CVID (*Online Supplementary Figure S4*). We found no important differences in regulatory T cells and double negative T cells (*Online Supplementary Figure S6*).

Follicular helper T (Tfh) cells orchestrate the formation of high-affinity antibody-producing plasma cells and memory B cells, and inducible T-cell co-stimulator (ICOS) is a key molecule in the differentiation and function of Tfh cells.[19] In our cohort, mean levels of circulating Tfh (cTfh) cells were significantly higher in CVID patients than in HC (Figure 5A). Within the CVID group, a higher proportion of cTfh cells expressed ICOS in the resting condition (Figure 5A). Upon stimulation, however, ICOS expression on CD4⁺ T cells was markedly lower in CVID patients than in HC, despite adequate T-cell activation as evidenced by the normal CD69 upregulation (Figure 5B). A similarly decreased ICOS upregulation was also seen on CD8⁺ T cells in CVID patients (*Online Supplementary Figure S7*). IPH and IgGSD patients and AFM did not demonstrate alterations in mean cTfh cell percentages or ICOS expression (Figure 5A,B).

cTfh cells in CVID patients displayed a highly significant reduction in mean CCR7 expression (Figure 5C). Additionally, CVID patients had decreased CCR7 expression on total T cells and naïve, RTE and central memory T-cell subsets (*Online Supplementary Figure S8*), analogous to the decrease in mean CCR7 expression levels on B cells (Figure 3). IPH and IgGSD patients and AFM demonstrated normal mean CCR7 expression on cTfh cells and naïve and memory T-cell subsets (Figure 5C and *Online Supplementary Figure S8*). Interestingly, CCR7 expression on cTfh cells was significantly correlated with the level of cTfh cells (Figure 5D). Furthermore, CCR7 expression on total T cells was positively correlated with levels of naïve T-cell subsets, and negatively correlated with levels of memory T-cell subsets except for CD8⁺ TCM cells (*Online Supplementary Figure S9*).

Abnormalities in natural killer cells and dendritic cells

Besides B and T lymphocytes, innate immune cells have also been shown to be affected in CVID patients.[3] We, therefore, screened for relevant innate immune cell subsets (the gating strategy is shown in *Online Supplementary Figure S10*). On average, levels of total natural killer (NK) cells were significantly lower in all PAD groups compared

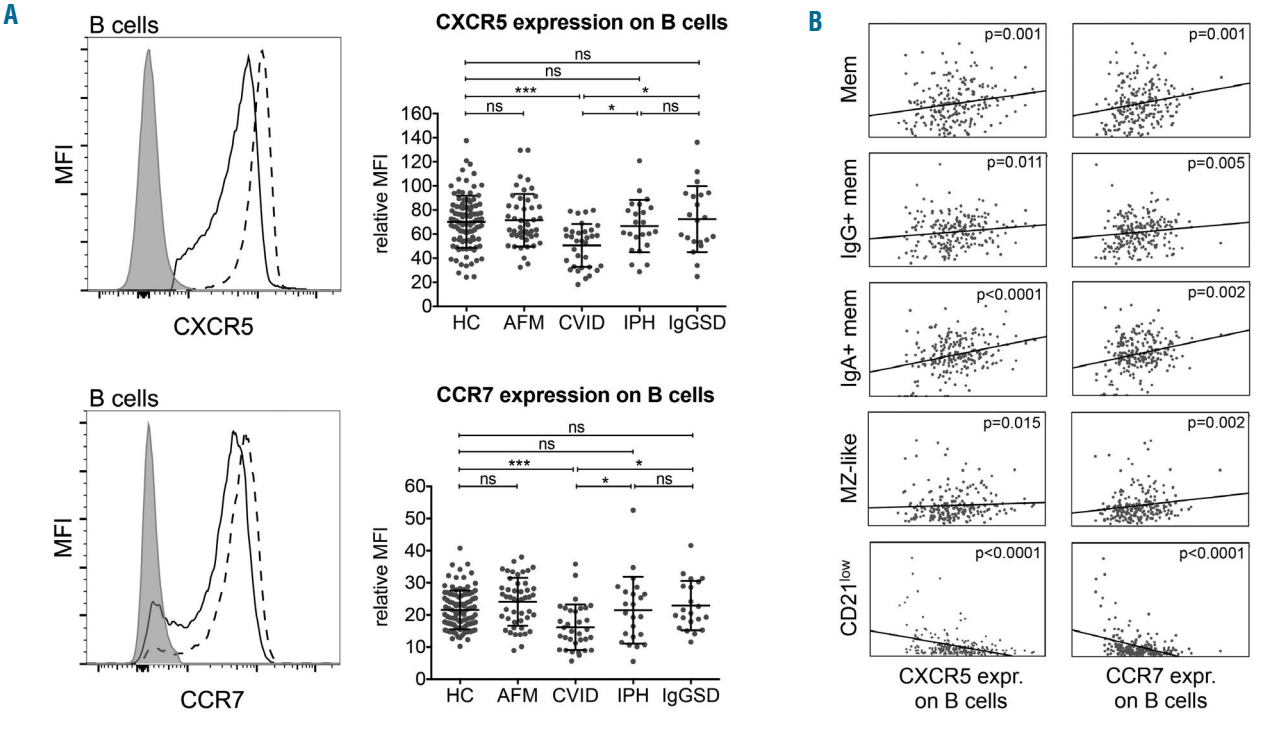

Figure 3. CXCR5 and CCR7 expression on B cells. (A) Graphs represent mean ± SD. Representative flow cytometric analysis is shown on the left. Full black lines represent CVID patients, dashed black lines represent HC. Relative mean fluorescence intensity (MFI) was calculated by dividing the MFI of the positive population (black line) by the MFI of the "fluorescence minus one" (FMO) population (gray). AFM, asymptomatic family member; CVID, common variable immunodeficiency; HC, healthy control; IgGSD, IgG subclass deficiency; IPH, idiopathic primary hypogammaglobulinemia. *$P \leq 0.05$, ***$P \leq 0.001$, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons). (B) Significant correlations between CXCR5 and CCR7 expression on B cells and levels of B-cell subsets. Correlations were calculated with the Spearman rank correlation. Expr, expression; mem, IgD⁻CD27⁺ memory B cells; MZ-like, IgD⁺CD27⁺ marginal zone-like B cells; CD21^{low}, CD21^{low} B cells.

to the group of HC, while NK-cell subsets were only aberrant in CVID (Figure 6). In our cohort, CVID patients had relatively increased levels of CD56^{bright} NK cells (a more immature NK phenotype) and relatively decreased levels of CD16^{bright} NK cells (a more effector NK phenotype) (Figure 6). This suggests a more immature NK-cell profile in CVID patients, analogous to their immature B-cell profile but opposite to their exhausted T-cell profile. Mean NKT- and invariant NKT-cell percentages were similar across groups (Figure 6). The levels of dendritic cells were, however, significantly decreased in all groups of patients (Figure 6). The relative proportion of conventional and plasmacytoid dendritic cells was not significantly altered in any of the patients (Figure 6). On average, there were no significant differences in the examined innate immune cells between AFM and HC, contrasting with the findings in the B- and T-cell compartments (Figure 6).

Unsupervised computational clustering analysis

Since significant associations between clinical features and immunological parameters had been found (see *Online Supplementary Results* and *Online Supplementary Table S4*), we investigated whether subgroups of the study subjects could be distinguished based on immunophenotypic data. We performed unsupervised computational clustering analysis of flow cytometric parameters by means of hierarchical clustering with heatmaps and principal component analysis. We could neither distinguish entire patient groups from healthy groups, nor distinguish subgroups among patients stratified according to diagnosis or clinical phenotype (*Online Supplementary Figure S11*). Furthermore, there was no clustering according to age or between members of the same family (*data not shown*). Similarly, principal component analysis could not reveal patient subgroups from the immunological data and could not distinguish patients from AFM or HC (*data not shown*). Different combinations of immunological parameters or patient stratifications did not improve the clustering (*data not shown*).

Discussion

Here we show for the first time in a relatively large cohort of subjects that there is a spectrum of peripheral immunophenotypic abnormalities in PAD, ranging from those present in AFM across those found in IgGSD and IPH patients to those in CVID patients, with the differences being most notable in naïve and memory T- and B-lymphocyte subsets. A summary of significantly different parameters in the patient and AFM groups compared to

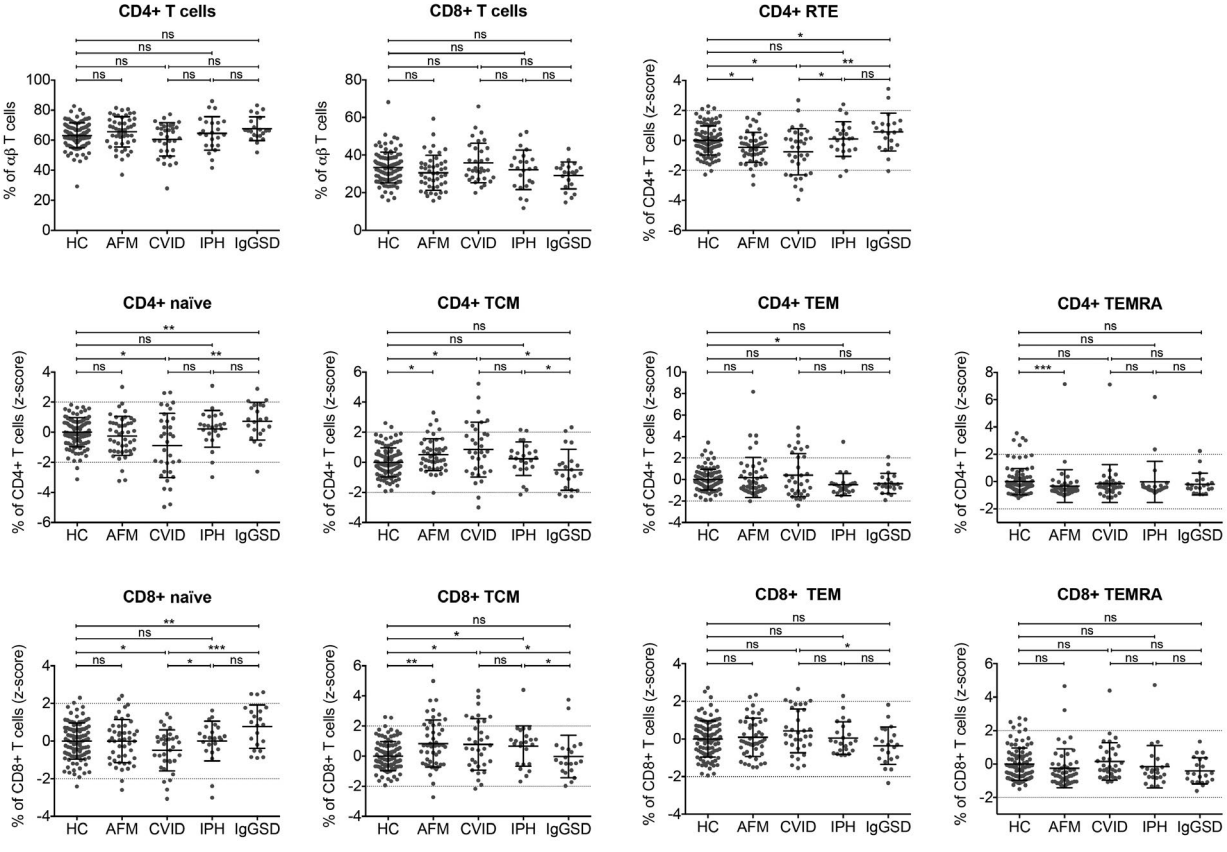

Figure 4. Naïve and memory T-cell subsets. Total T cells were gated as CD3$^+$ in alive cells. CD4$^+$ and CD8$^+$ T cells were gated in αβ T cells. Within CD4$^+$ and CD8$^+$ T cells, naïve cells were gated as CD45RO$^-$CCR7$^+$, central memory cells (TCM) as CD45RO$^+$CCR7$^+$, effector memory cells (TEM) as CD45RO$^+$CCR7$^-$, and terminally differentiated cells (TEMRA) as CD45RO$^-$CCR7$^-$. In naïve CD4$^+$ T cells, recent thymic emigrants (RTE) were gated as CD31$^+$. Graphs represent mean ± SD. Naïve and memory T-cell subsets were expressed as z-scores to adjust for age. Values normal for age have a z-score between -2 and 2 (dotted lines). AFM, asymptomatic family member; CVID, common variable immunodeficiency; HC, healthy control; IgGSD, IgG subclass deficiency; IPH, idiopathic primary hypogammaglobulinemia. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons).

the HC group is shown in Figure 7. The results presented support the notion of a complex basis of CVID and related milder PAD disorders, in which an accumulation of multiple genetic and/or environmental factors contributes to the final phenotype.[3,15,16] Monogenic defects have only been identified in a minority of cases with CVID.[14] Remarkably, some relatives with the same monogenic defect were found to be asymptomatic or suffer from a milder PAD phenotype such as IgGSD.[20-23] A multifactorial basis in the majority of CVID, and in extension PAD, would explain the vast variations in the clinical and immunological landscape seen in these patients.[2,14]

The immunophenotypic characteristics found in our CVID group are comparable to those reported previously in the literature: increased naïve and transitional B cells, CD21^{low} B cells and cTfh cells, and decreased memory B-cell subsets, total CD4$^+$ T-cell counts, naïve CD4$^+$ T cells and CD4$^+$ RTE;[24-30] heterogeneous distribution of peripheral B-cell patterns;[9,17,31] decreased proportions of NK cells and dendritic cells;[32,33] lower ICOS upregulation on activated T cells;[34] increased HLA-DR expression on B and T cells;[35,36] decreased BAFF-R, CXCR5 and CCR7 expression on B cells;[37,38] and decreased CCR7 expression on T cells, especially on cTfh cells.[38,39] It should be noted that the observed reduction of BAFF-R in CVID might be an overestimation because BAFF-R expression levels are lower on CD27$^-$ B cells, the predominant B-cell subset in these patients.

While CVID has been studied in depth over the past four decades, there are few immunophenotypic reports on milder forms of PAD, such as IPH and IgGSD. Our findings in IPH and IgGSD patients are compatible with previous published information on milder forms of PAD.[9,10,40,41] IPH and IgGSD patients showed an abnormal distribution of naïve and memory B- and T-cell subsets, similar to that observed in CVID patients, albeit to a lesser extent with a

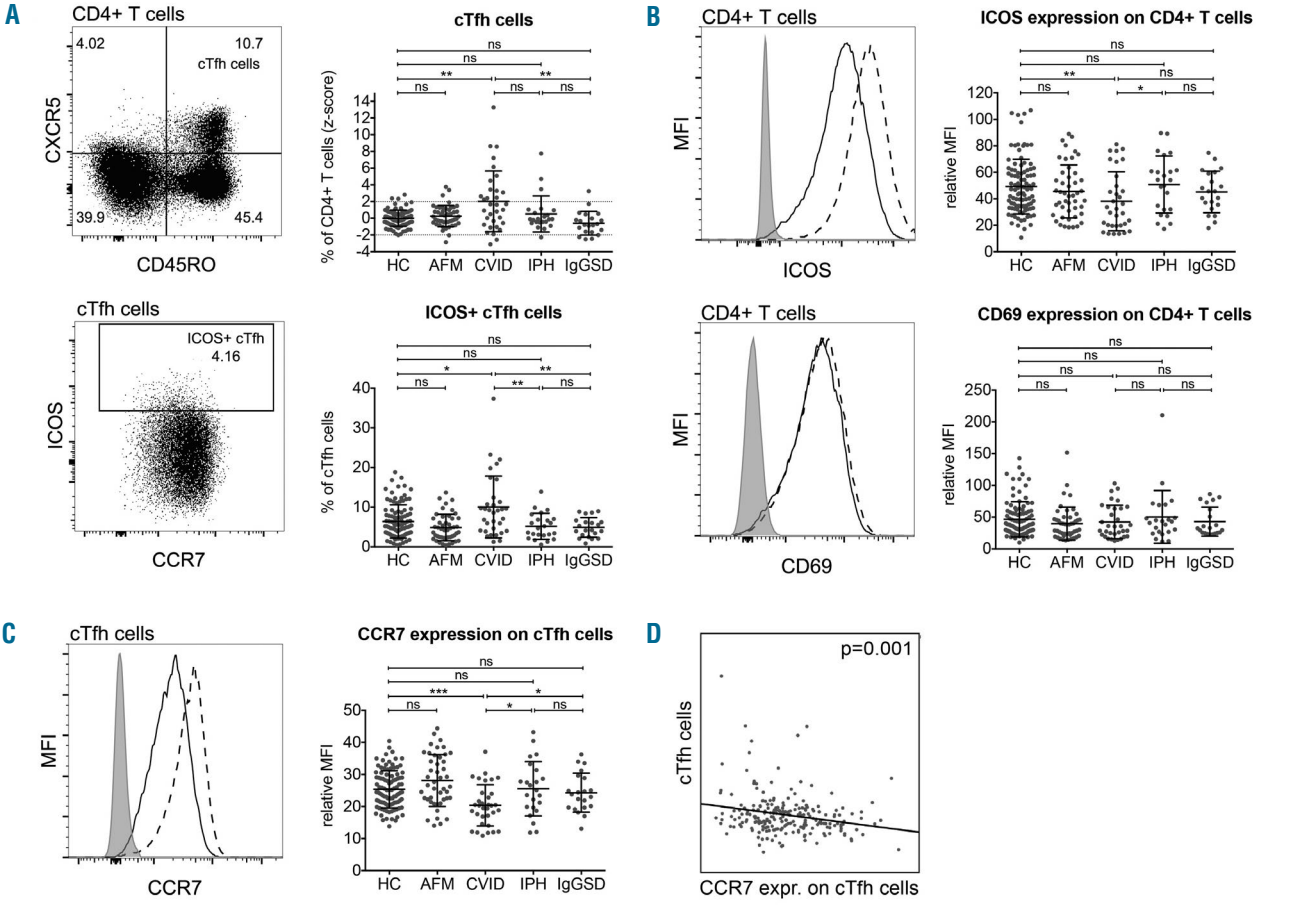

Figure 5. Circulating follicular helper T (cTfh) cells and expression of ICOS and CCR7 on cTfh cells. (A) Circulating follicular helper T (cTfh) cells were gated as CXCR5$^+$CD45RO$^+$ in CD4$^+$ T cells. ICOS$^+$ cTfh cells were gated based on "fluorescence minus one" (FMO). A representative gating strategy is shown on the left. Graphs represent mean ± SD. cTfh cells were expressed as z-scores to adjust for age-related differences in memory cells. Values normal for age have a z-score between -2 and 2 (dotted lines). (B) ICOS and CD69 expression on CD4$^+$ T cells stimulated with phytohemagglutinin for 72 h. CD69 was used as a positive control for T-cell activation. Graphs represent mean ± SD. Representative flow cytometric analysis is shown on the left. Full black lines represent CVID patients, dashed black lines represent HC. Relative mean fluorescence intensity (MFI) was calculated by dividing the MFI of the positive population (black line) by the MFI of the FMO population (gray). (C) CCR7 expression on cTfh cells. The graph represents mean ± SD. Representative flow cytometric analysis is shown on the left. The full black line represents a CVID patient, the dashed black line represents a HC. Relative mean MFI was calculated by dividing the MFI of the positive population (black line) by the MFI of the FMO population (gray). AFM: asymptomatic family member; CVID: common variable immunodeficiency; HC: healthy control; IgGSD: IgG subclass deficiency; IPH: idiopathic primary hypogammaglobulinemia. *P≤0.05, **P≤0.01, ***P≤0.001, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons). (D) Correlation between CCR7 expression on cTfh cells and level of cTfh cells (Spearman rank correlation). Expr: expression.

block later in B-cell development and a less pronounced T-cell skewing towards memory subsets. Furthermore, compared to the CVID group, IPH and IgGSD patients had less severely decreased serum levels of all Ig isotypes. Like the CVID group, the IPH and IgGSD groups in our cohort had significantly reduced proportions of NK cells and dendritic cells. Moreover, they had a similar though less pronounced decrease in BAFF-R and increase in HLA-DR and CD40 expression on B cells. In contrast to CVID patients, IPH and IgGSD patients did not have significant alterations in mean cTfh cell levels, in mean chemokine receptor expression on B and T cells, or in mean ICOS upregulation on activated T cells. To our knowledge, this is the first report on co-stimulatory molecules and chemokine receptors on B and T cells in milder forms of PAD. While the immunophenotypic parameters of CVID, IPH and IgGSD patients appear to form a continuous spectrum, we observed small differences in the type of infections they developed. In particular, compared to CVID and IgGSD patients, a higher proportion of IPH patients had unusual infections (i.e. warts, fungal infections), although the difference was not statistically significant.

There have been no detailed immunophenotypic studies in cohorts of asymptomatic relatives of patients with PAD. Two previous studies investigated serum Ig levels in first-degree relatives of Iranian (n=64) and Turkish (n=63) CVID patients.[42,43] The proportion of asymptomatic relatives with reduced total IgG, IgM and/or IgA in the Iranian (13%) and Turkish (19%) cohorts was comparable to that in our cohort (8/47, 17%).[42,43] In our cohort, mean IgG and IgM levels were significantly lower in AFM than in HC suggesting that AFM are genetically and/or environmentally predisposed to the development of PAD. This hypothesis is further supported by the fact that several flow cytometric abnormalities detected in PAD patients were also found in AFM (Figure 7). Especially, AFM showed a similar trend regarding the profile of naïve and memory B and T cells, with significantly increased transitional B cells, $CD4^+$ TCM cells and $CD8^+$ TCM cells and significantly decreased IgD^-CD27^+ memory B cells, plasmablasts and $CD4^+$ RTE compared to HC. Moreover, the distribution of peripheral B-cell patterns among AFM was similar to that among IPH and IgGSD patients and indicated that some AFM have a defect in later stages of

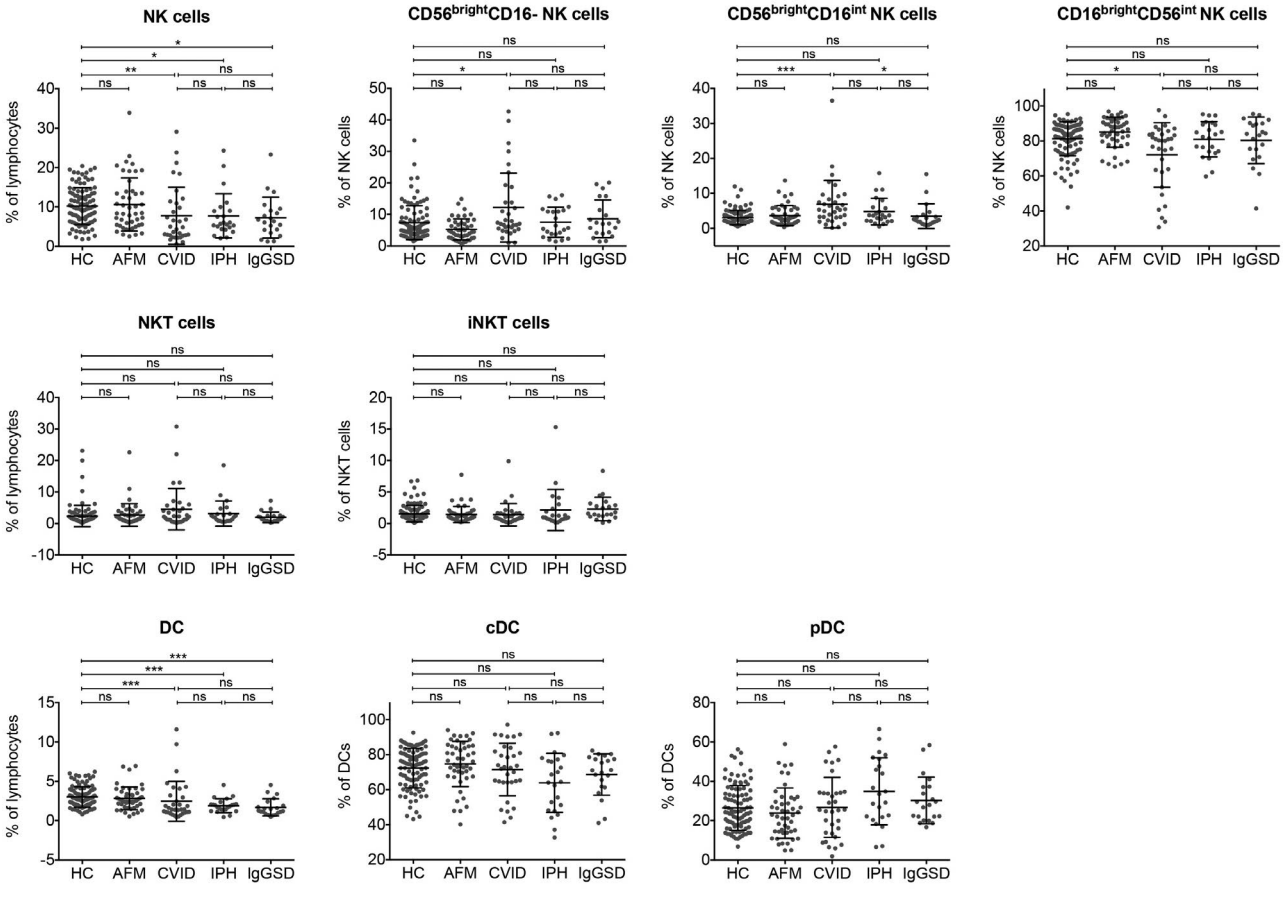

Figure 6. Innate immune cells: natural killer cells, natural killer T cells, dendritic cells and subsets. Natural killer (NK) cells were gated as $CD3^-CD56^+$ in $CD19^-HLA\text{-}DR^-$ lymphocytes. NK-cell subsets were determined by their relative expression of CD56 and CD16. Natural killer T (NKT) cells were gated as $CD3^+CD56^+$ in $CD19^-HLA\text{-}DR^-$ lymphocytes, and invariant NKT (iNKT) cells as invariant TCR (TCR Vα24-Jα18) positive in NKT cells. Dendritic cells (DC) were gated as lineage$^-CD4^+HLA\text{-}DR^+$ cells. Within DC, conventional DC (cDC) were gated as $CD11c^+CD123^-$ and plasmacytoid DC (pDC) as $CD123^+CD11c^-$. Graphs represent mean ± SD. AFM: asymptomatic family member; CVID: common variable immunodeficiency; HC: healthy control; IgGSD: IgG subclass deficiency; IPH: idiopathic primary hypogammaglobulinemia. *P≤0.05, **P≤0.01, ***P≤0.001, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons).

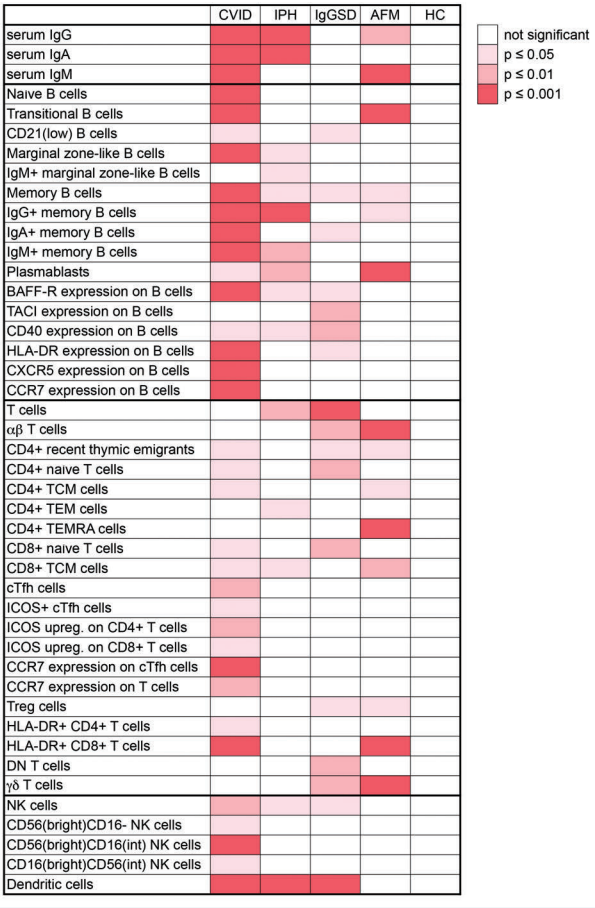

Figure 7. Summary of significant differences in the study. All parameters that tested significantly different in the study groups compared to HC are shown in a heat map with colours representing the corresponding *P*-values as indicated. AFM: asymptomatic family member; CVID: common variable immunodeficiency; HC: healthy control; IgGSD: IgG subclass deficiency; IPH: idiopathic primary hypogammaglobulinemia.

peripheral B-cell development. As opposed to B- and T-cell naïve and memory subsets, mean expression levels of the here-examined co-stimulatory molecules and chemokine receptors on B and T cells were not significantly altered in AFM compared to HC. Furthermore, AFM had, on average, normal levels of cTfh cells, NK cells, and dendritic cells (total and subsets). Together, our data suggest that a broader impairment of the immune system, including alterations in co-stimulatory molecules, lymphocyte trafficking and innate immune cells, is required to develop a clinical phenotype. It would be interesting to follow IPH and IgGSD patients as well as AFM over time (especially young individuals), to see whether their immunophenotype approaches that of patients with CVID. It should be noted that early Ig replacement therapy might interfere with the natural progression of the disease.

Several classifications have been proposed to distinguish subgroups among CVID patients, mainly using peripheral B- and/or T-cell immunophenotyping.[24-27,44,45] However, the plethora of immunological abnormalities and their unequal distribution among different cohorts have made it challenging to uniformly identify subgroups in the CVID population.[10,46-48] In our cohort, unsupervised computational clustering techniques were unable to reveal subgroups of subjects using different combinations of immunological and/or clinical parameters. The inability to distinguish clusters might be due to the relatively small groups. More probably, however, this reflects the heterogeneous nature of CVID and related PAD, and is in line with the fact that mild immunophenotypic abnormalities are already present in AFM. Possibly, genetic and/or environmental elements predisposing to the development of PAD may be more prevalent in the general community than initially thought. Depending on the degree to which these elements congregate, a wide range of phenotypes arises.

While we recognize that the small sample size of our study means that the findings warrant confirmation in larger cohorts, we conclude that the immunophenotypic landscape in CVID and other PAD comprises a varied and overlapping spectrum in which asymptomatic relatives show an intermediate immunophenotype, supporting the notion of a complex etiological basis of PAD.

Acknowledgments

The authors would like to thank the patients and their families and the healthy blood donors for participating in the study. We also thank the nurses for their assistance in blood sampling and Kelly Heyns and Nancy De Cabooter for their help with isolating peripheral blood mononuclear cells from blood samples. Finally, we are deeply grateful to Prof. Dr. Elfride De Baere for her constructive discussions.

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