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

Delfien J.A. Bogaert,[1,2,3,4] Marieke De Bruyne,[1,3] Veronique Debacker,[1,5] Pauline Depuydt,[3,6] Katleen De Preter,[3,6] Carolien Bonroy,[7] Jan Philippé,[7,8] Victoria Bordon,[9] Bart N. Lambrecht,[4,10,11] Tessa Kerre,[8,10,12] Andrea Cerutti,[13,14] Karim Y. Vermaelen,[5,10,11] Filomeen Haerynck[1,2,*] and Melissa Dullaers[1,4,10,*]

[1]Clinical Immunology Research Laboratory, Department of Pulmonary Medicine, Ghent University Hospital, Belgium; [2]Department of Pediatric Immunology and Pulmonology, Centre for Primary Immunodeficiency, Jeffrey Modell Diagnosis and Research Centre, Ghent University Hospital, Belgium; [3]Center for Medical Genetics, Ghent University and Ghent University Hospital, Belgium; [4]Laboratory of Immunoregulation, VIB Inflammation Research Center, Ghent, Belgium; [5]Tumor Immunology Laboratory, Department of Pulmonary Medicine, Ghent University Hospital, Belgium; [6]Cancer Research Institute, Ghent University, Belgium; [7]Department of Laboratory Medicine, Ghent University Hospital, Belgium; [8]Department of Clinical Chemistry, Microbiology and Immunology, Ghent University, Belgium; [9]Department of Pediatric Hematology, Oncology and Stem Cell Transplantation, Ghent University Hospital, Belgium; [10]Department of Internal Medicine, Ghent University, Belgium; [11]Department of Pulmonology, Ghent University Hospital, Belgium; [12]Department of Hematology, Ghent University Hospital, Belgium; [13]Department of Medicine, The Immunology Institute, Mount Sinai School of Medicine, New York, NY, USA and [14]B Cell Biology Laboratory, Hospital del Mar Medical Research Institute, Barcelona, Spain

FH and MD contributed equally to this work.

 doi:10.3324/haematol.2016.149112

Received: May 13, 2016.

Accepted: September 8, 2016.

Pre-published: September 15, 2016.

Correspondence: melissa.dullaers@ugent.be

Supplementary Online Materials

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

Delfien J.A. Bogaert[1,2,3,4], Marieke De Bruyne[3], Veronique Debacker[1,5], Pauline Depuydt[3,6], Katleen De Preter[3,6], Carolien Bonroy[7], Jan Philippé[7,8], Victoria Bordon[9], Bart N. Lambrecht[4,10,11], Tessa Kerre[8,10,12], Andrea Cerutti[13,14], Karim Y. Vermaelen[5,10,11], Filomeen Haerynck[$,1,2] and Melissa Dullaers[$,1,4,10]

Contents:

SUPPLEMENTARY METHODS

Serum immunoglobulin levels

Serum samples of AFM and HC were cryopreserved at -80°C and immunoglobulin (Ig)G, IgG2, IgG3, IgA and IgM concentrations were measured on thawed serum by nephelometry (Behring Nephelometer Analyzer II). Ig levels of patients had been previously determined at time of diagnosis, on fresh serum samples using nephelometry (Behring Nephelometer Analyzer II).

Absolute white blood cell counts

Absolute white blood cell counts and differentiations were determined on EDTA whole blood of patients during routine lab evaluations by means of a Sysmex XE-5000 (Sysmex), within a six-month range around time of inclusion in the study. On the same sample, B, T and NK cells were measured using a BD FACSCanto II flow cytometer (BD Biosciences) and FACSDiva software version 8 (BD Biosciences).

Due to practical reasons, absolute white blood cell counts on EDTA whole blood could not be assessed in asymptomatic family members (AFM) or healthy controls (HC).

Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from EDTA whole blood by Ficoll-Paque density gradient centrifugation and cryopreserved at -150°C for batch analysis. Thawed PBMCs were stained with fixable viability dye 506 (eBioscience) and fluorescently labeled monoclonal antibodies under saturation conditions. Following monoclonal antibodies were used (clones specified between brackets): CD8 (RPA-T8), CD14 (MΦP9), CD16 (3G8), CD19 (HIB19), CD20 (2H7), CD21 (B-LY4), CD27 (M-T271), CD31 (WM59), CD40 (5C3), CD138 (MI15), CXCR5 (RF8B2), IgD (IA6-2), IgM (G20-127), γδTCR (11F2) (all BD Biosciences); CD3 (SK7), CD4 (SK3), CD11c (BU15), CD19 (HIB19), CD20 (2H7), CD24 (ML5), CD25 (BC96), CD34 (581), CD45RO (UCHL1), CD123 (6H6), CD197(CCR7) (G043H7), CD267(TACI) (1A1), CD268(BAFF-R) (11C1), CD278(ICOS) (C398.4A), HLA-DR (L243) (all Biolegend); CD4 (RPA-T4), CD38 (HIT2), CD56 (TULY56), Foxp3 (PCH101) (all eBioscience); IgA (IS11-8E10), IgG (IS11-3B2.2.3), iNKT (6B11) (all Miltenyi Biotec).

To analyze ICOS upregulation on T cells, thawed PBMCs were incubated with 1% PHA (Life Technologies) for 72 hours at 37°C in 5% CO_2 at a density of 1.25 x 10^6 PBMCs/mL in supplemented

RPMI medium (Gibco). Afterwards, cells were stained with fixable viability dye 506 (eBioscience) and fluorescently labeled monoclonal antibodies under saturation conditions: CD69-BV605 (FN50) (BD Biosciences); CD3-PerCP-Cy5.5 (SK7), CD4-Pacific Blue (SK3), CD278(ICOS)-FITC (C398.4A) (all Biolegend); CD8-APC (BW135/80) (Miltenyi Biotec). CD69 expression was used as a positive control for T cell activation.

Cells were acquired on an LSR Fortessa flow cytometer with 3 lasers (488nm blue laser, 405 nm violet laser, 640nm red laser) that can measure up to 12 colors simultaneously (BD Biosciences). At least 100 000 events per sample were recorded. Data were analyzed with FlowJo version X (Tree Star Inc.).

Unsupervised computational clustering analysis

Unsupervised clustering analysis was performed on log2 transformed serum Ig levels and flow cytometry variables using the R programming language (v3.1.1) (1). The immunological data was studied for sample subgroups by means of hierarchical clustering – implementing the "manhattan" distance method and the "ward.D2" clustering method – through the R package "pheatmap" (v1.0.7) (2) to provide the accompanying heatmaps. Additionally, principal component analysis (PCA) was performed using the base R function "prcomp()". Both clustering methods were evaluated on data from (i) all samples included in the study, (ii) patients and AFM, (iii) patients only and (iv) each individual PAD entity.

Statistics

Statistical analysis was performed with SPSS Statistics version 22 (IBM®). Variables were not normally distributed; therefore non-parametrical statistical tests were used throughout. Continuous variables between multiple groups were compared using the Kruskal-Wallis test. If the former indicated significant differences, pairwise comparison of groups was done using the Mann-Whitney test with Bonferroni's post hoc correction for multiple comparisons. Categorical variables between multiple groups were compared using the chi square test. If this indicated significant differences, pairwise comparison of groups was done using the Fisher's Exact test with Bonferroni's post hoc correction for multiple comparisons. Correlations were calculated with Spearman Rank Correlation. A two-sided p value ≤ 0.05 was considered statistically significant.

Continuous variables were converted into z-scores to adjust for age-dependent differences when required. A z-score is the number of standard deviations (SD) the measured value is above or below the normal mean for age: $z = \frac{x - \mu}{\sigma}$, x being the measured value, μ the mean value of the age-based reference population, and σ the SD of the age-based reference population (3). Values normal for age have a z-score between -2 and +2 (i.e. 2 SD below and above the age-adjusted mean). Z-scores of Ig levels and absolute white blood cell counts were calculated using age-based reference values from the routine lab. Z-scores of naive and memory PBMC subsets were calculated using age-based reference values derived from the HC group. The division of the healthy controls into age groups and the corresponding reference values are given in Table S1.

SUPPLEMENTARY RESULTS

Characteristics of the study population: absolute white blood cell counts.

Absolute white blood cell counts were only determined in patients during routine lab evaluations. These routine lab evaluations were done within a six-month range around time of analysis for the study.

Mean lymphocyte, B cell, and CD4+ T cell counts were significantly lower in CVID compared to IPH and IgGSD (Figure S1). At individual level, lymphopenia was observed in six of 33 CVID patients (Table S2). Eleven CVID patients and one IgGSD patient had reduced B cell counts (Table S2). Total absolute T cell counts were mildly decreased in five CVID patients of whom two had decreased CD4+ T cells, one decreased CD8+ T cell numbers and two decreased CD4+ and CD8+ T cell numbers (Table S2). Note that for B, T, CD4+ T, CD8+ T, and NK cells, absolute counts measured in the routine lab were strongly correlated with percentages determined on flow cytometry (all $p<0.001$; data not shown).

Associations between clinical features and the immunophenotype in patients.

Flow cytometric B and/or T cell phenotyping is frequently used to discriminate CVID patients at risk for non-infectious complications (4, 5). Therefore, associations between clinical features and immunological parameters were examined in all PAD patients together and in CVID, IPH and IgGSD patients separately. Statistical data on the here-discussed associations are provided in Table S4.

In our cohort, PAD patients with chronic lung disease (i.e. bronchiectasis, lung granulomata and/or lymphocytic interstitial pneumonitis; n=22) (Table S3) had significantly increased $CD21^{low}$ B cells as well as significantly decreased IgG, IgM, IgA, absolute B cell numbers and IgD-CD27+ memory B cells compared to PAD patients without chronic lung disease. Within the separate groups of CVID, IPH and IgGSD patients, we could not detect a significant link between chronic lung disease and any of the immunological parameters, which could be due to the small group sizes.

Polyclonal lymphoproliferative disease was defined as the presence of benign lymphadenopathy (cervical, mediastinal and/or abdominal lymph nodes > 1 cm diameter, detected at least twice on medical imaging), hepatomegaly (as protocolled upon abdominal ultrasound), and/or splenomegaly (as protocolled upon abdominal ultrasound) (Table S3). PAD patients with polyclonal lymphoproliferative disease (n=17) had significantly higher naive B cells, $CD21^{low}$ B cells, CD4+ TCM

cells and cTfh cells compared to those without. Furthermore, PAD patients with lymphoproliferative disease had significantly lower IgG, IgM, IgA, IgD-CD27+ memory B cells, naive CD4+ T cells, CD4+ RTE, naive CD8+ T cells, CXCR5 and CCR7 expression on B cells, and CCR7 expression on cTfh cells. Within CVID patients separately, those with polyclonal lymphoproliferation (n=14) were found to have significantly increased CD4+ TCM cells and cTfh cells and significantly decreased IgG, naive CD4+ T cells, IgD-CD27+IgA+ memory B cells, and CXCR5 expression on B cells compared to those without lymphoproliferative disease. There were no immunological differences between IPH patients with and without polyclonal lymphoproliferative disease. None of the IgGSD patients had developed lymphoproliferative disease at time of analysis.

PAD patients with autoimmunity (i.e. symptoms related to autoimmune disease; n=19) (Table S3) had significantly increased CD4+ TCM cells and cTfh cells and significantly decreased IgA, naive CD4+ T cells and CD4+ RTE. PAD patients with autoimmunity also showed a trend towards increased TACI expression on B cells but this did not reach statistical significance. However, in PAD patients with autoimmune cytopenia (n=6), TACI expression was significantly higher compared to those without autoimmune cytopenia. Within CVID patients separately, those with autoimmunity (n=13) showed significantly higher CD4+ TCM cells and TACI expression on B cells compared to those without autoimmune manifestations. The increased TACI expression on B cells was even more significant in CVID patients with autoimmune cytopenias (n=5) compared to those without. IPH patients with autoimmunity (n=3) also demonstrated a non-statistically significant trend towards higher CD4+ TCM cells compared to those without autoimmunity. IgGSD patients with and without autoimmunity did not have significantly different immunological parameters.

SUPPLEMENTARY REFERENCES

1. R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

2. Kolde R (2015). pheatmap: Pretty Heatmaps. R package version 1.0.7. http://CRAN.R-project.org/package=pheatmap

3. Clark-Carter D (2005). Encyclopedia of Statistics in Behavioral Science. Hoboken: John Wiley and Sons, Ltd.

4. Wehr C, Kivioja T, Schmitt C, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood. 2008;111(1):77-85.

5. Giovannetti A, Pierdominici M, Mazzetta F, et al. Unravelling the complexity of T cell abnormalities in common variable immunodeficiency. J Immunol. 2007;178(6):3932-3943.

SUPPLEMENTARY TABLES

Table S1. Age-based reference values derived from the healthy control (HC) group

Age group (years)	N	Naive B cells (% B cells)	Transitional B cells (% B cells)	$CD21^{low}$ B cells (% B cells)	IgD-CD27+ memory B cells (% B cells)	IgD+CD27+ marginal zone-like B cells (% B cells)	Plasmablasts (% B cells)	Plasma cells (% alive)
4.5 - 10.0	11	54.80 - 73.72	1.80 - 6.17	0.00 - 10.75	11.31 - 24.09	3.04 - 15.23	0.04 - 1.85	0.20 - 0.61
10.1 - 16.0	15	45.55 - 92.68	0.06 - 13.67	0.60 - 3.34	1.81 - 29.95	0.00 - 19.32	0.00 - 2.66	0.00 - 1.09
16.1 - 20.0	10	57.64 - 83.58	0.20 - 5.82	0.12 - 3.50	6.37 - 25.71	1.49 - 12.31	0.14 - 1.57	0.08 - 0.91
20.1 - 30.0	21	40.26 - 87.39	0.00 - 6.20	0.00 - 5.65	3.05 - 37.64	0.61 - 15.91	0.10 - 2.02	0.00 - 1.99
30.1 - 40.0	14	25.81 - 78.79	0.23 - 4.88	0.22 - 7.25	5.81 - 52.36	2.15 - 21.56	0.52 - 1.47	0.12 - 1.30
40.1 - 50.0	12	25.26 - 88.85	0.00 - 4.06	0.00 - 6.96	2.84 - 52.59	0.60 - 17.51	0.00 - 1.88	0.00 - 1.68
50.1 - 90.0	18	0.00 - 82.40	1.18 - 6.60	7.56 - 15.93	17.64 - 48.17	8.51 - 25.31	0.97 - 1.68	0.81 - 2.25

Age group (years)	N	Naive CD4+ T cells (% CD4+ T cells)	CD4+ RTE (% CD4+ T cells)	CD4+ TCM (% CD4+ T cells)	CD4+ TEM (% CD4+ T cells)	CD4+ TEMRA (% CD4+ T cells)	Naive CD8+ T cells (% CD8+ T cells)	CD8+ TCM (% CD8+ T cells)	CD8+ TEM (% CD8+ T cells)	CD8+ TEMRA (% CD8+ T cells)	cTfh cells (% CD4+ T cells)
4.5 - 10.0	11	47.61 - 79.07	38.81 - 66.77	15.13 - 30.42	3.29 - 22.40	0.00 - 2.20	36.15 - 81.45	0.00 - 10.29	8.55 - 34.48	0.00 - 30.76	5.04 - 12.46
10.1 - 16.0	15	42.54 - 82.41	28.47 - 64.34	9.15 - 34.41	3.64 - 25.02	0.00 - 5.02	29.80 - 96.09	1.18 - 9.36	0.00 - 39.58	0.00 - 29.57	1.97 - 11.06
16.1 - 20.0	10	45.55 - 68.95	31.99 - 57.49	16.00 - 34.67	4.57 - 28.25	0.00 - 2.45	37.78 - 84.72	1.27 - 8.14	9.23 - 33.72	0.00 - 29.50	3.44 - 12.10
20.1 - 30.0	21	35.32 - 74.69	20.09 - 58.55	14.88 - 46.60	3.19 - 23.65	0.00 - 2.05	28.27 - 82.58	2.67 - 13.31	7.93 - 43.52	0.00 - 22.30	4.49 - 14.64
30.1 - 40.0	14	15.67 - 63.65	7.10 - 44.73	23.82 - 57.75	3.29 - 33.55	0.00 - 2.85	10.40 - 69.52	5.43 - 16.97	8.60 - 56.61	0.00 - 39.99	4.86 - 23.45
40.1 - 50.0	12	25.82 - 70.36	10.25 - 47.73	19.45 - 56.57	4.98 - 19.58	0.00 - 4.39	7.73 - 61.28	3.99 - 20.98	6.78 - 61.64	0.00 - 46.61	2.09 - 21.11
50.1 - 90.0	18	0.00 - 66.33	0.00 - 43.45	21.88 - 57.36	13.54 - 37.18	3.32 - 11.89	0.00 - 54.35	14.42 - 30.61	23.53 - 55.37	30.98 - 68.78	5.51 - 15.16

Healthy controls (HC) were divided into seven age groups. Reference values were calculated as mean ± two times the standard deviation of the corresponding age group. Negative values for the lower limit were set at 0.00. cTfh, circulating follicular helper T; N, number of HC in age group; RTE, recent thymic emigrants; TCM, central memory T; TEM, effector memory T; TEMRA, effector memory RA T.

Table S2. White blood cell counts of the patients

Patient	Diagnosis	Gender	Age (y)	Neutro (/μL)	Mono (/μL)	Lympho (/μL)	T cells (/μL)	CD4+ T (/μL)	CD8+ T (/μL)	B cells (/μL)	NK cells (/μL)
P1	CVID	Male	61.9	6690	690	2010	1670	362	1250	121	221
P2	CVID	Male	14.3	2470*	700	1700	1260	663	442	255	153
P3	CVID	Female	17.0	3860	550	2060	1774	1296	476	99	46*
P4	CVID	Male	13.6	5290	910	1190*	940	643	274	36*	179
P5	CVID	Female	10.8	6080	960	2410	1740	1080	603	48*	554
P6	CVID	Female	14.1	1870*	260*	1580	1200	664	427	253	126
P7	CVID	Female	49.0	4070	362	1538	1170	769	369	246	108
P8	CVID	Male	14.6	1490*	350*	580*	447*	290*	122*	87*	23.2*
P9	CVID	Male	18.2	2230	420	3610	2100	1360	679	1050	170
P10	CVID	Male	7.8	6980	690*	3990	2630	1600	838	798	479
P11	CVID	Male	7.8	3150	570*	3480	2580	1640	800	592	244
P12	CVID	Male	7.7	1550*	560*	1430	849	449	349	200	175
P13	CVID	Female	24.7	2340	330	1380	1310	804	459	115	180
P14	CVID	Female	14.0	4060	980	2350	1370	659	575	17*	304
P15	CVID	Female	9.4	3280	470*	2150	1700	862	583	117*	489
P16	CVID	Female	33.1	2601	213*	1160	1170	471	638	152	197
P17	CVID	Female	23.4	10400	2000	2580	2000	942	667	184	115
P18	CVID	Male	71.8	3368	590	868*	590*	174*	434	0*	234
P19	CVID	Male	43.7	5050	510	860*	559*	430	112*	155	120
P20	CVID	Female	83.2	4900	740	3020	2473	1389	1084	255	130
P21	CVID	Male	16.6	2780	530	1880	2270	1010	982	706	92.1
P22	CVID	Female	28.8	2884	831	4642	2880	1440	1390	1440	232
P23	CVID	Male	17.3	3090	760	2030	1400	974	365	386	223
P24	CVID	Male	16.6	2750	420*	2200	1780	781	805	317	293
P25	CVID	Male	32.3	3230	430	2480	1440	521	843	50*	918
P26	CVID	Female	45.0	3865	475	1966	1320	923	308	325	68.4*
P27	CVID	Male	53.6	6350	630	1370	740	480	219	41*	534
P28	CVID	Male	11.6	6210	1400	1680	1100	389	443	142*	478
P29	CVID	Male	13.8	780*	300*	1300*	552*	256*	280	200	32*
P30	CVID	Male	17.8	4000	580	1640	935	476	394	246	426
P31	CVID	Male	80.8	5890	680	1280	1210	604	574	30*	227
P32	CVID	Female	36.7	3800	390	1870	1570	617	879	243	37.4*
P33	CVID	Female	14.6	1130*	360*	650*	496*	306*	132*	174*	149
P34	IPH	Female	20.2	2200	320	1410	1640	1010	560	314	269
P35	IPH	Male	15.2	2860	260*	2080	2110	1300	572	312	156
P36	IPH	Female	22.6	1700	320	2210	2819	1879	766	487	139
P37	IPH	Female	37.4	7380	690	2070	1770	1230	517	492	172
P38	IPH	Female	37.5	3555	289	1879	939	543	352	132	358
P39	IPH	Female	30.5	4030	390	2010	1880	1010	746	265	241
P40	IPH	Female	68.6	4590	390	1240	982	742	241	149	75*
P41	IPH	Male	13.4	1890*	560	1950	1270	761	312	332	312
P42	IPH	Female	52.8	3520	440	1309	1140	940	198	379	115
P43	IPH	Male	14.2	3330	310*	1640	1000	623	279	476	131
P44	IPH	Female	50.9	4181	212*	2552	1910	1580	332	434	179
P45	IPH	Male	37.3	4600	730	2260	1780	725	681	220	198
P46	IPH	Male	86.5	2840	530	1570	1046	600	446	94	320
P47	IPH	Female	38.2	3929	315	2874	2302	1517	785	316	139
P48	IPH	Female	30.2	2440	350	1630	1320	848	424	147	163
P49	IPH	Male	50.1	2890	370	2770	2350	1270	942	249	139
P50	IPH	Male	11.9	1660*	320*	1810	1320	796	416	253	199
P51	IPH	Male	10.4	1800*	460*	2730	1100	713	356	300	146
P52	IPH	Female	81.6	8930	860	3930	3152	2727	424	150	393
P53	IPH	Female	28.7	3788	302	5490	877	525	352	99	58*
P54	IPH	Female	52.8	5770	1000	3020	2140	1540	604	513	302
P55	IPH	Male	10.3	3730	350*	2840	2240	909	1160	227	341
P56	IPH	Female	17.3	3010	300*	3010	2468	1355	903	361	151
P57	IgGSD	Female	49.8	4130	580	2600	2132	1482	624	182	260
P58	IgGSD	Female	11.9	3550	460*	4040	2200	1170	722	361	452
P59	IgGSD	Female	21.5	3820	1080	4190	3430	2300	1090	609	304
P60	IgGSD	Female	34.1	2800	650	2700	1660	1100	430	301	172
P61	IgGSD	Female	46.4	3280	890	2920	2316	1559	756	225	208
P62	IgGSD	Female	35.2	7469	747	2241	2960	1810	1020	165	165
P63	IgGSD	Female	34.7	5790	930	3360	2130	1450	568	454	227
P64	IgGSD	Female	42.9	2270	500	1620	2070	1540	498	249	174
P65	IgGSD	Female	57.5	3070	550	2300	1750	1290	437	322	230
P66	IgGSD	Female	42.2	4720	490	1490	1200	909	271	232	483
P67	IgGSD	Male	40.6	2434	667	2240	2120	1290	647	441	323
P68	IgGSD	Female	51.3	2843	202*	1444	1140	881	260	188	87
P69	IgGSD	Female	65.0	6969	481	1362	1140	708	409	41*	163
P70	IgGSD	Female	51.3	4045	502	1772	1520	1050	472	328	185
P71	IgGSD	Female	31.5	7669	327	1374	1060	715	289	206	96
P72	IgGSD	Female	56.4	5766	255	1123	831	550	269	123	157
P73	IgGSD	Female	32.5	3930	550	1420	852	650	308	270	209
P74	IgGSD	Female	46.0	5070	300	1750	1420	910	490	140	175
P75	IgGSD	Female	35.1	6465	172*	1853	1480	982	463	204	167
P76	IgGSD	Male	17.9	3090	460*	1990	1510	856	537	179	279
P77	IgGSD	Male	15.3	1590*	320*	1900	1060	494	494	380	437

CVID, common variable immunodeficiency; IgGSD, IgG subclass deficiency; IPH, idiopathic primary hypogammaglobulinemia.

* Below age-based reference values.

Table S3. Clinical characteristics of the patients

	CVID	IPH	IgGSD
Infections and infection-related complications			
Recurrent upper respiratory tract infections	32/33 (97.0%)	23/23 (100%)	21/21 (100%)
Recurrent and/or severe lower respiratory tract infections	25/33 (75.8%)	20/23 (87.0%)	13/21 (61.9%)
Recurrent gastrointestinal infections	6/33 (18.2%)	3/23 (13.0%)	1/21 (4.8%)
Bacterial skin infections	2/33 (6.1%)	3/23 (13.0%)	2/21 (9.5%)
Deep abscesses (organ/muscle)	2/33 (6.1%)	1/23 (4.3%)	0/21 (0%)
Recurrent or invasive herpes simplex infections	1/33 (3.0%)	1/23 (4.3%)	1/21 (4.8%)
Recurrent herpes zoster infections	2/33 (6.1%)	3/23 (13.0%)	0/21 (0%)
Recurrent warts	1/33 (3.0%)	2/23 (8.7%)	0/21 (0%)
Recurrent fungal infections	1/33 (3.0%)	4/23 (17.4%)	2/21 (9.5%)
Bronchiectasis on HRCT thorax	13/24° (54.2%)	4/13° (30.8%)	2/12° (16.7%)
Non-infectious complications			
Unexplained enteropathy	13/33 (39.4%)	7/23 (30.4%)	9/21 (42.9%)
Benign lymphadenopathy on medical imaging[$]	11/33 (33.3%)	3/23 (13.0%)	2/21 (9.5%)
Lymphocytic interstitial pneumonitis	2/33 (6.1%)	0/23 (0%)	0/21 (0%)
Granulomata on CT thorax and/or coloscopy	6/32° (18.8%)	0/23 (0%)	0/21 (0%)
Splenomegaly on abdominal ultrasound	8/28° (28.6%)	1/14° (7.1%)	0/10° (0%)
Splenectomy	1/33 (3.0%)	0/23 (0%)	0/21 (0%)
Hepatomegaly on abdominal ultrasound	6/28° (21.4%)	2/14° (14.3%)	0/10° (0%)
Acute non-infectious hepatitis	2/33 (6.1%)	1/23 (4.3%)	1/21 (4.8%)
Liver transplantation	1/33 (3.0%)	0/23 (0%)	0/21 (0%)
Solid organ tumor	3/33 (9.1%)	0/23 (0%)	1/21 (4.8%)
Autoimmune manifestations	13/33 (39.4%)	3/23 (13.0%)	3/21 (14.3%)
- Autoimmune cytopenia	5/33 (15.2%)	1/23 (4.3%)	0/21 (0%)
- Inflammatory bowel disease	1/33 (3.0%)	0/23 (0%)	0/21 (0%)
- Autoimmune thyroid disease	1/33 (3.0%)	1/23 (4.3%)	1/21 (4.8%)
- Rheumatic disease (JIA, RA)	2/33 (6.1%)	0/23 (0%)	1/21 (4.8%)
- Pernicious anemia	1/33 (3.0%)	0/23 (0%)	0/21 (0%)
- Raynaud phenomenon	1/33 (3.0%)	0/23 (0%)	0/21 (0%)
- Sicca syndrome	0/33 (0%)	1/23 (4.3%)	0/21 (0%)
- Alopecia	1/33 (3.0%)	1/23 (4.3%)	0/21 (0%)
- Vitiligo	1/33 (3.0%)	1/23 (4.3%)	0/21 (0%)
- Lichen ruber planus	0/33 (0%)	0/23 (0%)	1/21 (4.8%)
Other			
Growth delay	5/33 (15.2%)	1/23 (4.3%)	0/21 (0%)

° Missing data (no medical imaging performed).
[$] Cervical, mediastinal and/or abdominal lymph nodes > 1 cm diameter, detected at least twice on medical imaging.
CVID, common variable immunodeficiency; (HR)CT, (high-resolution) computed tomography; IgGSD, IgG subclass deficiency; IPH, idiopathic primary hypogammaglobulinemia; JIA, juvenile idiopathic arthritis; RA, rheumatoid arthritis.

Table S4. Most important associations between clinical features and immunological parameters in patients

	PAD with chronic lung disease* (n=22)	PAD without chronic lung disease* (n=27)	P value
Serum IgG (z-score, mean ± SD)	-3.2 ± 1.1	-2.0 ± 1.6	0.005
Serum IgM (z-score, mean ± SD)	-2.2 ± 1.2	-1.0 ± 1.6	0.003
Serum IgA (z-score, mean ± SD)	-2.5 ± 0.6	-1.3 ± 1.7	0.004
Absolute B cell numbers (z-score, mean ± SD)	-1.4 ± 1.6	0.28 ± 2.1	0.021
IgD-CD27+ memory B cells (z-score, mean ± SD)	-1.7 ± 1.5	-0.63 ± 1.1	0.007
$CD21^{low}$ B cells (z-score, mean ± SD)	5.8 ± 11.7	1.5 ± 5.1	0.016
	PAD with polyclonal lymphoproliferation[$] (n=17)	PAD without polyclonal lymphoproliferation[$] (n=60)	P value
Serum IgG (z-score, mean ± SD)	-3.6 ± 0.82	-2.0 ± 1.4	<0.001
Serum IgM (z-score, mean ± SD)	-2.1 ± 0.75	-1.3 ± 1.4	0.003
Serum IgA (z-score, mean ± SD)	-2.8 ± 0.38	-1.5 ± 1.7	<0.001
Naive B cells (z-score, mean ± SD)	1.5 ± 1.3	0.53 ± 1.6	0.01
IgD-CD27+ memory B cells (z-score, mean ± SD)	-1.8 ± 1.1	-0.95 ± 1.4	0.005
$CD21^{low}$ B cells (z-score, mean ± SD)	6.6 ± 14.1	0.81 ± 3.7	0.037
Naive CD4+ T cells (z-score, mean ± SD)	-1.6 ± 2.1	0.31 ± 1.5	<0.001
CD4+ RTE (z- score, mean ± SD)	-1.18 ± 1.5	0.15 ± 1.3	0.005
CD4+ TCM cells (z-score, mean ± SD)	1.6 ± 1.6	-0.07 ± 1.4	<0.001
cTfh cells (z-score, mean ± SD)	3.5 ± 3.7	0.12 ± 2.2	<0.001
Naive CD8+ T cells (z-score, mean ± SD)	-0.61 ± 1.1	0.17 ± 1.2	0.019
CXCR5 expression on B cells (rMFI, mean ± SD)	44.8 ± 20.4	66.0 ± 22.6	0.002
CCR7 expression on B cells (rMFI, mean ± SD)	15.0 ± 6.4	20.9 ± 8.9	0.01
CCR7 expression on cTfh cells (rMFI, mean ± SD)	19.7 ± 6.9	23.9 ± 7.2	0.027
	CVID with polyclonal lymphoproliferation[$] (n=14)	CVID without polyclonal lymphoproliferation[$] (n=19)	P value
Serum IgG (z-score, mean ± SD)	3.7 ± 0.82	-2.7 ± 1.5	0.032
IgD-CD27+IgA+ memory B cells (mean ± SD)	6.3% ± 10.8%	16.6% ± 9.7%	0.002
Naive CD4+ T cells (z-score, mean ± SD)	-1.8 ± 2.2	-0.22 ± 1.9	0.036
CD4+ TCM cells (z-score, mean ± SD)	1.7 ± 1.7	0.22 ± 1.7	0.032
cTfh cells (z-score, mean ± SD)	3.9 ± 3.8	0.63 ± 2.8	0.006
CXCR5 expression on B cells (rMFI, mean ± SD)	42.9 ± 19.4	56.3 ± 14.5	0.045
	PAD with autoimmunity (n=19)	PAD without autoimmunity (n=58)	P value
Serum IgA (z-score, mean ± SD)	-2.4 ± 1.1	-1.5 ± 1.7	0.012
Naive CD4+ T cells (z-score, mean ± SD)	-0.93 ± 1.76	0.16 ± 1.75	0.012
CD4+ RTE (z- score, mean ± SD)	-0.78 ± 1.3	0.06 ± 1.4	0.033
CD4+ TCM cells (z-score, mean ± SD)	1.4 ± 1.6	-0.06 ± 1.4	0.001
cTfh cells (z-score, mean ± SD)	2.8 ± 3.9	0.21 ± 2.2	0.002
TACI expression on B cells (rMFI, mean ± SD)	166.3 ± 78.7	128.3 ± 67.1	0.052 (ns)
	CVID with autoimmunity (n=13)	CVID without autoimmunity (n=20)	P value
CD4+ TCM cells (z-score, mean ± SD)	1.8 ± 1.6	0.20 ± 1.7	0.018
TACI expression on B cells (rMFI, mean ± SD)	175 ± 64	122 ± 38	0.022
	IPH with autoimmunity (n=3)	IPH without autoimmunity (n=20)	P value
CD4+ TCM cells (z-score, mean ± SD)	1.2 ± 0.86	0.10 ± 1.1	0.061 (ns)
	PAD with autoimmune cytopenia (n=6)	PAD without autoimmune cytopenia (n=71)	P value
TACI expression on B cells (rMFI, mean ± SD)	213.3 ± 65.4	131.3 ± 68.7	0.003
	CVID with autoimmune cytopenia (n=5)	CVID without autoimmune cytopenia (n=72)	P value
TACI expression on B cells (rMFI, mean ± SD)	217 ± 72	130 ± 41	0.008

* Bronchiectasis, lung granulomata and/or lymphocytic interstitial pneumonitis.

[$] Benign lymphadenopathy, hepatomegaly, and/or splenomegaly.

cTfh, circulating follicular helper T; CVID, common variable immunodeficiency; Ig, immunoglobulin; IPH, idiopathic primary hypogammaglobulinemia; ns, not significant; PAD, primary antibody deficiency; rMFI, relative mean fluorescence intensity; RTE, recent thymic emigrants; SD, standard deviation; TCM, central memory T.

SUPPLEMENTARY FIGURES

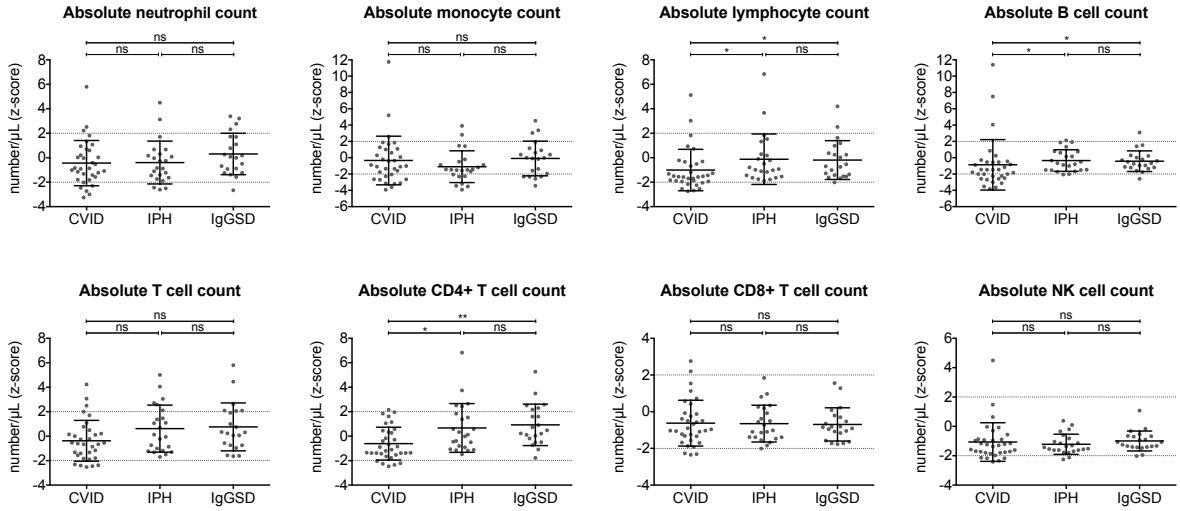

Figure S1. Absolute white blood cell counts in patients.

Absolute white blood cell counts in CVID, IPH and IgGSD patients determined in routine lab evalutions. Graphs represent mean ± SD. Cell counts were expressed as z-scores to adjust for age. Values normal for age have a z-score between -2 and 2 (dotted lines). CVID, common variable immunodeficiency; IgGSD, IgG subclass deficiency; IPH, idiopathic primary hypogammaglobulinemia. * p≤0.05, ** p≤0.01, *** p≤0.001, ns not significant (Mann-Whitney test with Bonferonni's correction for multiple comparisons).

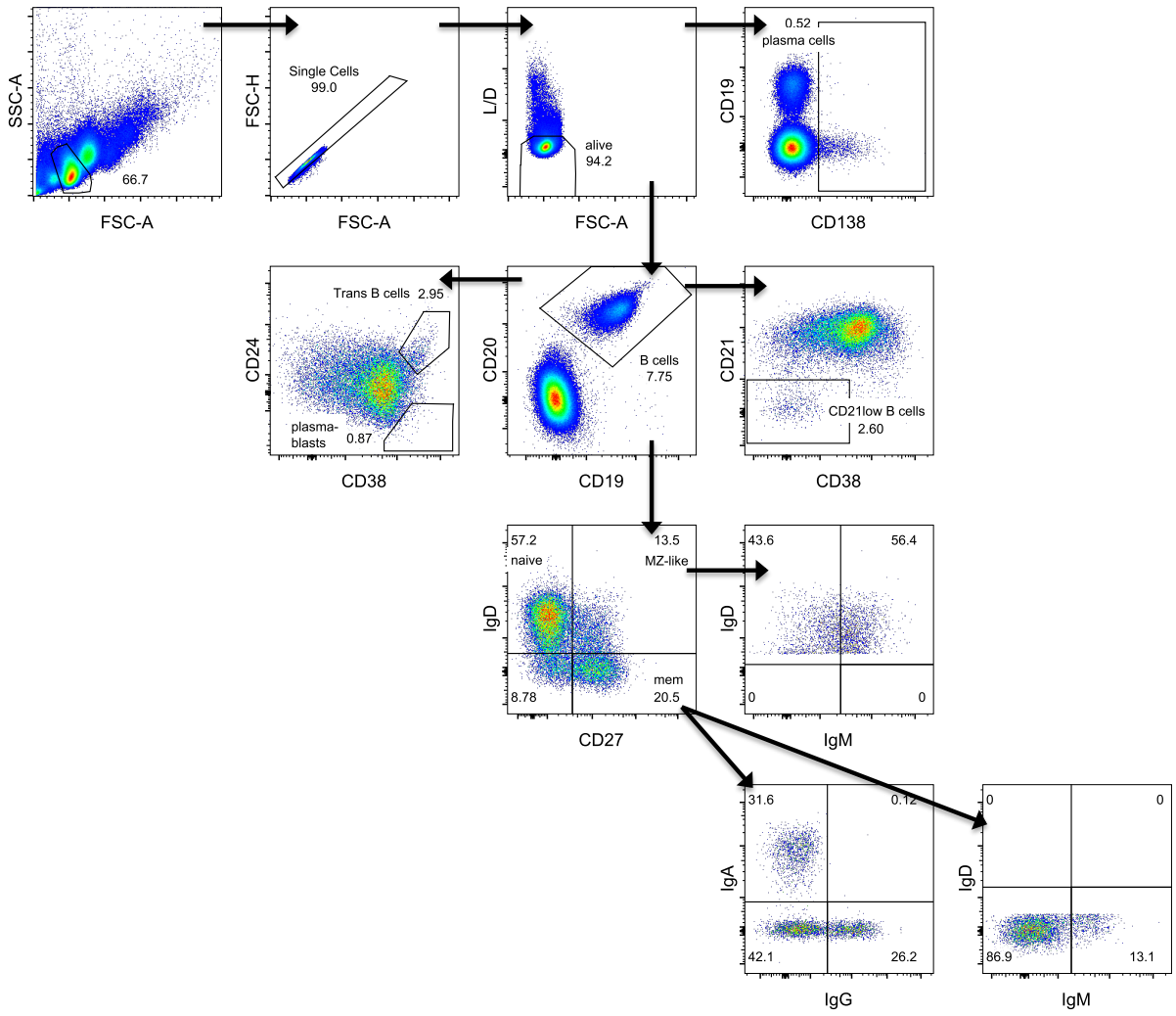

Figure S2. Representative flow cytometric analysis of B cell subsets in an adult healthy control.

PBMCs were sequentially gated on lymphocytes (gating out debris and monocytes), single cells (gating out doublets) and alive cells (gating out dead cells). B cells were gated as CD19+CD20+ in alive cells. Plasma cells were gated as CD138+CD19^{low} in alive cells. Within B cells, transitional (trans) B cells were gated as CD24^{high}CD38^{high}, plasmablasts as CD38^{high}CD24-, CD21^{low} B cells as CD21^{low}CD38^{low}, memory (mem) B cells as IgD-CD27+, naive B cells as IgD+CD27-, and marginal zone (MZ)-like B cells as IgD+CD27+. Within IgD+CD27+ marginal zone-like B cells, IgM+ marginal zone-like B cells were gated based on a Fluorescence Minus One (FMO). Within IgD-CD27+ memory B cells, IgG+, IgA+ and IgM+ memory B cells were gated based on an FMO. L/D, live/dead marker.

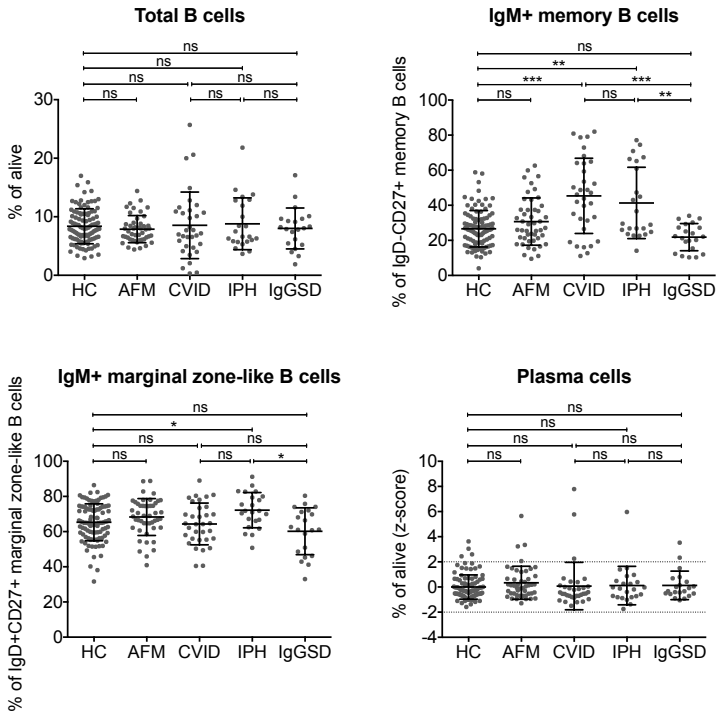

Figure S3. Total B cells, IgD-CD27+IgM+ memory B cells, IgM+IgD+CD27+ marginal zone-like B cells, and plasma cells.

B cells were gated as CD19+CD20+ and plasma cells as CD138+CD19^{low} in alive cells. IgD-CD27+ memory B cells and IgD+CD27+ marginal zone-like B cells were gated within B cells. Graphs represent mean ± SD. Plasma cells 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≤0.05, ** p≤0.01, *** p≤0.001, ns not significant (Mann-Whitney test with Bonferonni's correction for multiple comparisons).

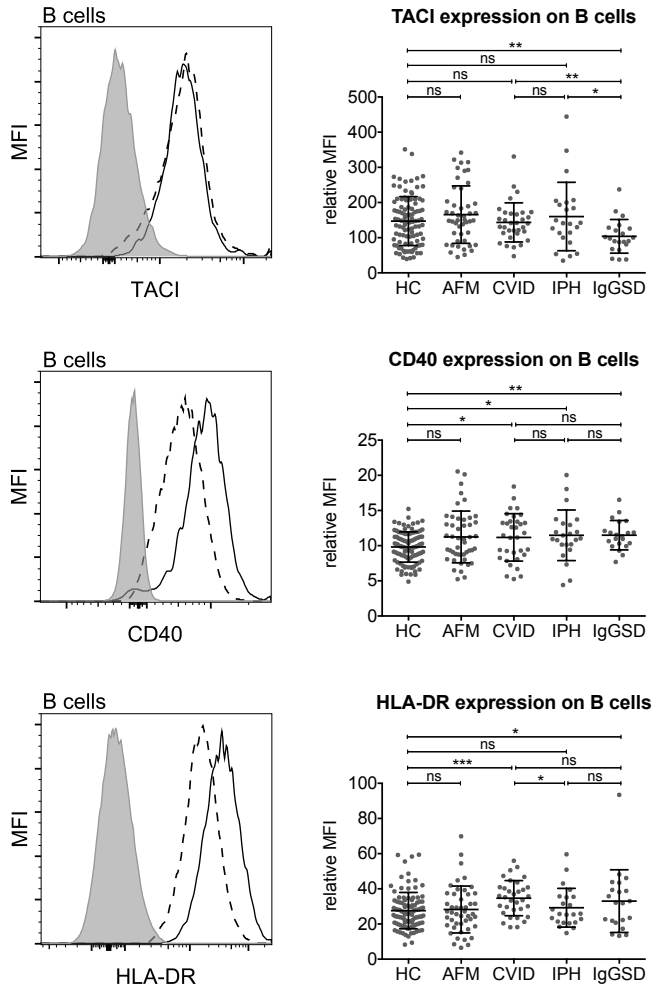

Figure S4. TACI, CD40 and HLA-DR expression on B cells.

Graphs represent mean ± SD. Representative flow cytometric analysis is shown on the left. Full black line represents CVID patient, dashed black line represents 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≤0.05, ** p≤0.01, *** p≤0.001, ns not significant (Mann-Whitney test with Bonferonni's correction for multiple comparisons).

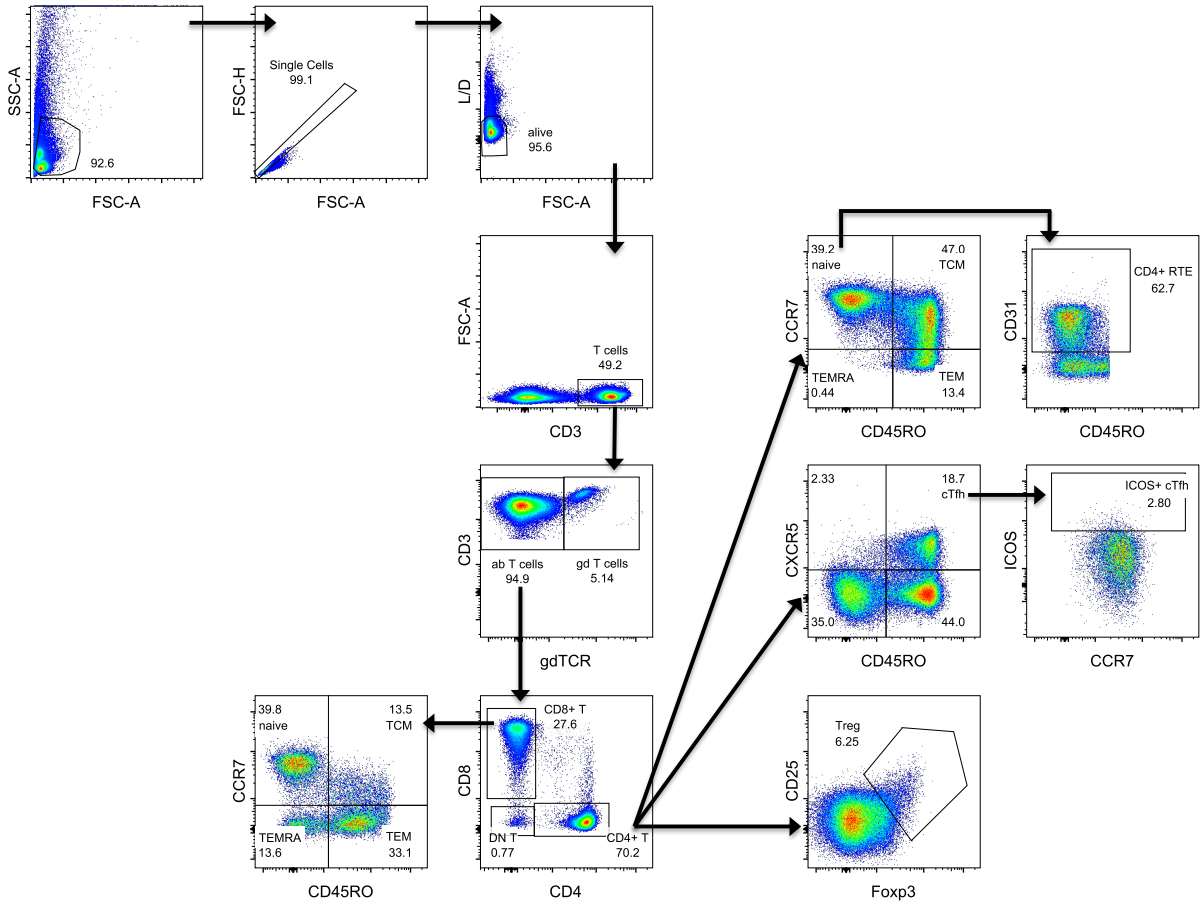

Figure S5. Representative flow cytometric analysis of naive and memory T cell subsets, DN T cells and Treg cells in an adult healthy control.

PBMCs were sequentially gated on cells (gating out debris), single cells (gating out doublets) and alive cells (gating out dead cells). Total T cells were gated as CD3+ in alive cells. αβ T cells were gated as γδTCR- and γδ T cells as γδTCR+ in total T cells. CD4+ and CD8+ T cells were gated in αβ T cells. Double negative (DN) T cells were gated as CD4-CD8- in αβ T cells. In CD4+ and CD8+ T cells, naive 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 naive CD4+ T cells, recent thymic emigrants (RTE) were gated as CD31+. Regulatory T (Treg) cells were gated as CD25+Foxp3+ in CD4+ T cells based on Fluorescence Minus One (FMO). Circulating follicular helper T (cTfh) cells were gated as CXCR5+CD45RO+ in CD4+ T cells. Within cTfh cells, ICOS+ cTfh cells were gated based on FMO. L/D, live/dead marker.

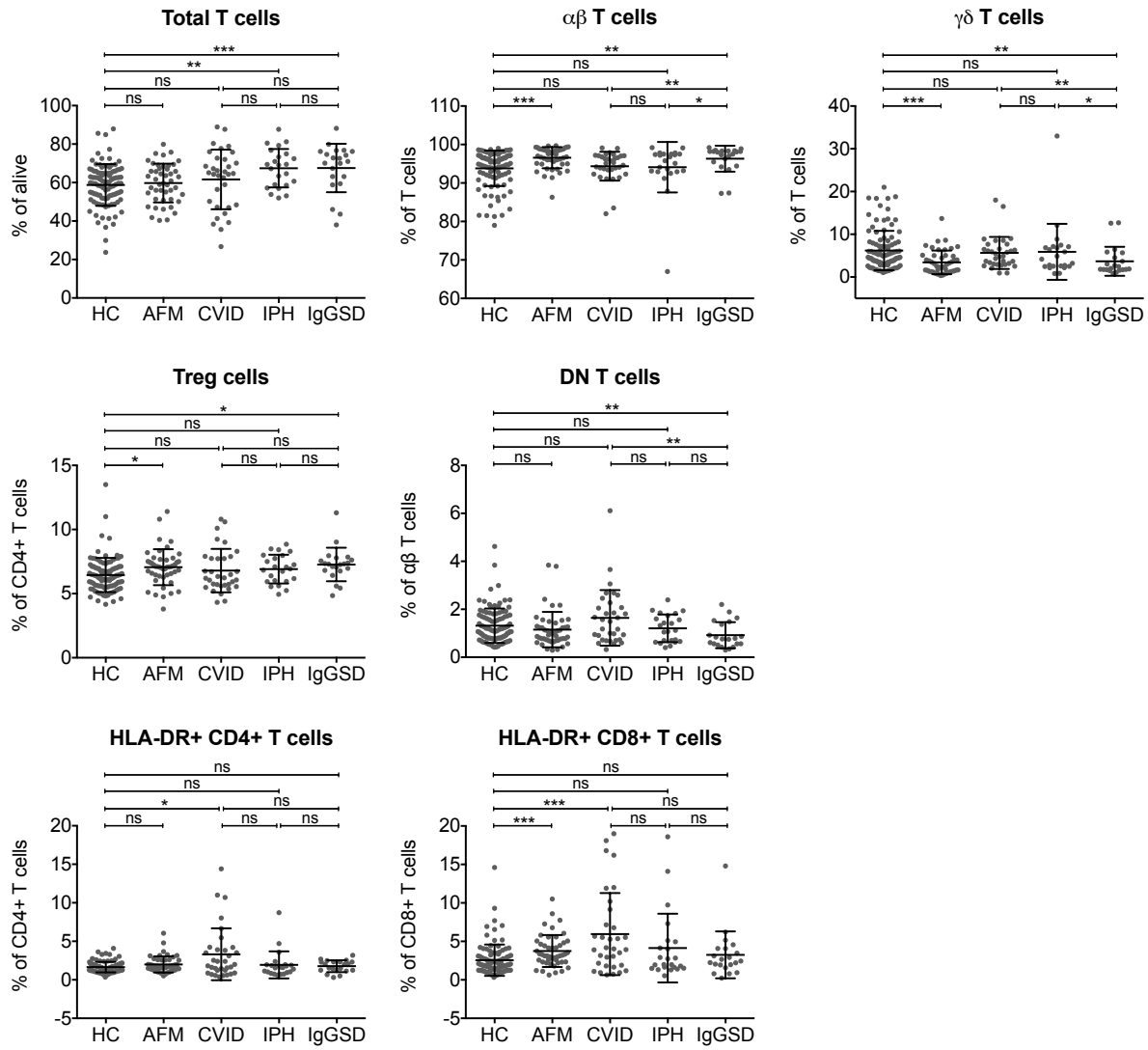

Figure S6. T cell subsets: total T cells, αβ T cells, γδ T cells, Treg cells, DN T cells, HLA-DR+CD4+ and HLA-DR+CD8+ T cells.

Total T cells were gated as CD3+ in alive cells. αβ T cells were gated as γδTCR- and γδ T cells as γδTCR+ in total CD3+ T cells. Regulatory T (Treg) cells were gated as CD25+Foxp3+ in CD4+ T cells. Double negative (DN) T cells were gated as CD4-CD8- in αβ T cells. HLA-DR+ cells were gated in CD4+ and CD8+ T cells. 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 Bonferonni's correction for multiple comparisons).

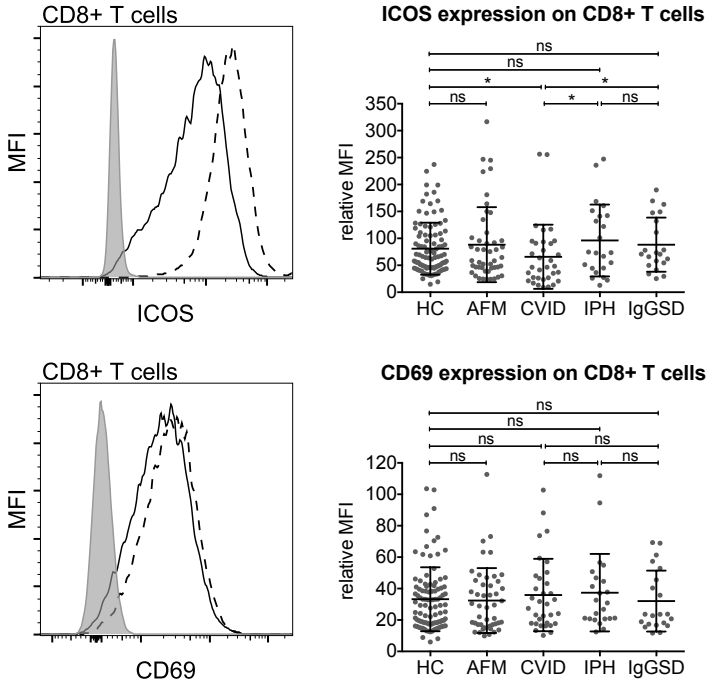

Figure S7. ICOS upregulation on stimulated CD8+ T cells.

ICOS and CD69 expression on CD8+ T cells stimulated with PHA for 72 hours. 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 line represents CVID patient, dashed black line represents 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≤0.05, ns not significant (Mann-Whitney test with Bonferonni's correction for multiple comparisons).

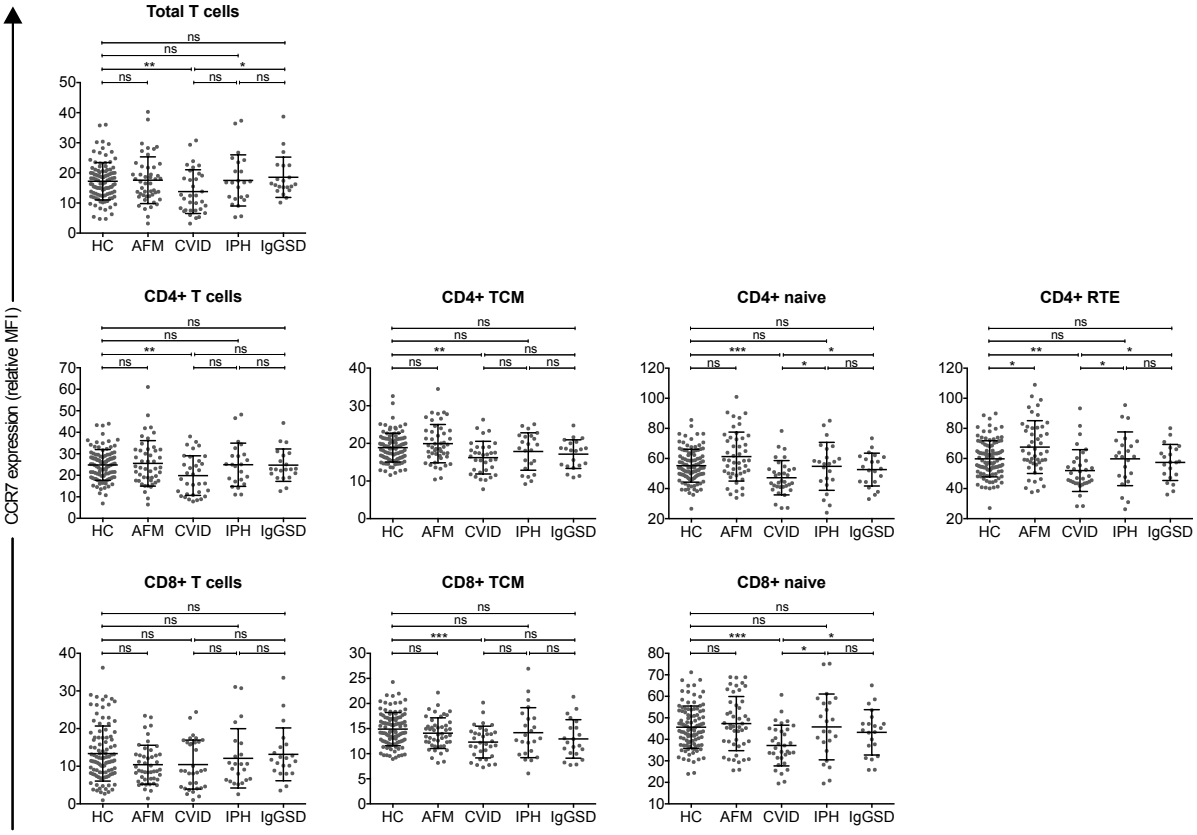

Figure S8. CCR7 expression on T cell subsets.

Total T cells were gated as CD3+ in alive cells. CD4+ and CD8+ T cells were gated in $\alpha\beta$ T cells. Within CD4+ and CD8+ T cells, central memory cells (TCM) were gated as CD45RO+CCR7+ and naive cells as CD45RO-CCR7+. In naive CD4+ T cells, recent thymic emigrants (RTE) were gated as CD31+. Relative mean fluorescence intensity (MFI) of CCR7 was calculated by dividing the MFI of the positive population by the MFI of the Fluorescence Minus One (FMO) population. Graph represents mean ± SD. 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 Bonferonni's correction for multiple comparisons).

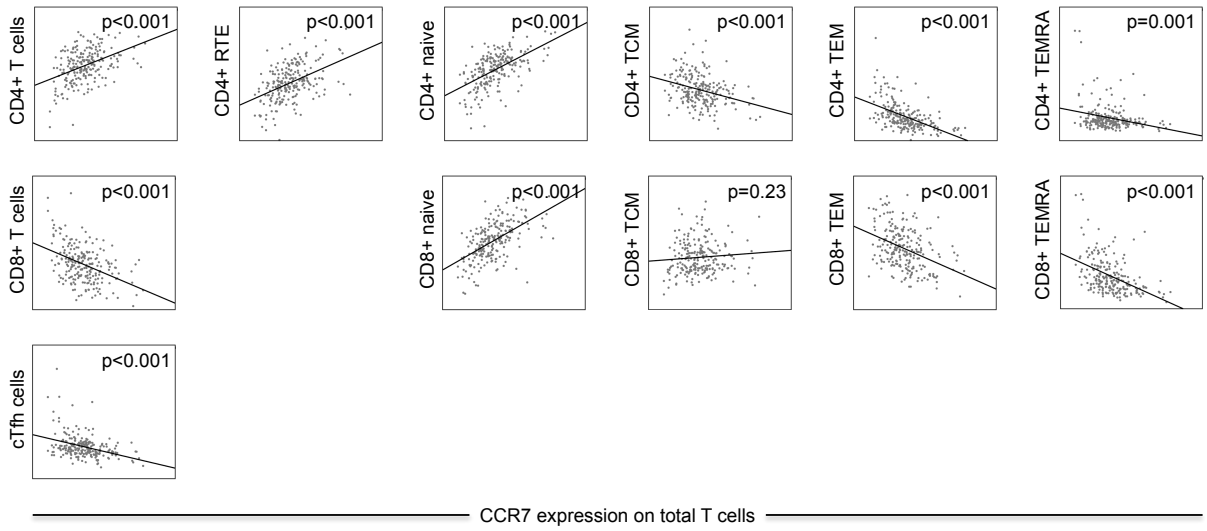

Figure S9. Correlation between CCR7 expression on total T cells and levels of T cell subsets.

Total T cells were gated as CD3+ in alive cells. CD4+ and CD8+ T cells were gated in $\alpha\beta$ T cells. Within CD4+ and CD8+ T cells, naive 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 naive CD4+ T cells, recent thymic emigrants (RTE) were gated as CD31+. Circulating follicular helper T (cTfh) cells were gated as CXCR5+CD45RO+ in CD4+ T cells. All naive and memory T cell subsets, including cTfh cells, were expressed as z-scores to adjust for age. CCR7 expression on total T cells is the relative mean fluorescence intensity (MFI) of CCR7 on total T cells, calculated by dividing the MFI of the positive population by the MFI of the Fluorescence Minus One (FMO) population. Correlations were calculated with Spearman Rank Correlation.

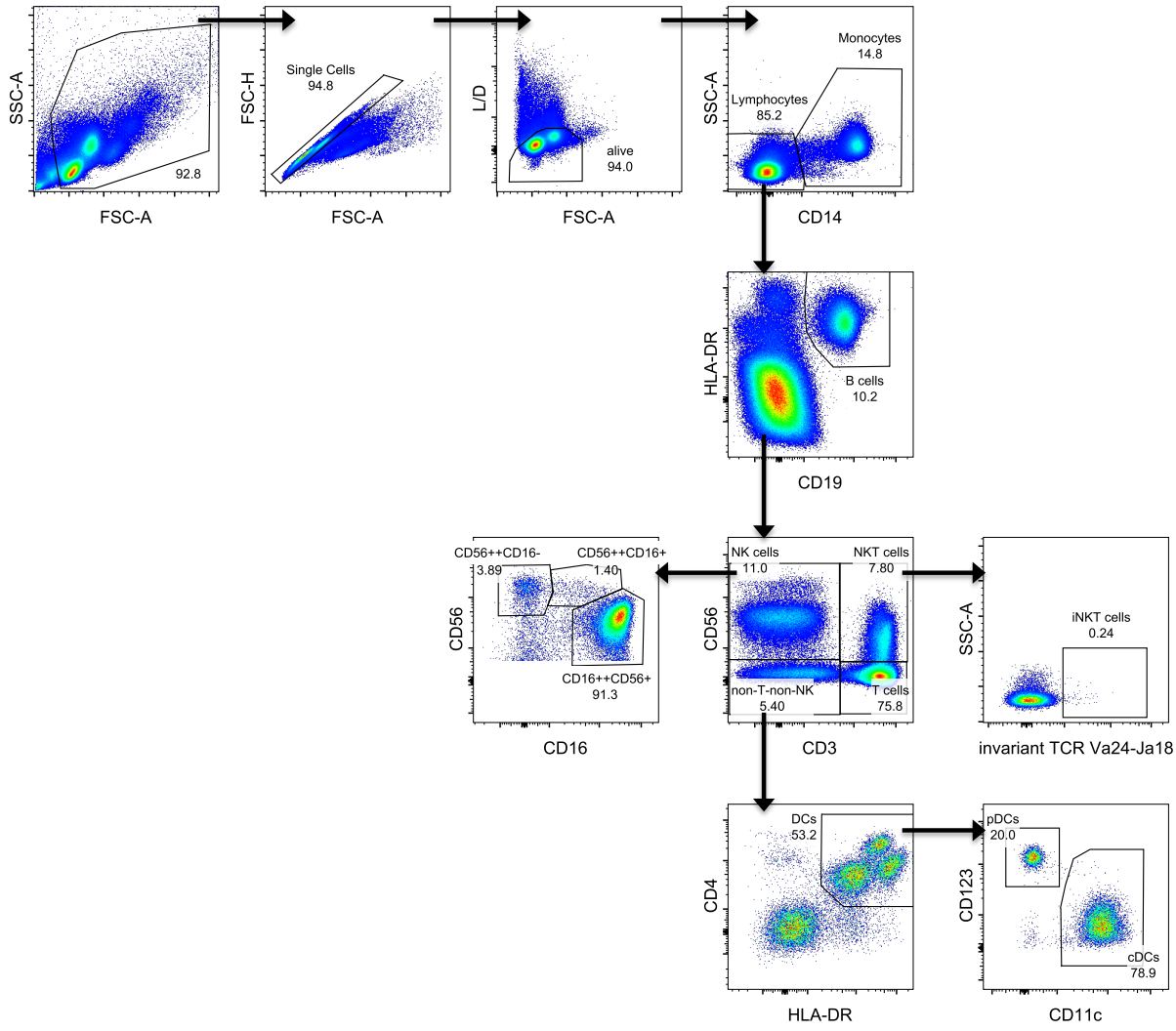

Figure S10. Gating strategy for innate immune cell subsets in an adult healthy control.

PBMCs were sequentially gated on cells (gating out debris), single cells (gating out doublets) and alive cells (gating out dead cells). Lymphocytes and monocytes were gated on SSC and CD14. B cells were gated as CD19+HLA-DR+ in lymphocytes; thereafter gating was continued on the NOT-gate. T cells were gated as CD56-CD3+, natural killer (NK) cells as CD3-CD56+ and natural killer T (NKT) cells as CD3+CD56+ in CD19-HLA-DR- lymphocytes. NK cell subsets were determined by their relative expression of CD56 and CD16. Invariant NKT (iNKT) cells were gated as invariant TCR (TCR Vα24-Jα18) positive in NKT cells. Dendritic cells (DCs) were gated as CD4+HLA-DR+ in non-T-non-NK cells. Within DCs, conventional DCs (cDCs) were gated as CD11c+CD123- and plasmacytoid DCs (pDCs) as CD123+CD11c-. L/D, live/dead marker.

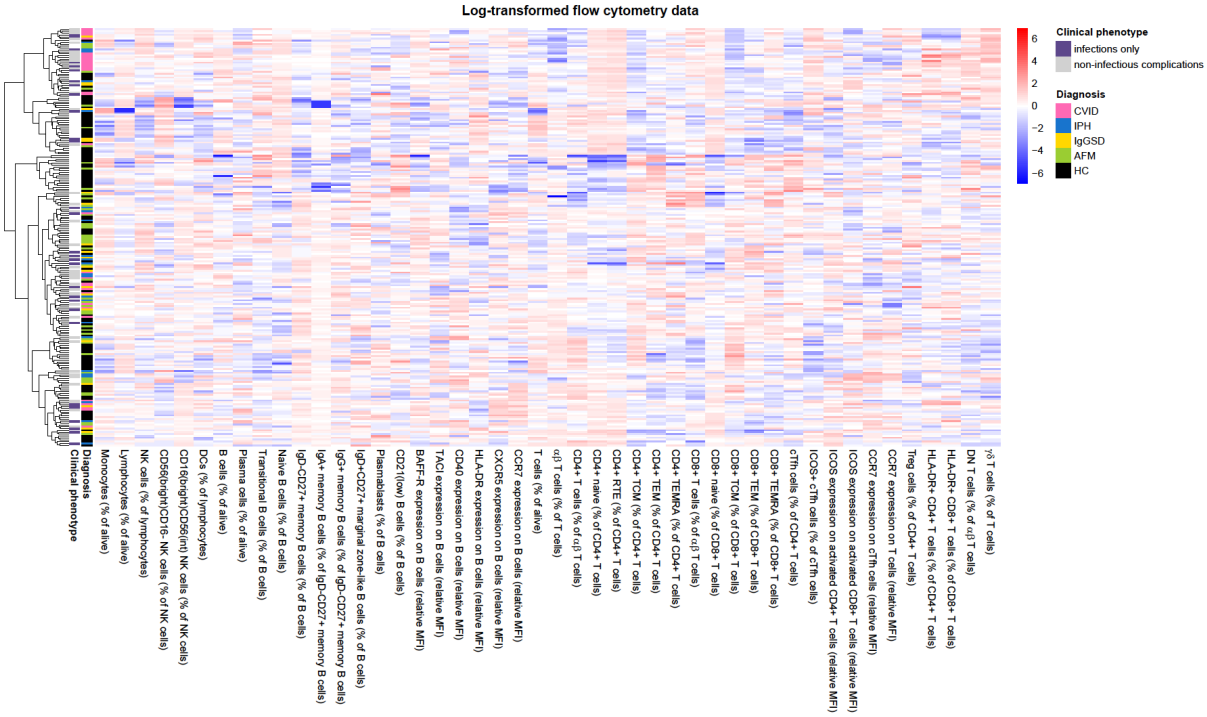

Figure S11. Unsupervised computational clustering analysis: hierarchical clustering and heatmap.

Unsupervised clustering of the study cohort based on 46 flow cytometric parameters. Study subjects are stratified according to diagnosis and patients are also stratified according to clinical phenotype (infections only versus non-infectious complications). Hierarchical clustering, plotted in the dendrogram on the left hand side, shows that subjects do not cluster according to diagnosis or clinical phenotype. The heatmap of log-transformed flow cytometric data does not reveal subgroups among patients nor distinguish patient from AFM or HC. AFM, asymptomatic family member; cTfh, circulating follicular helper T; CVID, common variable immunodeficiency; DCs, dendritic cells; DN T, double negative T; HC, healthy control; IgGSD, IgG subclass deficiency; IPH, idiopathic primary hypogammaglobulinemia; MFI, mean fluorescence intensity; NK, natural killer; RTE, recent thymic emigrants; TCM, central memory T; TEM, effector memory T; TEMRA, effector memory RA T; Treg, regulatory T.