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Comprehensive flow cytometric reference intervals of leukocyte subsets from six study centers across Europe

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Summary

A group of European FOCIS Centers of Excellence adapted panels of the Human Immunophenotyping Consortium (HIPC) for whole blood analysis. Using four core panels [T/regulatory T cell/B/natural killer (T/T_{reg}/B/NK) and myeloid cells] the main leukocyte populations were analyzed in a clinical–diagnostic setting in a harmonized manner across different platforms. As a first step, the consortium presents here the absolute and relative frequencies of the leukocyte subpopulations in the peripheral blood of more than 300 healthy volunteers across six different European centers.

Keywords: blood leukocyte counts, flow cytometry, immunophenotyping, reference intervals

Introduction

For decades, flow cytometry has been the standard technique for phenotyping immune cells in suspension. While, in the last 15 years, due to advances in instrumentation and introduction of novel fluorochromes, polyspectral approaches are standard techniques in basic science laboratories, their utilization in clinical immunology is not yet common practice. Furthermore, the analysis of three major lymphocyte

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subpopulations T, B and natural killer (NK) is, in most cases, not sufficient for clinical decision-making. Therefore, the characterization of differentiation and activation stages of the different lymphocyte subpopulations, as well as the analysis of other subpopulations of the innate immune system (monocytes, dendritic cells and granulocytes) is not only important to understand individual physiology of the immune system but also for differential diagnosis of immunodeficiency, activity in immune-mediated diseases and response towards immune-modulating therapies.

So far, different approaches have been utilized. As outlined by Maecker *et al.* [1], there are several opinions regarding suitable markers and different staining strategies have their pros and cons. In order to compare results across different sites, it is important to reach consensus regarding markers, reagents, instrument settings and gating/enumeration strategies, whereas minor site-to-site variations are of limited practical importance. Unlike in other areas of laboratory diagnostics, analysis of subpopulations of immune cells does not rely upon fixed reference intervals, but upon the overall pattern of differentiation/activation cell status and upon changes of these parameters during therapy. In addition, these laboratory values have to be stringently discussed in the clinical context of the individual patient.

In 2011, under the lead of the Federation of Clinical Immunology Societies (FOCIS), a minimal set of multispectral panels to evaluate the major leukocyte subsets in the peripheral blood was proposed [1]. So far, these panels have been evaluated in a standardized form by several centers [2]. However, as starting material this consortium favored frozen/lyophilized peripheral blood mononuclear cells (PBMC). In a clinical–diagnostic setting this approach seems to be problematic. First, original proportions of subpopulations – not biased due to isolation and manipulation techniques – and secondly, absolute cell counts are required. Therefore, a group of European FOCIS Centers of Excellence with support of the COST Action European Network for Translational Immunology, Research and Education (ENTIRE) adapted the proposed Human Immunophenotyping Consortium (HIPC) panels for whole blood analysis. The basic panels were compatible throughout different platforms. The emphasis was put upon harmonization, as due to the lack of suitable calibrators, the existence of a growing variety of cytometer platforms in different laboratories and the technical variations inherited to clinical material, standardization seems unfeasible. As a first step, the consortium presents here the percentages and absolute frequencies of major subpopulations in the peripheral blood of more than 300 healthy volunteers among six different European centers.

Materials and methods

A total of 330 healthy individuals (134 males, 196 females; age range = 20–81 years) who met the criteria for the study were recruited at six different study centers participating in the COST ENTIRE initiative, including Barcelona (BCN), Tartu (TAY), Heidelberg (HDB), London (LHR), Stockholm (ARL) and Halle (HAL). Age distribution of study participants across study centers is shown in Fig. 1. All study subjects gave their informed consent according to the approved protocols of research ethics committees of the participating study centers (Supporting information, Table S1).

After venipuncture, fresh anti-coagulated lithium heparinized whole blood of the study subjects was aliquoted in 100 µl amounts into polystyrene/polypropylene tubes and incubated with the appropriate fluorochrome-conjugated monoclonal antibodies (Supporting information, Table S2) to cell surface markers for 30 min at room temperature (RT) in the dark. After incubation, stained samples were subjected to red blood cell lysis using 2 ml 1× BD lysing solution (Cat. no 349202). After erythrocyte lysis for 10 min, the samples were washed and acquired on the flow cytometer. Prepared samples were stored at RT in the dark until analyzing (maximum 6 h) and for each sample, a maximum of 300 000 leukocyte events were recorded as a rule. Using appropriate thresholds, the collection of an excess of debris was avoided.

Flow cytometric analysis was performed on a flow cytometer available at the specific study center [either LSR Fortessa, FACS Canto (BD Biosciences, San Jose, CA, USA) or Navios (Beckman Coulter, Brea, CA, USA)] using a common set-up for the proposed markers (Supporting information, Fig. S1). The configuration of cytometers and the laser specifications are included in the Supporting information, Table S3. Briefly, BD cytometer set-up and tracking (CST) beads (Cat. no. 656505) were used to set the mean fluorescence form each fluorescence channel.

Data were analyzed with the FlowJo software (BD Biosciences) according to the gating strategy developed by the EU COST-ENTIRE Consortium working group (Supporting information, file: HIPC version 3.3 protocol). Gating was performed by each center separately using predesigned FlowJo gating templates. Gating strategy was published previously [3]. Relative numbers of T cells, regulatory T cells (T_{regs}), B cells, dendritic cells (DC), NK cells, monocytes, granulocytes and their subsets were determined. For the determination of absolute cell counts, different centers used different approaches: (1) single platform: (a) the BD Multitest™ six-color TBNK reagent and TruCount tubes (Cat. no. 337166) (HDB, TAY, HAL, LHR) or (b) Perfect Count Microspheres™ (Cat. no. CYT-PCM-100) (BCN); and (2) double platform, where the absolute numbers were calculated by the results of hematological analyzer results (ARL).

Using Trucount tubes, the absolute cell count was determined by comparing cellular events to bead events and the absolute counts were calculated according to the manufacturer's instructions, as follows:

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Fig. 1. Violin plots showing age distribution of immunophenotyped subjects across the centers (Kruskal–Wallis test). ARL = laboratory in Stockholm, Sweden; BCN = laboratory in Barcelona, Spain; HDB = laboratory in Heidelberg, Germany; HAL = laboratory in Halle, Germany; LHR = laboratory in London, UK; TAY = laboratory in Tartu, Estonia.

$$\frac{\text{events in cell population}}{\text{events in beads region}} \times \frac{\text{beads per tube}}{\text{sample volume } (50\mu l)} = \text{absolute cell count}$$

For quantification of absolute cell counts using Perfect Count Microspheres, 25 µl of peripheral blood samples was incubated with CD45-peridinin chlorophyll (PerCP) (BD Biosciences) for 15 min at room temperature in darkness. Erythrocytes were removed using 450 µl lysis buffer (BD FACS™ lysing Solution; BD Biosciences). Twenty-five microliters of Perfect Count Microspheres was added to each sample and acquired on a flow cytometer. The absolute counts were calculated as follows: (%subset/100) × counts of the main subpopulation.

In the T cell panel, following markers were used: CD45, CD3, CD4, CD8, CD45RA, chemokine receptor type 7 (CCR7) (CD197), C-X-C chemokine receptor type 3 (CXCR3) (CD183), CCR6 (CD196), human leukocyte antigen D-related (HLA-DR) and CD38. $CD4^+$ and $CD8^+$ T cells were further characterized: using the differential expression of CD45RA and CCR7 naive, central memory (CM), effector memory (EM) and effector memory RA (TEMRA) compartments were analyzed. Anti-CXCR3 and anti-CCR6 antibodies were used to identify T helper type 1 (Th1) ($CXCR3^+CCR6^-$), Th2 ($CXCR3^-CCR6^-$) and Th17 ($CXCR3^-CCR6^+$) subpopulations from CM and EM $CD4^+$ T cells. Activation of $CD4^+$ and $CD8^+$ T cells was examined by CD38 and HLA-DR expression.

In the T_{reg} panel, the following surface markers were utilized: CD45, CD3, CD4, CD127, CD25, CD45RO, CCR4 (CD194) and HLA-DR markers, following the consensus of the Human Immunology Consortium using whole blood. The T_{reg} subset was identified as $CD4^+CD25^+CD127^-$ T cells, and according to the differential expression of CD45RO and CCR4 were characterized as memory T_{reg}, and the expression of HLA-DR to define activated T_{reg}.

In the B cell panel, the following subsets were studied: $CD19^+$ B cells, naive B cells, transitional B cells, preswitch memory B cells, switched memory B cells, plasmablasts and exhausted memory B cells were identified, which were distinguished according to differential expression of CD45, CD3, CD19, CD20, CD27, CD24, CD38 and immunoglobulin (Ig)D.

In the NK cell/ myeloid cell panel the following markers were used: CD45, CD3, CD19, CD20, CD11c, CD123, CD14, CD16, CD56, HLA-DR and sulfo-LacNac (SLAN).

Table 1. Descriptive statistics of T cell frequencies and absolute counts

	Number of samples	Minimum	Maximum	Median	Mean	10% Percentile	90% Percentile	25% Percentile	75% Percentile	s.d.	s.e.	Lower 90% CI of mean	Upper 90% CI of mean
CD3^{+} (%)	270	35·00	90·00	67·00	66·31	55·00	76·00	61·00	72·00	8·69	0·53	65·44	67·18
CD3^{+} (cell/μl)	330	487·00	3204·00	1292·00	1361·00	791·00	2014·00	1022·00	1638·00	483·30	26·60	1308·00	1413·00
CD4^{+} T cell (%)	330	29·00	90·00	60·00	60·73	48·00	73·90	54·00	68·00	10·27	0·57	59·80	61·66
CD4^{+} T (cell/μl)	330	266·00	2318·00	782·00	823·10	454·50	1249·00	617·30	993·80	319·60	15·59	788·50	857·70
CD4^{+} T-naive (%)	330	8·00	93·00	44·00	44·22	26·00	62·00	34·00	54·00	14·70	0·81	42·88	45·55
CD4^{+} T-naive (cell/μl)	330	42·00	1590·00	330·00	378·70	146·00	673·90	218·00	465·00	232·20	1·30	353·60	403·90
CD4^{+} T-EMRA (%)	330	0·00	18·00	1·00	2·35	0·00	6·00	0·00	3·00	3·02	0·17	2·07	2·62
CD4^{+} T-EMRA (cell/μL)	330	0·00	146·00	8·00	18·55	2·00	49·80	3·00	22·00	23·55	4·15	16·00	21·10
CD4^{+} T-EM (%)	330	2·00	72·00	15·00	17·53	7·00	31·00	10·00	22·00	10·51	0·58	16·58	18·48
CD4^{+} T-EM (cell/μl)	330	7·00	445·00	116·00	133·40	58·00	234·00	78·50	167·00	75·44	2·07	125·30	141·60
CD4^{+} T-EM Th1 from CD4^{+}(%)	303	0·00	29·00	6·00	6·87	2·00	13·00	3·00	9·00	4·87	0·28	6·41	7·33
CD4^{+} T-EM Th1 (cell/μl)	303	1·00	231·00	42·00	51·94	17·40	102·60	25·00	71·00	35·98	0·94	47·88	56·01
CD4^{+} T-EM Th2 from CD4^{+}(%)	303	0·00	25·00	1·00	1·87	0·00	4·00	1·00	2·00	2·36	0·14	1·65	2·09
CD4^{+} T-EM Th2 (cell/μl)	303	0·00	133·00	9·00	14·10	4·00	28·60	6·00	16·00	16·44	0·73	12·24	15·95
CD4^{+} T-EM Th17 from CD4^{+} (%)	303	0·00	13·00	2·00	2·60	1·00	5·00	1·00	3·00	1·98	0·11	2·41	2·79
CD4^{+} T-EM Th17 (cell/μl)	303	1·00	85·00	17·00	19·44	7·00	36·00	10·00	26·00	12·66	7·87	18·01	20·87
CD4^{+} T-CM (%)	330	3·00	73·00	35·00	35·87	21·10	52·00	27·00	44·00	12·11	0·67	34·78	36·97
CD4^{+} T-CM (cell/μl)	330	12·00	852·00	269·00	292·60	142·10	488·90	189·80	372·50	142·90	2·04	277·10	308·00
CD4^{+} T-CM Th1 from CD4^{+} (%)	303	1·00	18·00	8·00	8·44	4·00	13·00	6·00	10·00	3·22	0·19	8·13	8·74
CD4^{+} T-CM Th1 (cell/μl)	303	3·00	216·00	65·00	68·74	28·00	116·60	46·00	87·00	35·58	2·03	64·71	72·76
CD4^{+} T-CM Th2 from CD4^{+} (%)	303	1·00	23·00	8·00	8·25	4·00	13·00	6·00	10·00	3·71	0·21	7·90	8·60
CD4^{+} T-CM Th2 (cell/μl)	303	2·00	181·00	57·00	64·99	27·40	117·20	41·00	81·00	35·35	2·14	61·00	68·99
CD4^{+} T-CM Th17 from CD4^{+} (%)	303	0·00	23·00	8·00	8·89	5·00	14·00	6·00	11·00	3·75	0·22	8·54	9·25
CD4^{+} T-CM Th17 (cell/μl)	303	1·00	247·00	64·00	70·69	31·00	117·20	45·00	92·00	37·23	0·43	66·48	74·90
CD4^{+} T-activated (%)	330	0·00	7·00	1·00	1·50	1·00	3·00	1·00	2·00	0·95	0·05	1·42	1·59
CD4^{+} T-activated (cell/μl)	330	0·00	47·00	10·00	12·02	4·00	22·00	7·00	15·00	7·79	0·43	11·17	12·86
Non-activated (CD38^{-} HLA-DR^{-}) CD4^{+} T cells (%)	330	16·00	101·00	59·00	58·33	36·00	82·00	47·00	70·00	16·59	0·91	56·83	59·83
Non-activated (CD38^{-} HLA-DR^{-}) CD4^{+} T cells (cell/μl)	330	107·00	1298·00	432·00	464·90	246·10	750·80	321·50	577·30	196·50	10·82	443·70	486·20
CD4^{+} CD38^{-} HLA-DR^{+} (%)	330	1·00	22·00	3·00	3·99	2·00	7·00	2·00	5·00	2·57	0·14	3·75	4·22
CD4^{+} CD38^{-} HLA-DR^{+} (cell/μl)	330	3·00	116·00	25·00	30·76	13·00	54·90	18·00	38·25	19·26	1·06	28·68	32·85
CD4^{+} CD38^{+} HLA-DR^{-} (%)	330	1·00	80·00	37·00	36·22	12·00	58·00	24·75	48·00	16·99	0·94	34·68	37·75
CD4^{+} CD38^{+} HLA-DR^{-} (cell/μl)	330	2·00	1643·00	262·50	315·90	81·00	651·20	148·80	401·50	237·70	13·08	290·10	341·60
CD8^{+} T (%)	330	7·00	67·00	32·00	31·66	20·00	42·00	26·00	37·00	8·73	0·48	30·87	32·45
CD8^{+} T cell (cell/μl)	330	61·00	1527·00	388·50	435·30	218·10	690·70	295·00	547·00	217·00	11·95	411·80	458·90
CD8^{+} T-naive (%)	330	3·00	88·00	35·50	37·67	14·00	62·00	23·00	52·00	18·34	1·01	36·01	39·33
CD8^{+} T-naive (cell/μl)	330	9·00	631·00	136·50	159·10	42·10	307·90	82·00	210·00	105·80	5·82	147·60	170·50
CD8^{+} T-EMRA (%)	330	1·00	85·00	33·00	33·80	12·00	59·00	19·00	47·00	17·60	0·97	32·20	35·39

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Table 1. (Continued)

	Number of samples	Minimum	Maximum	Median	Mean	10% Percentile	90% Percentile	25% Percentile	75% Percentile	s.d.	s.e.	Lower 90% CI of mean	Upper 90% CI of mean
$CD8^+$ TEMRA (cell/µl)	330	1·00	820·00	120·00	157·10	34·10	288·60	61·75	210·00	138·10	7·60	142·10	172·00
$CD8^+$ T-EM (%)	330	0·00	58·00	19·00	19·95	9·00	33·90	13·00	25·00	9·63	0·53	19·08	20·83
$CD8^+$ T-EM (cell/µl)	330	0·00	602·00	68·50	84·92	30·00	161·00	47·00	105·00	62·36	3·43	78·16	91·67
$CD8^+$ T-CM (%)	330	0·00	34·00	7·00	8·51	2·00	17·00	4·00	12·00	5·89	0·32	7·98	9·04
$CD8^+$ T-CM (cell/µl)	330	0·00	251·00	29·00	34·04	8·10	59·90	17·00	44·00	27·62	1·52	31·05	37·03
$CD8^+$ T-activated (%)	330	0·00	40·00	2·00	3·16	1·00	6·00	1·00	4·00	3·57	0·20	2·84	3·49
$CD8^+$ T-activated (cell/µl)	330	0·00	349·00	9·00	13·40	2·00	27·00	5·00	16·00	22·12	1·22	11·00	15·79
Non-activated ($CD38^-$ $HLA\text{-}DR^-$) $CD8^+$ T cells (%)	330	24·00	112·00	82·00	78·05	59·00	92·00	70·00	88·00	13·47	0·74	76·83	79·26
Non-activated ($CD38^-$ $HLA\text{-}DR^-$) $CD8^+$ T cells (cell/µl)	330	43·00	1329·00	301·50	340·60	154·40	568·80	217·50	413·00	186·90	10·29	320·40	360·90
$CD8^+$ $CD38^-$ $HLA\text{-}DR^+$ (%)	330	0·00	46·00	7·00	8·98	3·00	19·90	4·00	12·00	7·04	0·39	8·34	9·62
$CD8^+$ $CD38^-$ $HLA\text{-}DR^+$ (cell/µl)	330	3·00	317·00	25·00	38·69	8·00	84·00	15·00	50·00	37·73	2·08	34·60	42·78
$CD8^+$ $CD38^+$ $HLA\text{-}DR^-$ (%)	330	0·00	65·00	6·00	9·90	1·00	25·00	3·00	13·00	10·32	0·57	8·97	10·84
$CD8^+$ $CD38^+$ $HLA\text{-}DR^-$ (cell/µl)	330	1·00	407·00	23·50	42·99	5·00	106·80	9·00	57·00	52·70	2·90	37·28	48·69
T DP (%)	330	0·00	50·00	1·00	1·37	0·00	2·00	1·00	1·00	2·98	0·16	1·10	1·64
T DP (cell/µl)	330	2·00	848·00	11·00	18·96	5·00	31·90	7·00	18·00	50·26	2·77	13·52	24·41
T DN (%)	324	0·00	37·00	5·00	6·30	2·00	8·00	3·00	8·00	4·88	0·27	5·85	6·74
T DN (cell/µl)	324	5·00	639·00	65·00	84·33	24·00	157·50	41·25	106·00	73·57	4·09	76·29	92·37

Centers: ARL, Stockholm, Sweden; BCN, Barcelona, Spain; HDB, Heidelberg, Germany; HAL, Halle, Germany; LHR, London, UK; TAY, Tartu, Estonia.

EM = effector memory; CM = central memory; DP = double-positive; DN = double-negative; s.d. = standard deviation; s.e. = standard error; CI = confidence interval; CM = central memory; EM = effector memory; Th = T helper; HLA-DR = human leukocyte antigen D-related.

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Table 2. Descriptive statistics T_{reg} cell frequencies and absolute counts

	Number of samples	Minimum	Maximum	Mean	Median	10% Percentile	90% Percentile	25% Percentile	75% Percentile	s.d.	s.e.	Lower 90% CI of mean	Upper 90% CI of mean
T_{reg} (%)	263	3	13	7·74	8	5	10	6	9	1·87	0·12	7·55	7·93
T_{reg} cells (cell/µl)	263	18·00	173·00	64·00	67·18	36·00	104·60	48·00	80·00	26·80	1·65	63·92	70·43
Memory T_{reg} from $CD4^+$ (%)	263	2	11	4·45	4	3	6	4	5	1·42	0·09	4·31	4·60
Memory T_{reg} (cell/µl)	263	10·00	88·00	36·00	37·78	22·00	59·00	27·00	46·00	14·47	0·89	36·02	39·54
HLA-DR^+ from $CD4^+$ (%)	263	0	6	1·70	2	1	3	1	2	0·81	0·05	1·62	1·78
HLA-DR^+ T_{reg} (cell/µl)	263	2·00	46·00	13·00	14·37	6·40	25·00	9·00	18·00	7·51	0·46	13·45	15·28

Centers: BCN, Barcelona, Spain; HDB, Heidelberg, Germany; HAL, Halle, Germany; LHR, London, UK; TAY, Tartu, Estonia.
s.d. = standard deviation; s.e. = standard error; CI = confidence interval; T_{reg} = regulatory T cell; HLA-DR = human leukocyte antigen D-related.

Table 3. Descriptive statistics B cell frequencies and absolute counts

	Number of samples	Minimum	Maximum	Median	Mean	10% Percentile	90% Percentile	25% Percentile	75% Percentile	s.d.	s.e.	Lower 90% CI of mean	Upper 90% CI of mean
B cells (cell/µl)	260	53·00	628·00	211·00	229·00	106·00	364·60	151·50	271·80	106·60	3·65	216·00	242·00
Switch memory B cells (%)	260	2·06	82·07	19·90	17·27	8·38	33·03	12·00	24·62	11·39	0·71	18·74	21·06
Switched memory B cells (cell/µl)	260	3·00	349·00	37·00	44·23	15·00	76·80	25·00	54·00	34·06	11·39	40·07	48·39
Plasmablasts from B cell (%)	260	0·00	10·67	1·04	0·76	0·06	2·09	0·31	1·27	1·19	0·07	0·92	1·16
Plasmablasts (cell/µl)	260	0·00	13·00	1·50	2·07	0·00	4·00	1·00	3·00	2·09	1·19	1·81	2·32
Preswitch memory B cells (%)	260	0·82	79·93	16·75	14·98	4·86	29·59	8·00	22·59	12·05	0·75	15·52	17·98
Preswitch memory B cells (cell/µl)	260	1·00	471·00	29·00	37·31	9·00	68·60	16·25	44·00	39·97	12·05	32·43	42·19
Exhausted memory B cells (%)	260	0·79	39·37	4·61	3·00	1·19	9·81	1·98	5·17	4·93	0·31	4·11	5·11
Exhausted memory B cells (cell/µl)	260	1·00	63·00	7·00	10·07	2·00	26·90	4·00	12·00	10·05	4·93	8·85	11·30
Naive B cells (%)	260	1·23	87·68	58·81	61·00	36·27	78·00	50·00	69·67	16·56	1·03	57·12	60·50
Naive B cells (cell/µl)	260	1·00	475·00	126·50	137·50	45·10	232·50	81·50	177·00	81·04	16·56	127·60	147·40
B-transitional from B cell (%)	260	0·00	19·09	6·36	5·80	2·04	11·63	3·78	9·00	3·87	0·24	5·97	6·76
B-transitional (cell/µl)	260	0·00	75·00	12·00	14·66	3·00	30·90	6·00	20·00	12·06	3·87	13·19	16·13

Centers: BCN, Barcelona, Spain; HDB, Heidelberg, Germany; HAL, Halle, Germany; LHR, London, UK; TAY, Tartu, Estonia.
s.d. = standard deviation; s.e. = standard error; CI = confidence interval.

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Statistical analysis was carried out in two centers (Tartu and Barcelona) using the R language and environment and Graphpad Prism version 7. For analysis of differences between females and males the Mann–Whitney *U*-test was used. Association between age and cell counts was examined using Spearman's rank correlation test and age groups were compared using the Kruskal–Wallis test. Participants were divided into three groups aged 20–39, 40–59 and 60–81 years. The results of statistical tests were considered statistically significant when $P \leq 0{\cdot}05$. For the calculation of reference intervals, a non-parametric method was applied because of the non-normal distribution.

Results

T cell panel: centers ARL, BCN, HDB, HAL, LHR and TAY

Table 1 shows the distribution of cell percentages and total counts of T cell subpopulations. The data divided for gender groups are presented in Supporting information, Tables S3 and S4 and for age groups in Supporting information, Tables S5 and S6.

Females had a higher CD4⁺ T cell count than males (878 ± 325 *versus* 744 ± 296/μl). This difference was statistically significant in both the naive (416 ± 241 *versus* 325 ± 208/μl) and central memory (CM) (310 ± 147 *versus* 267 ± 134/μl), but not in the effector memory (EM) compartments. In-depth analysis of CM T cells revealed a higher count of CM Th1 cells in females *versus* males (75 ± 38; 59 ± 30/μl).

As expected, there was a clear age-relation in the distribution of naive and memory subsets of T cells. The cell frequencies and the absolute count of naive $CD4^+$ and $CD8^+$ T cells decreased with age (Supporting information, Tables S5 and S6), while the CM and EM $CD4^+$ T cell compartments, as well as the CM $CD8^+$ T cells, increased. In our cohort, we also observed an increase of EM Th1 and CM Th2 cells (Supporting information, Tables S5 and S6). In addition, there was a marked increase of activated HLA-DR⁺ $CD4^+$ and $CD8^+$ T cells in both the $CD38^+$ and $CD38^-$ compartments. We also observed an age-dependent decline of the DN population (Supporting information, Tables S5 and S6).

T_{reg} cell panel: centers BCN, HDB, HAL, LHR and TAY

Table 2 shows the distribution of the absolute and relative counts of T_{reg} subpopulations.

Although, in comparison between male and female subjects, we identified no differences in any of the T_{reg} subsets when absolute counts were analyzed (Supporting information, Table S7), sex-related differences appeared in the relative distribution of all T_{reg} subsets: males have significantly higher proportions compared to female subjects (Supporting information, Table S8).

Age-associated differences were analyzed in the different T_{reg} subpopulations divided into three groups (Supporting information, Tables S9 and S10). Absolute and relative cell frequencies of the total T_{reg} subpopulation did not show age-specific anomalies (Supporting information, Tables S9 and S10). However, the absolute number of memory T_{regs} and the absolute and relative count of activated T_{reg} increased with age (Supporting information, Tables S9 and S10).

B cell panel: centers BCN, HDB, HAL, LHR and TAY

Table 3 shows the absolute and relative counts of the total B cell population and their subpopulations.

The absolute number and the relative amount of the total population of peripheral blood $CD19^+$ B cells remained stable throughout adulthood and did not show sex-related (Supporting information, Tables S11 and S12) or age-related differences (Supporting information, Tables S13 and S14) The sex-related differences appeared only in the absolute count and frequency of preswitch memory B cells, showing significantly higher values in males compared to female participants.

We used anti-IgD and anti-CD27 antibodies to separate B cells into four subsets: naive B cells (IgD^+CD27^-), preswitch memory B cells (IgD^+CD27^+), switched memory B cells (IgD^-CD27^+) and exhausted double-negative B cells (IgD^-CD27^-). Interestingly, the proportion of naive B cells does not decrease with age and remains the predominant B cell subset throughout adulthood. None of the three memory B cell subsets (preswitch memory, switched memory, exhausted memory) showed statistically significant age-related changes. However, the absolute count and frequency of the developmentally earliest B cell subset in the periphery, termed transitional B cells ($IgD^+CD27^-CD38^+CD24^+$), as well as the frequency of plasmablasts ($CD20^-IgD^-CD27^+$ $CD38^+$), decreased with age.

NK cell and myeloid cell panel: centers BCN, HDB, HAL, LHR and TAY

Table 4 shows the relative distribution and the absolute counts of NK cell, monocyte and DC cell subpopulations.

NK cells and myeloid cells were identified by the absence of T and B cell-specific lineage markers (CD3, CD20 and CD19). NK cells were then divided into four subsets based on the differential expression of CD56 and CD16. $CD56^+CD16^+$ ($CD56^{dim}CD16^+$) subset represent the predominant NK cell subset in all age groups followed by $CD56^{++}CD16^-$ ($CD56^{bright}CD16^-$), $CD56^+CD16^-$ ($CD56^{dim}CD16^-$) and $CD56^{++}CD16^+$ ($CD5^{bright}CD16^+$) NK cell subsets. The proportion of the total NK cell population increases with age (Supporting information, Table S17),

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Table 4. Descriptive statistics NK cells, monocytes, dendritic cells and granulocyte frequencies and absolute counts

	Number of samples	Minimum	Maximum	Median	Mean	10% Percentile	90% Percentile	25% Percentile	75% Percentile	s.d.	s.e.	Lower 90% CI of mean	Upper 90% CI of mean
NK cells (%)	270	2·00	55·00	12·00	12·64	6·00	20·00	8·00	16·00	6·74	0·41	11·97	13·32
NK cells (cell/μl)	270	33·00	1345·00	242·00	264·10	98·10	441·20	154·50	327·30	166·60	10·14	244·10	284·10
$CD56^{bright}$ $CD16^{-}$ (%)	270	0·00	20·00	4·00	5·07	2·00	9·00	3·00	7·00	3·22	0·20	4·75	5·40
$CD56^{bright}$ $CD16^{-}$ (cell/μl)	270	1·00	34·00	10·00	10·83	4·00	18·00	7·00	14·00	5·89	0·36	10·13	11·54
$CD56^{bright}$ $CD16^{+}$ (%)	270	0·00	15·00	2·00	2·19	1·00	4·90	1·00	3·00	1·93	0·12	1·99	2·38
$CD56^{bright}$ $CD16^{+}$ (cell/μl)	270	0·00	28·00	4·00	4·90	1·00	10·00	2·00	6·25	4·21	0·26	4·40	5·40
$CD56^{dim}$ $CD16^{-}$ (%)	270	0·00	95·00	4·00	5·89	1·00	10·00	2·00	6·00	9·53	0·58	4·93	6·84
$CD56^{dim}$ $CD16^{-}$ (cell/μl)	270	1·00	318·00	8·00	12·71	3·10	20·90	5·00	13·00	22·47	1·37	10·02	15·41
$CD56^{dim}$ $CD16^{+}$ (%)	270	0·00	99·00	89·00	86·87	79·00	95·00	84·00	93·00	11·07	0·67	85·76	87·98
$CD56^{dim}$ $CD16^{+}$ (cell/μl)	270	0·00	1288·00	208·00	235·70	74·40	413·80	132·50	297·30	163·00	9·92	216·20	255·20
NT-cells (%)	270	0·00	48·00	5·00	6·51	1·00	13·00	3·00	8·00	6·06	0·37	5·90	7·11
NT-cells (cell/μl)	270	7·00	1052·00	95·00	140·10	28·10	300·10	48·00	168·80	150·70	9·17	122·00	158·20
Dendritic cells (DC) (%)	269	0·30	5·11	1·16	1·27	0·70	1·88	0·89	1·56	0·56	0·03	1·21	1·32
Dendritic cells (DC) (cell/μl)	269	16·00	404·00	72·00	79·80	35·00	137·00	49·50	98·00	45·31	2·76	74·36	85·23
$CD11c^{-}CD123^{-}$ DC (%)	229	0·00	75·00	10·00	14·90	2·00	38·00	4·00	20·00	14·13	0·93	13·37	16·44
$CD11c^{-}CD123^{-}$ DC (cell/μl)	229	0·00	274·00	7·00	12·13	1·00	28·00	3·00	16·00	20·73	1·37	9·43	14·83
Plasmacytoid DC (%)	229	1·00	44·00	11·00	13·43	5·00	24·00	7·00	18·00	7·79	0·51	12·58	14·27
Plasmacytoid DC (cell/μl)	229	2·00	32·00	8·00	9·20	4·00	17·00	5·00	12·00	5·54	0·37	8·48	9·92
Myeloid DC (mDC) (%)	229	20·00	95·00	73·00	71·11	49·00	89·00	62·50	83·00	15·29	1·01	69·45	72·77
Myeloid DC (mDC) (cell/μl)	229	7·00	222·00	49·00	55·49	23·00	99·00	32·00	74·00	31·44	2·08	51·40	59·58
$Slan^{+}$ $CD16^{+}$ mDC (%)	204	0·00	87·00	45·50	45·42	23·00	68·50	34·00	57·00	17·30	1·21	43·43	47·41
$Slan^{+}$ $CD16^{+}$ mDC (cell/μl)	204	0·00	129·00	21·00	25·27	6·00	49·00	12·00	34·00	19·04	1·33	22·64	27·90
$Slan^{-}$ $CD16^{-}$ mDC (%)	204	3·00	79·00	26·00	29·35	14·00	51·00	19·00	37·00	14·15	0·99	27·72	30·98
$Slan^{-}$ $CD16^{-}$ mDC (cell/μl)	204	1·00	43·00	12·00	13·77	6·00	24·00	8·25	17·00	7·52	0·53	12·73	14·81
$Slan^{-}$ $CD16^{+}$ mDC (%)	204	0·00	73·00	22·50	23·96	7·00	41·50	14·25	31·00	13·74	0·96	22·37	25·54
$Slan^{-}$ $CD16^{+}$ mDC (cell/μl)	204	0·00	83·00	9·00	14·46	2·00	34·00	5·00	17·75	14·98	1·05	12·39	16·53
Monocytes (%)	270	0·28	19·04	5·81	5·99	3·75	8·25	4·66	7·14	1·97	0·12	5·79	6·19
Monocytes (cell/μl)	270	48·00	896·00	354·50	370·60	185·40	568·40	261·80	477·00	148·20	9·02	352·80	388·30
$CD14^{+}$ $CD16^{-}$monocytes (%)	270	62·00	100·00	96·00	95·22	92·00	98·00	94·00	97·00	3·58	0·22	94·86	95·58
$CD14^{+}$ $CD16^{-}$ monocytes (cell/μl)	270	30·00	892·00	337·00	353·50	176·20	538·90	247·80	451·30	143·30	8·72	336·30	370·60
$CD14^{+}$ $CD16^{+}$ monocytes (%)	270	0·00	37·00	4·00	4·71	2·00	8·00	3·00	6·00	3·54	0·22	4·36	5·07

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Table 4. (Continued)

	Number of samples	Minimum	Maximum	Median	Mean	10% Percentile	90% Percentile	25% Percentile	75% Percentile	s.d.	s.e.	Lower 90% CI of mean	Upper 90% CI of mean
CD14⁺ CD16⁺ monocytes (cell/μl)	270	0·00	113·00	14·50	16·91	5·00	31·00	9·00	21·00	13·13	0·80	15·34	18·48
Granulocytes (%)	270	16·26	83·31	60·38	59·43	45·12	71·30	52·75	67·08	10·34	0·63	58·40	60·47
Granulocytes (cell/μl)	270	287·00	13414·00	3602·00	3875·00	1967·00	6176·00	2664·00	4653·00	1808·00	110·00	3659·00	4092·00

Centers: BCN, Barcelona, Spain; HDB, Heidelberg, Germany; HAL, Halle, Germany; LHR, London, UK; TAY, Tartu, Estonia.
s.d. = standard deviation; s.e. = standard error; CI = confidence interval; mDC = myeloid dendritic cells; NK = natural killer; Slan = sulfo-LacNac.

while the absolute count and proportion of CD56^{bright}CD16^{-} and CD56^{dim}CD16^{-} NK cells show an age-related reduction (Supporting information, Tables S17 and S18). In contrast, the absolute and relative count of NK T cells are inversely correlated with age. When we studied the effect of sex, we observed that there is a difference between cell proportion and/or absolute count in male and female in the total NK cell population and the in CD56^{bright}CD16^{-} NK cell subset (Supporting information, Tables S15 and S16).

The parameters of the whole population ofs DC (LIN^{-}CD14^{-}CD56^{-}LA^{-}DR^{+}) are relatively stable throughout adulthood (Supporting information, Tables S17 and S18). In DC subsets, age has a differential effect. Absolute counts of the relative proportion of myeloid DCs (mDC) (CD123^{-}CD11c^{+}), which is the major subset of DC in the peripheral blood, are expanding in parallel with increasing age, accompanied by a decrease in the absolute count and frequency of plasmacytoid DCs (pDC) (CD123^{+}CD11c^{-}) (Supporting information, Tables S17 and S18). We also studied mDC subsets divided based on the differential expression of CD16 and Slan. The majority of mDCs express CD16. Interestingly, the proportion of CD16^{+} single-positive mDC increases with age, while CD16^{+}Slan^{+} cells show a reverse trend. The sex-specific difference appeared only in the proportion of DC, as males have a slightly higher frequency of DCs than females (Supporting information, Table S15).

It seems that the absolute number of cells in the monocyte population remain unchanged over age, whereas the relative proportion of monocytes slightly decreases with age (Supporting information, Tables S17 and S18). As in the total population of DCs, males also show a slightly higher proportion of monocytes when compared with females (Supporting information, Table S15). When we divided monocytes were into two subsets based on the expression of CD16 (also known as FcγRIII): (1) CD14^{+}CD16^{-} classical monocytes, which represent the majority of monocytes, and (2) a minor subset of CD14$^{+/low}$CD16^{+} non-classical monocytes, we did not notice any difference between them. Absolute counts and relative size of both monocytes subsets are comparable between males and females (Supporting information, Tables S15 and S16) and over age (Supporting information, Tables S17 and S18). The absolute count of granulocytes remains strongly stable through adulthood and is not influenced by the sex of the study participants; the relative size of the total granulocyte population is positively correlated with age (Supporting information, Table S17).

Discussion

The diversity in the healthy human immune system has been only partially described, and the focus has been more upon characterizing disorders related to the immune system. The reason is that the data concerning

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interindividual variation are limited and the metrics of immune health are not clearly defined [4].

Since its invention in the 1960s, flow cytometry has undergone rapid development and has become the first-choice method in the evaluation, prognosis and diagnosis of a variety of clinical conditions, including autoimmune diseases, immunodeficiency, infection, malignancy and transplantation [1]. The availability of accurate reference intervals obtained from a healthy population, against which the patients' results can be evaluated, is essential for all diagnostic methods [5].

Further, the accurate measurement of variations in the immune system depends upon a standardized assessment procedure, which is critical to demarcate between true biologically important changes from artifacts [4].

In this study, we used a standardized protocol for flow cytometric analysis to describe sex- and age-related variations of cellular components of the adaptive and innate immune system in more than 300 healthy volunteers from six different study centers.

T cells

Sex-specific differences in relation to the total $CD4^+$ T cell numbers, as well as both the naive and central memory $CD4^+$ populations, have been previously reported [6]. However, considering the large spread and overlap in and between the groups this observation seems to be not relevant in a diagnostic setting. Therefore, both cohorts were combined for further analysis.

As previously described [7], there is a clear relation to age in the distribution of naive and memory T cell subsets. Using age as a continuous parameter, the strongest correlation was found between the decline of the number of naive $CD8^+$ T cells and age (Fig. 2). However, there was no significant accumulation of memory type $CD8^+$ T lymphocytes. Therefore, the reported relative accumulation of the latter with age is only due to the decline of naive $CD8^+$ T lymphocytes. This confirms the findings of Wertheimer *et al.*, although a different staining strategy was employed in this study [7].

The enumeration of naive and the different memory populations based on the expression of CCR7 and CD45RA is straightforward for $CD4^+$ cells; however, in the $CD8^+$ population the border between effector-memory and T_{EMRA} cells requires a consensus opinion. The re-expression of CD45RA is a continuous process, and depending on the instrument settings a distinguished intermediate population appears. The use of additional markers such as CD27, CD28, CD57 and CD127 suggested that the intermediate population relates more to the T_{EMRA} population (data not shown). Naive cells were defined as $CD45RA^{++}/CCR7^+$; CM cells as $CD45RA^-/CCR7^+$; EM cells as $CD45RA^-/CCR7^-$; and T_{EMRA} cells as $CD45RA^{+/++}/CCR7^-$. The proportion of $CD8^+$ T_{EMRA} is highly dependent upon the CMV-status of healthy individuals [7]; however, these data were not available in our cohort and is a limitation of our study.

Our core panel for analysis of peripheral T cells provides various opportunities to add markers that answer specific questions. In one of our study sites, 17 different markers were studied in this panel for diagnostic purposes. For example, an important question is the expression of CD28 on the different T cell subpopulations, as CD28-negative cells are, for example, potentially resistant to cytotoxic T lymphocyte antigen 4 (CTLA-4) blockade. Naive and CM $CD8^+$ T cells are considered CD28-positive, EM-variable, and T_{EMRA} CD28 mainly negative [8,9]. In the CD4 compartment, the number of $CD28^-$ cells is much less frequent [10].

In our cohort, we observed infrequently a high number of $CD4^-CD8^-$ double-negative T cells (DN). Most of these cells turned out to be T cell receptor (TCR)-γδ cells. Therefore, we considered it important to also include an anti-TCR-γδ antibody into the panel. We observed an age-dependent decline of the DN population, which might reflect the lower counts of γδ T cells observed with age [11]. The presence of TCR-αβ DN T cells above 2·5% of the $CD3^+$ T cells is a criterion for the diagnosis of an autoimmune lymphoproliferative syndrome (ALPS) [12], and the addition of this marker might be meaningful in the clinical immunology laboratory.

For the estimation of activated T cells a combination of HLA-DR and CD38 was used, as this has been suggested for the monitoring of HIV-positive patients [13]. For further characterization of Th cells a combination of anti-CCR6 and anti-CXCR3 antibodies were employed [14]. Here, Th1 cells were defined as $CD4^+/CXCR3^+/CCR6^-$, Th2 as $CD4^+/CXCR3^-/CCR6^-$ and Th17 as $CD4^+/CXCR3^-/CCR6^+$.

In the originally proposed HIPC panels three tubes were utilized to characterize peripheral T lymphocytes: T basic, T regulatory and T helper. In the present study, the basic and the helper panels were combined resulting in a 10-color panel. At study sites that lack the equipment above eight-color detection, the T_{helper} panel can still be measured separately in case this information is required.

T_{reg} cells

T_{regs} play a crucial role in immune homeostasis and are associated with prevention of autoimmune diseases, allergies and allogenic responses in organ transplantation [15–17].Characterization of T_{reg} cells was initially difficult because they do not depict specific markers, and no consensus standardized phenotypical panel exists for them.

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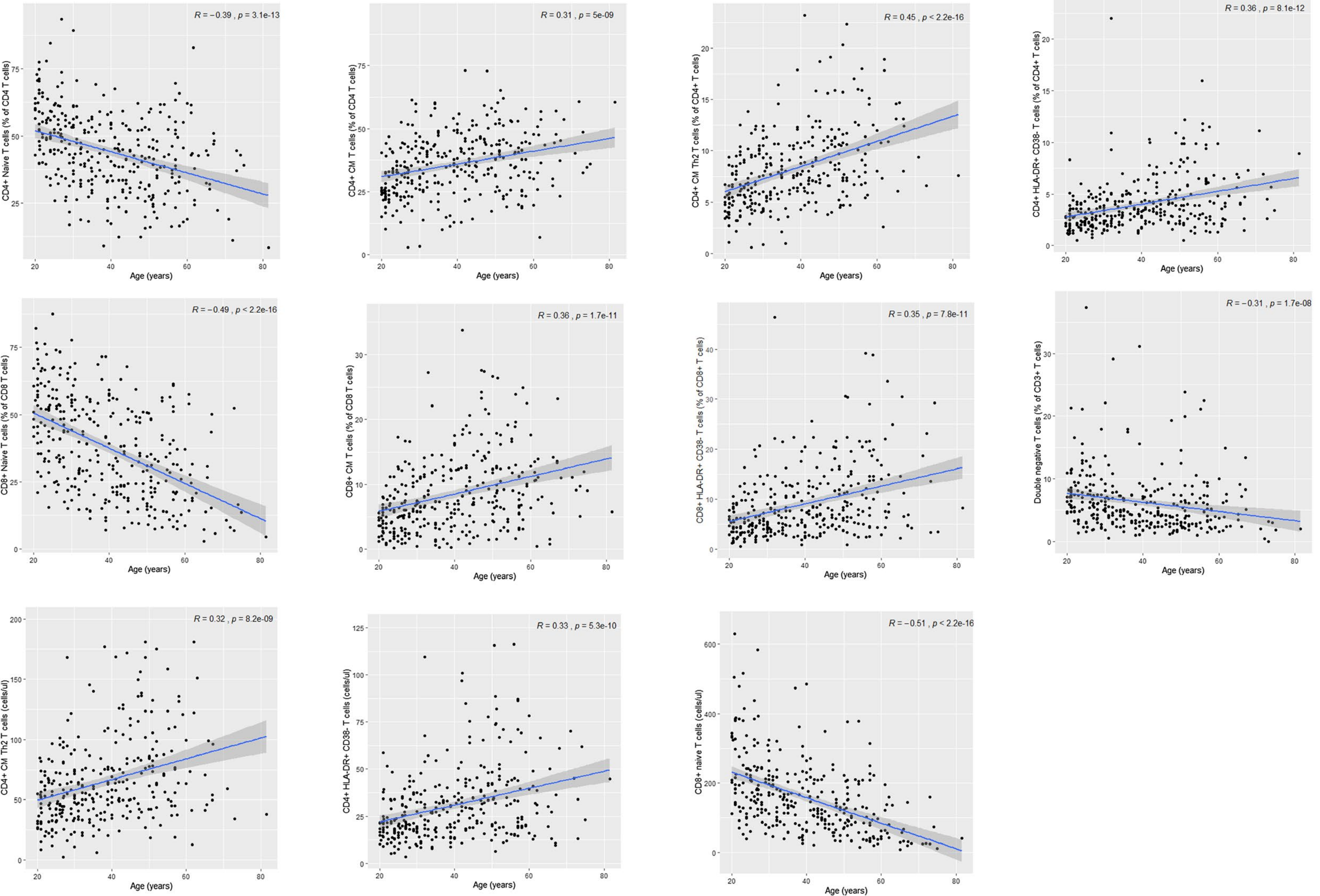

Fig. 2. Correlation between age and relative or absolute cell counts in T cell panel (only data with correlation coefficient ≥ 0·3 or ≤ −0·3 are shown). Spearman's rank correlation was performed and the cell subset dependency with age is illustrated by regression lines and confidence intervals.

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The heterogeneity in utilized monoclonal antibodies made it difficult to compare studies, either in healthy individuals or in patients with immune-mediated diseases [18].

T_{reg} can also be subdivided into naive and memory T_{reg} through the expression of CD45 isoforms, together with chemokine receptors [20,21]. Expression of CCR4 is restricted to circulating memory T_{reg} [22]. Most of the T_{reg} in the peripheral blood of adults show a $CCR4^+CD45RO^+$ effector/memory phenotype. Moreover, most research to date on T_{reg} focuses upon this subset [22–24].

Interestingly, memory T_{reg} cells can be further subdivided on the basis of HLA-DR expression. Mature and highly activated T_{reg} express HLA-DR [25,26].

Therefore, in our study, we characterized T_{reg} subsets (memory and activated) by the surface expression of CD4, CD25, CD127, CD45R0, CCR4 and HLA-DR following the consensus of the Human Immunology Consortium [1], but using whole blood. Our results show that there is an increase in the percentage of memory T_{reg} and activated T_{reg} with age, in accordance with previous results in which these populations of T_{reg} have been analyzed, although using different markers [26]. Nevertheless, no age- or sex-specific differences in the total count of T_{reg} lymphocytes were found in our cohort.

B cells

B cells play an essential role in adaptive humoral immunity, as they are the only cells capable of differentiang into antibody producing plasma cells. In addition, they can act as antigen-presenting cells and initiate an immune response. B cells also have regulatory and effector functions mediated by cytokines and chemokines [27]. Different marker combinations have been proposed for the identification of memory and naive B cells [28,29]. Here, we used anti-IgD and anti-CD27 antibodies for dividing B cells into four subsets. CD27 is considered to be a memory B cell marker and its expression is in correlation with cell size, proliferative capacity, antigen presentation efficacy and ability to differentiate into antibody-secreting plasma cells [30]. Similarly, as has been shown by other studies [31], we also found that the majority of B cell subsets are not affected by sex.

As reviewed by Frasca and colleagues [32], there are conflicting results in the field of studies focusing upon the effect of aging on B cells. In our study, the absolute number and relative size of the total population of $CD19^+$ B cells, as well as naive and memory B cell subpopulations, showed a slight but not statistically significant age-associated trend of reduction, which is also shown by other authors [11,33]. In contrast to our study, other studies have not shown an age-dependent reduction of immature transitional B cells (here identified as $CD19^+IgD^+CD27^-CD38^+CD24^+$) in adults [28,31,33].

Plasmablasts (here identified as $CD19^+CD20^-IgD^-CD27^+CD38^+$) are short-lived precursor cells for long-lived plasma cells. Our study revealed an age-related reduction in the proportion of plasmablasts, which is consistent with previous studies [28,33,34] showing an age-associated decline in plasma cell absolute counts and/or proportions. These findings confirm the findings of Pritz *et al.*, who demonstrated a reduction of plasma cells in the bone marrow in relation to aging [34]. In contrast to our results, Caraux *et al.* demonstrated that memory cells are also in reverse relationship with age [28]. The controversy between these results might result from different marker choices, as they used CD27 in combination with CD10 and CD38, instead of IgD, which was used in the present study.

Double-negative (IgD^-CD27^-) B cells have been characterized as senescent/exhausted memory B cells, but so far their biological function remains elusive [35]. Expansion of these exhausted B cells has been associated with chronic antigenic stimulation and with diseases such as systemic lupus erythematosus [36] and Alzheimer's disease [37], but also with aging [38]. Our data did not support the latter finding, as in our study population we surprisingly observed the decline of exhausted B cells in elderly patients; however, this did not reach statistical significance. Conflicting results might be derived from the choice of study material, as Colonna-Romano *et al.* used separated PBMCs and our data are based on the analysis of fresh whole blood, which again illustrates the necessity of harmonization of flow cytometric methods in order to compare results from different studies in a meaningful way. Many authors have emphasized the critical part of methodical differences as the main source of variability in flow cytometric immunophenotyping [1,39,40].

NK cells, dendritic cells, monocytes and granulocytes

In concordance with several other studies, we found that the number of NK cells increases with age [41,42]. We used the expression of CD56 and CD16 to study four NK cell subsets. Immature NK cells, forming approximately 10% of the peripheral blood NK cells, have high levels of CD56 on their surface and lack expression of CD16 ($CD56^{bright}CD16^-$). As NK cells mature they start to express CD16, while the expression of CD56 is down-regulated ($CD56^{dim}CD16^+$) [43]. In relation to age we observed an increase in the proportion of whole NK cells accompanied by the decrease in both $CD16^-$ NK cell subsets ($CD56^{bright}CD16^-$ immature NK cells and $CD56^{dim}CD16^-$). Numbers of mature NK cells ($CD56^{dim}CD16^+$) had a tendency for age-related increase, but this was not statistically significant. In contrast to our observation, a previous study reported a significant difference in the absolute number of mature NK cells; however, they were comparing participants ranging from 18 to 60 years and participants over 80 years [43]. Unfortunately, we were not able to include a higher number of older participants in our study populations.

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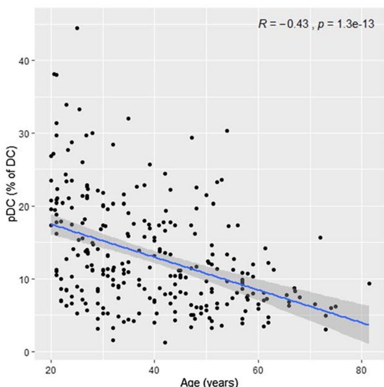

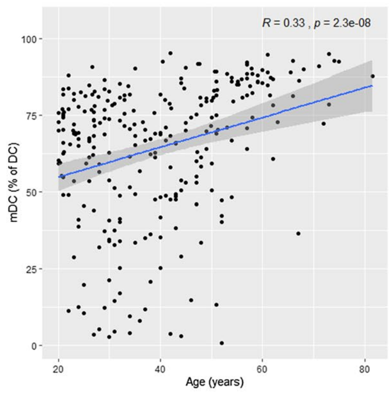

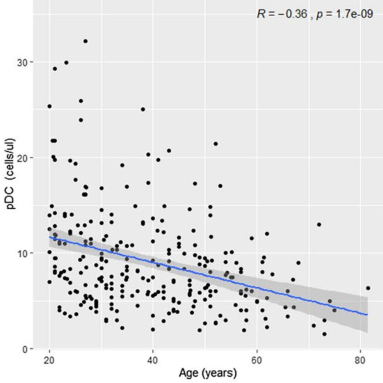

Fig. 3. Correlation between age and relative or absolute cell counts in natural killer (NK) cell and myeloid cell panel (only data on cell subsets with correlation coefficient ≥ 0·3 or ≤ −0·3 are shown). Spearman's rank correlation was performed and the cell subset dependency with age is illustrated by regression lines and confidence intervals.

Results of previous reports show conflicting results regarding the effect of age to DC populations [44–47]. We observed a strong association between age and the subsets of DC (Fig. 3). Despite similar marker choice in some previous studies, we observed a significant age-dependent increase in mDC and its CD16 expressing subsets [31,48] (Fig. 3). On pDCs, aging has the opposite effect, as the number of pDCs decreases in elderly patients, both absolutely and relatively (Fig. 3). Although Stervbo *et al.* [44] used a combination of BDCA1 (CD1c), BDCA2 (CD303) and BDCA3 (CD141) for distinguishing DC subsets, they also observed the age-dependent decline in pDC and expansion of mDC.

We next investigated the total population of peripheral blood monocytes and its two main subsets: classical ($CD14^+CD16^-$) and non-classical monocytes ($CD14^+CD16^+$). CD16-expressing non-classical monocytes have been described as proinflammatory cells, and accumulation of these cells has been associated with aging [47,49,50]. However, our results do not support these findings, and suggest that age does not significantly affect the composition of the peripheral blood monocyte compartment. The difference from other studies may be partly explained by the fact that we divided monocytes into two subsets, and others have also distinguished a third subset called 'intermediate monocytes' [47,49].

Although the number of most cells representing components of the innate immune system cells do not differ between males and females, we found that absolute and/or relative proportions of some subsets are significantly higher in men. In accordance with previous studies [41], we also detected a higher proportion of total NK cells in males and absolute numbers and frequency of immature ($CD56^{bright}CD16^-$) NK cells in females. The relative size of the monocyte population was also significantly smaller in females, as reported previously [42]. These differences might be hormone-related, as has been shown by several studies [51].

Conclusions

In summary, the presented core panel is well applicable to analyze the main leukocyte subpopulations in whole blood in a clinical–diagnostic setting. This panel serves as a comprehensive starting-point for characterization of main peripheral blood cells populations, which can be expanded to resolve specific questions.

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Disclosures

Authors have no conflicts of interest to declare.

Author contributions

D. P., O. W., D. R., B. S., E. M. C., R. U. and T. G. participated in the design of the study and/or supervised the study sites. A. O., B. Q. S., S. T., S. H., M. C., D. R. and T. G. recruited the donors and collected the primary data. A. O., B. Q. S., D. P. and T.G. analyzed the data and performed the statistics. A. O., B. Q. S., E. M. C., R. U. and T. G. wrote the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Statistic view to see the means of fluorescence for each fluorochrome channel used. Instrument settings to make sure that we all have the same configurations.

Table S1. Overview of study centers, method used and studied immune cell populations.

Table S2. Antibody panels used in the different cytometers.

Table S3. Configuration of cytometers and filters used for fluorochromes.

Table S4. Comparison between male and female in T cell frequency, Mann-Whitney Test.

Table S5. Comparison of T cell panel absolute counts between male and female.

Table S6. Comparision between age groups for T cell frequencies, Kruskal-Wallis Test.

Table S7. Comparison between age groups for absolute count of T cell. Kruskal -Wallis test.

Table S8. Comparison of Treg absolute counts between male and female. Mann-Whitney test.

Table S9. Comparison of Treg frequencies between male and female. Mann-Whitney Test.

Table S10. Comparison between age groups for Treg frequencies. Kruskal-Wallis Test.

Table S11. Comparison between age groups for Treg absolute counts. Kruskal-Wallis test.

Table S12. Comparison of B cell frequency between male and female. Mann-Whitney Test.

Table S13. Comparison of B cell absolute count (cells/μl) between male and female. Mann-Whitney Test.

Table S14. Comparison between age groups for B cell frequencies. Kruskal-Wallis Test.

Table S15. Comparison between age groups for absolute count of B cell. Kruskal-Wallis Test.

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Table S16. Comparison between male and female for NK cells, Monocytes, Dendritic cells and Granulocyte frequencies, Mann-Whitney Test.

Table S17. Comparison of NK cell, dendritic cell, monocytes and granulocyte absolute counts between male and female. Mann-Whitney test.

Table S18. Comparison between age groups for NK cells, Monocytes, Dendritic cells and Granulocyte frequencies. Kruskal-Wallis Test.

Table S19. Comparison between age groups for absolute count of NK cells, Monocytes, Dendritic Cells and Granulocytes. Kruskal-Wallis test.

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