Age-associated distribution of normal B-cell and plasma cell subsets in peripheral blood

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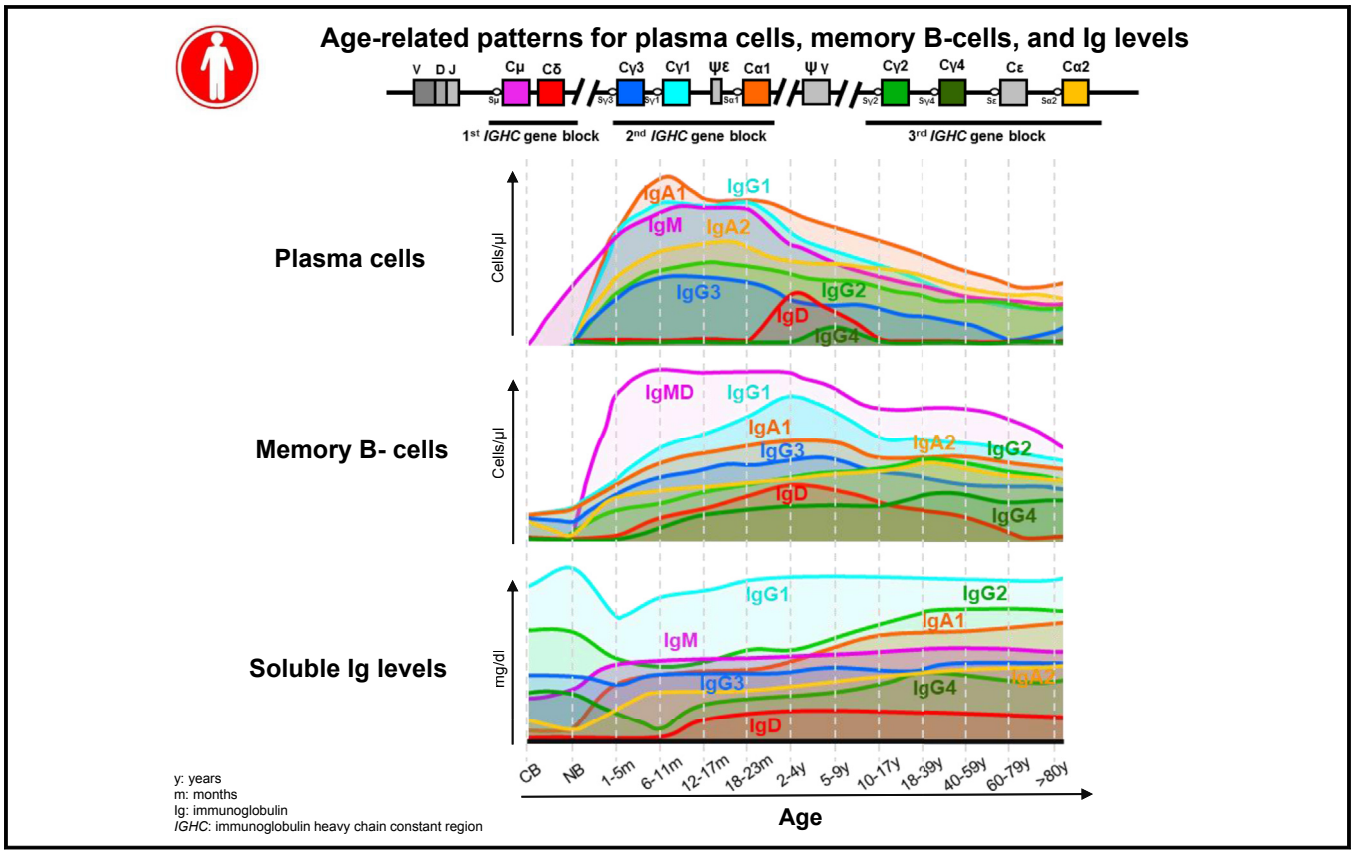
GRAPHICAL ABSTRACT



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E.B. was supported by a grant from Junta de Castilla y León (Fondo Social Europeo, ORDEN EDU/346/2013, Valladolid, Spain). This work was supported by the CB16/12/00400 grant (CIBERONC, Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, Madrid, Spain, and FONDOS FEDER) and the FIS PI12/00905-FEDER grant from the Fondo de Investigaciones Sanitarias of Instituto de Salud Carlos III (Madrid, Spain). O.P. and T.K. were supported by the Ministry of Education, Youth and Sports (NPU I no. LO1604 and 15-28541A). The coordination and innovation processes of this study were supported by the EuroFlow Consortium.

Disclosure of potential conflict of interest: E. Blanco, M. Pérez-Andrés, E. López-Granados, T. Kalina, M. van Zelm, M. van der Burg, J. J. M. van Dongen, and A. Orfao each report being one of the inventors on the EuroFlow-owned patent PCT/NL 2015/050762 (Diagnosis of primary immunodeficiencies), which is licensed to Cytognos, a company that pays royalties to the EuroFlow Consortium. M. van Zelm reports grants from the NHMRC and has a patent issued (EP 2780711 B1). J. J. M. van Dongen and A. Orfao report an Educational Services Agreement from BD Biosciences The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication July 27, 2017; revised December 15, 2017; accepted for publication February 5, 2018.

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0091-6749

https://doi.org/10.1016/j.jaci.2018.02.017

Background: Humoral immunocompetence develops stepwise throughout life and contributes to individual susceptibility to infection, immunodeficiency, autoimmunity, and neoplasia. Immunoglobulin heavy chain (IgH) isotype serum levels can partly explain such age-related differences, but their relationship with the IgH isotype distribution within memory B-cell (MBC) and plasma cell (PCs) compartments remains to be investigated.

Objective: We studied the age-related distribution of MBCs and PCs expressing different IgH isotypes in addition to the immature/transitional and naive B-cell compartments.

Methods: B-cell and PC subsets and plasma IgH isotype levels were studied in cord blood (n = 19) and peripheral blood (n = 215) from healthy donors aged 0 to 90 years by using flow cytometry and nephelometry, respectively.

Results: IgH-switched MBCs expressing IgG_1, IgG_2, IgG_3, IgA_1, and IgA_2 were already detected in cord blood and newborns at very low counts, whereas $CD27^+IgM^{++}IgD^+$ MBCs only became detectable at 1 to 5 months and remained stable until 2 to 4 years, and IgD MBCs peaked at 2 to 4 years, with both populations decreasing thereafter. MBCs expressing IgH isotypes of the second immunoglobulin heavy chain constant region *(IGHC)* gene block (IgG_1, IgG_3, and IgA_1) peaked later during childhood (2-4 years), whereas MBCs expressing third *IGHC* gene block immunoglobulin isotypes (IgG_2, IgG_4, and IgA_2) reached maximum values during adulthood. PCs were already detected in newborns, increasing in number until 6 to 11 months for IgM, IgG_1, IgG_2, IgG_3, IgA_1, and IgA_2; until 2 to 4 years for IgD; and until 5 to 9 years for IgG_4 and decreasing thereafter. For most IgH isotypes (except IgD and IgG_4), maximum plasma levels were reached after PC and MBC counts peaked.

Conclusions: PC counts reach maximum values early in life, followed by MBC counts and plasma IgH isotypes. Importantly, IgH isotypes from different *IGHC* gene blocks show different patterns, probably reflecting consecutive cycles of IgH isotype switch recombination through life. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: *Immunoglobulins, IgH isotype, subclass, memory B cells, plasma cells, flow cytometry, reference ranges, normal B cells, age-related values*

Abbreviations used
BM: Bone marrow
CB: Cord blood
IgH: Immunoglobulin heavy chain
IGHC: Immunoglobulin heavy chain constant region
MBC: Memory B cell
PB: Peripheral blood
PC: Plasma cell
RT: Room temperature
sm: Surface membrane
SSC: Side scatter

Serum immunoglobulin levels are widely accepted as the most reliable surrogate marker for B-cell functionality in healthy donors and patients with different immune-related diseases.[1-6] Thus, reference serum immunoglobulin values per age group have been generally adopted as the measure of changes in B-cell immunocompetence through life.[7-17]

Overall, increasing serum immunoglobulin levels are observed with age, although patterns vary for the distinct immunoglobulin heavy chain (IgH) isotypes and subclasses. At birth, serum contains low IgM and IgA levels, whereas maternal immunoglobulin molecules provide near-adult levels of serum IgG.[13-15,18] Once maternal antibodies are cleared, the infant's antibody responses are initially dominated by IgM, and serum IgM, IgG, and IgA values reach approximately 100%, 60%, and 30% of adult levels by the first year of life, respectively.[14,15] Total serum IgG levels gradually increase at early ages; however, different production patterns are observed for the 4 IgG subclasses.[16,17] Although IgG_3 and IgG_1 levels increase faster and reach adult-like concentrations at 5 to 10 years of age, IgG_2 production is delayed, with maximum levels at adulthood.[16,17] Similarly, production of IgG_4 appears to be delayed, but reference values per age group vary significantly among healthy subjects.[16,17] Furthermore, both IgA_1 and IgA_2 levels increase gradually during childhood,[8,19,20] reaching maximum IgA_1 production around the second decade of life, whereas IgA_2 serum levels keep increasing throughout adulthood.[12] Finally, IgE serum levels peak around 10 years and decrease thereafter to adult levels.[21] A common feature for all serum IgH isotype subclasses (except for IgD, IgG_4, and IgE) is that their levels accumulate with age.[7,8,11,12,16] Once maximum levels are reached, serum IgH isotype subclass levels remain remarkably stable, without significant changes in older subjects.[18]

In contrast to serum immunoglobulin levels, more dynamic changes have been observed for total B-cell numbers and the composition of the B-cell compartment in peripheral blood (PB). Early studies reported that total B-cell counts increase 2-fold immediately after birth, remain high until 2 years, and gradually decrease approximately 6.5-fold until adulthood.[22] Detailed immunophenotypic analyses demonstrated that the initial increase is mostly due to an increased B-cell production in bone marrow (BM) and release of high numbers of immature/transitional and naive B cells into PB, with maximum values at 0 to 12 months of age.[23,24] This early wave of recently produced B cells is followed by increased numbers of memory B cells (MBCs) that rapidly increase from 2 months of life, remain high until age 5 years, and gradually decrease thereafter to adult-like values.[23-26] During adulthood, the number of immature/transitional and naive B cells remains stable, whereas MBC and newly generated plasma cell (PC) counts gradually decrease in subjects older than 60 years.[27,28]

Thus far, accurate and robust detection of low PC counts in PB (eg, in infants) appeared to be a challenge,[23,25,26] and information on IgH isotypes and subclasses within MBC and PC subsets remains limited,[27] with absence of age-related reference values. Likewise, recent studies on antigen-experienced B cells expressing different IgH isotypes did not discriminate between MBCs and PCs[25] and/or did not provide data about the IgG_1 to IgG_4 and IgA_1 and IgA_2 subclasses.[24,26,27]

Here we dissected the PB compartments of MBCs and PCs into 38 distinct subsets expressing different IgH isotypes and their subclasses and investigated their distribution in a large series of normal cord blood (CB) and PB from healthy European donors aged 0 to 90 years. Our ultimate goal was to define the kinetics of the different B-cell and PC subsets

through life and their correlation with plasma IgH isotype subclass levels.

METHODS

Samples

EDTA-anticoagulated CB (n = 19) and PB samples from 215 healthy European Caucasian donors (aged 0-90 years) with no past history of immunologic or hematologic diseases (including allergy) were studied: newborns (<12 days; 14 cases); children aged 1 to 5 months (11 cases), 6 to 11 months (7 cases), 12 to 17 months (12 cases), 18 to 23 months (12 cases), 2 to 4 years (25 cases), 5 to 9 years (22 cases), and 10 to 17 years (14 cases); and adults aged 18 to 39 years (32 cases), 40 to 59 years (27 cases), 60 to 79 years (27 cases), and greater than 80 years (12 cases). All samples were collected after informed consent was provided by the subjects, their legal representatives, or both, according to the Declaration of Helsinki. The study was approved by the local ethics committees of the participating centers (University of Salamanca, Salamanca, Spain; Charles University, Prague, Czech Republic; University of Lisbon, Lisbon, Portugal; and La Paz Hospital, Madrid, Spain).

All subjects were vaccinated following similar national vaccination schedules (European Centre for Disease Prevention and Control; http://vaccine-schedule.ecdc.europa.eu/Pages/Scheduler.aspx). CB was from Spain, Portugal, and the Czech Republic, newborns and children aged 1 to 23 months were from Spain and the Czech Republic, and all cases older than 2 years were from Spain. Donors with any sign or suspicion of immunologic or hematologic diseases (including an abnormal infection rate or a known history of allergies) were excluded from the study. In addition, a questionnaire with environmental factors that could potentially affect development of the immune system,[25,29,30] was conducted in children less than 4 years of age (n = 36), with no differences observed in the frequency of these factors among the age groups evaluated (see Table E1 in this article's Online Repository at www.jacionline.org). Also, the exact dates of different vaccinations received were collected in a subset of 72 children. Elderly donors (>80 years) were representative of a broader group of elderly persons in the Salamanca region (median life expectancy, 82 and 87 years for men and women, respectively).

Multiparameter flow cytometric identification of B cells and their maturation-associated and immunoglobulin isotype subclass subsets

Samples were processed within less than 24 hours after collection; 10^7 cells per sample were stained with the EuroFlow 12-color IgH-isotype B-cell tube (see Table E2 in this article's Online Repository at www.jacionline.org). IgH isotypes and subclasses were assessed in every sample by using surface membrane staining; in addition, in a subset of 6 children (age, 3 ± 5 years) and 6 adults (age, 33 ± 8 years) surface membrane plus cytoplasmic staining was done in parallel.

Both protocols were performed strictly according to the EuroFlow BulkLysis standard operating procedure, as previously described (a detailed protocol is provided in the Methods section in this article's Online Repository at www.jacionline.org and www.EuroFlow.org).[31,32] A minimum of 5×10^6 leukocytes (including $\geq 10^5$ B cells) was acquired per tube with Fortessa X-20 or LSR II Flow Cytometers (BD Biosciences, San Jose, Calif), and FACSDiva software (BD Biosciences) was used. Instrument setup was standardized across laboratories according to the EuroFlow standard operating procedure[33] adapted for 12-color measurements. For data analysis, Infinicyt software (Cytognos, Salamanca, Spain) was used. Absolute counts were calculated by using a dual-platform assay.[34]

Analysis of plasma IgH isotype subclass levels

Plasma from 18 CB and 201 PB samples from all age groups was obtained by means of sequential centrifugation of PB (800*g* for 10 minutes) and platelet-rich plasma (2000*g* for 5 minutes) and immediately stored at −80°C until analysis. Soluble IgM, IgE, and IgG_1 to IgG_4 subclass levels were measured by using conventional nephelometry (Dimension Vista; Siemens Healthcare, Erlanger, Germany), whereas IgA_1, IgA_2, and IgD levels were measured by using the SPA$_{PLUS}$ turbidimetric system (Binding Site, Birmingham, United Kingdom). For samples with IgH isotype subclass concentrations of greater than the measurable range, appropriate dilutions were made.

Statistical analyses

To assess statistical significance (set at $P < .05$) of differences observed between distinct age groups, Mann-Whitney *U* and Wilcoxon tests for unpaired and paired (continuous) variables were used, respectively. For categorical variables, the Fisher exact test was applied (SPSS software, version 23; IBM, Armonk, NY).

RESULTS

Identification of maturation and isotypic subpopulations of PB B cells

Based on their staining pattern for CD19, CD27, CD38, CD5, CD24, CD21, surface membrane (sm) IgM, smIgD, and side scatter (SSC), B cells ($CD19^+SSC^{lo/int}$ forward $scatter^{lo/int}$ lymphocytes) were classified into maturation-associated subsets (Fig 1, *A* and *B*, and see Fig E1 in this article's Online Repository at www.jacionline.org).[27,35] First, PCs were defined by a $CD38^{++}CD27^+CD24^-CD21^-CD5^-CD19^{lo}SSC^{int}$ phenotype. Then pre–germinal center B cells were identified by their unique $CD27^-smIgM^+smIgD^+CD5^{het}$ features; this subset was subdivided subsequently into immature/transitional $CD38^{hi}CD24^{hi}smIgM^{++}IgD^+$ B cells and $CD38^-CD24^{het}smIgM^+IgD^{++}$ naive B lymphocytes. Afterward, unswitched MBCs were identified by their unique $CD38^{lo}CD5^-CD27^+smIgM^{++}IgD^+$ phenotype, and switched MBCs were defined as $CD38^{lo}CD5^-smIgM^-IgD^-$ B cells (see Fig E1). Despite CD10 being reported to identify immature/transitional B cells, the combination of markers used here has proved sufficient to discriminate reproducibly between mature and immature B cells.[27,36]

MBCs and PCs were further subclassified according to their smIgH phenotype into (1) $smIgM^{++}IgD^+$, $smIgD^+$, $smIgA_1^+$, $smIgA_2^+$, $smIgG_1^+$, $smIgG_2^+$, $smIgG_3^+$, and $smIgG_4^+$ MBCs and (2) $smIgM^+$, $smIgD^+$, $smIgA_1^+$, $smIgA_2^+$, $smIgG_1^+$, $smIgG_2^+$, $smIgG_3^+$, and $smIgG_4^+$ PCs, respectively (Fig 2, *A* and *B*, and Fig 2, *D* and *E*). All subsets of naive cells and MBCs were further subclassified according to CD21 expression, and the subset of switched MBCs was also further subdivided based on CD27 expression. Although a minor fraction of PCs did not show smIgH, no statistically significant differences were observed in the IgH isotype and subclass distribution of PCs when staining for smIgH was compared with cytoplasmic IgH, either in children (n = 6) or adults (n = 6), except for the percentage of IgH^- PCs, which was lower when cytoplamsic versus Sm staining was used (see Fig E2 in this article's Online Repository at www.jacionline.org), which is in line with previous findings.[32]

Kinetics of total B cells and maturation-associated B-cell subsets through life

Total lymphocyte and B-cell counts reached maximum levels at approximately 1 to 5 months of age. The total lymphocyte count increased progressively from CB to newborns and 1- to 5-month-old children, whereas B cells showed a slightly delayed

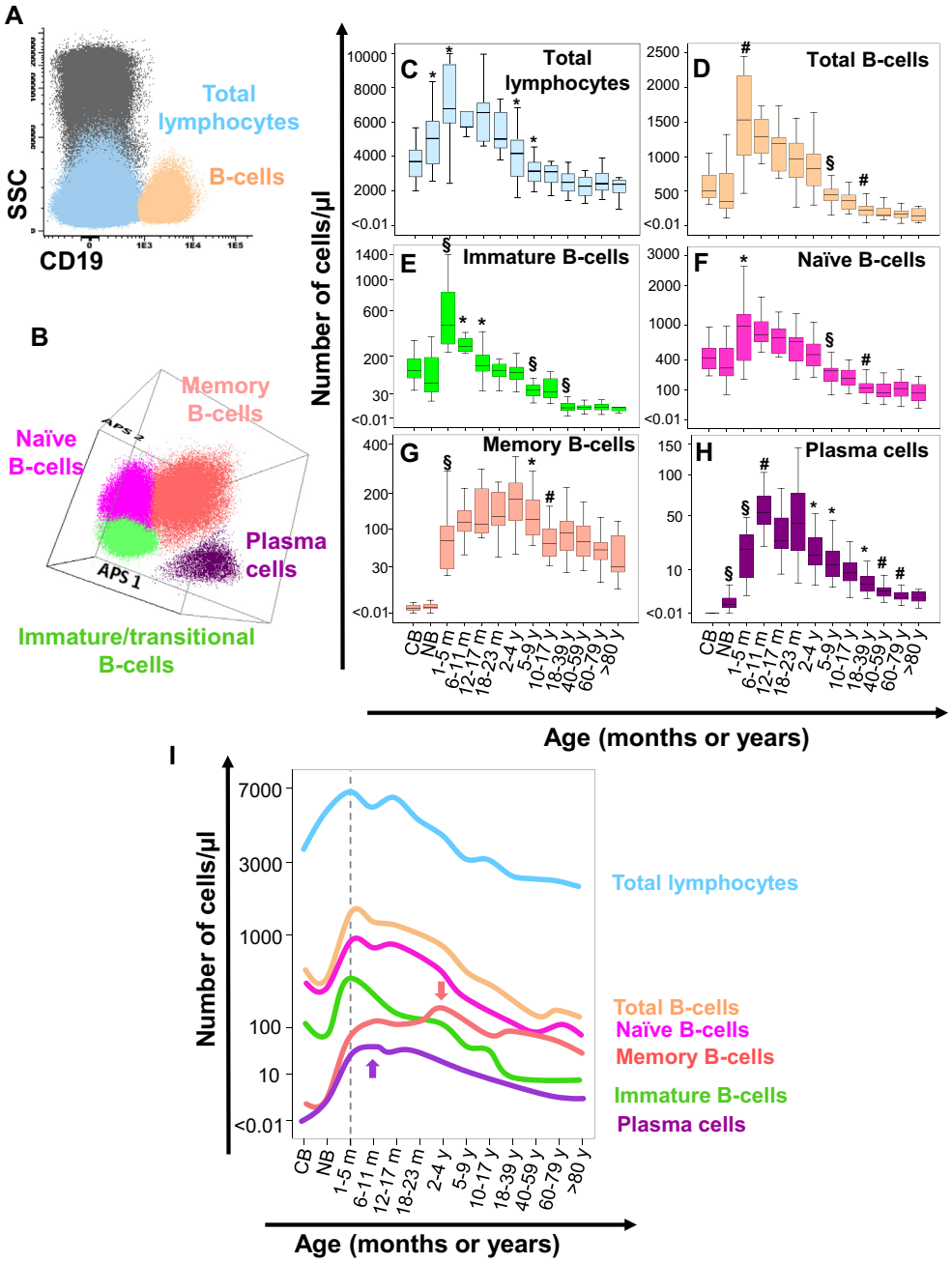

FIG 1. Age distribution of PB lymphocytes, total B cells, and maturation-associated B-cell subsets. **A,** Dot plot representation of total lymphocytes (blue) and B cells (light orange). **B,** Three-dimensional Automated Population Separator *(APS)* view: principal component 1 versus principal component 2 versus principal component 3. Graphic representation of the B-cell maturation–associated subsets is shown as follows: immature/transitional (green), naive (pink), and MBCs (orange), and PCs (violet). The APS representation, which generates automatic separation of clusters of cells based on their immunophenotype, was used as a visualization tool. The gating strategy is described in Fig E1. **C-H,** Absolute counts (cells per microliter) of lymphocytes (Fig 1, *C*), total B cells (Fig 1, *D*), and maturation-associated B-cell subsets (Fig 1, *E-H*) present in CB and PB distributed by age. *Notched boxes* represent 25th and 75th percentile values; *middle line* corresponds to median values, and *vertical lines* represent the highest and lowest values that are neither outliers nor extreme values. **I,** Lines link median values of total lymphocytes; total B cells, immature/transitional naive MBCs, and PCs. *$P < .05$, #$P < .01$, and §$P < .001$ versus the previous age group, respectively. *NB,* Newborn.

significant increase during this period ($P > .05$ for CB vs for newborns; $P = .001$ for newborns vs 1- to 5-month-old children). Thereafter, absolute B-cell counts progressively decreased until adulthood (18 years), remaining relatively stable afterward (Fig 1, *C*, *D*, and *I*, and see Table E3 in this article's Online Repository at www.jacionline.org).

Most CB B cells corresponded to immature/transitional and naive B cells (Fig 1, *E*, *F*, and *I*, and see Table E3). Still, a minor

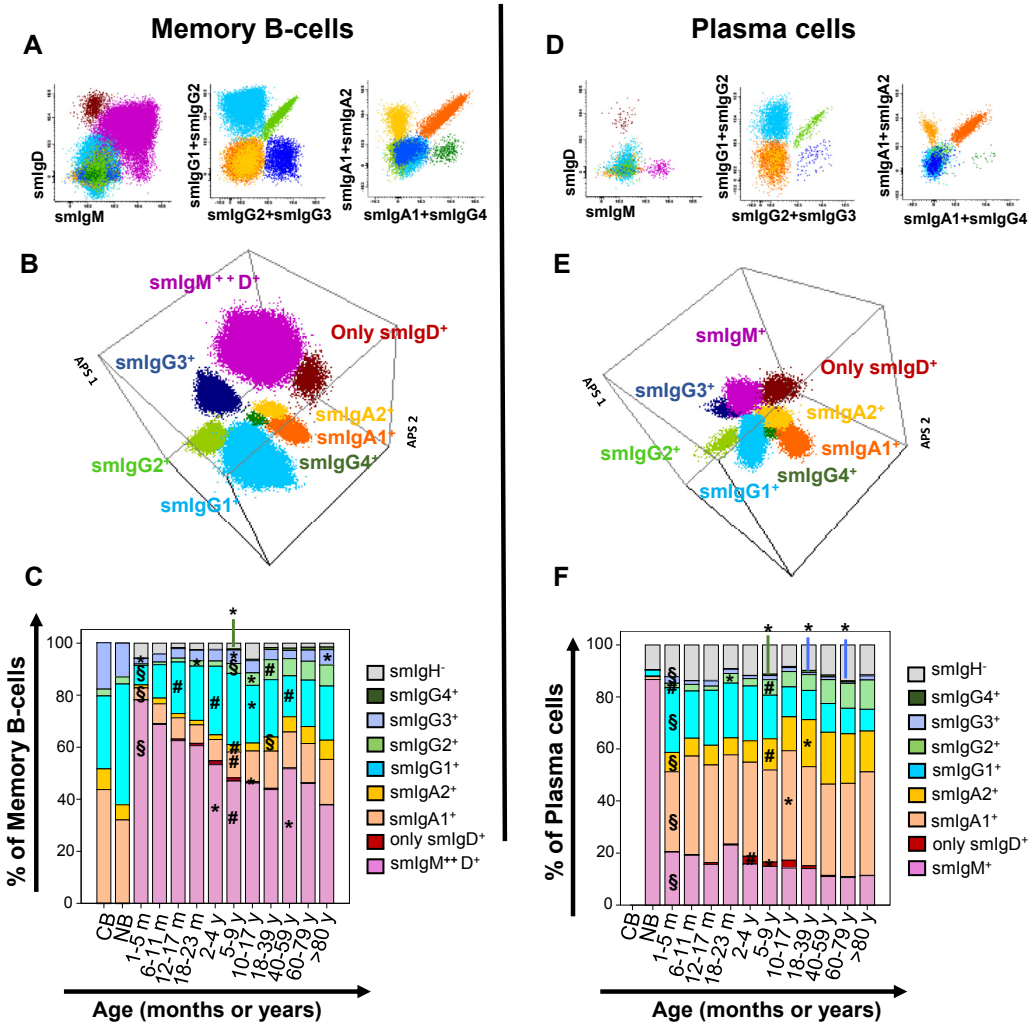

FIG 2. Distribution of different IgH isotype and subclass subsets of PB MBCs and PCs by age. **A** and **D,** Dot plot graphic representation of MBC (Fig 2, *A*) and PC (Fig 2, *D*) subsets expressing distinct smIgH isotypes and isotype subclasses: IgM(D+) (violet), IgG_1 (light blue), IgG_2 (light green), IgG_3 (dark blue), IgG_4 (dark green), IgA_1 (orange), IgA_2 (yellow), and IgD (brown); $smIgH^-$ B cells are highlighted in gray. **B** and **E,** Three-dimensional Automated Population Separator *(APS)* view: principal component 1 versus principal component 2 vs principal component 3 of the IgH isotype and subclass subsets of MBCs (Fig 2, *B*) and PCs (Fig 2, *E*) identified by the same color code as described above. The APS graphic representation, which generates automatic separation of clusters of cells based on their immunophenotype, was used as a visualization tool. **C** and **F,** Relative distribution of the distinct IgH isotype and subclass subsets of MBCs and PCs in the different age groups analyzed. $*P < .05$, $\#P < .01$, and $\S P < .001$ versus the previous age group, respectively. *NB*, Newborn.

fraction of IgH-switched MBCs was detected in most CB (95% of subjects) in the absence of PCs (≤0.01 PCs/μL; Fig 1, *G-I*, and see Table E3). Interestingly, although no significant differences were observed in immature/transitional, naive, and MBC numbers between CB and newborn samples, PCs became detectable at low numbers in most newborns ($P < .001$). Through life, the number of PB immature/transitional and naive B cells showed a similar profile to that of total B cells, with a peak at 1 to 11 months ($P \leq .001$ vs CB) and a progressively significant decrease until adulthood ($P < .001$ vs 18- to 39-year-old subjects). Subjects older than 18 years showed relatively stable numbers of both immature/transitional and naive B cells (Fig 1, *E*, *F*, and *I*, and see Table E3).

MBC and PC counts increased from CB and newborn samples to 1- to 5-month-old children ($P \leq .001$). PC counts already started to decrease in children older than 23 months ($P = .03$), with a gradually continued decrease through adulthood. In contrast, MBC counts increased gradually until age 2 to 4 years ($P < .001$ vs 1- to 5-month-old children) followed by a decrease until a plateau was reached that lasted through adolescence (10-17 years) until adulthood (40-59 years); afterward, MBC counts progressively decreased ($P = .02$ vs >80 years; Fig 1, *G* and *H*, and see Table E3).

Age distribution of MBCs expressing distinct IgH isotypes and subclasses

Relative and absolute counts of MBCs expressing different IgH isotypes and subclasses varied significantly through life (Figs 2 and 3). $smIgM^{++}IgD^+$ MBCs represented the largest fraction of MBCs (approximately 80%) in 1- to 5-month-old

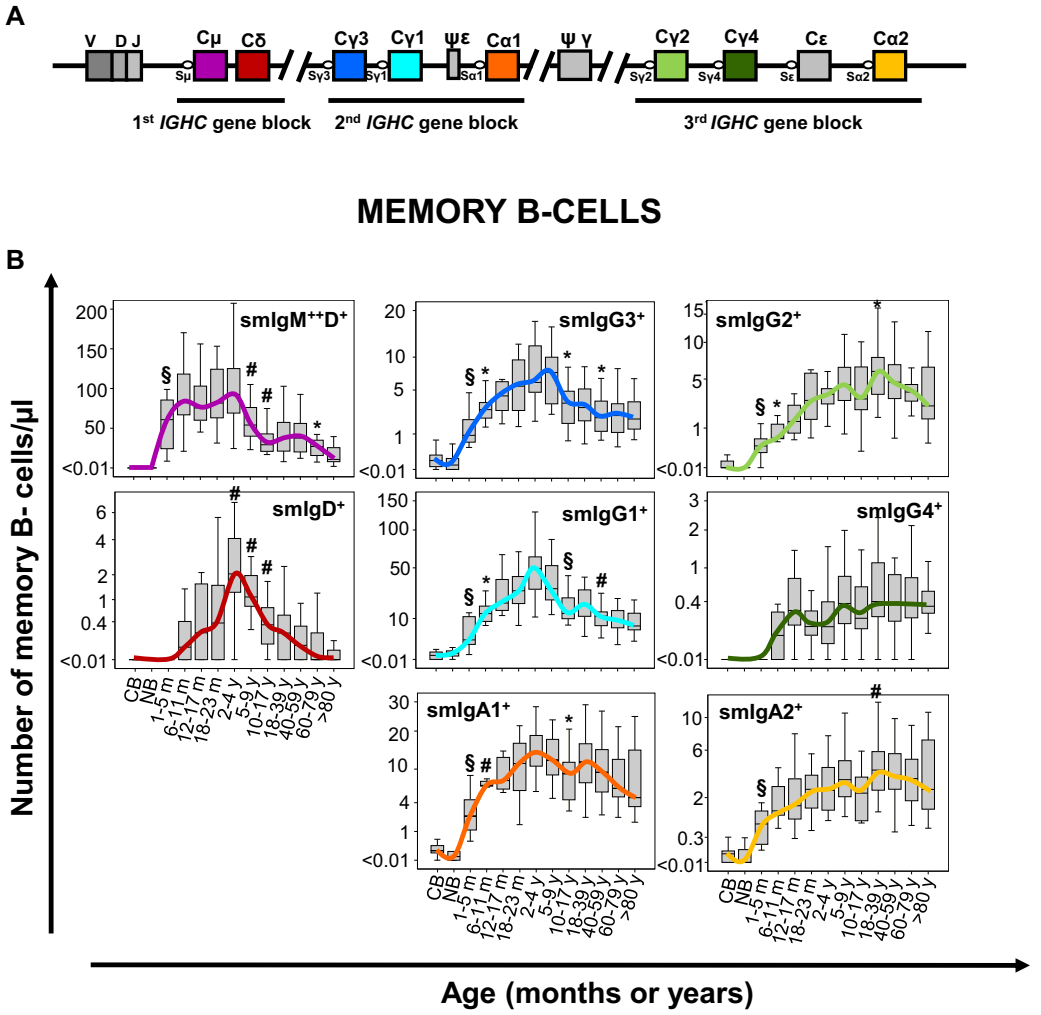

FIG 3. Absolute count of CB and PB IgH isotype and subclass subsets of MBCs in healthy subjects grouped by age. **A,** Schematic illustration of the human *IGH* gene constant region. **B,** Absolute number of different subsets of MBCs expressing distinct isotypes and subclasses in CB and PB according to age. *Notched boxes* represent 25th and 75th percentile values; *middle line* corresponds to median values, and *vertical lines* represent the highest and lowest values that are neither outliers nor extreme values. *Colored lines* link median MBC absolute count values. **P* < .05, #*P* < .01, and §*P* < .001 versus the previous age group, respectively. *NB,* Newborn.

children, being significantly expanded versus those in CB and newborns ($P < .001$), with a subsequent decrease to 40% to 50% of MBCs. Conversely, $smIgG_1^+$, $smIgG_3^+$, and $smIgA_1^+$ switched MBCs showed a transient relative decrease among 1- to 5-month-old children versus newborns ($P \leq .01$), with their relative numbers increasing significantly at 6 to 11 months until 2 to 4 years for $smIgG_1^+$ ($P = .001$ for 6-11 months vs 1-5 and 2-4 years vs 18-23 months) and at 5 to 9 years for $smIgG_3^+$ ($P = .02$), when they started to decrease, particularly in the transition to adulthood. In turn, the percentage of $smIgA_1^+$ MBCs increased progressively from 2 to 4 years until 10 to 17 years ($P = .003$ for 2-4 years vs 5-9 years and $P < .001$ for 2-4 years vs 10-17 years), remaining rather stable from then onward. In contrast, both $smIgG_2^+$ and $smIgA_2^+$ MBCs reached their highest representation in young (18-39 years) adults ($P \leq .001$). During adulthood, no significant changes were observed in the relative distribution of most IgH subsets of MBCs, with a few exceptions (Fig 2, *C*).

Regarding absolute counts, very low numbers of MBCs were observed in CB and newborns, with similar frequencies of subjects with detectable IgH subsets of MBCs (see Table E4 in this article's Online Repository at www.jacionline.org). Subsequently, nonswitched $smIgM^{++}IgD^+$ MBCs became detectable at significantly increased counts among 1- to 5-month-old children ($P = .001$), with numbers remaining fairly stable until 4 years and subsequently decreasing significantly to adult levels at 10 to 17 years ($P < .002$, Fig 3). After 60 years, progressively lower numbers of $smIgM^{++}IgD^+$ MBCs were observed ($P = .002$ for 40-59 years vs >80 years). The $smIgD^+IgM^-$ MBCs showed a very similar profile: increased counts until 2 to 4 years of age, which progressively decreased thereafter (Fig 3 and see Table E4).

In contrast, MBCs expressing $smIgG_3$, $smIgG_1$, and $smIgA_1$ (IgH isotype subclasses of the second immunoglobulin heavy chain constant region *[IGHC]* gene block) showed progressively higher counts until 2 to 4 years ($P \leq .03$) and remained relatively

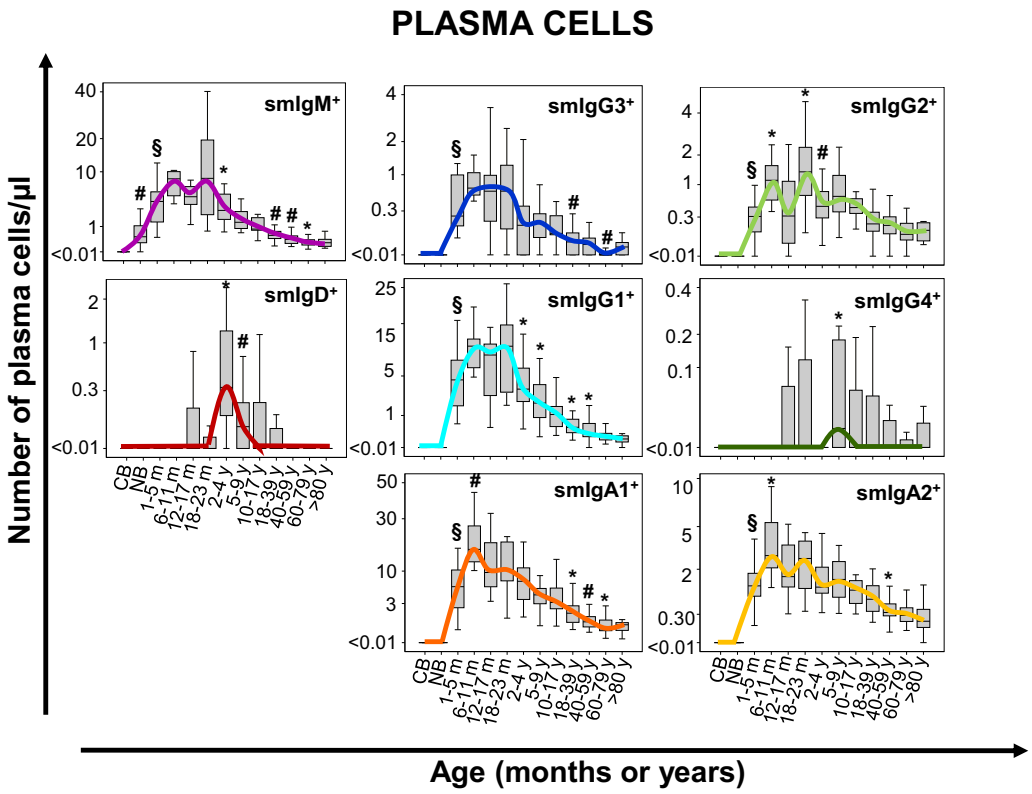

FIG 4. Absolute CB and PB counts of IgH isotype and subclass subsets of PCs in healthy subjects grouped by age. Absolute number of different subsets of PCs expressing distinct IgH isotypes and subclasses in CB and PB according to age. *Notched boxes* represent 25th and 75th percentile values; *middle line* corresponds to median values, and *vertical lines* represent the highest and lowest values that are neither outliers nor extreme values. *Colored lines* link median PC absolute count values. $^{**}P < .05$, $\#P < .01$, and $\S P < .001$ versus the previous age group, respectively. *NB*, Newborn.

stable until 10 to 17 years of age. Interestingly, although MBC counts with $smIgG_2^+$ and $smIgA_2^+$ expression (IgH isotype subclasses of the third *IGHC* gene block) also increased in childhood, these counts continued increasing until adulthood, with significantly higher counts among 18- to 39-year-old adults versus younger subjects ($P = .03$ and $P = .005$, respectively). Despite representing a minor subset (<1% and <1 cell/µL for most donors), particularly among children, $smIgG_4^+$ MBCs peaked at 40 to 59 years (Fig 3 and see Table E4).

Interestingly, the immunophenotype of switched MBCs differed significantly between CB/newborns versus older children and adults. Thus although switched MBCs in CB and newborns lacked CD27, most MBCs from children older than 1 month and adults (80% to 90%) were $CD27^+$. The percentage of $CD27^+smIgG_3^+$ MBCs also increased after the first month (40% to 60%), but the fraction of $CD27^-$ cells remained significantly greater (≥40%) in $smIgG_3^+$ MBCs than in MBCs with other IgH isotype subclasses (see Fig E3 in this article's Online Repository at www.jacionline.org). Moreover, although CB and newborn MBCs were mostly $CD21^+$, a significantly higher proportion of $CD21^-$ switched MBCs was observed among 1- to 11-month-old children versus all other age groups. In contrast, the percentage of $CD21^-$ naive B lymphocytes and $CD21^-$ $smIgM^{++}IgD^+$ MBCs remained low and relatively stable among all age groups. Similar to CD27, the percentage of $CD21^-$ cells within $smIgG_3^+$ MBCs was also greater than among MBCs expressing other smIgH isotype subclasses (see Fig E4 in this article's Online Repository at www.jacionline.org).

Age distribution of PC subsets expressing different IgH isotypes and subclasses

Despite their absence in normal CB, PCs were detected in most newborn samples (79%) and in all childhood and adult PB samples analyzed. In contrast to MBCs, PCs from newborns were mostly IgM^+ (68% to 96% of PCs), with only 7% to 25% of NBs showing $smIgG1^+$ (range, 0% to 13%), $smIgA_1^+$ (range, 0% to 6.8%), $smIgG_2^+$ (range, 0% to 2.3%) and/or $smIgA_2^+$ PCs (range, 0% to 2.3%; Fig 2, *F*, and see Table E4).

Children at 1 to 5 months had greater percentages of $smIgG_3^+$, $smIgG_1^+$, $smIgA_1^+$, $smIgG_2^+$, and $smIgA_2^+$ PCs than newborns ($P \leq .003$), whereas the proportion of $smIgM^+$ PCs decreased at this age ($P \leq .001$). Thereafter, the relative distribution of circulating PCs expressing different IgH isotypes and subclasses remained relatively stable, except for a transient increase in IgA_1^+ PC counts at 10 to 17 years, a decrease in IgG_3^+ PC counts in adults, and a progressive increment of $smIgG_2^+$ and $smIgA_2^+$ cell counts until 10 to 17 and 18 to 39 years, respectively (Fig 2, *F*). Finally, the relative number

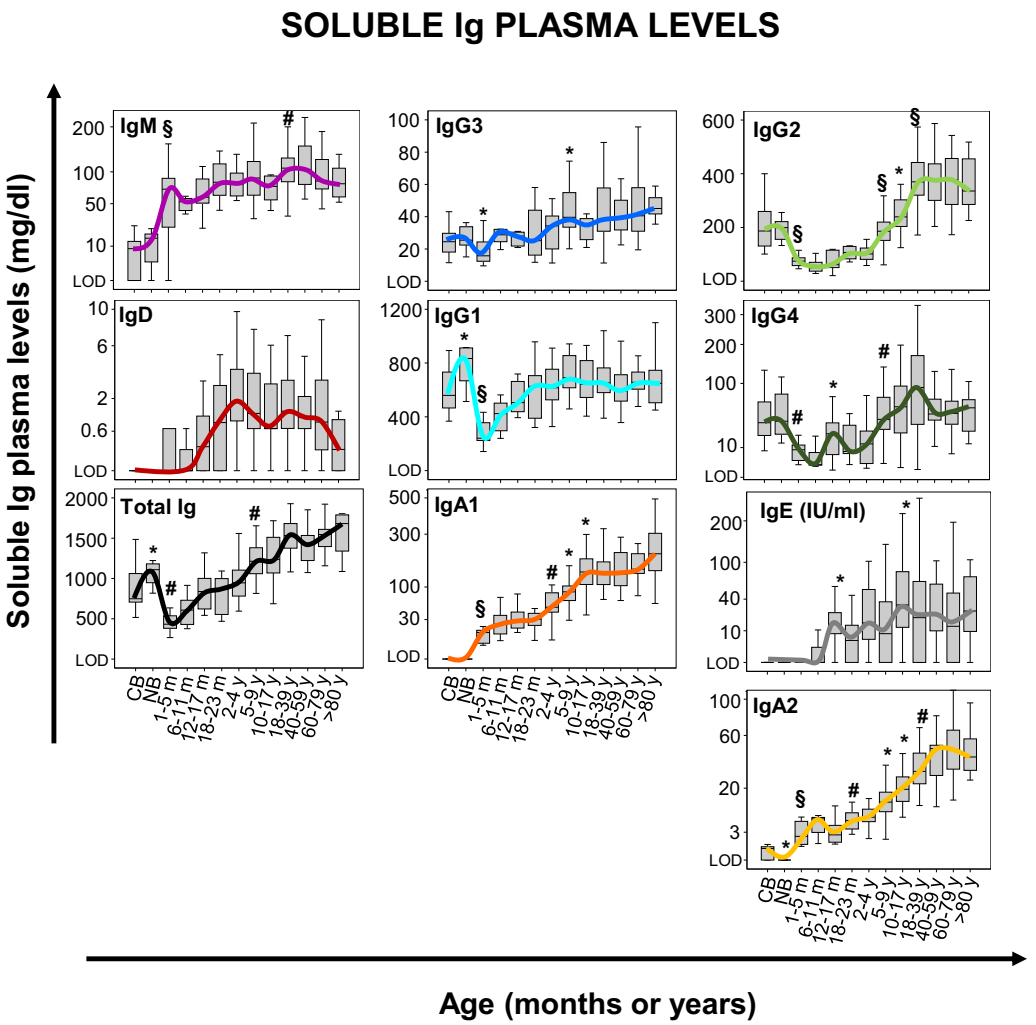

FIG 5. Plasma levels of distinct soluble IgH isotypes and subclasses through life. Plasma levels of the distinct soluble IgH isotypes and subclasses are shown per age group. Total immunoglobulin *(Ig)*, IgM, IgD, IgG, and IgA subclass concentrations are expressed in milligrams per deciliter, and IgE levels are expressed in international units per milliliter. *Notched boxes* represent 25th and 75th percentile values; *middle line* corresponds to median values, and *vertical lines* represent the highest and lowest values that are neither outliers nor extreme values. *Colored lines* link median values of sequential age groups. **P* < .05, #*P* < .01, and §*P* < .001 versus the previous age group, respectively. *NB*, Newborn.

of $smIgD^+$ and $smIgG_4^+$ PCs was greater among 2- to 4-year-old ($P = .001$) and 5- to 9-year-old children ($P = .01$), respectively (Fig 2, *F*).

Regarding absolute counts, the number of PB $smIgM^+$ PCs increased significantly during the first year of life ($P < .001$). Thereafter, $smIgM^+$ PCs decreased gradually from 1 to 2 years onward, reaching the lowest levels among adults older than 60 years. In contrast, IgD^+ PC numbers peaked at 2 to 4 years, progressively decreasing thereafter (Fig 4). Increased $smIgG_3^+$ and $smIgG_1^+$ PC counts were already detected among 1- to 5-month-old children ($P \leq .001$). In turn, PCs expressing $smIgA_1$, $smIgA_2$, and $smIgG_2$ showed significantly increased counts from newborns to 6- to 11-month-old children ($P \leq .02$). All these IgH-switched PC subsets decreased gradually in the transition from late childhood to adulthood. Finally, $smIgG_4^+$ PC numbers peaked at 5 to 9 years, decreasing thereafter (Fig 4 and see Table E4).

Plasma levels of distinct IgH isotypes and subclasses through life

All IgG subclasses (IgG_1-IgG_4) were systematically detected already in CB and newborn plasma. In contrast, IgM and IgA_2 were detected in only a fraction of the CB (61% and 65% cases, respectively) and newborn (60% and 10% cases, respectively) plasma samples investigated; no IgA_1 or IgD plasma levels of greater than the limit of detection (>3.58 and >0.66 mg/dL, respectively) were found in newborn samples, and IgA_1 was found in only 1 CB sample. Later, soluble IgM, IgA_1, and IgA_2 plasma levels became detectable systematically (except IgM in 1 donor) at higher levels among 1- to 5-month-old children ($P \leq .004$); IgA_1 and IgA_2 plasma levels gradually increased thereafter, whereas IgM plasma levels started to (slightly) decrease among 40- to 59-year-old adults. IgD plasma levels remained relatively stable through life until the age of 80 years. Regarding

the different IgG subclasses, a transient decrease in IgG_1, IgG_2, and IgG_4 (but not IgG_3) levels, as well as total immunoglobulin plasma levels, was observed during the first year of life, with the levels of all IgG subclasses and total immunoglobulin levels increasing thereafter until elderly age. Finally, IgE plasma levels peaked at 10 to 17 years, remaining relatively stable thereafter (Fig 5 and see Table E5 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Serum/plasma immunoglobulin levels have been used classically to define the ability (eg, protection against infection) or inability (eg, immunodeficiency) of B cells to mount effective and sustained immune responses. However, in recent years, increasing evidence indicates that a subgroup of long-lived PCs accumulate in the BM over years (or even decades) and are the major contributor to immunoglobulin plasma levels.[18,27,37] Moreover, comparison of B-cell numbers and serum antibody levels in infection models has shown that specific MBCs are more affected by age than serum antibody levels.[38] Therefore immunoglobulin levels in plasma might not reflect the actual status of the B-cell compartment in real time. Nevertheless, this hypothesis has not been fully confirmed, and direct detailed evaluation of the B-cell compartment through life has been only partially achieved.

Here we analyzed the distribution of 38 subsets of MBCs and PCs, including those expressing different IgH isotype subclasses, through life and their relationships with the corresponding IgH plasma levels. Only IgE MBCs and PCs, identification of which in PB of healthy (nonallergic) donors remains controversial,[39-41] could not be studied because of the absence of reliable and sensitive antibody reagents to detect them on the cell surface membrane in healthy subjects at very low levels,[32] despite several anti-IgE clones being tested here for analyses that included more than 100,000 B cells (data not shown).

In line with previous observations,[42] increased production of immature/transitional and naive B cells was observed during the first year of life. Afterward, BM production decreased progressively until adulthood, when immature/transitional and naive B-cell counts in PB stabilized. No further age-related differences were observed when young versus elderly adults were compared, as also reported by others.[27]

Although serologic studies in steady-state[7-10,14-16,18] and immunized[43] subjects suggest that children have a lower ability to produce PCs,[44] direct quantification of circulating PCs showed that PCs become detectable in newborns and are produced at massive levels in infants, when their PC counts are 10-fold greater than in young adults. Of note, no correlation was observed between the number of PCs and the vaccination schedule (data not shown), despite all infant but newborn samples were analyzed more than 8 days apart from the last vaccine. These results suggest that previously used methods to evaluate B-cell effector functions are probably biased by long-lived BM PC immunoglobulin production, and flow cytometry provides direct evaluation of the actual PC counts.[28] In this regard, circulating PCs are a very dynamic cell population with a high turnover rate[27,28] that might offer a closer view into ongoing B-cell responses. In fact, our results indicate that PC production precedes (the peak of) production of both MBCs and serum immunoglobulin levels by years or even decades, depending on the IgH isotype subclass evaluated (see Fig E5).

In line with this, new preliminary observations from our group indicate that the number of circulating normal PCs correlates with hypogammaglobulinemia, risk of infection, and BM PC production in both B-cell and PC neoplasms (Criado et al, unpublished data; Sanoja-Flores et al[45]). Therefore evaluation of PC counts in PB emerges as a surrogate marker for (future) PC production in BM and potentially also an early predictor of primary antibody deficiencies, particularly because the diagnosis of many primary antibody deficiencies is currently delayed to children 4 years old or older because of the slower constitution of antibody levels in serum.[46,47]

Of note, massive PC production associated with early (first) antigen stimulation of naive B cells does not translate into a parallel increase in corresponding antibody serum levels. Thus accumulation of long-lived PCs in BM, which are required to produce significant antibody serum levels, might still be limited in infants because of the intrinsic (pro)-apoptotic susceptibility of newly generated PCs,[27,28] the immaturity of the BM environment after birth,[48] and/or overfilling of BM by highly proliferating B-cell precursors during childhood,[42] which might occupy the BM survival niches for PCs. In turn, when significant levels of serum immunoglobulins are generated, decreased PC counts of the corresponding IgH isotype were observed, probably because of antibody-dependent modulation of B-cell responses.[49,50] Interestingly, immature/transitional and naive B-cell counts and IgH plasma levels remained stable through adulthood, whereas the number of PCs and MBCs decreased in the elderly versus younger adults, which is in line with previous findings.[27,28] The reduced ability to produce antigen-experienced B cells might explain why elderly subjects more frequently have severe infections and a lower capacity to produce antigen-specific antibodies after vaccination, despite having similar (even slightly higher) serum antibody levels versus younger adults[44,51-54]

Overall, the observed kinetics of the sequential waves of PCs, MBCs, and plasma IgH levels throughout life were influenced by the relative position of the IgH isotype/subclass gene segments within the *IGHC* locus. Thus IgG_3 and IgG_1 production peaks appear to precede those of IgG_2 and IgG_4 for PCs (1-5 months vs 6-11 months and 5-9 years), MBCs (2-4 years vs 18-39 years), and serum antibody levels (5-9 years vs 18-39 years; see Fig E6 in this article's Online Repository at www.jacionline.org). Among IgA subclasses, no age-related differences were observed for the PC peaks (both detected in 6- to 11-month-old children), which could be due to the fast rate of IgA responses induced by massive gut bacterial colonization after birth.[55] Nevertheless, when IgA MBCs and IgA plasma levels were analyzed, we confirmed that IgA_1 MBC and serum IgA_1 levels increased faster than levels of their IgA_2 counterparts (2-4 years vs 18-39 years and 10-17 years vs 40-59 years, respectively). In line with these findings, previous reports indicate that IgG_2, IgG_4, and IgA_2 show a greater number of mutated sequences and greater levels of antigen selection than IgG_3, IgG_1, and IgA_1,[56,57] supporting the notion that IgH isotypes encoded by the third block of the *IGHC* locus might, at least in part, be generated by secondary class-switch recombination events. Sequential production of different IgH isotypes might also influence the pattern of B-cell responses during life. Thus although IgH molecules from the first and second *IGHC* gene blocks (IgM and IgG_3, IgG_1, and IgA_1) show a higher ability for complement binding, opsonization, and triggering of natural killer cell–mediated cytotoxicity, those from the third block of the *IGHC* locus (IgG_2, IgG_4, and IgA_2)

have increased neutralization activity.[58-60] If such differences hold functional relevance, sequential production of distinct IgG and IgA subclasses through life might lead to a more tolerogenic response in adults versus children, with a potential for lower expansions of terminally switched MBCs with increasing age.

In addition to massive PC production during the first year of life, vaccination does not mount a sustained immune response in infants, particularly against encapsulated bacteria.[44] This suggests that the immaturity of the secondary B-cell response in infants might be related not only to a lower number of circulating MBCs but also to the ability of these MBCs to respond against different stimulatory conditions. In fact, MBCs from CB, newborns, and infants less than 2 years of age showed significant phenotypic differences to those of children older than 2 years and adults; such differences involved functional markers potentially associated with maturation of other cellular and soluble components of the immune system. Thus in line with the absence of organized germinal centers during fetal life and in newborns,[61-63] the very few switched MBCs detected in CB and newborns lacked CD27, a molecule that has been previously associated with T-dependent germinal center B-cell responses.[35,64] Further expansion of $CD27^+$ MBCs during the first year of life occurred in parallel with an increased proportion of $CD21^-$ (C3d receptor) MBCs with a limited ability to respond to polysaccharide-complement complexes.[65,66] The fact that within naive B cells the percentage of $CD21^-$ cells remains stable with age and shows a comparable *in vitro* response to polysaccharide antigens in children and adults[67] suggests that external factors affecting antigen recognition might increase the production of C3d receptor–deficient MBCs in children of around 1 year of age when serum C3 levels reach adult-like values.[68] Thus the limited availability of C3d and C3d-antigen complexes in infants less than 1 year old because low serum C3 levels[69] might contribute to reduced signaling for expression of this receptor during antigen recognition. In line with this, other age-related phenotypic subsets of MBCs have been previously reported, such as the "atypical" MBCs and the minor population of $CD11c^+$ (iC3b receptor) B cells,[70-72] which overlapped here with $CD21^-$ cells both in children and adults (see Fig E7 in this article's Online Repository at www.jacionline.org), as well as in other studies.[27] Although we did not confirm here in human subjects previous observations about the potential expansion of atypical $CD27^-CD21^-$ MBCs or other $CD21^-$ subsets of B cells in elderly subjects (see Fig E8 in this article's Online Repository at www.jacionline.org), there might be additional phenotypic markers that might contribute to better understand B-cell immunity at advanced ages.

Altogether, these results suggest that maturation of other components of the immune response (eg, complement levels and germinal centers in lymphoid tissues) potentially contribute to determine the number of MBCs generated and their phenotype and consequently also their functional abilities. However, the effect of these environmental costimulatory signals might also be influenced by the position of the IgH isotype in the *IGHC* gene because, independent of age, a higher proportion of $CD27^-$ and $CD21^-$ MBCs was observed among those B-cell compartments expressing upstream IgH isotypes (eg, IgG_3) versus those displaying IgH subclasses located at the end of the *IGHC* gene (eg, IgG_2).

Overall, our results support the notion that despite serum IgH levels possibly provide important information about the long-lived steady-state PCs that accumulate in BM, they are not enough to fully understand the humoral immunocompetence status of a subject. PCs in PB emerge as a robust earlier sensor of actual ongoing immune responses, whereas progressive accumulation of MBCs expressing IgH isotypes and subclasses encoded by the downstream part of the *IGHC* locus reflect potential terminal sequential class-switching and accumulation of MBCs expressing more tolerogenic isotypes at more advanced ages, as also confirmed by very preliminary data on longitudinal changes in children (see Fig E9 in this article's Online Repository at www.jacionline.org). Additionally, we provide normal reference values for PCs and MBCs expressing different IgH isotypes and subclasses that, once confirmed in larger longitudinal series of subjects from different genetic and geographic backgrounds, might be of value in future studies in patients with multiple disease conditions, particularly immunodeficiency, inflammation, allergy, autoimmunity, and infection.

Key messages

- **Distinct age-related production patterns are observed for PCs, MBCs, and immunoglobulin plasma levels.**
- **PCs, MBCs, and antibodies of different IgH isotypes corresponding to the different *IGHC* gene blocks peak at distinct ages, likely reflecting consecutive cycles of IgH class-switch recombination through life.**

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METHODS

Sample preparation

Forty-eight milliliters of an ammonium chloride solution was mixed with 2 mL of PB in a 50-mL tube and incubated for 15 minutes at room temperature (RT) while gently shaking in a roller to lyse nonnucleated red cells. Then the cell suspension was centrifuged for 10 minutes at 800*g*, and the supernatant was removed by using a Pasteur pipette or a vacuum system without disturbing the cell pellet. Thereafter, cells were washed with 50 mL of PBS containing 0.5% BSA and 0.09% sodium azide (PBS-BSA; pH 7.6) and centrifuged at 800*g* for 5 minutes, and the supernatant was removed. Subsequently, 4 mL of PBS plus 0.09% of NaN_3 plus 0.5% of BSA was added, and the cell suspension was transferred to a 5-mL tube, which was centrifuged at 540*g* for 5 minutes. After removal of the supernatant, sample cellularity was counted in a hematologic analyzer. Ten million nucleated cells were stained subsequently with the 12-color EuroFlow antibody combination (30 minutes at RT). When only smIgH staining was performed, 2 mL of FACS lysing solution (BD Biosciences, San Jose, Calif) diluted 1:10 (vol/vol) in distilled water was added, and cells were sequentially incubated for another 10 minutes at RT, washed, and resuspended in 500 μL of PBS-BSA. In turn, when surface membrane plus cytoplasmic IgH staining was performed, after surface membrane staining, the cell suspension was washed and incubated with solution A of the Fix & Perm Reagent Kit (An der Grub, Vienna, Austria) for 15 minutes at RT. Afterward, washed cells were incubated for another 15 minutes at RT with solution B of the Fix & Perm Reagent Kit and antibodies against IgM, IgD, IgG_1 to IgG_4, and IgA_1 and IgA_2; then stained cells were washed and resuspended in 500 μL of PBS-BSA. In both protocols cells were acquired immediately after sample preparation was completed or stored at 4°C for a maximum of 1 hour.

CD11c staining

In 6 adults (35 ± 8 years) and 6 children (3 ± 5 years), in addition to the EuroFlow 12-color IgH isotype B-cell tube (Table E2), a similar tube that contained an anti-CD11c PE-Cy7 (clone B-ly6; BD) reagent instead of CD5 PE-Cy7 was stained in parallel. The protocol used was the same as described above, and 5×10^6 leukocytes were measured per sample.

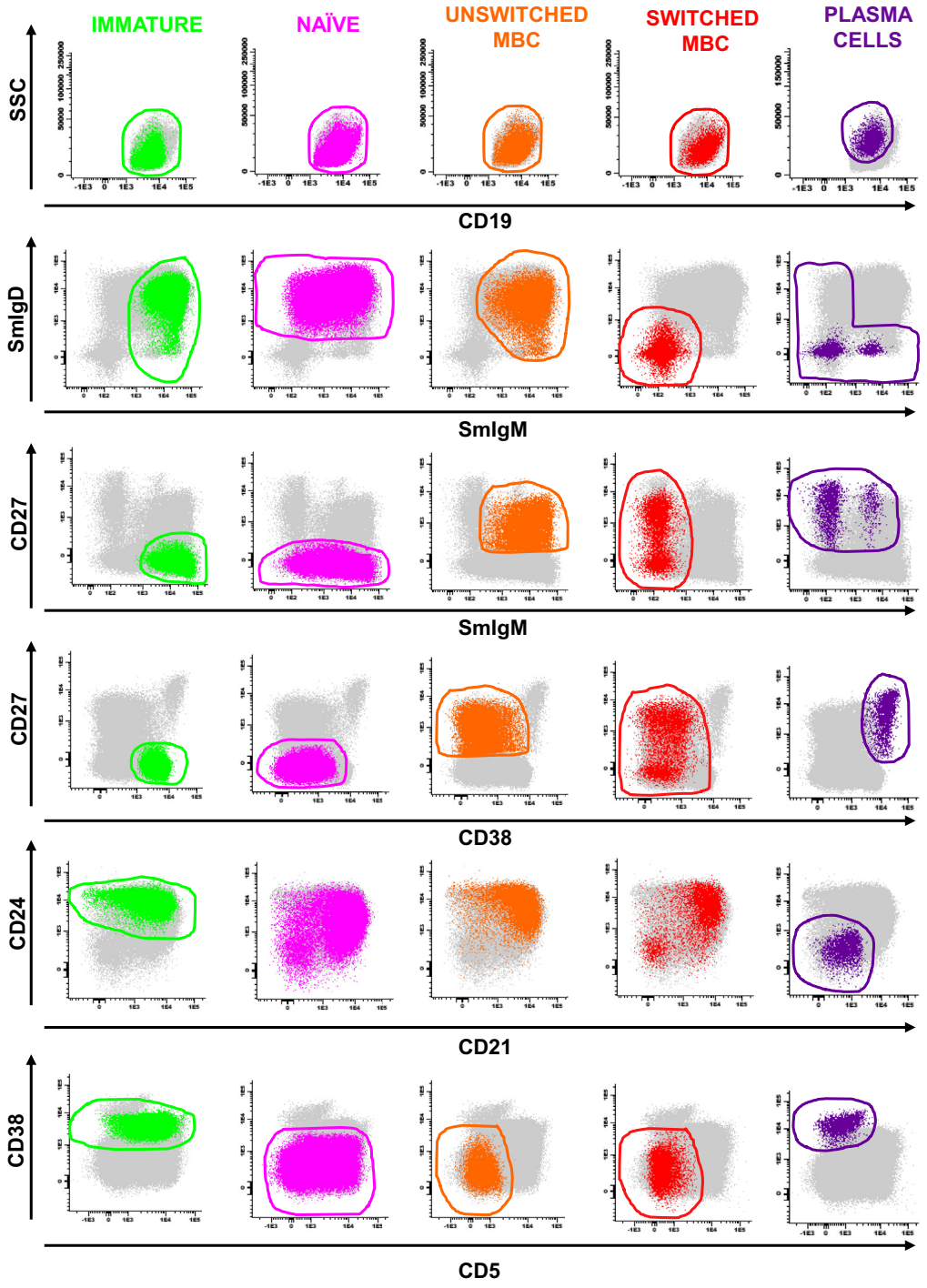

FIG E1. Gating strategy used for identification of maturation-associated B-cell subsets. Bivariate dot plot graphic representations of the gating strategy used to identify immature, naive, switched, and unswitched MBCs and PCs, according to their pattern of expression of CD19, CD27, CD38, CD5, CD24, CD21, smIgM, and smIgD and their side-scatter *(SSC)* features are shown.

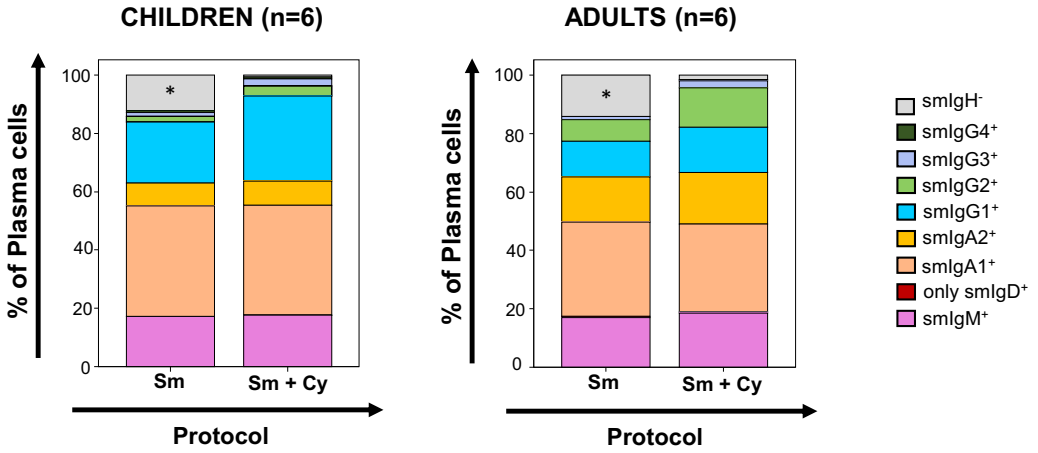

FIG E2. Comparison of the relative distribution in PC of subsets expressing distinct IgH isotypes and subclasses when surface membrane only versus surface membrane plus cytoplasmic staining of aliquots of the same sample (n = 12) was performed. *Bars* represent relative distribution of the distinct IgH isotype and subclass subsets of PCs with the sm-only versus surface membrane plus cytoplasmic IgH staining in children (n = 6) and adults (n = 6). **P* < .05, surface membrane only versus surface membrane plus cytoplasmic staining.

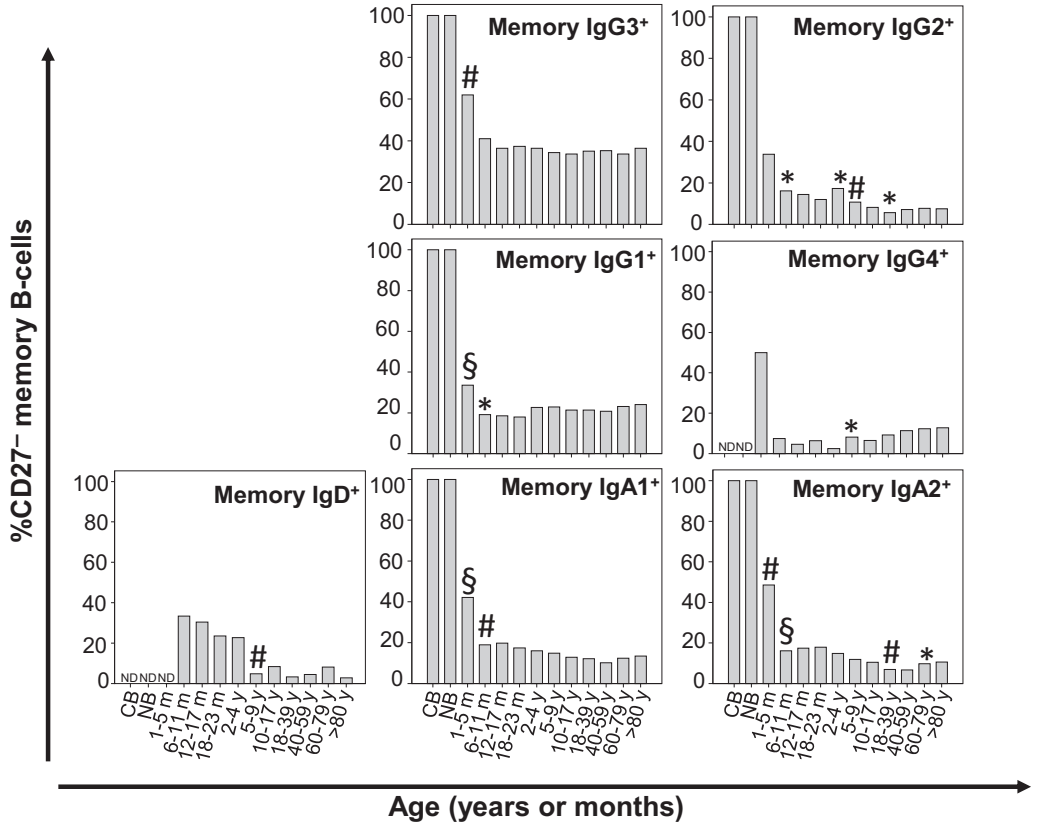

FIG E3. Relative distribution of $CD27^-$ B cells within each subset of CB and PB memory B lymphocytes expressing different IgH isotypes and isotype subclasses through life. *Bars* represent percentage values of $CD27^-$ B cells within each MBC compartment. $^*P < .05$, $\#P < .01$, and $\S P < .001$ versus the previous age group, respectively. *NB*, Newborn.

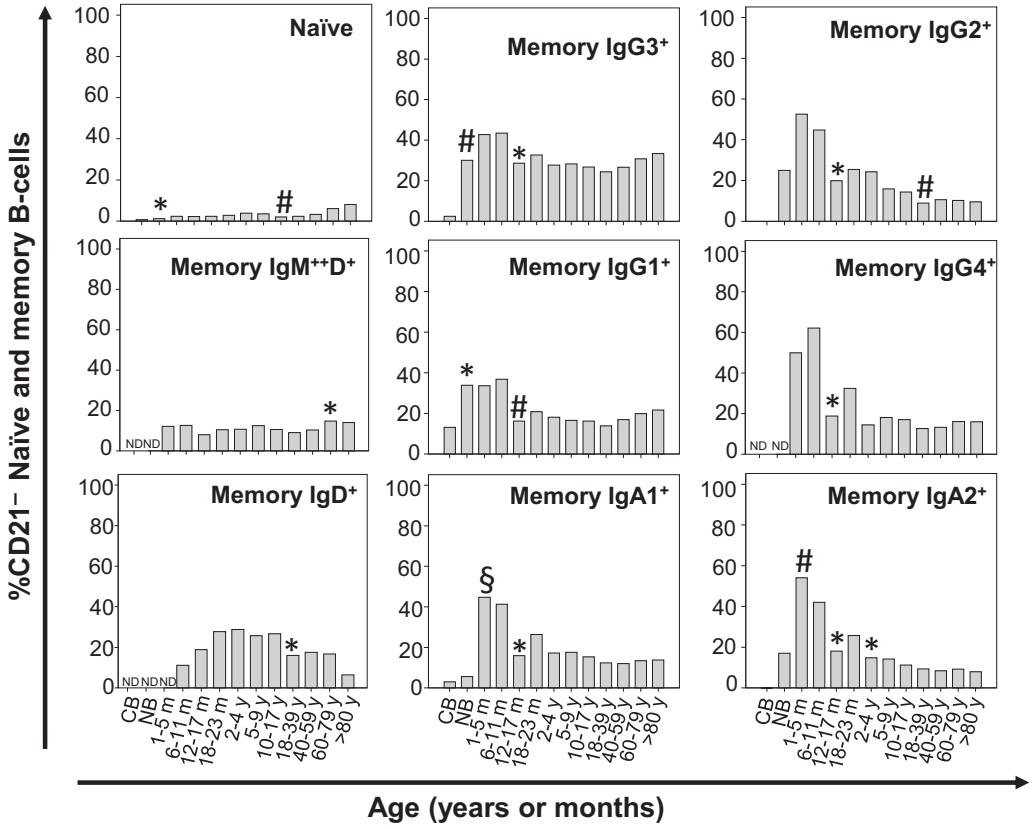

FIG E4. Relative distribution of CD21$^-$ B cells within each subset of CB and PB naive B lymphocytes and MBCs expressing different IgH isotypes and isotype subclasses through life. *Bars* represent percentage values of CD21$^-$ B cells within each B-cell compartment. *$P < .05$, #$P < .01$, and §$P < .001$ versus the previous age group, respectively. *NB*, Newborn.

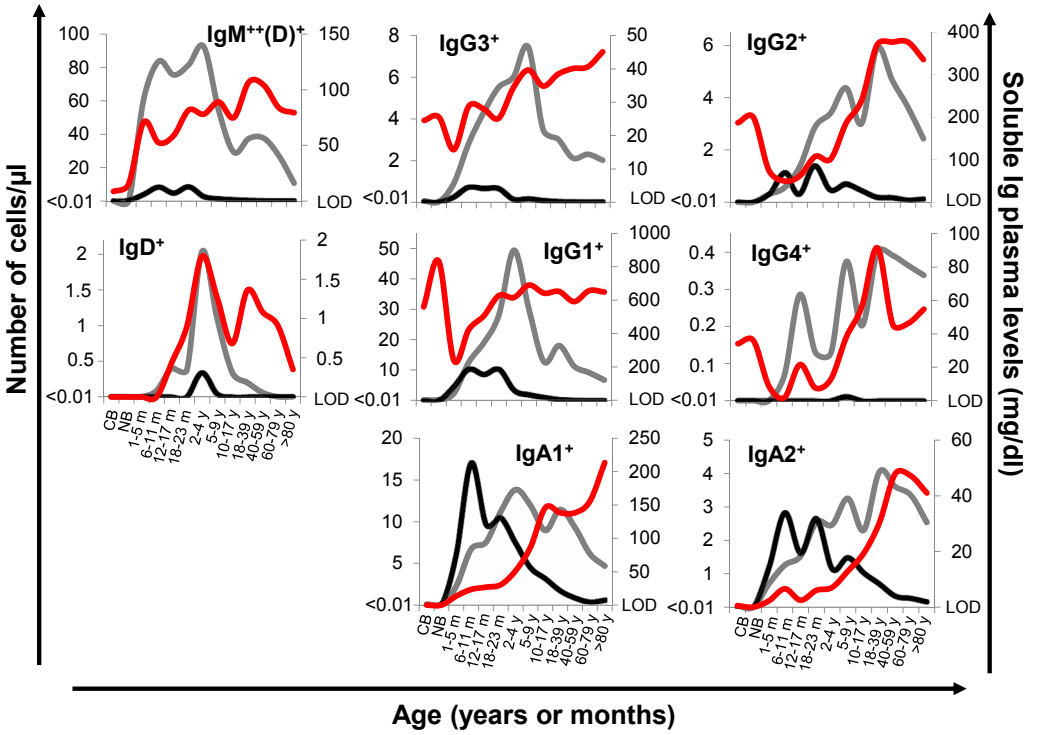

FIG E5. Direct comparison among median numbers of CB and PB MBCs, PCs, and immunoglobulin *(Ig)* soluble levels of the same IgH isotype and isotype subclasses through life. *Lines* correspond to median absolute numbers of MBCs (gray), PCs (black), and soluble immunoglobulin plasma levels (in milligram per deciliter; red) per age group. *LOD*, Limit of detection; *NB*, newborn.

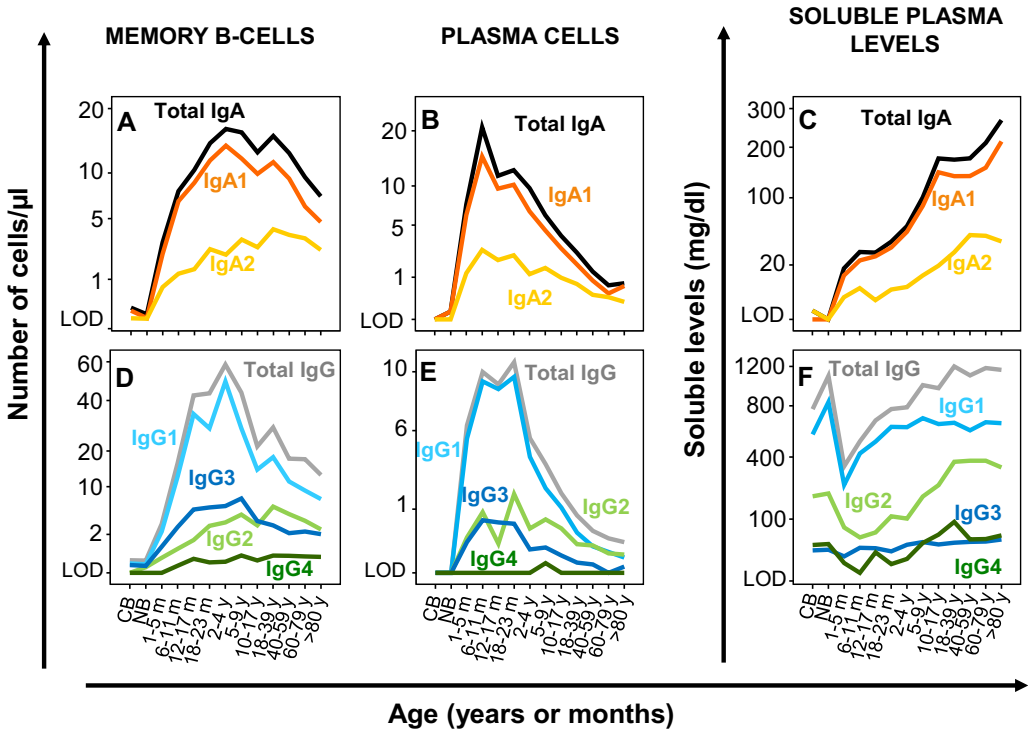

FIG E6. Comparison of the distribution of CB/PB MBCs and PCs, as well as soluble immunoglobulin plasma levels, through life: total IgA and IgG versus individual cellular and soluble compartments of IgA and IgG subclasses. *Lines* link median values of total IgA (black), IgA_1 (orange), and IgA_2 (yellow) MBCs **(A)**, PCs **(B)**, and soluble immunoglobulin plasma levels **(C)** plus total IgG (gray), IgG_1 (light blue), IgG_2 (light green), IgG_3 (dark blue), and IgG_4 (dark green) MBCs **(D)**, PCs **(E)**, and soluble immunoglobulin plasma levels **(F)**. *LOD*, Limit of detection; *NB*, newborn.

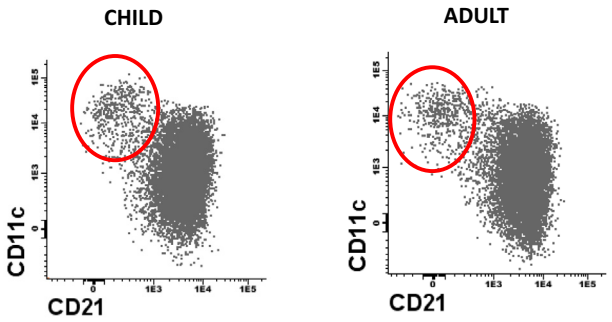

FIG E7. Pattern of expression of CD21 in $CD11c^+$ in MBCs. Dot plots illustrating CD11c expression with MBCs of a representative healthy child and a healthy adult are shown. In both types of samples, $CD11c^+$ MBCs systematically corresponded to $CD21^-$ MBCs in line with previous findings reported in the literature.[27,71]

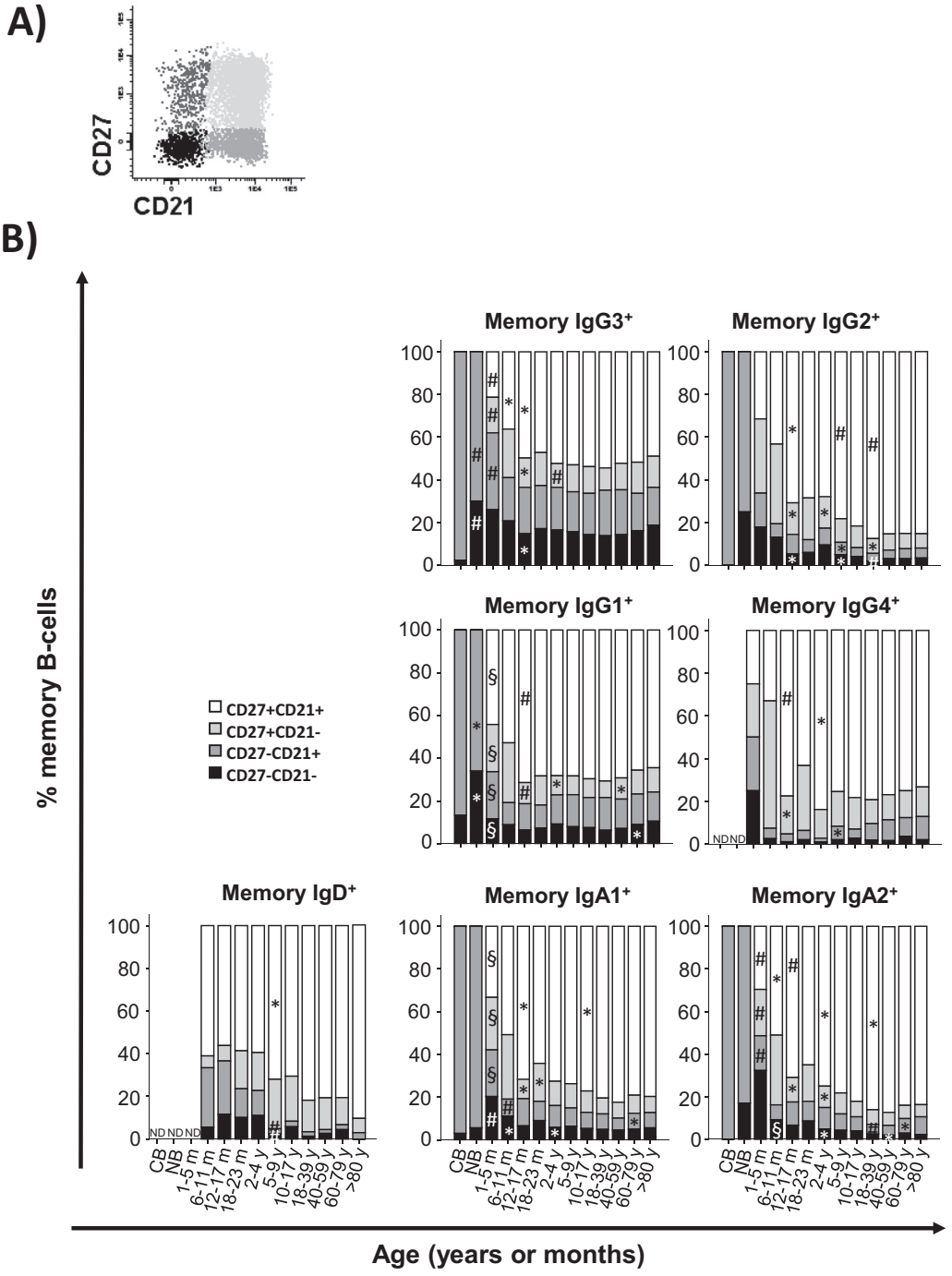

FIG E8. Relative distribution of typical, atypical, and other CD21$^-$ B cells within each compartment of MBCs expressing different IgH isotypes and subclasses through life. **A,** Representative dot plot of MBCs grouped according to CD21 and CD27 expression. **B,** *Bars* represent percentage values of typical (CD27$^+$CD21$^+$), atypical (CD27$^-$CD21$^-$), other CD21$^-$ B cells (CD27$^+$CD21$^-$) and CD27$^-$CD21$^+$ MBCs within each subset of MBCs expressing different IgH isotypes and subclasses. *$P < .05$, #$P < .01$, and §$P < .001$ versus the previous age group, respectively. *NB*, Newborn.

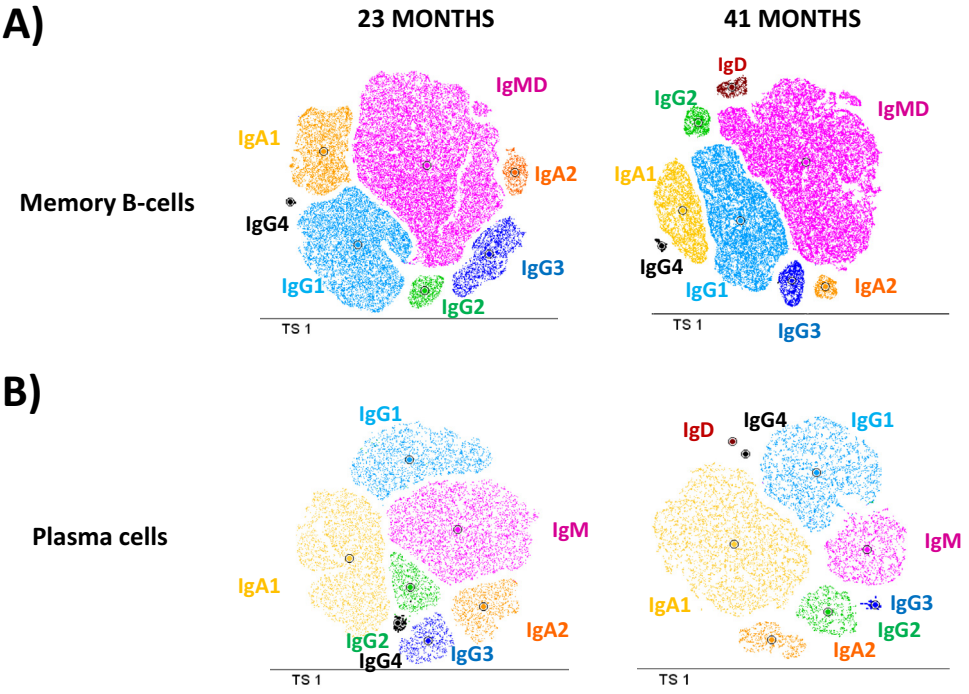

FIG E9. t-Distributed stochastic neighbor embedding plots showing the relative size of different subsets of MBCs and PCs expressing distinct IgH isotypes and subclasses in a single child at 2 different ages (23 and 41 months). Please note the relative decrease in, for example, IgG_3 MBCs **(A)** and both IgM and IgG_3 PCs at the expense of a larger IgA_1 and IgG_1 PC area **(B)**.

TABLE E1. Demographics of children younger than 4 years included in the study

	Age group				
Environmental factors	**0-5 mo**	**6-11 mo**	**12-17 mo**	**18-23 mo**	**2-4 y**
Breast-feeding ever	80%	100%	71%	86%	92%
Breast-feeding until 6 mo	—	60%	30%	50%	50%
Day care	20%	40%	71%	43%	83%
Pets	20%	20%	14%	0%	25%
Rural environment	0%	40%	43%	29%	17%
Smoking parents	60%	20%	14%	29%	25%
Smoking during pregnancy	20%	40%	14%	14%	17%
Delivery					
Vaginal	100%	60%	86%	71%	92%
Caesarean	0%	20%	14%	29%	8%

Percentage of subjects analyzed presenting each environmental factor per age group.

TABLE E2. EuroFlow 12- color IgH isotype B-cell tube

Fluorochrome conjugated to antibody reagent	BV421	BV510	BV711	BV650	BV786	FITC	PerCP-Cy5.5	PE	PE-CF594	PE-Cy7	APC	APC-H7
Antibody marker	CD27	smIgM	CD21	CD24	CD19	smIgG_3 + smIgG_2	smIgA_1 + smIgA_2	smIgG_1 + smIgG_2	smIgD	CD5	smIgG_4 + smIgA_1	CD38
Antibody clone	M-T271	MHM-88	B-ly4	ML5	SJ25C1	SAG3/SAG2	SAA1/SAA2	SAG1/SAG2	IA6-2	L17F12	SAG4/SAA1	HB7
Antibody manufacturer	BD Biosciences, San Jose, Calif	BioLegend, San Diego, Calif	BD Biosciences	BD Biosciences	BD Biosciences	Cytognos, Salamanca, Spain	Cytognos	Cytognos	BD Biosciences	BD Biosciences	Cytognos	BD Biosciences

Combination of fluorochrome-conjugated antibodies used for the immunophenotypic analysis of PB samples by flow cytometry.
APC, Allophycocyanin; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; *PerCP*, peridinin-chlorophyll-protein complex.

TABLE E3. Absolute number of PB lymphocytes, total B cells, and maturation-associated B-cell subsets in healthy control subjects distributed per age group

	CB (n = 19)	NB (n = 14)	1-5 mo (n = 11)	6-11 mo (n = 7)	12-17 mo (n = 12)	18-23 mo (n = 12)	2-4 y (n = 25)	5-9 y (n = 22)	10-17 y (n = 14)	18-39 y (n = 32)	40-59 y (n = 27)	60-79 y (n = 27)	>80 y (n = 12)
Lymphocytes	3,710 (2,041-5,687)	5,049 (2,581-8,360)	6,771 (2,443-9,955)	5,709 (5,150-8,531)	6,547 (4,611-9,924)	5,014 (3,798-11,033)	4,184 (1,620-6,856)	3,134 (1,967-4,564)	3,109 (1,749-3,739)	2,504 (1,479-3,681)	2,273 (1,280-3,192)	2,408 (1,531-4,891)	2,386 (938-2,793)
Total B cells	502 (313-1,280)	357 (108-1,322)	1533 (470-4,327)	1,294 (869-2,316)	1,186 (692-1,737)	965 (268-1,559)	825 (302-1,637)	451 (157-725)	360 (174-630)	220 (41-470)	148 (91-413)	173 (36-384)	141 (42-283)
Immature B cells	116 (40-316)	65 (15-343)	449 (228-1,398)	268 (111-384)	157 (105-389)	120 (39-300)	108 (36-314)	41 (12-84)	37 (11-111)	5.6 (0.25-24)	6.0 (0.84-23)	6.1 (0.69-36)	5.7 (1.3-23)
Naive B cells	426 (214-962)	298 (71-976)	981 (186-2,620)	812 (508-1,686)	677 (232-1,293)	678 (181-1,254)	473 (204-1,064)	265 (68-505)	189 (75-401)	111 (13-288)	83 (26-244)	109 (20-280)	80 (15-210)
Memory B cells	0.3 (<0.01-3.0)	0.4 (<0.01-2.3)	73 (20-283)	115 (48-219)	111 (79-291)	131 (44-241)	182 (48-343)	123 (64-282)	68 (31-160)	91 (23-221)	70 (25-173)	56 (13-128)	30 (8.1-119)
PCs	<0.01	0.3 (<0.01-4.2)	21.2 (1.6-48)	53 (24-104)	28 (8.1-82)	43 (4.8-144)	18 (4.1-52)	13 (3.5-45)	8.5 (1.3-27)	4.4 (1.1-25)	2.7 (0.6-9.7)	1.2 (0.3-7.1)	1.5 (0.14-18)

Results are expressed as median number of cells per microliter (range).
NB, Newborn.

TABLE E4. Percentage of cases with detectable numbers of PB MBCs and PCs expressing different IgH isotypes and isotype subclasses and their absolute numbers per age group

	CB (n = 19)	NB (n = 14)	1-5 mo (n = 11)	6-11 mo (n = 7)	12-17 mo (n = 12)	18-23 mo (n = 12)	2-4 y (n = 25)	5-9 y (n = 22)	10-17 y (n = 14)	18-39 y (n = 32)	40-59 y (n = 27)	60-79 y (n = 27)	>80 y (n = 12)
MBC subsets													
$IgMD^+$													
Cases	0%	0%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Cells/μL	<0.01	<0.01	61 (8.6-245)	84 (21-170)	76 (45-186)	81 (31-153)	92 (24-207)	54 (23-147)	29 (17-78)	38 (7.9-122)	36 (12-114)	27 (7.4-72)	11 (2-56)
IgD^+													
Cases	0%	0%	9%	57%	50%	78%	96%	95%	79%	69%	57%	48%	25%
Cells/μL	<0.01	<0.01	<0.01	0.1 (<0.01-1.4)	0.4 (<0.01-2.1)	0.4 (<0.01-5.6)	2.0 (<0.01-6.9)	1.1 (<0.01-2.9)	0.3 (<0.01-1.7)	0.2 (<0.01-2.4)	0.06 (<0.01-0.9)	0.01 (<0.01-1.2)	0.01 (<0.01-0.4)
IgG_1^+													
Cases	63%	86%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Cells/μL	0.1 (<0.01-0.6)	0.2 (<0.01-0.8)	2.3 (0.1-13)	12.5 (6.9-28)	19 (11-65)	28 (6.9-69)	49 (11-130)	30 (12-86)	18 (7-42)	18 (3.2-40)	11 (2.8-30)	9.1 (1.3-22)	6.7 (2.0-31)
IgG_2^+													
Cases	37%	21%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Cells/μL	0 (<0.01-0.2)	0.04 (<0.01-0.2)	0.3 (<0.01-1.2)	0.6 (0.4-3.5)	1.4 (0.5-3.4)	2.9 (0.6-6.1)	3.4 (0.8-11)	4.4 (0.7-15)	3.0 (0.7-10)	5.9 (1.6-30)	4.7 (0.6-14)	3.6 (1.0-11)	2.4 (0.4-12)
IgG_3^+													
Cases	74%	50%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Cells/μL	0.06 (<0.01-0.7)	0.03 (<0.01-0.5)	0.9 (0.4-4.7)	2.8 (1.4-6.2)	4.3 (1.6-14)	5.5 (1.5-12)	6.0 (1.8-17)	7.4 (2.4-16)	3.0 (1.1-8.3)	3.0 (0.5-8.4)	2.1 (0.7-6.6)	2.3 (0.4-8.1)	2.0 (0.7-6.5)
IgG_4^+													
Cases	0%	0%	18%	71%	83%	92%	76%	86%	86%	91%	89%	92%	92%
Cells/μL	<0.01	<0.01	<0.01 (<0.01-0.6)	0.07 (<0.01-0.4)	0.3 (<0.01-1.4)	0.1 (<0.01-0.5)	0.1 (<0.01-1.9)	0.4 (<0.01-2.0)	0.2 (<0.01-2.9)	0.4 (<0.01-2.4)	0.4 (<0.01-4.1)	0.4 (<0.01-2.1)	0.3 (<0.01-2.3)
IgA_1^+													
Cases	90%	79%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Cells/μL	0.1 (<0.01-1.2)	0.1 (<0.01-1.1)	2.3 (0.4-8.6)	6.7 (3.9-13)	7.5 (5.5-33)	11 (1.5-22)	14 (5.7-28)	12 (4.6-24)	9.0 (2.9-21)	11 (2.1-43)	8.1 (2.5-27)	6.2 (2.2-22)	4.7 (1.7-25)
IgA_2^+													
Cases	63%	36%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Cells/μL	0.04 (0-0.3)	<0.01 (<0.01-0.3)	0.7 (0.07-1.7)	1.3 (0.6-4.3)	1.5 (0.3-7.9)	2.5 (0.5-5.6)	2.5 (0.8-18)	3.2 (1.0-13)	2.7 (0.8-5.9)	4.1 (1.2-18)	3.6 (0.4-14)	3.4 (0.7-9.0)	2.5 (0.6-11)
PC subsets													
IgM^+													
Cases	0%	79%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Cells/μL	<0.01	0.3 (<0.01-2.9)	3.9 (0.3-12)	8.3 (4.8-52)	4.7 (1.1-8)	8.5 (0.6-40)	2.6 (0.6-7.2)	1.4 (0.6-14)	0.8 (0.2-5.7)	0.4 (0.05-4.7)	0.2 (0.04-1.0)	0.1 (0.01-0.8)	0.1 (0.02-1.1)
IgD^+													
Cases	0%	0%	0%	14%	50%	33%	84%	55%	36%	31%	19%	15%	17%
Cells/μL	<0.01	<0.01	<0.01 (<0.01-0.2)	<0.01 (<0.01-0.7)	<0.01 (<0.01-0.8)	<0.01 (<0.01-2.4)	0.3 (<0.01-2.3)	0.04 (<0.01-0.8)	<0.01 (<0.01-2.0)	<0.01 (<0.01-1.1)	<0.01 (<0.01-0.1)	<0.01 (<0.01-0.2)	<0.01 (<0.01-0.02)
IgG_1^+													
Cases	0%	14%	100%	100%	100%	100%	100%	100%	100%	100%	93%	100%	92%
Cells/μL	<0.01	<0.01 (<0.01-0.5)	4.5 (0.5-16)	10 (4.7-19.3)	8.5 (0.4-14)	8.5 (1.7-26)	3.3 (0.3-13)	1.9 (0.1-7.7)	1.1 (0.1-4.8)	0.4 (0.05-4.4)	0.2 (<0.01-1.7)	0.1 (0.01-0.6)	0.1 (<0.01-1.6)

(Continued)

TABLE E4. *(Continued)*

	CB (n = 19)	NB (n = 14)	1-5 mo (n = 11)	6-11 mo (n = 7)	12-17 mo (n = 12)	18-23 mo (n = 12)	2-4 y (n = 25)	5-9 y (n = 22)	10-17 y (n = 14)	18-39 y (n = 32)	40-59 y (n = 27)	60-79 y (n = 27)	>80 y (n = 12)
IgG_2^+													
Cases	0%	25%	82%	100%	75%	100%	100%	100%	100%	97%	96%	96%	100
Cells/μL	<0.01	<0.01 (<0.01-0.1)	0.3 (<0.01-1.0)	1.1 (0.4-2.4)	0.3 (<0.01-2.4)	1.3 (1.3-4.7)	0.5 (0.02-1.5)	0.7 (0.07-2.3)	0.5 (0.08-0.8)	0.2 (<0.01-2.6)	0.2 (<0.01-0.7)	0.09 (<0.01-1.6)	0.1 (0.03-1.6)
IgG_3^+													
Cases	0%	0%	100%	100%	93%	92%	68%	76%	86%	59%	63%	27%	67%
Cells/μL	<0.01	<0.01	0.2 (0.05-1.3)	0.7 (0.1-1.5)	0.6 (<0.01-3.4)	0.6 (<0.01-2.5)	0.1 (<0.01-2.1)	0.2 (<0.01-1.3)	0.08 (<0.01-0.4)	0.03 (<0.01-0.3)	0.02 (<0.01-0.2)	<0.01 (<0.01-0.2)	<0.01 (<0.01-0.23)
IgG_4^+													
Cases	0%	0%	9%	14%	48%	50%	20%	50%	43%	44%	30%	27%	33%
Cells/μL	<0.01	<0.01	<0.01 (<0.01-0.15)	<0.01 (<0.01-0.1)	<0.01 (<0.01-0.4)	<0.01 (<0.01-0.3)	<0.01 (<0.01-0.2)	0.02 (<0.01-0.2)	<0.01 (<0.01-0.2)	<0.01 (<0.01-0.4)	<0.01 (<0.01-0.1)	<0.01 (<0.01-0.1)	<0.01 (<0.01-0.02)
IgA_1^+													
Cases	0%	14%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100
Cells/μL	<0.01	<0.01 (<0.01-0.3)	6.1 (0.3-17)	17 (10-44)	9.6 (3.4-33)	10 (1.2-80)	7.3 (1.3-20)	4.4 (0.6-16)	3.1 (0.5-14)	1.7 (0.3-6.9)	0.9 (0.2-3.8)	0.4 (0.04-3.3)	0.6 (0.03-7.1)
IgA_2^+													
Cases	0%	7%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	92%
Cells/μL	<0.01	<0.01 (<0.01-0.1)	1.2 (0.04-4.0)	2.8 (1.6-9.0)	1.6 (0.3-15)	2.6 (0.4-4.5)	1.1 (0.3-4.4)	1.5 (0.3-3.5)	1.0 (0.3-3.6)	0.7 (0.2-4.2)	0.3 (0.04-2.9)	0.3 (0.06-1.2)	0.2 (<0.01-1.2)

Results are expressed as percentages of cases and median absolute numbers of cells per microliter (range).
NB, Newborn.

TABLE E5. Soluble plasma levels of different immunoglobulin isotypes and isotype subclasses through life

IgH isotypes/ subclasses	CB (n = 18)	NB (n = 10)	1-5 mo (n = 9)	6-11 mo (n = 7)	12-17 mo (n = 12)	18-23 mo (n = 12)	2-4 y (n = 25)	5-9 y (n = 22)	10-17 y (n = 14)	18-39 y (n = 31)	40-59 y (n = 27)	60-79 y (n = 21)	>80 y (n = 11)
Total immunoglobulin	748 (516-1484)	1108 (818-1223)	433 (267-634)	607 (377-915)	837 (542-1317)	866 (469-1092)	946 (596-1559)	1213 (814-1654)	1234 (686-1716)	1532 (1082-2182)	1430 (1073-1852)	1546 (978-1922)	1682 (1087-1804)
IgM	8.6 (<LOD-142)	15 (<LOD-23)	71 (<LOD-158)	52 (37-193)	59 (6-110)	82 (42-142)	89 (54-135)	89 (32-210)	75 (42-94)	107 (35-200)	104 (56-225)	84 (43-240)	79 (52-245)
IgD	<LOD	<LOD	0.3 (<LOD-0.7)	LOD (<LOD-3.7)	0.4 (<LOD-3.1)	0.9 (<LOD-5.2)	1.8 (<LOD-22)	1.3 (<LOD-96)	0.7 (<LOD-6)	1.4 (<LOD-13)	1.1 (<LOD-5.9)	1.0 (<LOD-8.7)	0.4 (<LOD-1.4)
IgG_1	560 (368-893)	833 (514-915)	241 (142-434)	424 (142-563)	506 (394-718)	628 (321-958)	616 (328-1200)	691 (459-942)	642 (405-931)	652 (390-1040)	592 (357-963)	657 (477-986)	649 (451-1100)
IgG_2	187 (101-400)	200 (133-256)	75 (46-115)	50 (29-104)	62 (21-632)	108 (72-223)	102 (59-223)	186 (61-403)	240 (125-361)	370 (172-771)	377 (204-587)	377 (173-543)	336 (226-518)
IgG_3	25 (12-66)	26 (15-36)	16 (9.6-38)	29 (20-70)	28 (21-236)	25 (12-58)	34 (11-86)	40 (20-74)	34 (21-72)	38 (11-86)	40 (23-188)	41 (20-96)	45 (24-85)
IgG_4	34 (8-222)	34 (13-114)	9.0 (1.8-20)	1.8 (1.3-19)	22 (0.6-74)	8 (1.8-46)	13 (0.9-138)	38 (2.2-138)	56 (1.1-198)	92 (0.7-335)	45 (3.4-127)	46 (6.2-174)	55 (13-104)
IgA_1	<LOD (<LOD-48)	<LOD	13 (3.7-20.4)	23 (6.5-73)	27 (14-81)	31 (7-101)	52 (7-308)	87 (29-233)	146 (37-299)	138 (70-327)	139 (66-286)	156 (77-377)	214 (60-493)
IgA_2	0.5 (<LOD-9)	<LOD (<LOD-0.6)	2.3 (0.7-7.2)	6.6 (0.7-7.7)	2.5 (1.0-11)	6.0 (2.6-35)	7.1 (1.4-39)	13 (1.0-35)	19 (7.1-44)	30 (11-131)	48 (14-80)	47 (14-122)	41 (25-114)
IgE	<LOD	<LOD	<LOD (<LOD-11)	<LOD (<LOD-10)	14 (<LOD-202)	4.8 (<LOD-45)	16 (<LOD-102)	8.4 (<LOD-139)	33 (<LOD-444)	20 (<LOD-269)	22 (<LOD-45)	13 (<LOD-196)	28 (<LOD-105)

Results are expressed as medians (ranges). Total immunoglobulin and IgM, IgD, IgG, and IgA subclasses are expressed in milligrams per deciliter. IgE levels are expressed in international units per milliliter.
LOD, Limit of detection; *NB*, newborn.