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Comprehensive phenotyping of human peripheral blood B lymphocytes in healthy conditions

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Abstract

The B cell compartment provides innate and adaptive immune defenses against pathogens. Different B cell subsets, reflecting the maturation stages of B cells, have non-interchangeable functions and roles in innate and adaptive immune responses. In this review, we provide an overview of the B cell subsets present in peripheral blood of healthy individuals. A specific gating strategy is also described to clearly and univocally identify B cell subsets based on the their phenotypic traits by flow cytometric analysis.

KEYWORDS
B cell subsets, flow cytometry, human B cells, phenotyping

1 | B CELLS IDENTITY CARD

B cells have the function of producing antibodies for protection against infectious diseases. Protective antibodies are highly specific for the antigen that they recognize and are produced at the end of a complex differentiation pathway that requires weeks. Whereas the early phases of the development of the B lineage occur in the bone marrow, the several types of B cells detected in the peripheral blood represent the different steps of maturation mostly triggered by antigenic experience [1].

The B cell function was discovered in 1960 by Max Cooper who demonstrated that the production of antibody was completely depleted in irradiated chickens after surgical removal of the primary site of B-cell development in birds, named the *Bursa of Fabricius* [2], hence the name *B* cells.

The B-cell development occurs in steps, that are tightly controlled by the expression and function of the B-cell receptor (BCR). In the bone marrow, the B-lineage includes phenotypically distinct cell types in their different developmental stages. The success of the stepwise rearrangement of the Heavy and Light Chain is indispensable for the progression along the early phases of the developmental pathway and for the exit to the periphery. B cells emigrate from the bone marrow as transitional B cells when they express a functional BCR, composed of membrane-bound antibody, capable of recognizing the antigen, associated to the B-cell signaling module represented by the Igα-Igβ heterodimer [3, 4]. Transitional B cells expressing IgM and IgD on the cell surface represent an intermediate stages between immature and mature-naïve circulating B-cells and can be found both in the bone marrow and in the peripheral blood [5, 6] (Figure 1).

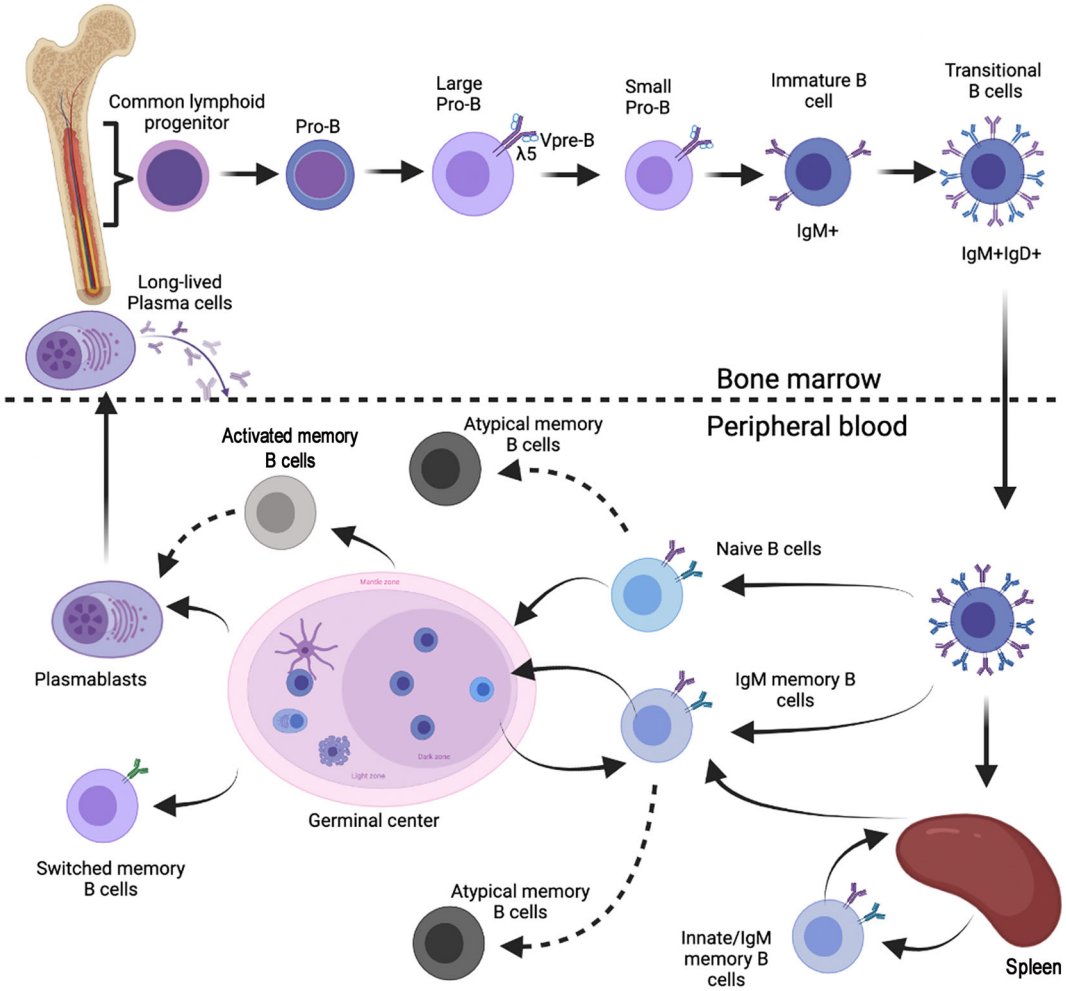

FIGURE 1 Schematic representation of B cell development in the bone-marrow and in the peripheral blood. The top part of the figure illustrates the step-wise development of the cells of the B lineage in the bone marrow. Transitional B cells are generated in the bone marrow, but can be also found in the peripheral blood (lower part of the figure). In the bone marrow B-cell development is driven and controlled by the rearrangement of immunoglobulin heavy and light chain. In the periphery B-cell antigen and TLR signals determine the progression to the memory B cells and plasma cells stage. Long-lived memory plasma cells represent the final stage of B-cell development and home to a dedicated niche in the bone marrow [Color figure can be viewed at wileyonlinelibrary.com]

Thanks to the phenomenon of allelic exclusion, immunoglobulin rearrangement results in the expression of a unique receptor by each B cell. Thus, every B cell has its own specificity, and the high number of possible rearrangements (>10^{12}) provides humans with a potentially infinite repertoire capable of recognizing any encountered antigen [7].

In the periphery B cells continue their development in order to fulfill their ultimate function: protection through the production of specific antibodies. Transitional B cells are short-lived and rapidly differentiate into mature-naïve B cells [8], that represent a major population in the peripheral blood and populate the primary lymphoid follicles in lymph nodes and spleen. Mature-naïve B cells continuously recirculate with the lymph and blood scrutinizing the environment in search of antigen. If they encounter the antigen that their BCR recognizes, the next phase of development initiates in the germinal centers. Activated mature-naïve B cells proliferate, introduce mutation in their immunoglobulin genes and are selected for their affinity to the antigen [9]. Only high-affinity B cells become either memory B cells or plasma cells [10, 11] (Figure 1). An alternative T- and germinal center-independent pathway leads to the generation of innate/IgM memory B cells in the spleen (Aranburu et al.[12] and see below).

The different types of B cells in peripheral blood can be distinguished by the combined expression of few clusters of differentiation (CD) markers.

In the adult peripheral blood, B cells account for ~10% of the total lymphocytes. The identification of the different B-cell type has been addressed by several research groups during the last years. The usage of different markers and classifications has led to a controversial terminology. We propose a simple nine-color staining (Table 1) to define B-cell populations with different functions in healthy donors. Among circulating $CD19^{pos}$ B-cells we can identify six major populations: transitional, mature-naïve, memory, atypical memory, activated B cells, and plasmablasts (Figure 2 and Tables 1 and 2).

$CD24^{bright}CD38^{bright}$ B cells in the blood correspond to transitional B cells (Figure 2, in red); they represent the most abundant

TABLE 1 Cluster of differentiation expressed on the different B-cell populations

	Transitional	Mature-Naïve	Memory	Plasmablasts	Atypical memory	Activated memory
CD45	++	++	++	++	++	++
CD19	++	++	++	++	+++	++
CD24	+++	++	+++	−	−/+	+ and ++
CD21	+ and ++	++	++	+	−	−
CD27	−	−	+ and +++	+++	−	++
CD38	+++	+	−	+++	−	−
Igs	IgM + IgD+	IgM + IgD+	IgM + IgD+ IgG+ IgA+ IgM only IgE+	Intracellular Staining	IgM + IgD+ IgG+ IgM only	IgM + IgD+ IgG+

population in the newborns and decrease to ~3% of total B cells in adults [13]. $CD24^{bright}CD38^{bright}$ transitional B cells coexpress high levels of IgM and IgD ($IgM^{bright}IgD^{bright}$). CD21, CD23 (not shown), and L-selectin (not shown) are also expressed in transitional B cells [5]. CD21 expression differentiates transitional B cells in two populations, $CD21^{bright}$ and $CD21^{dull}$ (Figure 2, in red). $CD21^{dull}$ have been called Transitional 1 (T1) and are the most recent bone marrow emigrant. They still express the marker CD10, which is present in all developing B-cell in the bone marrow [6]. T1 develop into $CD21^{bright}$ T2 cells, a population increased in autoimmune disease [14].

The two largest B-cell subsets found in the peripheral blood are mature-naïve and memory B cells that can be identified by the expression of CD24 and CD27. Mature-naïve B cells are $CD24^{pos}CD27^{neg}$ (Figure 2, in light blue) and correspond to the circulating B cells that migrate through the lymphoid follicles. If they are activated by the antigen, mature-naïve B cells, in collaboration with T cells and dendritic cells, become the major actors of the adaptive immune response [15].

In healthy adults, 30%–50% of the B cells are $CD24^{bright}CD27^{pos}$ $CD21^{pos}$ memory B cells (Figure 2, in blue). This population includes IgM memory B cells, expressing IgM and IgD, and IgD^{neg} that includes switched memory B cells, carrying immunoglobulins of different isotypes, and IgM-only memory B cells (Figure 2, in green). Most of the switched memory B cells express IgG (~2/3; Figure 2) and IgA (1/3) [16]. A subset of IgM-only memory B cells has also been described [17] (Figure 2).

We recently demonstrated that $CD27^{pos}$ memory B cells can be divided in $CD27^{dull}$ and $CD27^{bright}$ memory B cells [18] (Figure 2). The $CD27^{dull}$ memory B cells are the precursor of the $CD27^{bright}$ subset. The two populations are related be but have distinct molecular signatures and functions. $CD27^{dull}$ are mostly of IgM isotype, whereas the majority of $CD27^{bright}$ memory B cells express switched isotypes [18].

Somatic mutations indicating antigen selection are rare in the $CD27^{dull}$ population and increase at the $CD27^{bright}$ stage. The proportions of $CD27^{dull}$ and $CD27^{bright}$ change during life. $CD27^{bright}$ MBCs become detectable at 3–4 years of age, increase to a median value of 13% of total B cells in peripheral blood at 6–9 years of age, represent the 23% (median value) of B cells in adults and reach 38% (median value) of total B cells in the elderly. $CD27^{dull}$ memory B cells, which are the major population in children, are a minority in adults and rare in older individuals [18].

IgM memory B cells ($IgM^{pos}IgD^{pos}CD27^{pos}$) are a heterogeneous population [19, 20] composed by innate memory B cells [12] and IgM memory B cells that have been remodeled in the germinal centers, where they acquired somatic mutations [12, 21, 22, 23].

Innate or natural memory B cells develop in the absence of germinal centers and T cells [12, 24], generate extra-follicular, T-independent responses, and produce natural antibodies [15, 25]. IgM memory B cells act as a first line of defense against infections [12, 15] whereas switched memory B cells embody the highly specific adaptive memory. We have shown that innate IgM memory B cells can be remodeled and acquire somatic mutations in the germinal centers thus participating to the adaptive immune response [12].

It has been shown that memory B cells, IgM and switched, are stored in the spleen, but only IgM memory B cells are significantly reduced in both asplenic and splenectomized patients [26, 27]. Due to their origin, function, and location in the spleen, IgM memory B cells have been considered the human equivalent of murine marginal zone or B-1 B cells [28–30].

IgM memory B cells are the first population of memory B cells that develop after birth. Remodeled IgM memory and switched memory B cells number increases slowly in infants [13]. The presence in the blood of switched memory B cells is an indicator of a perfectly functional germinal center reaction [12]. The immunoglobulins expressed by switched memory B cells are always more mutated than those of IgM memory B cells, indicating their role as highly specialized B cells for antigen recognition [12].

Recently, two subsets of IgM memory B cells have been described: IgM^{hi} and IgM^{lo} B cells [30]. IgM^{hi} B cells have similarities with the previously described marginal zone B cells that have been remodeled through the germinal centers [12]. They have higher expression of CD27 and are more mutated compared to IgM^{lo}. Based on our results [18], IgM^{hi} memory B cells coincide with remodeled

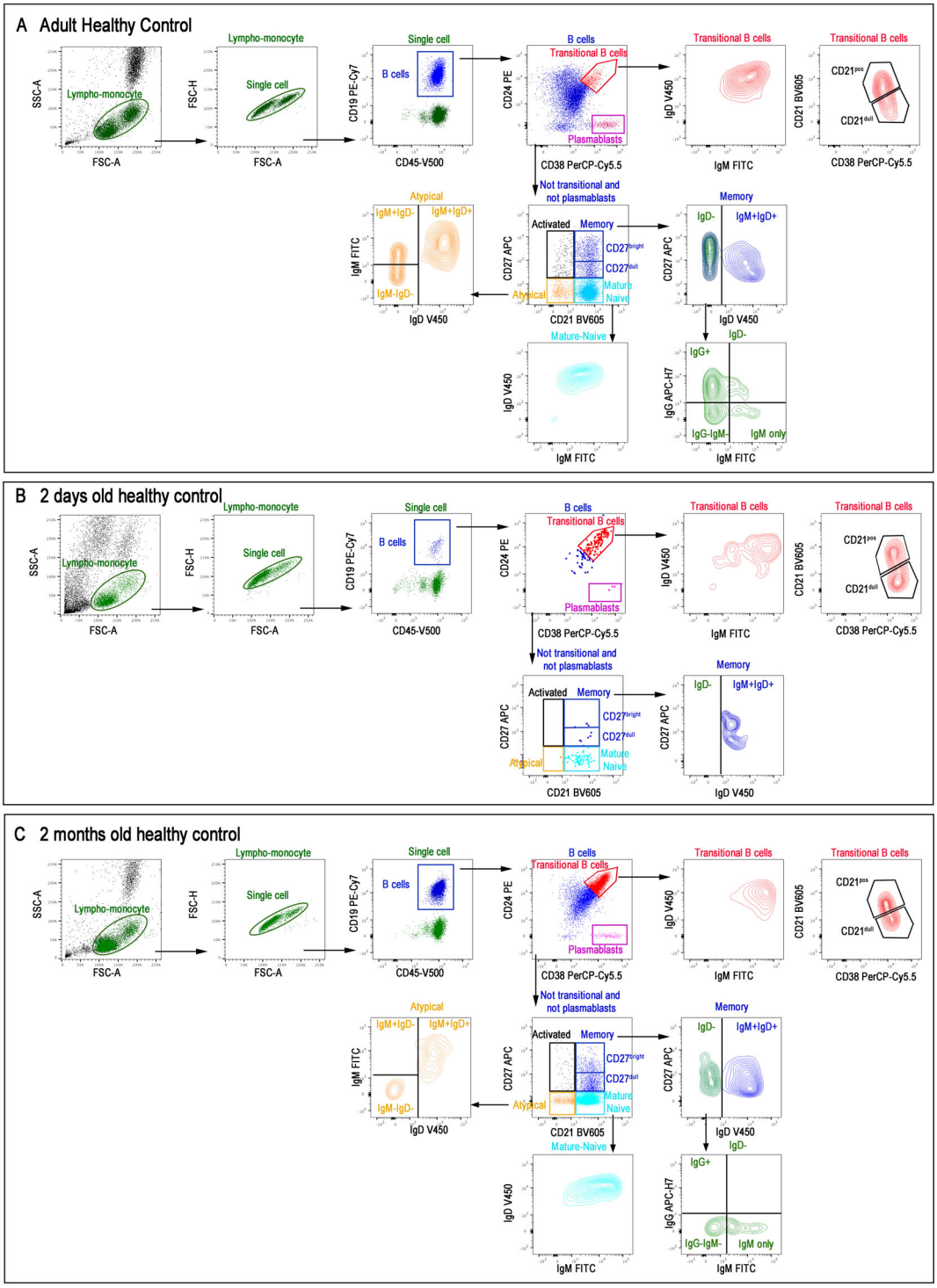

FIGURE 2 Total blood of adult (A), 2 days old (B), and 2 months old infant (C) stained with antibodies against CD19, CD24, CD27, CD38, IgM, IgG, IgD, and CD21. Live cells were identified based on FSC/SSC lympho-monocyte gate and then selected as $CD45^{pos}CD19^{pos}$ B cells. We identified transitional ($CD24^{pos}CD38^{bright}$) and plasmablasts ($CD24^{neg}CD38^{bright}$). We showed IgM, IgD, and CD21 expression in transitional B cells ($CD21^{dull}$ and $CD21^{bright}$). In not transitional/not plasmablasts population, we discriminated mature-naïve ($CD24^{neg}CD21^{pos}$), memory ($CD24^{pos}CD27^{pos}CD21^{pos}$), atypical memory ($CD21^{neg}CD27^{neg}$), and activated B cells ($CD21^{neg}CD27^{pos}$). $CD27^{pos}$ memory B cells can be also divided into $CD27^{dull}$ and $CD27^{bright}$ based on CD27 expression. In the $CD27^{pos}$ memory B-cell population, we showed IgD^{pos} and IgD^{neg} memory B cells. IgD^{neg} memory B cells were further divided into IgG^{pos}, $IgG^{neg}IgM^{neg}$ (that are mostly IgA^{pos}), and IgM-only. Among atypical memory B cells, IgM and IgD expression identify different subtypes [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Differentially markers expressed on B-cell populations

	Transitional	Mature-Naïve	Memory	Plasmablasts	Atypical memory	Activated memory	Bibliography
CD10	+/−	−	−	−	−	−	Carrion et al. [6]
CD11c+	−	+/−	+/−	−	++	++	Golinski et al. [51, 52]
CD20	+	+	+	−	+	+	Pavlasova and Mraz [53]
CD22	++	+	+	−	+	+	Martin et al. [8, 54]
CD23	+++	++	+	+	+/−	−	Grimsholm et al. [18, 31, 47, 48]
CD40	+	+	+	−	+/−	+	Ubillos et al. [43, 52, 55]
CD44	++	+	+	−	+/−		Martin et al. [8, 56–58]
CD55		+	+	+	−	−	Pascual et al. [57, 59–61]
CD62L	+/−	+	+	+/−	+/−		Caraux et al. [31, 52, 59, 62]
CD63		+/−	+/−	+/−	+	++	Holla et al. [59, 63, 64]
CD72	+	++	+	−		++	Glass et al. [56, 59, 65]
CD80	−	−	+		−	++	Sanz et al. [47, 62, 66]
CD84	++	+/−	++	−	+	++	Tangye et al. [67]
CD86	+/−	+/−	+		+/−	++	Sanz et al. [47, 52, 59]
CD95	+	−	++	+	++	++	Grimsholm et al. [18, 47, 52, 56, 59, 62, 66]
CD138	−	−	−	+/−	−	−	Caraux et al. [31]
CD151		+/−	+/−		++	+	Holla et al. [59, 64]
CD200		++	+				Grimsholm et al. [18]
BAFF receptor	++	+	+	−	+/−	+	Smulski et al. [68]
β7 integrin		−	+	++	++	+	Caraux et al. [31, 59]
FCGR2B	++	++	++		++	+	Holla et al. [59, 69]
FCRL4	+	+	+		+++	+	Li et al. [70]
TACI	+	−	+	+			Cuss et al. [62]
TCL1	+++	++	+				Grimsholm et al. [18]
TLR1		++	+	+			Grimsholm et al. [18, 71]
TLR2		+	+	+++			Grimsholm et al. [18, 71]
TLR3		+/−	+/−	+			Meyer-Bahlburg and Rawlings [71]
TLR4		+	+	+			Grimsholm et al. [18, 71]
TLR5		+/−	+/−	+			Meyer-Bahlburg and Rawlings [71]
TLR6		+	++	+			Meyer-Bahlburg and Rawlings [71]
TLR7		+	++	+	−		Meyer-Bahlburg and Rawlings [71]
TLR8		+/−	+/−	+	−		Meyer-Bahlburg and Rawlings [71]
TLR9	+++	+	+++	+	−		Meyer-Bahlburg and Rawlings [26, 71]
TLR11		+	+				Meyer-Bahlburg and Rawlings [71]
CXCR3		+	++	++	++		Grimsholm et al. [18, 38, 52]
CXCR4	+	++	+	−	−		Grimsholm et al. [18, 31, 38, 62]
CXCR5	++	++	++	−	−		Wang et al. [38, 52, 59, 62]
CCR6		+	++		++		Grimsholm et al. [18, 72]
CCR7		+	+		−		Grimsholm et al. [18, 38, 59]
ACKR3		+	++				Grimsholm et al. [18]
CCR9		+	++	++			Grimsholm et al. [18, 38]
CCR10			+	+++			Caraux et al. [31]
IL2R		+	+		+	+	Grimsholm et al. [18, 38]
IL4R		++	+		+	+	Grimsholm et al. [18, 38]

(Continues)

TABLE 2 (Continued)

	Transitional	Mature-Naïve	Memory	Plasmablasts	Atypical memory	Activated memory	Bibliography
IL10R	+	+	++	++	+	++	Grimsholm et al. [18, 73]
IL13R		++	+				Grimsholm et al. [18]
IL21R	+	++	+		++	++	Grimsholm et al. [18, 38, 59]

IgM memory B cells that belong to the $CD27^{bright}$ pool. IgM^{lo} memory B cells have few somatic mutations and most likely overlap with the $CD27^{dull}$ innate memory B cells.

Plasmablasts represent ~3%–5% of $CD19^{pos}$ B cells in the peripheral blood of healthy adults. They are defined as $CD24^{neg}CD27^{bright}$ $CD38^{bright}$ (Figure 2, in pink) and down-regulate CD20 expression, which, in contrast, is expressed by all other B-cells in the periphery. Circulating plasmablasts can be further differentiated by the expression of CD138. CD138+ plasmablasts represent a more mature stage and are characterized by the absence of surface immunoglobulin, lower CD45 expression, and higher amounts of cytoplasmic immunoglobulin [31]. Plasmablasts are differentiated B cells that provide protective immunity thanks to the continuous secretion of antibodies. Plasmablasts are precursor cells of short- and long-lived plasma cells which are the terminally differentiated elements of the B lineage [32]. Normally, plasma cells are not found in the circulation; all antibody-secreting cells in the blood en route to, for example, the bone marrow, are plasmablasts and are still considered as a proliferating fraction of antibody-secreting cells.

Plasmablasts have been extensively studied in the context of the response to influenza virus vaccination and infection. In the course of this response, another group of B cells was identified, the activated memory B cells. These cells have been found to be different from plasmablasts from the transcriptional point of view and still committed to the memory lineage [33]. Activated memory B cells are identified as $CD27^{pos}CD21^{neg}$ (Figure 2, in black) and can be either IgG^{pos} or $IgM^{pos}IgD^{pos}$. Following influenza vaccination, hemagglutinin (HA)-reactive clones are shared between activated memory B cells and antibody secreting cells [10].

Atypical memory B cells (aMBCs; Figure 2, in orange) represent approximately 5% of B cells in the peripheral blood of healthy individuals [34, 35]. aMBCs have been described in aged mice and humans [36, 37], increase during autoimmune diseases [37–39] and in viral infections [40–42] and are thought to reflect a failure or impairment of the germinal center reaction [43]. These cells have also been described as age associated B cells (ABCs) [44–46], tissue-based memory B cells [47], $CD11c^{+}$ B cells, T-bet^{+} memory B cells [48], $CD21^{-/low}$ B cells or double negative (DN) B cells [49]. They express high level of CD19, lack CD21 and CD27 and are characterized by the expression of CD11c and the transcription factor T-bet [48]. Inside aMBCs it is possible to identify $IgM^{pos}IgD^{pos}$, IgG^{pos}, and IgM-only cells [47].

In our staining, we did not use CD5 which is a very important marker for mouse B cells and in human for the diagnosis and follow-up of CD5 + B lymphoproliferative chronic diseases (B-CLPDs).

In our experience, CD5 is not useful for the identification of B cell populations in peripheral blood of control adults and children, because transitional, mature-naïve, and memory B cells can be identified in both the CD5+ and CD5− gate. CD5 can be useful only to identify plasmablasts. When B cells are gated as CD19+CD5+ or as CD19+-CD5−, plasmablasts can be found exclusively in the CD19+CD5+ gate and are absent in the CD19+CD5− gate.

Regulatory B cells (Bregs) were originally described as transitional B cells able to produce IL-10 [50]. Later, it has been demonstrated that the capacity to secrete IL-10 can be acquired by different B cell populations depending on the culture conditions. Thus, Bregs do not have a defined phenotype but represent a functional stage with the immune-modulatory activity. A reliable methods to discriminate Bregs is to perform an intracellular staining for IL-10 following an in-vitro stimulation [47].

2 | IDENTIFICATION OF B-CELL SUBSETS IN PERIPHERAL BLOOD OF HEALTHY DONORS BY FLOW CYTOMETRY

The cytometric analysis of the B cell surface markers is the easiest way to segregate all the above-described subsets. This analysis should be always preceded by a proper gating strategy, in order to identify living cells and exclude nonviable cells and doublets (Figure 2).

To correctly detect the different B cell populations, it is necessary to stain an adequate number of cells and use the right concentration of antibodies. An optimal antibody concentration should be established via antibody titration assays.

An appropriate selection of the best-performing clones of monoclonal antibodies and most appropriate fluorescence for each marker is of primary importance for the correct phenotypic identification of the different B-cell subsets in the peripheral blood. The choice of the brightness of the fluorochrome is particularly important when within the same B-cell population, cells may express low or high amount of a certain marker. For example, in order to discriminate between $CD27^{dull}$ and $CD27^{bright}$ memory B cells we used anti-CD27 labeled with a fluorochrome with a very bright (e.g., PE) or bright (APC) fluorescence index. On the other side, we used a very dim fluorophore for CD45 (V-500) because of its homogenous expression on all B cells.

It is essential to minimize the time between blood collection in the clinic and running stained samples on the flow cytometer. We normally process the blood within maximum 24 h from collection to ensure a reliable quantification of the B-cell populations.

In order to achieve high level of standardization, reagents for B-cell multicolor panel are used in a dried format (BD Bioscience, see Table 3). Bulk lysing is performed to lyse the entire blood sample to be stained. Briefly, 500 μL of fresh total peripheral blood (EDTA) are

TABLE 3 Antibodies used for the staining of the peripheral blood and the identification of the B-cell population

	Fluorochrome	Clone
CD45	V500-C	2D1
CD19	PE-Cy7	SJ25C1
CD24	PE	ML5
CD27	APC	L128
CD38	PerCP-Cy5.5	HIT2
CD21	BV605	B-ly4
IgM	FITC	G20-127
IgG	APC-H7	G18-145
IgD	V450	IA6-2

incubated for 10 min at room temperature with 9.5 mL of the lysing solution Pharm Lyse (1×; BD Biosciences) to remove red blood cells. Afterwards, cells are washed twice with 10 mL of phosphate-buffered saline (PBS) containing 1% of bovine serum albumin (BSA). Our staining is performed on whole blood following red blood cell lysis. In the plasma, IgG antibodies are present in high concentration. The two washing steps after lysis are essential because they wash-off the free IgGs that, by sequestering the labeled anti-IgG antibody, prevent the identification of memory B cells expressing IgG on the surface. Cells are resuspended in 200 μL of PBS and added to a B-Cell lyotube (BD Bioscience). CD21 BV605 is added as drop-in. After 20 min incubation at room temperature in the dark, the samples are washed in PBS 1% BSA and finally resuspended in 300 μL of PBS 1% BSA. Flow cytometric data are acquired on a BD FACSLyric™ cytometer (BD Biosciences) and analyzed by FlowJo ver. 10.7 (Becton, Dickinson & Company) (See supporting informations).

In Figure 2 it is shown an example of a B cells staining in an adult control (A), and in a newborn at 48 h after delivery (B) and after 2 months of life (C). In the staining performed in the adult we can observe all the B cells described above and distributed in a normal way (Figure 2A). On the contrary, at 2 days of life B cells are essentially composed by transitional cells, a few mature B cells and very few memory B cells that are all of the IgM isotype (Figure 2B). At 2 months of age, an expansion in different B cell populations can be detected. In particular, mature B cells, plasmablasts, and memory B cells tend to increase (Figure 2C).

The distribution of the different B cell populations significantly changes over the course of life [13]. In the first years of life, the B cell populations with the highest frequency are transitional B cells and memory cells of the IgM type. Thereafter the transitional B cells are reduced and the switched memory B cells increase [12]. Aging is associated with an increase of memory and aMBCs. Knowing the normal distribution of B cell populations based on age, allow to understand whether the variation observed by flow cytometry is associated with age or a disease state.

A correct staining allows to identify the different B-cell populations and may be of extreme importance and utility in the diagnosis of diseases associated with B cells, as discussed in the accompanied paper.

AUTHOR CONTRIBUTIONS

Writing—original draft: Rita Carsetti, Eva Piano Mortari, and Maria Giulia Conti. *Writing—review and editing*: Rita Carsetti, Eva Piano Mortari, Sara Terreri, Maria Giulia Conti, and Francesco Corrente. *Methodology*: Francesco Corrente, Claudia Capponi, Ane Fernandez Salinas, and Christian Albano. *Formal analysis*: Eva Piano Mortari, Claudia Capponi, Francesco Corrente, and Ane Fernandez Salinas. *Funding acquisition*: Rita Carsetti. *Supervision*: Rita Carsetti.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/cyto.a.24507.

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