Journal of Immunological Methods 475 (2019) 112372

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Selection and validation of antibody clones against IgG and IgA subclasses in switched memory B-cells and plasma cells

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ARTICLE INFO

Keywords:
IgG and IgA subclasses
Flow cytometry
EuroFlow
Antibody clones
Immune monitoring
Immunophenotyping

ABSTRACT

The clinical value of assessing immunoglobulin (Ig)G and IgA subclasses in addition to the isotypes of soluble Igs in serum has been well established. > 20 years ago, the International Union of Immunological Societies and the World Health Organization performed collaborative studies in order to validate antibody (Ab) clones for the detection of IgG and IgA subclasses for a broad range of laboratory assays, except for flow cytometry. Here we analyzed the performance of commercially available Ab clones to detect IgG and IgA subclasses in memory B-cells and plasma cells (PCs) by flow cytometry. In a first step, 28 Ab clones were evaluated in peripheral blood from healthy donors. Only 17/28 clones showed reactivity against IgG and IgA subclasses expressed on the B-cell and PC surface membrane, including Ab clones for IgG_1 (SAG1, HP6188, HP6001 and HP6186), IgG_2 (SAG2, HP6014 and HP6002), IgG_3 (SAG3, HP6095 and HP6050), IgG_4 (SAG4), IgA_1 (SAA1, H69-11.4 and B3506B4) and IgA_2 (SAA2, 2E2, and A9604D2). In a second step, for each Ig subclass a single clone was selected according to its specificity and fluorescence intensity (resolution power), for further more detailed validation (SAG1, SAG2, SAG3, SAG4, SAA1 and SAA2). This validation process was carried out in 4 different laboratories by testing the selected Ab clones in human peripheral blood, bone marrow and tonsil samples, using different staining protocols (*e.g.* surface membrane and/or cytoplasmic staining). All selected Ab clones displayed strong positivity, high specificity and optimal resolution between negative and positive cells. Alternative Ab clones were also validated. Thus, our results show the feasibility of using the validated Ig subclass Ab clones in combination with other B cell-associated markers for detailed dissection of the memory B-cell and PC compartments that express distinct Ig subclasses in different human tissues.

1. Introduction

Four immunoglobulin (Ig)G and two IgA subclasses have been identified so far based on distinct characteristic primary amino acid sequences of their Ig heavy chains (IgH), each of which displays unique immunogenic properties and effector functions. Although IgH subclasses of the same isotype display a high sequence homology (> 95%), small variations in their functional domains are observed (Hamilton,

Abbreviations: Ab, antibody; MoAb, monoclonal antibody; Ig, immunoglobulin; IgH, immunoglobulin heavy chain; Sm, surface membrane; Cy, cytoplasmic; PC, plasma cell; PB, peripheral blood; BM, bone marrow; SI, stain index; SD, standard deviation

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http://dx.doi.org/10.1016/j.jim.2017.09.008
Received 21 March 2017; Received in revised form 18 July 2017; Accepted 15 September 2017
Available online 28 September 2017
0022-1759/

1987; Schroeder and Cavacini, 2010; Vidarsson et al., 2014). These confer differences in their effector functions such as in antibody (Ab) binding, formation of immune complexes, complement activation, triggering of effector cells, regulation of further Ab production, Ig half-life and both their placental transport and secretion/excretion into different body fluids (Hamilton, 1987; Kerr, 1990; Schroeder and Cavacini, 2010; Vidarsson et al., 2014). Such differences in the biological role of the different IgH subclasses also translate into different disease patterns and clinical behavior, including i) different susceptibility to infections in primary and secondary Ab deficiencies (Grimbacher, 2014; Spickett et al., 1991; Vale and Schroeder, 2010; Yazdani et al., 2016), ii) distinct responses against red blood cells and platelets in transfused subjects and pregnancy (Abramson and Schur, 1972; Meulenbroek, 2008), iii) different ability to neutralize microorganisms at the mucosa level (Schroeder and Cavacini, 2010), and iv) variable functional regulation of exacerbated immune responses in chronic immune stimulation or during immunotherapy (Aalberse et al., 1993; Shakib, 1986), among other conditions.

Based on all the above, over 20 years ago the International Union of Immunological Societies (IUIS) and the World Health Organization (WHO) promoted validation studies of those Ab clones specific for IgH subclasses, for a broad range of laboratory assays (Jefferis et al., 1992, 1985; Mestecky et al., 1996). Such studies showed that the affinity and performance of most Ab clones directed against unique IgH subclasses evaluated, varied among different groups of assays, probably because of differences in the Ig epitopes available depending on the format of the targeted (soluble *vs.* cellular) Ig, and the way the sample was collected, studied and prepared (Jefferis et al., 1992, 1985; Mestecky et al., 1996). In fact, although these studies proposed candidate reagents for different applications, they also showed that it was not possible to select an ideal set of reference Ab for quantitation of IgH subclasses across all protocols and assays (Jefferis et al., 1992, 1985; Mestecky et al., 1996).

Despite the well-established clinical utility of assessing IgG and IgA subclasses in human serum and other body fluids, in addition to the isotypes of soluble Ig (Berth et al., 1999; Freeman et al., 2013; Grimbacher, 2014; Jefferis and Kumararatne, 1990; Ladomenou and Gaspar, 2016), no study has been reported so far in which the performance of Ab clones directed against different IgH subclasses has been evaluated by flow cytometry, for the immunophenotypic dissection of switched memory B-cells and plasma cells (PCs). In fact, lack of reliable reagents for quantification of human memory B-cells and PCs expressing distinct IgH subclasses has hampered detailed *ex vivo* investigation of their functional capacities. Therefore, alternative functional assays have been developed to dissect B-cells according to the IgH subclasses they produce (Berkowska et al., 2011; Jackson et al., 2014; Jahnmatz et al., 2016; Martin et al., 2015). However, these methods require either *in vitro* stimulation and differentiation to generate Ab-secreting cells (*e.g.* ELISPot) (Jahnmatz et al., 2016; Rösel et al., 2015), or molecular sequencing of the IgH repertoire (Berkowska et al., 2011; Jackson et al., 2014; Martin et al., 2015). Therefore, this has translated into limited clinical application of IgH subclass detection, mostly because the assays that are available are time-consuming (*e.g.* require several days of culture) and/or they require extensive sample manipulation, before IgH subclass assessment.

A limited number of studies have previously shown that flow cytometry can contribute to an improve dissection of B-cells according to their IgH subclasses, in cultured B-cells and purified mononuclear cells (Avery et al., 2008; He et al., 2007; Lighaam et al., 2014; Wirths and Lanzavecchia, 2005). However, these studies did not prove to be routinely applicable to whole peripheral blood (PB) samples, and no information has been provided about the different staining patterns that might exist among B-cells at different maturation stages, B-cells derived from different human tissues, and Igs that have different cellular localization -*e.g.* surface membrane (Sm) *vs.* cytoplasmic (Cy) Igs-.

Here, we summarize the results of a multicenter evaluation performed by the EuroFlow consortium aimed at selecting optimal Ab clones for detection of IgG and IgA subclasses on human PB memory B-lymphocytes and PCs by flow cytometry. In addition, those variables that most frequently had an impact on IgH subclass staining profiles are also discussed, and recommendations for their assessment in daily routine are provided.

2. Material and methods

2.1. Samples

Overall, 39 EDTA-anti-coagulated PB samples from healthy volunteers (18 females, 21 males; mean age of 53 ± 29 years) were collected and used to evaluate the reactivity of the Ab clones (≥ 3 donors per Ab clone), for titration experiments and to assess reagent specificity and cross-reactivity. The subset of well-performing clones selected was further validated in PB samples from 19 healthy donors (9 females and 10 males; mean age of 42 ± 17 years) at four different laboratories (University of Salamanca, Salamanca, Spain; La Paz Hospital, Madrid, Spain; Erasmus MC, Rotterdam, The Netherlands; and Charles University, Prague, Czech Republic). In addition, these later Ab clones were also tested in B-cells from tissues other than PB such as tonsils (n = 3) and bone marrow (BM) (n = 12) samples (with paired PB in 7 of these samples). For this purpose, 3 normal/reactive tonsils (extracted due to infectious tonsillitis and sleeping apnea from a 5-year boy, and two 24 and 37 year-old adults, respectively), and 12 normal BM samples (mean age of 53 ± 29 years) were analyzed. All samples were collected after informed consent was given by the donor or his/her legal representative, according to the recommendations of the local ethics committees and the Declaration of Helsinki.

2.2. Selection and evaluation of IgG and IgA subclass-specific Ab clones

Twenty-eight commercially available Ab clones, that had been previously validated for IgG (IgG_1, IgG_2, IgG_3 and IgG_4) and IgA (IgA_1 and IgA_2) heavy chain subclass detection in different laboratory assays –*e.g.*: direct haemagglutination, inhibition of haemagglutination and enzyme-linked immunoabsorbent assays (ELISA), immunocytochemistry, immunohistochemistry immunoblotting, immunoprecipitation and latex agglutination assays (Jefferis et al., 1992, 1985; Mestecky et al., 1996)-, were evaluated in this study (Table 1). Although the validation was carried out mostly using Ab clones conjugated with fluorochromes, four unconjugated Ab were also evaluated, two of which had previously been shown to be reactive with a subset of B-cells (Wirths and Lanzavecchia, 2005).

All PB and BM samples were stained within < 24 h after they were obtained, using the EuroFlow bulk lysis standard operating procedure (SOP) (Flores-Montero et al., 2017a, b; Kalina et al., 2012) (for more detailed protocol steps, please see www.EuroFlow.org). Briefly, 2 mL of PB or BM was mixed with 50 mL of an ammonium chloride solution (NH_4CL) and incubated for 15 min at room temperature (RT) to lyse non-nucleated red cells. Then, nucleated cells were washed in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.09% sodium azide (NaN_3); for Sm staining only, washed cells were subsequently stained with the different Ab for 30 min at RT in the darkness. Afterward, 2 mL of the FACS lysing solution –Becton/Dickinson Biosciences (BD), San Jose, CA- was added to the cell pellet, mixed, and another incubation was performed for 10 min (RT). Identification of Cy Ig was performed according to the EuroFlow SOP for simultaneous staining of cell Sm and Cy markers (Kalina et al., 2012) using the Fix&Perm reagent kit (An der Grub, Vienna, Austria) (for more detailed protocol information see www.EuroFlow.org). Intracellular specificity of CyIgH stainings was validated in PB samples by blocking SmIgH with saturating concentrations of the same Ab clones (unconjugated), as previously described (Shingadia et al., 2001) (Supplementary Table S1). In turn, fresh tonsil samples were first placed in PBS containing 10% fetal bovine serum and dissociated into a single

Table 1
Antibody clones specific for different IgG and IgA subclasses evaluated.

Ig Subclass	Clone	Company	Epitope location	FCM B-cell reactivity profile	Cross-reactivity on other Ig subclass $^+$ B-cells
IgG_1	SAG1	Cytognos	CH2	IgG^+ MBC & PC	Negative
	HP6188	Sanquin	Fc	IgG^+ MBC & PC	NT
	4E3	Southern Biotech	Hinge	No staining	
	HP6091	Cytognos	CH2	No staining	
	HP6027	Cytognos	G1m(f) allotypic specific	No staining	
	HP6001	Southern Biotech	CH2	IgG^+ MBC & PC	Negative
	HP6186	Nordic-MUbio	Fc	IgG^+ MBC & PC	Negative
IgG_2	SAG2	Cytognos	CH2	IgG^+ MBC & PC	Negative
	HP6014	Southern Biotech	CH1/Hinge*	IgG^+ MBC & PC	Negative
	HP6002	Southern Biotech	CH2	IgG^+ MBC & PC	Negative
	HP6207	Nordic-MUbio	Hinge	IgG^+ MBC	NT
IgG_3	SAG3	Cytognos	Hinge	IgG^+ MBC & PC	Negative
	HP6095	Sanquin	Hinge	IgG^+ MBC & PC	NT
	HP6050	Southern Biotech	Hinge	IgG^+ MBC & PC	Negative
IgG_4	SAG4	Cytognos	CH2	IgG^+ MBC & PC	Negative
	HP6025	Southern Biotech	CH3	No staining	
	HP6023	Southern Biotech	CH3	No staining	
	HP6011	Cytognos	CH3	No staining	
	HP6206	Nordic-MUbio	CH3	No staining	
	HP6098	Sanquin	CH2	No staining	
IgA_1	SAA1	Cytognos	Fc	IgA^+ MBC & PC	Negative
	H69-11.4	Nordic-MUbio	CH2	IgA^+ MBC & PC	NT
	B3506B4	Southern Biotech	Fc	IgA^+ MBC & PC	Negative
IgA_2	SAA2	Cytognos	Fc	IgA^+ MBC & PC	Negative
	2E2	Cytognos	Hinge	IgA^+ MBC & PC	NT
	H194-3.1	Nordic-MUbio	IgA2 (m)2 allotypic specific	No staining	
	512-H5.1	Nordic-MUbio	CH2	IgA^+ MBC	NT
	A9604D2	Southern Biotech	Fc	IgA^+ MBC & PC	Negative

Antibodies provided or purchased from Cytognos SL (Salamanca, Spain), Sanquin (Amsterdam, NL), Southern Biotech (Birmingham, AL), Nordic-MUbio (Susteren, NL). FCM: flow cytometry; CH: constant Ig heavy chain domain; Fc: fragment crystallizable obtained by papain digestion of human Ig heavy chains that contains residual hinge, CH2 and CH3 Ig heavy chain regions; MBC: memory B-cells; PC: plasma cells. NT: Not tested. *HP6014 Ab clone epitope has been located in the neighborhood of the hinge/CH1 junction, close to the Thr^{214} residue (Harada et al., 1991).

cell suspension using conventional mechanical disaggregation procedures (Domingues et al., 2012), prior to staining with the Ab reagents; subsequently, single cell suspensions of the tonsil samples were stained using the EuroFlow SOPs, as described above. SmIgG and SmIgA subclass expression was evaluated in tonsils (n = 3) and PB (n = 19), while Cy expression of Ig subclasses was evaluated in BM (n = 12) and PB (n = 12) samples, including 7 paired (BM and PB) specimens.

Initially, the staining profile for the IgG and IgA subclass Ab clones was evaluated in combination with other monoclonal antibody (MoAb) reagents used for simultaneous identification of the major maturation-associated B-cell subsets, as defined by their previously reported immunophenotypic profiles (Perez-Andres et al., 2010). This included the following B cell-associated reagents: CD19 (J3-119)-phycoerythrin cyanine7 (PECy7) (Beckman/Coulter, Brea, CA), CD27 (M-T271)-brilliant violet (BV)421 (BD), CD38 (HB7)-allophycocyanine-hilite 7 (APC-H7) (BD), anti-IgM (MHM-88)-BV510 (Biolegend, San Diego, CA), anti-IgD (IA6-2)-peridinin chlorphyll protein (PerCP)/Cy5.5 or fluorescein isothiocyanate (FITC) (Biolegend) and anti-IgG (G18-145)-PE or FITC (BD) or anti-IgA (IS11-8E10)-PE or FITC (Miltenyi, Bergisch Gladbach, Germany). B-cells were classified into the following maturation-associated subsets based on the staining pattern for CD19, CD27, CD38, IgM and IgD (Fig. 1): immature/transitional B-cells ($CD19^+$, $CD27^-$, $CD38^{++}$, IgM^{++} D^+), naïve B-cells ($CD19^+$, $CD27^-$, $CD38^{lo}$ and IgM^+D^{++}), non-switched memory B-cells ($CD19^+$, $CD27^+$, $CD38^{lo}$ and $IgM^{++}D^+$), switched memory B-cells ($CD19^+$, $CD27^{+/-}$, $CD38^{lo}$ and IgM^-D^-), non-switched PB PCs ($CD19^{+\,low}$, SSC^{hi}, $CD27^+$, $CD38^{hi}$ and IgM^+) and switched PB PCs ($CD19^{+\,low}$, SSC^{hi}, $CD27^+$, $CD38^{hi}$ and IgM^-), and BM PC ($CD19^{+\,low/-}$, SSC^{hi}, $CD27^+$ and $CD38^{hi}$), as previously reported (Perez-Andres et al., 2010). Each IgA and IgG subclass Ab reagent was conjugated with PE, FITC, APC and PerCP/Cy5.5, except for the HP6188, HP6091, HP6027 and HP6095 Ab clones which were evaluated in an unconjugated format (*e.g.* purified Ab reagents). Thus, for these four later Ab clones an additional staining step with a rabbit anti-mouse immunoglobulin-FITC reagent (DAKO, Agilent technologies, Santa Clara, CA) was required as secondary Ab, which was performed *via* a standard indirect immunofluorescence technique. For cross-reactivity studies, those Ab clones specific for the distinct IgG and IgA subclasses selected in the initial screening phase of the study, were tested against each other in combination with the MoAb described above for identification of the major maturation-associated B-cell subsets. For the second phase of the study (multicenter validation study, cy staining and staining profiles in different tissues), the anti-IgG and IgA subclass Ab clones selected were conjugated with different fluorochromes and combined in a single tube with MoAb directed against CD19, CD27, CD38, smIgM, smIgD. In both phases of the study, a minimum of 5×10^6 leukocytes were measured per staining using FACSCanto II (BD) or LSR Fortessa X-20 (BD) flow cytometers, and the FACSDiva software (BD). Instrument set-up and data acquisition were performed according to the EuroFlow SOP for instrument set-up and fluorescence compensation, using the 8-peak Rainbow bead calibration particles (Spherotech, Lake Forest, IL) and either stained PB samples or CompBeads (BD), as previously described in detail (Kalina et al., 2012). For data analysis the Infinicyt software was used (Cytognos SL., Salamanca, Spain).

2.3. Statistical analyses

Stain index (SI) was calculated for each reagent and used for the comparison between the distinct Ab clones conjugated with the same fluorochrome reagent (in separate tubes), as well as for the different tissues evaluated and the distinct protocols tested, as previously described (Maecker et al., 2004); naïve B-cells were used as internal negative reference population. For continuous variables, mean values and their standard deviation (SD), were calculated. In order to assess the statistical significance of differences observed between groups, the Mann Whitney U test was used. For all statistical analyses, the SPSS

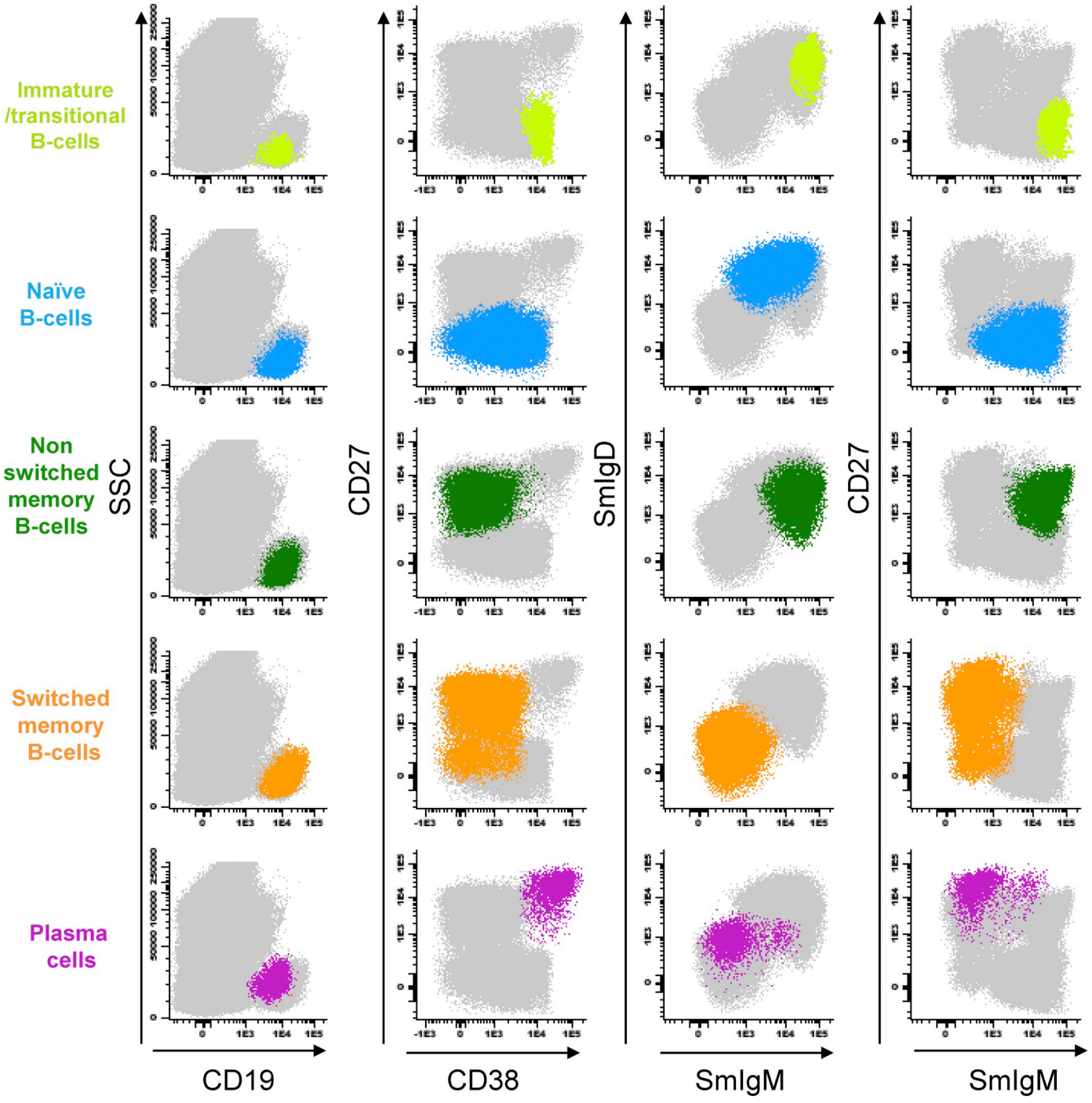

Fig. 1. Gating strategy used for the identification of maturation-associated B-cell subsets in a representative normal PB sample. Illustrating bivariate dot-plot graphical representations of the gating strategy used to identify immature, naïve, switched and non-switched memory B-cells and plasma cells, according to their expression profiles for CD19, CD27, CD38, SmIgD and SmIgM, as well as side-scatter (SSC).

software program (SPSS 18.0, IBM SPSS Statistics, IBM, Armonk, NY) was used. Statistical significance was set at p-values ≤ 0.05.

3. Results

3.1. Screening for optimal anti-IgA and anti-IgG MoAb reagents

A total of 28 Ab clones against the IgG_{1-4} and IgA_{1-2} subclasses were initially tested, such reagents including Ab against different epitopes of the same IgH subclass (Table 1 and Fig. 2). From the 28 anti-IgG and anti-IgA subclass Ab clones evaluated, 17 (61%) showed simultaneous staining for SmIg on subsets of both memory B-cells and PCs, while two additional reagents only stained memory B-cells (anti-IgG_2 HP6207 and anti-IgA_2 512-H5.1). The former 17 reagents included 4 Ab clones for IgG_1 (SAG1, HP6188 HP6186 and HP6001), 3 for IgG_2 (SAG2, HP6014 and HP6002), another 3 for IgG_3 (SAG3, HP6095 and HP6050), one for IgG_4 (SAG4), 3 for IgA_1 (SAA1, H69-11.4 and B3506B4) and 3 for IgA_2 (SAA2, 2E2 and A9604D2). No positive B-cells were observed for the other 9 Ab clones evaluated.

Among those reagents that were positive on B-cells and PCs, Ab clones conjugated with the same fluorochrome were compared for their SI (Table 2). No significantly different fluorescence profiles were

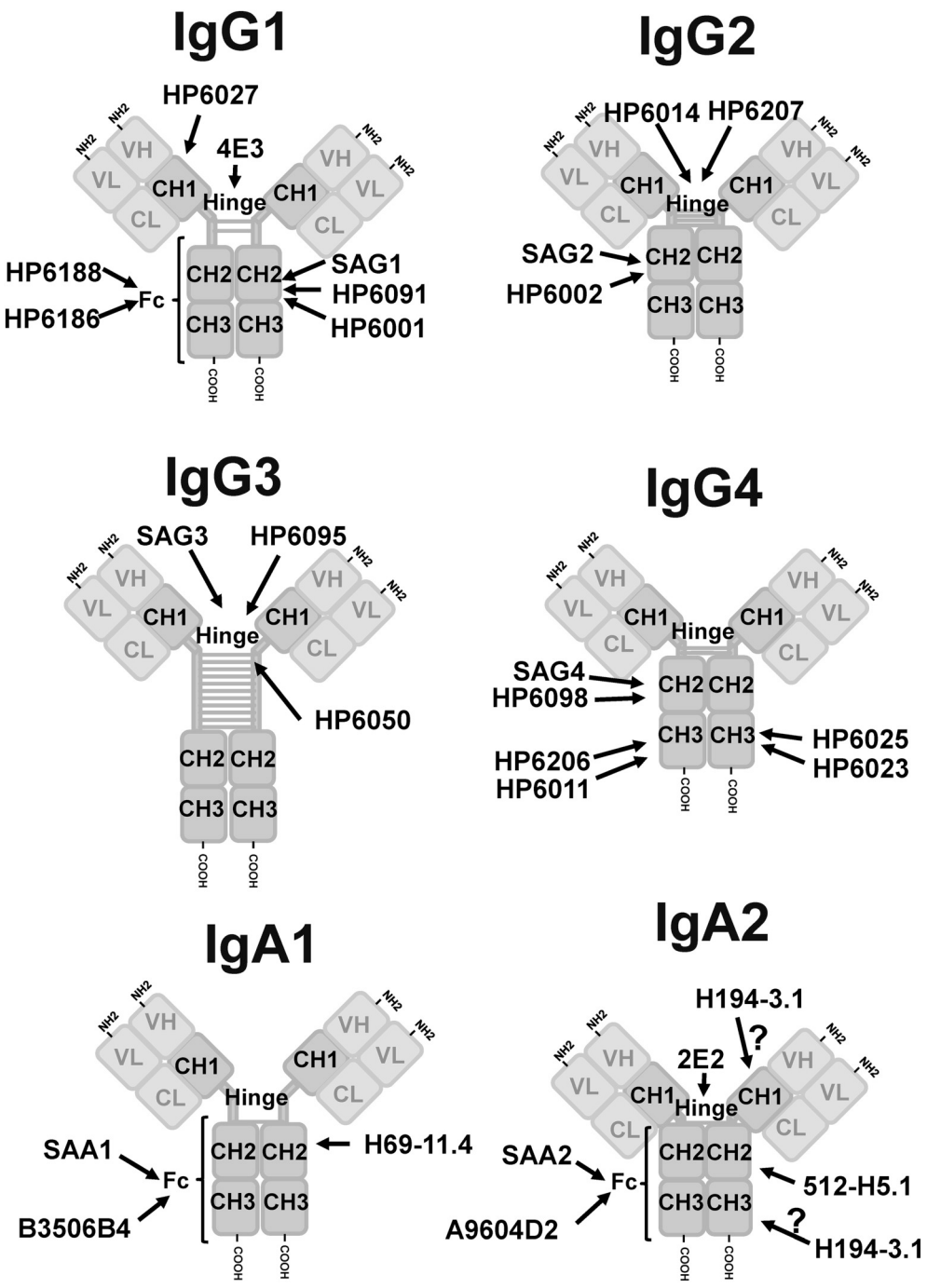

Fig. 2. Epitope location on the IgG_{1-4} and IgA_{1-2} subclasses for the antibody clones evaluated. Immunoglobulin domains targeted by the IgG and IgA subclass antibody clones tested (Hamilton and Morrison, 1993; Harada et al., 1991; Jefferis et al., 1992, 1985; Lighaam et al., 2014; Mestecky et al., 1996; Reimer et al., 1989). HP6027 and H194-3.1 are G1m(3) and A2m(2) allotype specific, their corresponding epitopes being located in the CH1 and CH1 or CH3 regions, respectively.

observed for the two Ab clones directed against the IgG_3 subclasses conjugated with the same fluorochrome (Table 2); in contrast, the anti-IgG_2 SAG2, the anti-IgA_1 SAA1 and B3506B4 and the anti-IgA_2 SAA2 subclass clones showed significantly higher ($p \leq 0.05$) SI in switched memory B-cells *vs.* HP6207 (20 ± 5 *vs.* 5 ± 0.5), H69–11.4 (11 ± 6 and 11 ± 4 *vs.* 5 ± 1, respectively) and 2E2 (18 ± 2 *vs.* 4 ± 0.4) Ab clones, respectively (Table 2). No statistically significant differences were found between the other Ab clones tested in terms of their fluorescence intensity profile (*i.e.* SI). Of note, although a large number of anti-IgG_4 clones was evaluated ($n = 6$ clones), the SAG4 Ab clone was the only one that showed reactivity on B-cells, despite individuals with high IgG_4 serum levels were specifically selected to test the anti-IgG4 Ab reagents. Of note, two allotype-specific Ab clones were evaluated -HP6027 (G1m(f) allotype), and H194 3.1 (A2m(2) allotype)- (Fig. 2 and Table 1), but none of them showed reactivity on B-cells, the specific allotype of the donors evaluated ($n = 4$) being unknown.

All clones here evaluated had been previously demonstrated to be IgG and IgA subclass-specific, when techniques other than flow cytometry were used (Jefferis et al., 1992, 1985; Mestecky et al., 1996). Despite this, here we evaluated also the B-cell and PC staining profiles for the IgG_{1-4} and IgA_{1-2} subclass-specific Abs selected, against previously validated total IgM, IgD, IgG and IgA-specific reagents (Berkowska et al., 2011; Perez-Andres et al., 2010). As expected, we observed that staining for IgG_{1-4} subclass Abs was specifically detected on B-cells and PCs that tested positive for total IgG staining and lacked reactivity for IgA, IgM and IgD; similarly IgA_{1-2} staining was also restricted to $IgG^-/IgA^+/IgM^-/IgD^-$ B-cells and PCs (data not shown).

Table 2
Mean fluorescence intensity (MFI) and stain index obtained for the IgH subclass-specific antibody clones showing reactivity on human memory B-cells and plasma cells.

IgH isotype detected	Ab clone	Fluorochrome	MFI	Stain index
IgG_1	SAG1	PE	MBC: 10,616 ± 802	37 ± 5
			PC: 5083 ± 1553	18 ± 10
	HP6188	Secondary FITC staining used	MBC: 8443 ± 1560	14 ± 3
			PC: 4999 ± 32,777	8 ± 6
	HP6001	PE	MBC: 10,228 ± 812	34 ± 6
			PC: 5303 ± 640	15 ± 3
	HP6186	FITC	MBC: 4088 ± 235	18 ± 1
			PC: 2282 ± 454	9 ± 3
IgG_2	SAG2	PE	MBC: 10,050 ± 1893	65 ± 33
			PC: 8364 ± 444	28 ± 3
	SAG2	FITC	MBC: 4749 ± 1029	20 ± 5*
			PC: 2223 ± 448	9 ± 2
	HP6014	PE	MBC: 20,170 ± 3610	64 ± 40
			PC: 10,808 ± 3412	32 ± 31
	HP6002	PE	MBC: 18,664 ± 2232	58 ± 15
			PC: 1866 ± 200	7 ± 2
	HP6207	FITC	MBC: 846 ± 106	5 ± 0.5
			PC: ND	ND
IgG_3	SAG3	FITC	MBC: 3617 ± 644	15 ± 4
			PC: 1972 ± 644	7 ± 6
	HP6095	Secondary FITC staining used	MBC: 4159 ± 368	15 ± 2
			PC: 3332 ± 200	15 ± 0.4
	HP6050	FITC	MBC: 6102 ± 1801	12 ± 4
			PC: 2371 ± 1033	5 ± 4
IgG_4	SAG4	FITC	MBC: 5269 ± 395	19 ± 2
			PC: 2209 ± 278	8 ± 1
	SAG4	APC	MBC: 16,387 ± 2968	38 ± 18
			PC: 2179 ± 598	8 ± 4
IgA_1	SAA1	FITC	MBC: 5187 ± 1204	11 ± 6*
			PC: 3963 ± 1431	9 ± 6
	SAA1	PerCP-Cy5.5	MBC: 19,078 ± 1876	36 ± 4
			PC: 9450 ± 1913	18 ± 4
	SAA1	APC	MBC: 46,939 ± 14,178	45 ± 22
			PC: 24,939 ± 6879	25 ± 16
	H69–11.4	FITC	MBC: 784 ± 134	5 ± 1
			PC: 685 ± 15	4 ± 1
	B3506B4	FITC	MBC: 5896 ± 809	11 ± 4*
			PC: 4605 ± 1081	8 ± 2
IgA_2	SAA2	PE	MBC: 11,592 ± 3256	36 ± 11
			PC: 6675 ± 1086	21 ± 6
	SAA2	PerCP-Cy5.5	MBC: 11,992 ± 81	18 ± 2*
			PC: 9754 ± 6671	8 ± 2
	2E2	PerCP-Cy5.5	MBC: 3012 ± 1400	4 ± 0.4
			PC: 1259 ± 297	2 ± 1
	512-H5.1	FITC	MBC: 704 ± 213	5 ± 0.5
			PC: ND	ND
	A9604D2	PE	MBC: 6504 ± 2555	27 ± 21
			PC: 3916 ± 833	15 ± 11

MFI and stain index are expressed as mean ± one standard deviation. Secondary FITC staining: Anti-IgH subclass clone not available in a fluorochrome conjugated format and a secondary staining step was performed with an anti-mouse immunoglobulins/FITC antibody. MFI and SI information was obtained from the staining of IgH subclasses individually. *$p < 0.05$ *vs.* other reagents directed against the same IgH subclass and conjugated with the same fluorochrome. MBC: memory B-cells; PC: plasma cells. ND: not detected.

3.2. Validation of the selected anti-IgG and anti-IgA subclass MoAb clones on PB B-cells and PCs

Identical reactivity was observed in the preliminary testing for several pairs of clones, as shown in Table 2. In such case, only one of the clones was selected based on the availability of reagents directly conjugated with compatible fluorochromes, for further validation in PB samples from 19 healthy donors at four different laboratories. The selected reagents included Abs for: IgG_1 (SAG1), IgG_2 (SAG2), IgG_3 (SAG3), IgG_4 (SAG4), IgA_1 (SAA1) and IgA_2 (SAA2). Alternative clones that are equivalent to the selected 6 Ig subclass Ab clones were also validated in a subset of the samples, and these results are summarized in Supplementary Table S2.

Subsequently, the potential existence of cross-reactivity among the selected clones for each IgG and IgA subclass (SAG1, SAG2, SAG3, SAG4, SAA1 and SAA2) was evaluated; as a result of these experiments, we confirmed that the staining for all six reagents was mutually exclusive as illustrated in Supplementary Fig. S1. In addition, when high-sensitivity techniques were used, IgH switched memory B-cells and PCs staining positive for the 6 Ab, were systematically detected in all donors tested ($n = 19$), except for $SmIgG_4$. These included $SmIgG_1^+$ (44 ± 12% and 16 ± 15% of all IgH switched memory B-cells and PCs, respectively), $SmIgG_2^+$ (15 ± 6% and 12 ± 6%), $SmIgG_3^+$ (7 ± 2% and 2 ± 3%), $smIgA_1^+$ (22 ± 7% and 39 ± 12%) and $smIgA_2^+$ (8 ± 4% and 18 ± 10%) memory B-cells and PCs, respectively; meanwhile, $SmIgG_4^+$ memory B-cells and PCs were only detected in a subgroup of subjects analyzed: (13/19 and 4/19 healthy donors, respectively) in whom they represented 2 ± 1% and 0.3 ± 0.5% of all IgH-switched memory B-cells and PCs, respectively (Fig. 3). Of note, a significant percentage of PB PCs from each sample evaluated was negative for SmIgH (15 ± 9%), while the percentage of SmIgH-negative memory B-cells was systematically < 2% of memory B-cells.

3.3. Validation of the selected anti-IgG and anti-IgA subclass Ab clones on BM and tonsil B-cells and PCs

Once validated in PB, the above selected Ab clones were also tested on B-cells and PCs from tonsils and BM (7 paired with PB) samples (Supplementary Fig. S2). Once again, strong staining was also found for each of the 6 reagents, with slight differences as regards their distribution in paired BM *vs.* PB. Thus, a higher percentage of IgG_3^+ PCs together with a lower proportion of IgA_2^+ PCs were detected in paired BM *vs.* PB: 5 ± 1% *vs.* 2 ± 2% and 6 ± 5% *vs.* 17 ± 4% ($p = 0.01$ and $p = 0.002$, respectively) (Table 3). Due to the limited number of tonsils analyzed ($n = 3$) no definitive conclusions could be made on the distribution of the distinct Ig subclass subsets of switched memory B-cells and PCs in tonsils *vs.* PB and BM (Table 3).

3.4. Validation of the selected anti-IgG and anti-IgA subclass Ab clones for the staining of intracellular Ig heavy chains

PB memory B-cells showed undetectable $CyIgG_1$ staining (Table 4), and a significantly weaker SI for cytoplasmic *vs.* surface membrane IgG_2 and IgG_3 staining (mean SI of 3 ± 2 and 5 ± 2 *vs.* 14 ± 9 and 14 ± 9, respectively; $p < 0.001$, and $p = 0.002$, respectively). Similarly, switched-memory B-cells did not show $CyIgG_1$ staining in any BM sample evaluated, and $CyIgG_2^+$ and $CyIgG_4^+$ switched-memory B-cells were detectable in only seven and four of the 12 donors tested, respectively. In contrast, no significant differences were observed for intracellular *vs.* surface membrane IgA_1, IgA_2 or IgG4 fluorescence levels in PB memory B-cells, despite for IgA1 a trend toward higher SI in Sm *vs.* Cy was observed (30 ± 17 *vs.* 18 ± 9; $p = 0.07$) (Table 4).

In contrast to memory B-cells, PB PCs showed higher SI when IgA_1, IgA_2, IgG_1 IgG_2, IgG_3 and IgG_4 subclasses were evaluated at the cytoplasmic *vs.* surface membrane levels: mean SI of 159 ± 66, 38 ± 19, 21 ± 9, 49 ± 41, 320 ± 133 and 64 ± 46 *vs.* 17 ± 8, 4 ± 2, 8 ± 3, 5 ± 4, 9 ± 8 and 8 ± 4, respectively ($p < 0.001$ for IgA_{1-2} and IgG_{1-3}, and $p = 0.04$ for IgG_4, respectively) (Fig. 4 and Table 4). SmIgH blocking experiments were run in parallel and confirmed CyIgH staining to be specific of intracellular IgH (Supplementary Table S1 and Supplementary Fig. S3). In contrast, no statistically significant differences were found between $CyIgG_{1-4}$ and $CyIgA_{1-2}$ staining in BM *vs.* PB PCs from paired samples (Fig. 4 and Table 4).

Of note, despite the percentage of IgH-negative PCs was lower when Cy *vs.* Sm staining was used (2 ± 2% *vs.* 13 ± 8%; $p = 0.001$) (Table 3), no significant differences were observed in the relative distribution of PCs expressing distinct IgG_{1-4} and IgA_{1-2} subclasses in PB,

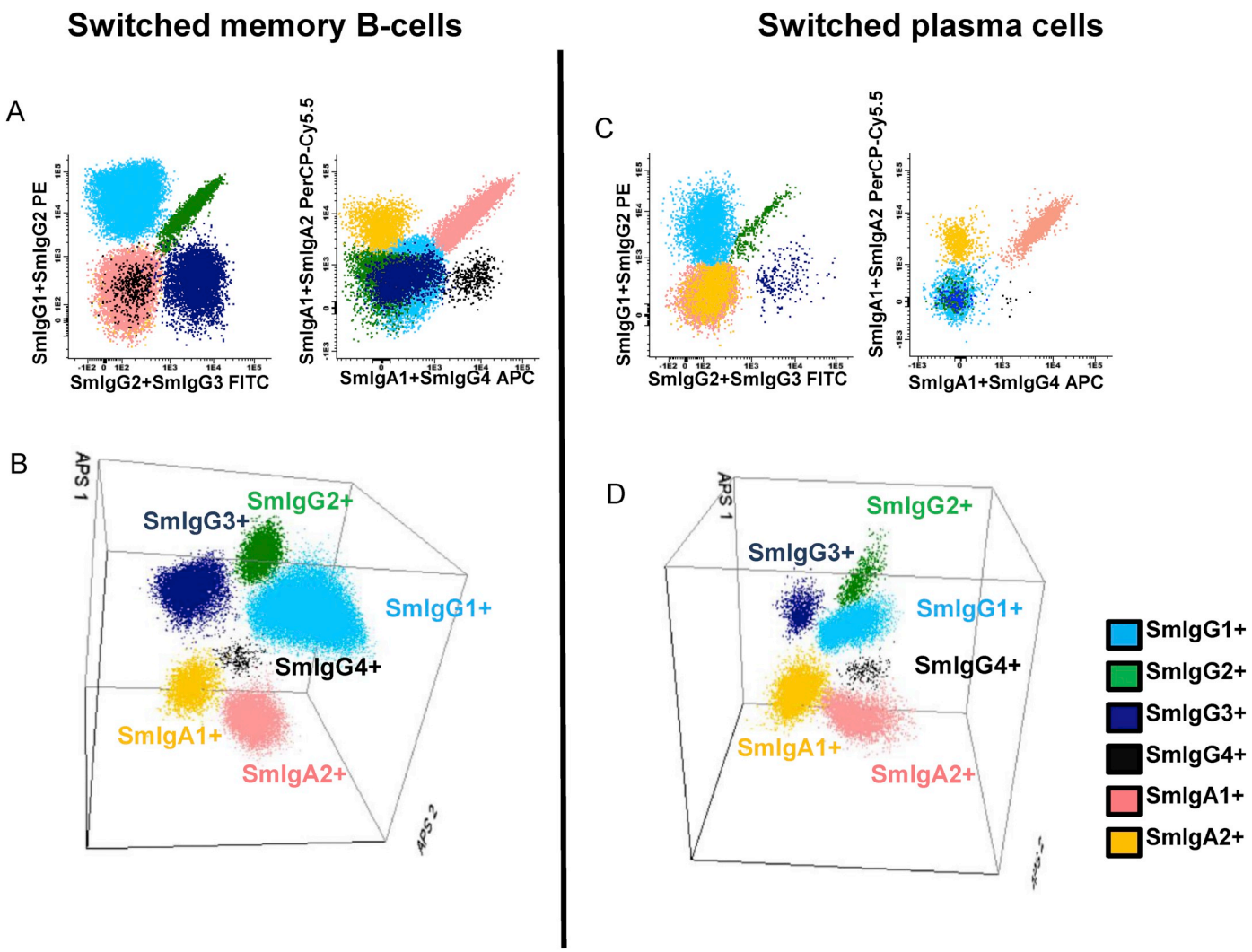

Fig. 3. Simultaneous identification of the different subsets of IgH switched memory B-cells and plasma cells expressing distinct IgA and IgG subclasses. Panels A and C show illustrating bivariate dot-plot graphical representations of the subsets of switched memory B-cells (A) and plasma cells (C) expressing different Ig subclasses after staining with the selected anti-IgG1 (light blue -SAG1-), IgG2 (green -SAG2-), IgG3 (purple -SAG3-), IgG4 (dark -SAG4-), IgA1 (light red -SAA1-) and IgA2 (yellow – SAA2-), antibody clones. Panels B and D: Illustrating examples of 3-dimensional Automated Population Separator (APS) -Principal Component 1 (PC1) *vs.* PC2 and PC3- graphical representations of the distinct subsets of switched memory B-cells (B) and plasma cells (D) expressing different IgG and IgA subclasses (identical color code as used in panels A and C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

when Cy *vs.* Sm-only IgH staining protocols were used.

3.5. Evaluation of anti-IgA and IgG staining on other leucocyte subsets

Ig subclass-specific Ab clones also showed positivity in populations of PB leukocytes other than B-cells and PCs. Thus, the IgG_1 and IgG_3 MoAb clones constantly showed bright reactivity on PB monocytes and neutrophils, while IgA_1, IgA_2 and IgG_4 were detected at lower levels in these two PB subsets of myeloid cells in only a subset of the samples tested (data not shown). In contrast, we did not find any evidence of IgG_2 Ab clone binding to PB monocytes and neutrophils in any of the samples. Interestingly, some of the anti-IgH subclass Ab clones which were tested in the first phase of the study, and not further evaluated because they did not react with B-cells and PCs (*e.g.* the anti-IgG_1 HP6091, HP6027 and 4E3 Ab clones), systematically showed positivity on PB neutrophils and/or monocytes (data not shown).

4. Discussion

More than two decades ago, both the IUIS and WHO promoted large multicenter studies to test and validate Ab clones directed against the IgG_{1-4} and IgA_{1-2} subclasses, due to their clinical utility (Jefferis et al., 1992, 1985; Mestecky et al., 1996). As a consequence, a relatively large number of Ab reagents have been produced and characterized which are specific for the distinct human IgG_{1-4} and IgA_{1-2} subclasses. These Abs have been validated for a broad range of assays such as direct haemagglutination, inhibition of haemagglutination and enzyme-linked immunoabsorbent assays (ELISA), immunocytochemistry, immunohistochemistry immunoblotting, immunoprecipitation and latex agglutination assays (Jefferis et al., 1992, 1985; Mestecky et al., 1996), but not for flow cytometry. Here, we tested and validated the specificity and staining profile of Ab clones specific for the distinct IgG_{1-4} and IgA_{1-2} subclasses on human B-cells and PCs from different tissues, by flow cytometry.

Although IgG_{1-4} and IgA_{1-2} subclasses share a high degree of homology in their sequences, structural differences exist among them that have an impact on their biological abilities and functions (Fig. 2). As a consequence of such structural differences, IgG_{1-4} and IgA_{1-2} subclass molecules also contain different epitopes that might react differently with a variety of anti-IgG and anti-IgA Ab clones. Similarly to other Ig isotypes, IgG_{1-4} and IgA_{1-2} subclasses also consist of two identical heavy (H) chains containing an N-terminal variable domain

Table 3
Distribution of IgG and IgA subclasses on switched memory B-cells and plasma cells from peripheral blood, bone marrow and tonsils.

		Tissue (staining)				
	IgH isotype	PB[¥] (Sm) *n* = 12	PB[¥] (Cy) n = 12	PB[§] (Cy) *n* = 7	BM[§] (Cy) n = 7	Tonsil (Sm) n = 3
IgH switched Memory B-cell	IgG_1	44 ± 12%	0 ± 0%	0 ± 0%	0 ± 0%	35 ± 13%
	IgG_2	15 ± 6%	11 ± 7%	10 ± 7%	7 ± 7%	5 ± 3%
	IgG_3	7 ± 2%	5 ± 3%	4 ± 3%	7 ± 4%	6 ± 3%
	IgG_4	2 ± 1%	1 ± 1%	1 ± 2%	1 ± 1%	NT
	IgA_1	22 ± 7%	19 ± 10%	18 ± 13%	21 ± 7%	39 ± 12%
	IgA_2	8 ± 4%	7 ± 5%	8 ± 6%	8 ± 4%	11 ± 2%
	IgH^-	2 ± 2%	57 ± 18%	59 ± 25%	57 ± 14%	NT
IgH switched Plasma cells	IgG_1	16 ± 15%	22 ± 9%	21 ± 12%	30 ± 11%	9 ± 4%
	IgG_2	12 ± 6%	17 ± 12%	17 ± 10%	19 ± 7%	9 ± 8%
	IgG_3	2 ± 3%	2 ± 2%	2 ± 2%	5 ± 1%[#]	10 ± 5%
	IgG_4	0.3 ± 0.5%	1 ± 2%	1 ± 2%	2 ± 1%	NT
	IgA_1	39 ± 12%	33 ± 14%	36 ± 13%	34 ± 9%	17 ± 4%
	IgA_2	18 ± 10%	17 ± 4%	17 ± 4%	6 ± 5%[#]	8 ± 11%
	IgH^-	13 ± 8%	2 ± 2%*	2 ± 3%	5 ± 3%	NT

Results expressed as percentage (mean ± one standard deviation) of positive cells from all switched memory B-cells or plasma cells. *$p < 0.05$ CyIgH *vs.* SmIgH staining, [#]$p < 0.05$ CyIgH BM PC *vs.* PB PC. Sm: surface membrane staining; Cy: intracellular cytoplasmatic staining; $SmIgH^-$: surface membrane staining negative; NT: not tested; PB: peripheral blood; BM: bone marrow. [¥]Paired PB samples. [§]BM with paired PB samples.

(VH), responsible for antigen-specificity, followed by three constant domains (CH1, CH2, CH3) responsible for the Ig effector functions (Fig. 2) (Schroeder and Cavacini, 2010; Vidarsson et al., 2014). The four IgG subclasses share > 90% amino acid sequence homology, most variations being localized in the hinge region and the amino acid residues of the CH2 domain that are most proximal to the hinge; this is where the binding sites for both C1q (complement 1q protein) and the IgG-fragment crystalline receptors (FcγR) responsible for the Ig effector functions of other immune effector cells (*e.g.* phagocytosis, Ab-dependent cell-mediated cytotoxicity, and release of inflammatory mediators and superoxide radicals), are mapped (Hamilton, 1987; Schroeder and Cavacini, 2010; Vidarsson et al., 2014). In turn, IgG_3 and IgA_1 show elongated hinge domains that have an impact on the orientation of the V domains, and confer a higher susceptibility to proteases, compared to other Ig subclasses of the same isotype (Schroeder and Cavacini, 2010; Vidarsson et al., 2014). In line with this, most epitopes targeted by the Ab clones that showed strong reactivity on human B-cells and PCs in our study, had been previously mapped in the CH2 and the hinge domains (Hamilton and Morrison, 1993; Harada et al., 1991; Jefferis et al., 1992, 1985; Lighaam et al., 2014; Mestecky et al., 1996; Reimer et al., 1989, 1984). In contrast, all Ab clones targeting the CH3 Igγ-heavy chain region, such as the HP6011, HP6023, HP6025 and HP6206 Ab clones (Hamilton and Morrison, 1993; Jefferis et al., 1992, 1985; Lighaam et al., 2014) systematically failed to show a specific, reproducible and robust staining profile on human B-cells and PCs. Although the specific domain in the Fc region targeted by six of the Ab clones evaluated still remains elusive, higher sequence homology has been reported for the CH3 domain among distinct Ig subclasses of the same isotype, including the highly-preserved N-linked glycosylation site located in the interface between the CH2 and CH3 heavy chain regions, responsible for changes in the quaternary structure of the Fc region (Vidarsson et al., 2014). Together with our results, this suggests that the hinge and CH2 domains are the most efficient domains to be targeted in producing anti-IgH subclass specific MoAb that react against IgH subclass molecules expressed on human B-cells and PCs.

At present, it is well-established that the staining profile of a given protein for different Ab clones that target the same region of that protein might vary because of several factors. Thus, such distinct Ab clones might target different epitopes due to post-transcriptional modifications (Lanza et al., 2001) and alternative splicing forms of the protein (Haralambidou et al., 1987), amino acid polymorphisms (Hodge et al., 1991) and the polymerization status of the targeted protein (Zapata et al., 1999). Among other factors, this might lead to variable patterns of expression of specific epitopes in different tissues,

Table 4
Stain index for IgG and IgA subclass antibody clones on memory B-cells and plasma cells from peripheral blood, bone marrow and tonsils.

		Tissue (staining)				
	IgH isotype	PB[¥](Sm) n = 12	PB[¥] (Cy) n = 12	PB[§] (Cy) n = 7	BM[§] (Cy) n = 7	Tonsil (Sm) n = 3
Memory B-cells	IgG_1	65 ± 43	0 ± 0	0 ± 0	0 ± 0	3 ± 1
	IgG_2	14 ± 9	3 ± 2*	4 ± 1	4 ± 2	9 ± 5
	IgG_3	14 ± 9	5 ± 2*	6 ± 2	6 ± 2	3 ± 2
	IgG_4	19 ± 8	11 ± 6	15 ± 4	16 ± 2	NT
	IgA_1	30 ± 17	18 ± 9	16 ± 10	16 ± 17	4 ± 2
	IgA_2	9 ± 7	6 ± 3	5 ± 3	5 ± 2	2 ± 0.2
Plasma cells	IgG_1	8 ± 3	21 ± 9*	21 ± 10	41 ± 19	2 ± 1
	IgG_2	5 ± 3	49 ± 41*	61 ± 46	79 ± 55	6 ± 0.4
	IgG_3	9 ± 8	320 ± 133*	270 ± 112	290 ± 136	4 ± 2
	IgG_4	8 ± 4	64 ± 46*	94 ± 15	63 ± 4	NT
	IgA_1	17 ± 8	159 ± 66*	117 ± 61	305 ± 138	2 ± 1
	IgA_2	4 ± 2	38 ± 19*	37 ± 17	62 ± 27	1 ± 0.04

Results expressed as mean stain index ± one standard deviation of positive cells from all switched memory B-cells and plasma cells. *$p < 0.05$ PB CyIgH *vs.* SmIgH staining; $p > 0.05$ for all BM *vs.* PB cytoplasmic staining. Sm: surface membrane staining; Cy: intracellular cytoplasmatic staining; NT: not tested; PB: peripheral blood; BM: bone marrow. [¥]Paired PB samples. [§]BM samples paired PB samples.

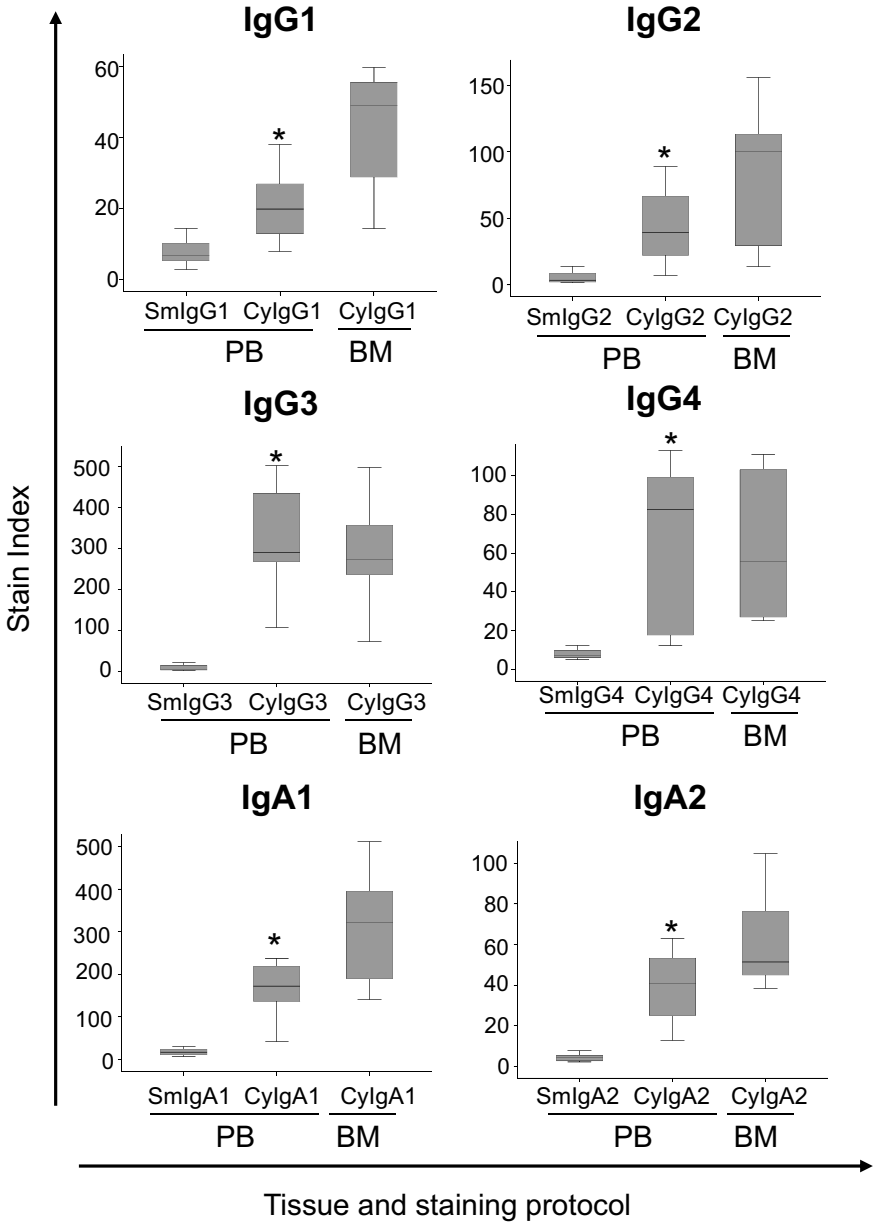

Fig. 4. Comparison between the cytoplasmic and surface Ig staining profile for Ig subclass antibodies on plasma cells from paired peripheral blood and bone marrow samples. Box plots represent stain index 25th and 75th percentile values for the different IgA and IgG subclasses; the line in the middle corresponds to median values and vertical lines represent the highest and lowest values that are neither outliers nor extreme values.*$p < 0.05$ *vs.* PB sm. Cy: cytoplasmic staining; Sm: surface membrane staining; PB: peripheral blood; BM: bone marrow.

cell lineages and maturation stages within a cell lineage (Haralambidou et al., 1987; Lanza et al., 2001). Also, epitopes (and therefore Ab binding to such epitopes) might be modified by technical issues such as the staining technique used (*e.g.* Sm *vs.* Cy staining), the localization of the protein (*e.g.* soluble, bound to a cell or immobilized to a solid phase substrate), the anticoagulant used and/or the affinity and avidity of the Ab clones for their epitopes (Leino and Sorvajärvi, 1992; Lighaam et al., 2014; Partridge et al., 1982; van Dongen et al., 1988). Consequently, comparative (multiparameter) direct evaluation of distinct clones targeting the same protein/marker is of utmost importance for selection of optimal reagents for a given assay (*e.g.* flow cytometry panel) and the standardization of their subsequent measurements. In fact, Ab clones targeting IgH subclasses have previously shown assay-restricted efficiency, including some of the clones selected in the present study for flow cytometry, (Jefferis et al., 1992, 1985; Lighaam et al., 2014; Mestecky et al., 1996; Partridge et al., 1982), which potentially reflects variations on the epitopes expressed of the targeted Ig under different assay conditions.

Here, we showed that 17/28 Ab clones, previously validated for serum IgG_{1-4} and IgA_{1-2} subclass quantitation, were also able to stain their corresponding SmIgG and IgA subclasses on human PB memory B-cells and PCs. In contrast, the other 11 Ab clones failed to detect IgG and IgA subclasses, except for the HP6207 and 512-H5.1 Ab clones that stained IgG_2 and IgA_2 memory B-cells, respectively, but failed to detect Ig^+ PCs. Whether or not those Ab clones that failed to stain B-cells and PCs were specific for soluble forms of their corresponding Ig because of being directed against Ig domains that are less accessible on smIgH (*e.g.* CH3), or they target epitopes that are dependent on specific serum Ig quantitation assay conditions (*e.g.* presented by antigens immobilized to a solid phase substrate), still remains to be elucidated.

Among those clones that tested positive on B-cells, SAG1, SAG2, SAG3, SAA1 and SAA2 were selected according to their specificity and SI for further studies. Despite this, other Ab clones might be considered as alternative reagents since they provided highly similar (and specific) staining profiles in memory B-cells and PCs, based on preliminary testing performed in a small subset of samples (Supplementary Table S2), whenever such identical reactivity is confirmed in more extensive studies. Of note, our selection of reagents might also favor some Ab clones *vs.* others that, *e.g.*, were not directly conjugated to a fluorochrome because comparisons based on SI are only reliable for

comparisons of fluorochrome-matched conjugated Ab reagents. Regarding IgG_4, only the SAG4 clone showed positivity on human B-cells and PCs while no IgG_4^+ cells were identified by the HP6098 clone (Supplementary Fig. S4). Of note, in contrast to our results, previous studies have reported positivity for the HP6098 anti-IgG4 Ab clone on FACS-sorted human B-cells (Lighaam et al., 2014).

Most importantly, our results showed that the 6 selected clones (SAG1, SAG2, SAG3, SAG4, SAA1 and SAA2) were highly specific for their corresponding IgH subclass (no cross-reactivity with other IgG or IgA subclasses), independently of the B-cell maturation stage, as they systematically stained specifically, both IgH switched memory B-cells and PCs. With the objective to evaluate all subclasses in one tube together with other maturation-associated B-cell markers, we used two Abs conjugated with two different fluorochromes each, an approach that has been previously validated by both the Euroflow consortium and others (Bellido et al., 1998; Tembhare et al., 2011; van Dongen et al., 2012). A possible limitation of the approach would be the potential existence of cross-reactivity among the markers stained simultaneously, but this possibility was ruled out by demonstrating that the 6 Ab clones selected against IgG_{1-4} and IgA_{1-2} were mutually exclusive. Thus, their combined use allowed for an efficient and detailed dissection of all IgG and IgA subclass subsets of memory B-cells and PCs in PB, BM and tonsils using either two 8-color or one 12 color-tube (van Dongen et al., 2016).

At present, it is well-known that not all Ab clones that bind to a given protein and epitope on the cell surface, also react and detect the same protein (and epitope) at the intracellular level (van Dongen et al., 1988). Here, we demonstrated that the selected IgH subclass reagents did detect both SmIgH and CyIgH on PCs, the later being associated with lower numbers of CyIgH-negative PCs in PB. These results support the notion that, as previously reported also for other BCR-associated molecules (Perez-Andres et al., 2010), membrane bound IgH levels are reduced on PCs, due to the fact that in PCs, newly generated IgH molecules mostly accumulate in the cytoplasm or they are secreted outside the cell (Perez-Andres et al., 2010). In contrast, memory B-cells showed strong Sm staining for the IgG and IgA subclass Abs, with either undetectable or low amounts of intracellular Ig. Altogether, these results indicate that CyIgH staining could be the preferred method for more precise identification of the distinct IgG and IgA subsets of PCs, while for memory B-cells Sm staining should be used.

A subgroup of the IgH subclass-specific Ab clones, mostly anti-IgG_1 and anti-IgG_3, also showed reactivity in PB on leucocyte populations other than B-cells and PCs. Such apparently "unspecific" staining profile appears to mimic the FcRγ expression patterns (Schroeder and Cavacini, 2010; Vidarsson et al., 2014), suggesting Ab binding to Ig-FcR complexes on the surface of both neutrophils and monocytes. Thus, it is well-known that both IgG_1 and IgG_3 bind to all FcγR, their avidity for FcγRI (CD64) and FcγRIII (CD16) being particularly high; CD64 and CD16 are both homogeneously expressed on monocytes and neutrophils, respectively, further supporting this hypothesis. In line with the lower ability of IgG_2, IgG_4, IgA_1 and IgA_2 to bind to the Fc on PB leucocytes other than B-cells, we observed no (low and/or variable) staining for the anti-IgG_2, anti-IgG_4, anti-IgA_1 and anti-IgA_2 Ab clones tested (Schroeder and Cavacini, 2010; Vidarsson et al., 2014). Interestingly, positivity on neutrophils and/or monocytes was also observed for some Ab clones which did not react with B-cells; since these clones have been previously reported to specifically detect Ig subclasses by other techniques (Gao et al., 2014; Jefferis et al., 1992, 1985) it can be hypothesized that they might target epitopes that only become available on secreted isoforms of the corresponding Ig subclasses, or when these are bound to antigens as part of immune complexes. Of note, B-cells also express FcγRIIb (CD32b); thus, IgG subclasses detected by Ab clones might theoretically be due to "unspecific" binding to this receptor. However, CD32b is expressed in all B-cell subsets (Karnell et al., 2014) at similar levels, and no staining for IgG_{1-4} subclasses was detected on B-cells other than the corresponding IgG-subclass$^+$ memory B-cells and PCs, such as other IgG-subclass$^+$ and IgA$^+$ memory B-cells/PCs and the naïve B-lymphocytes, ruling out the possibility for unspecific binding on B-cells and PCs.

In summary, here we provide strong evidence about the feasibility to investigate in detail the IgG_{1-4} and IgA_{1-2} subsets of human memory B-cells and PCs from different human tissues, together with information about the optimal Ab clones and staining protocols. Quantification of serum Abs by itself, usually does not provide an accurate evaluation of the memory B-cell compartment and thereby, detailed dissection of the distinct subsets of IgG_{1-4} and IgA_{1-2} memory B-cell and PC subsets might provide new insights in the understanding of memory formation, long-term protection and waning for specific Ig subclass-mediated effector functions in steady state *vs.* disease-associated conditions. The reagents and protocols here proposed provide a fast, robust and sensitive approach for the identification and quantitation of memory B-cells and PCs expressing distinct Ig isotypes and subclasses in a broad range of clinical and research applications, with limited sample manipulation. Further studies in large series of health controls and patients with different immunological conditions are required to establish the potential clinical value of these flow cytometry measurements *vs.* currently used assays.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2017.09.008.

Conflict of interest

The Euroflow consortium is an independent scientific consortium which aims at innovation and standardization of diagnostic flow cytometry. All acquired knowledge and experience will be shared with the scientific and diagnostic community after protection of the relevant Intellectual Property, for example by filling patents. The involved patents are owned by the Euroflow Consortium and licensed to companies, including Cytognos SL (Salamanca, Spain), Becton/Dickinson Biosciences (San José, CA) and Immunostep SL (Salamanca, Spain). The revenues of the patents are exclusively used for Euroflow Consortium activities, such as for covering (in part) the costs of the Consortium meetings, the Euroflow Educational Workshops and the purchase of custom-made reagents for collective experiments. MMA and GG are employees of Cytognos SL. The other authors declare no other conflict of interest.

Acknowledgments

We would like to thank all volunteers for their contribution to this work, Quentin Lecrevisse (University of Salamanca) for his continuous support with the infinicyt software tools and Cristina Teodosio (Leiden University), Andrea Mayado (University of Salamanca), Ignacio Criado (University of Salamanca) and all EuroFlow members for the fruitful discussions.

Funding

EB is supported by a grant from the *Junta de Castilla y León* (*Fondo Social Europeo*, ORDEN EDU/346/2013, Valladolid, Spain). This work was supported by the: CB16/12/00400 grant (CIBER/ONC, *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 Investigación Sanitaria of Instituto de Salud Carlos III*, Madrid, Spain).

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