

A novel B cell population revealed by a CD38/CD24 gating strategy: CD38⁻CD24⁻ B cells in centenarian offspring and elderly people

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Received: 21 June 2012 / Accepted: 25 October 2012 / Published online: 7 November 2012
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Abstract The B cell arm of adaptive immunity undergoes significant modifications with age. Elderly people are characterized by impaired B cell responses reflected in a reduced ability to effectively respond against viruses and bacteria. Alterations of immunity with advancing age (immunosenescence) have been widely studied in centenarians who are considered a good example of successful aging. In recent years, attention has shifted to centenarian offspring (CO) as a model of people genetically advantaged for healthy aging and longevity. Here, we describe the preliminary characterization of a proposed new population of memory B cells, defined as CD19⁺CD38⁻CD24⁻, which we find at higher frequencies in the elderly but less so in CO

than healthy age-matched random controls. In addition, we found a decreased expression of RP105 (CD180), a toll-like receptor-associated molecule, on these cells. CD180 downregulation may potentially be a marker of immunosenescence. Moreover, we show that these CD19⁺CD38⁻CD24⁻ B cells produce TNF and hypothesize that their observed expansion in the elderly might contribute to the increased inflammatory status sometimes designated “inflamm-aging.”

Keywords B cell · CD38 · CD24 · CD180 · Immunosenescence · Centenarian offspring

Introduction

B cells are key mediators of immunity. The humoral immune response includes production of antibodies against pathogens and cytokines interacting with other components of the immune system. Early stages of B cell development occur in the bone marrow from hematopoietic stem cells. The early progenitors of B lymphocytes develop into pro-, pre-, and immature B cells (LeBien and Tedder 2008) that, after they are controlled for autoreactivity (Carsetti et al. 1995; Palanichamy et al. 2009), leave the bone marrow and enter the blood as transitional B cells (Allman et al. 1993; Chung et al. 2003; Mauri and Ehrestein 2008). In humans, peripheral blood naïve and memory B cells have been described on the basis of the differential

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expression of IgD and CD27 (Anolik et al. 2004; Shi et al. 2005; Wei et al. 2007; Frasca et al. 2008), as follows: $\text{IgD}^+\text{CD27}^-$ cells are naïve; $\text{IgD}^+\text{CD27}^+$ cells are memory cells including the unswitched memory cells also known as marginal zone-like B cells (Weller et al. 2004) and the IgM^+ memory B cell population identified as $\text{IgM}^+\text{IgD}^+\text{CD27}^+$; $\text{IgD}^-\text{CD27}^+$ cells are classical switched memory B cells also including the “IgM-only” memory B cells identified as $\text{IgM}^+\text{IgD}^-\text{CD27}^+$, and finally, $\text{IgD}^-\text{CD27}^-$, double-negative memory B cells (Fecteau et al. 2006; Colonna-Romano et al. 2009).

More recently, a different flow cytometric approach has been used to distinguish naïve from memory B cells (Allman et al. 2001, 2004; Carsetti et al. 2004; Palanichamy et al. 2009; Blair et al. 2010). The use of two developmentally regulated markers, CD24 and CD38, in association with the B-lineage marker CD19, allowed the identification of three different B cell populations: $\text{CD19}^+\text{CD38}^{\text{high}}\text{CD24}^{\text{high}}$, the previously mentioned transitional B cells that also include immature B cells; $\text{CD19}^+\text{CD38}^{\text{int}}\text{CD24}^{\text{int}}$ defined as mature B cells; and the final step of maturation in the periphery, $\text{CD19}^+\text{CD38}^-\text{CD24}^{\text{high}}$ so-called “primarily memory B cells.” In a recent paper (Chaplin et al. 2011), differentiation from transitional to mature B cells was induced by stimulation with CD180 (RP105), a toll-like receptor (TLR)-4 homologue expressed by monocytes, macrophages, dendritic cells, and B lymphocytes which regulates TLR-4 signaling and induces B cell proliferation.

Many papers have focused on modifications of the immune system in the elderly (immunosenescence) likely to contribute to their increased morbidity and mortality. It has been widely reported that elderly people show changes in B cell number, low levels of antibody production, and poor responses to recall antigens, and a collapse in B cell receptor repertoire diversity correlated with poor health status and the impairment of antibody response (Miller and Cancro 2007; Kumar and Burns 2008; Cancro et al. 2009; Gibson et al. 2009; Dunn-Walters and Ademokun 2010; Frasca et al. 2010; Frasca and Blomberg 2011; McElhaney et al. 2012). Lower numbers and percentages of B cells also form part of the cluster of immune parameters collectively known as the “Immune Risk Profile” associated with 2-, 4-, and 6-year mortality of the very elderly in the Swedish OCTO/NONA longitudinal studies (Pawelec et al. 2005). We and others have previously demonstrated that in elderly people, $\text{IgD}^+\text{CD27}^-$ naïve B cells are significantly reduced (Gupta et al. 2005; Colonna-Romano et al. 2008).

In contrast, Chong et al. (2005) demonstrated that the percentage of circulating naïve B cells, identified as CD27^- , were significantly higher in the aged subjects than young subjects. This topic is still a somewhat controversial finding, as previously reviewed by us (Bulati et al. 2011). Moreover, double-negative (DN) B cells ($\text{IgD}^-\text{CD27}^-$) are significantly increased in the elderly (Colonna-Romano et al. 2009; Bulati et al. 2011), as well as under certain pathological conditions, such as systemic lupus erythematosus (SLE) (Anolik et al. 2004; Wei et al. 2007), chronic HIV infection (Cagigi et al. 2009), and in healthy subjects challenged with respiratory syncytial virus (Sanz et al. 2008).

We have recently focused on some characteristics of the naïve/memory B cell compartment of centenarian offspring (CO). This is a special population of elderly people that, like their centenarian parent(s), could have genetic and functional advantages that predispose them to healthy aging and longer survival (Terry et al. 2003, 2004). We have shown that CO fail to show the age-associated increase of DN ($\text{IgD}^-\text{CD27}^-$) B cells seen in the general elderly population. Consistent with this, the level of serum IgM in CO is within the range of the levels observed in young subjects (Colonna-Romano et al. 2010).

As outlined above, several distinct memory B cell populations have been identified in humans, but associations between their specific phenotype and their functions remain to be clarified. Here, we report the characterization of a proposed new population of memory B cells identified as $\text{CD19}^+\text{CD38}^-\text{CD24}^-$. Moreover, we show that these cells are expanded in the general elderly but not in CO who show similar levels to those observed in young donors, suggesting a more “youthful” B cell constellation. A more detailed examination of CO B cells was then performed to evaluate the expression of CD27, IgM, and IgD. It is known that IgM memory B cells are reduced in the elderly, thus predisposing them to pneumococcal infection (Shi et al. 2005; Buffa et al. 2011). Here again, we show that CO have a “younger” B cell profile. Indeed, the percentage of these cells are not as reduced in CO as in their age-matched controls and is more similar to the percentage observed in young people. Moreover, we have examined the expression of CD180 on total B cells and found that elderly people have a significant increase of the $\text{CD19}^+\text{CD180}^-$ B cell subset. Finally, we have evaluated the expression of CD180 on B cell populations, identified by the

differential expression of IgD/CD27 and CD38/CD24, in order to determine the age-related modulation of this marker on these cells.

Material and methods

Subjects

A total of 35 healthy Sicilians were included in the study. Twelve subjects aged 70.1 ± 8.3 years, who were the offspring of at least one centenarian parent, were compared with seven age-matched controls without a centenarian parent (aged 69.1 ± 9 years), eight young (28.5 ± 1.9 years), and eight old (86.4 ± 3.8 years). All subjects were in good health according to their clinical history, and none of them had neoplastic, infectious, or autoimmune diseases or received any medications influencing immune function at the time of the study. The study received approval from the local ethics committee, and all participants gave their informed consent.

Whole blood was collected by venepuncture in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Lympholyte (Cedarlane Laboratories Limited, Ontario, Canada) and stored frozen.

Flow cytometric immunophenotyping of B cell subsets from human peripheral blood of young, elderly, centenarian offspring, and age-matched controls

To characterize the phenotype of B lymphocyte subsets, extracellular labeling was performed with anti-CD19-PE, CD24-Pe-Cy7, CD5-V450, IgD-FITC, IgM-PerCP-Cy5.5 (BD Biosciences), CD27-Qdot605 (Invitrogen), and CD38-eFluor650 (eBioscience). Cell viability was determined with RedVid (Invitrogen). All staining steps were performed in PBS, 2 % FCS, 2 mM EDTA, and 0.01 % Na azide (PFEA) buffer. Blocking of nonspecific binding sites was accomplished using human immunoglobulin Gamunex (Bayer, Leverkusen, Germany) or mouse serum (Caltag/Invitrogen, Karlsruhe, Germany). For each experiment, cells or mouse/rat κ -chain Comp Beads (Becton Dickinson) were stained with the corresponding fluorochrome-labeled antibodies and incubated for 20 min at 4 °C in the dark. Human-unstained cells were used as negative controls. After washing with PFEA, the cells or beads were resuspended and

measured on a LSR-II flow cytometer using the acquisition software FACSDiva (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star, Portland, OR). For data analysis, dead cells (RedVid-positive) were excluded. CD19⁺ living cells were gated within the side/forward scatter (SSC/FSC) lymphocyte gate.

Stimulation of PBMC with cytosine guanine/phorbol myristate acetate/ionomycin for B cell IL-10, IL-6, and TNF production

PBMC (10^6 cells/ml) were suspended in X-vivo 15 (Lonza, Walkersville, MD, USA) with or without cytosine guanine (CpG)-B 2006 (3 μ g/ml, Tib Molbiol), phorbol myristate acetate (PMA) (50 ng/ml), ionomycin (1 mg/ml), and monensin sodium salt (2 mM, Sigma Aldrich) in 24-well flat-bottom plates for 5 h, at 37 °C (CO₂ 5 %). Cells were harvested, washed, and directly stained with anti-CD19-PerCP-Cy5.5, CD24-Pe-Cy7, IgD-FITC, CD180-PE (BD Biosciences), CD27-Qdot605 (Invitrogen), and CD38-eFluor650 (eBioscience). For indirect immunofluorescence, antihuman CD5 (clone UCHT2) culture supernatant hybridoma cell line (Zentrum für Medizinische Forschung, Tübingen, Germany) was used as primary antibody. The secondary antibody was rat anti-mouse PO (Invitrogen). Cells were permeabilized with Citofix/Citoperm (BD Biosciences). Finally, cells were stained with anti-IL-10-APC (Miltenyi Biotec), TNF-AlexaFluor700, and IL-6-V450 (BD Biosciences), washed, and analyzed.

Statistical analyses

All statistical analyses were performed with Graph Pad Prism 4.0 using the Mann–Whitney nonparametric *U* test to compare two independent groups. Statistical significance was expressed as $P < 0.05$, $P < 0.01$, and $P < 0.001$ as shown in the figures. All values are expressed as mean \pm standard error of the mean (SEM).

Results

Characterization of CD38[±]/CD24[±] B cell subsets

To better characterize the memory/naïve phenotype of CD38[±]/CD24[±] B cell subsets, we have gated CD19⁺CD38^{hi}CD24^{hi} “classical transitional,” CD19⁺CD38^{int}CD24^{int} “mature naïve,” and

CD19⁺CD38⁻CD24⁺ “primarily memory” B cells and further evaluated them on the basis of the expression of IgD and CD27, widely used to identify naïve and memory B cells (Fig. 1). Moreover, as a fourth population that lacks both CD24 and CD38 is clearly present, we also extended the IgD/CD27 characterization to this population. In the figure, CD19⁺CD38⁺⁺CD24⁻ plasma cells are also shown. Most of the CD19⁺CD38^{hi}CD24^{hi} cells (plot I) are IgD⁺CD27⁻ (85.4 %), thus showing that classical transitional B cells have a naïve phenotype, although about 12 % of them are IgD⁻. Again, CD19⁺CD38^{int}CD24^{int} cells (plot II) are principally IgD⁺CD27⁻, although about 15 % of them express CD27. Moreover, the CD19⁺CD38⁻CD24⁺ B lymphocytes (plot III), here referred to as primarily memory B cells, show a variety

of different phenotypes. Indeed, some of them (26.6 %) are IgD⁺CD27⁺ (unswitched memory); others (44 %) are IgD⁻CD27⁺ (switched memory); 25 % of them are naïve as they express IgD but are negative for CD27 expression, and those that have a very low percentage (4.3 %) are IgD⁻CD27⁻ (DN). Our analysis of the fourth population (CD19⁺CD38⁻CD24⁻) (plot IV) indicated that these cells were predominantly IgD⁻, including the IgD⁻CD27⁺ (switched memory) and the IgD⁻CD27⁻ (double negative) B cell subsets, although 8.3 % of them are naïve (IgD⁺CD27⁻). Therefore, during maturation, it seems that B cells progressively modulate both CD38/CD24 and IgD/CD27 expressions, although not synchronously. In order to characterize these populations in more detail, we have evaluated the expression of additional immunological markers (Fig. 2);

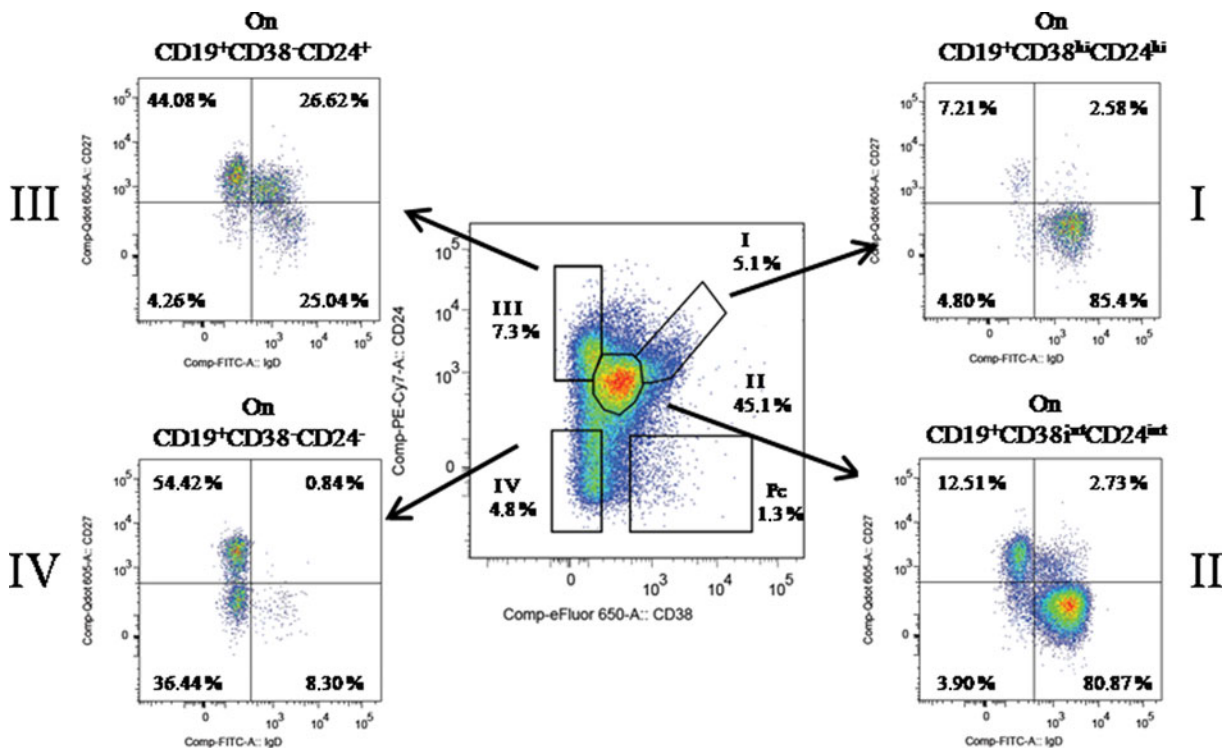


Fig. 1 Phenotypic characterization of CD38/CD24 B cell subsets. CD19⁺CD38^{hi}CD24^{hi} (I), CD19⁺CD38^{int}CD24^{int} (II), CD19⁺CD38⁻CD24⁺ (III), and CD19⁺CD38⁻CD24⁻ (IV) were evaluated on the basis of the expression of IgD and CD27. Moreover, it is also depicted the gate of CD19⁺CD38⁺⁺CD24⁻ plasma cells. In figure, the phenotypic characterization of an 82-year-old sample is shown. For data analysis, dead cells (RedVid positive) were excluded. CD19⁺ living cells were gated within the SSC/FSC lymphocyte gate. The evaluation of CD38 and CD24 within CD19⁺ B cells leads to the identification of four naïve/memory B cell subsets. This kind of characterization

confirmed that the majority of CD19⁺CD38^{hi}CD24^{hi} have a naïve (IgD⁺CD27⁻) phenotype, although about 12 % of them are IgD⁻. The CD19⁺CD38^{int}CD24^{int} B cells are principally naïve, whereas about 15 % of them express CD27. The analysis of CD19⁺CD38⁻CD24⁺ B cells reveals a variety of different phenotypes; some of them are IgD⁺CD27⁺ (unswitched memory); others are IgD⁻CD27⁺ (memory switched); about 25 % of them are naïve as they express IgD, but lack CD27 expression, and those that have a very low percentage (4 %) are IgD⁻CD27⁻ (DN). Moreover, CD19⁺CD38⁻CD24⁻ B cells, principally IgD⁻, are IgD⁻CD27⁺/IgD⁻CD27⁻

as no differences have been observed among the groups of age studied, the figure shows representative histograms of IgM, CD5, and CD180 expression. As shown, the largest proportion of CD19⁺CD38^{hi}CD24^{hi} cells ($\geq 80\%$ IgD⁺CD27⁻) was IgM⁺CD5⁺, whereas of CD19⁺CD38^{int}CD24^{int} cells ($\geq 80\%$ IgD⁺CD27⁻), IgM[±]CD5[±], and of CD19⁺CD38⁻CD24⁺ cells (IgD⁺CD27⁺), IgM⁺CD5⁻, as previously demonstrated (Carsetti et al. 2004; Palanichamy et al. 2009; Blair et al. 2010). Finally, the CD19⁺CD38⁻CD24⁻ population (IgD⁻CD27⁺) not thus far described was found to be IgM⁺CD5⁺. Moreover, the analysis of CD180 expression revealed that almost all CD180-negative B cells are gated within the CD19⁺CD38⁻CD24⁻ population, whereas classical transitional, mature naïve, and primarily memory cells were positive for this marker. Results depicted in Fig. 2 are representative of all subjects (young, old, centenarian offspring, and age matched) analyzed.

CpG+PMA/ionomycin stimulates intracellular cytokine production by human blood B cells in vitro

A typical feature of aging is a chronic, low-grade inflammation characterized by a general increase in the production of pro-inflammatory cytokines and inflammatory markers (Cevenini et al. 2010). Memory and naïve B cells can produce different cytokines and chemokines;

in particular, memory B cells produce high levels of the pro-inflammatory cytokines IL-1 α , IL-1 β , IL-6, and TNF- α , suggesting that B cells might take part in the generation or in the maintenance of the inflammatory environment of the elderly (Agrawal and Gupta 2011). To study the functional properties of peripheral blood B lymphocytes, we tested their ability to produce cytokines “in vitro” on stimulation with a combination of CpG, PMA, and ionomycin. We evaluated the production of IL-10, an anti-inflammatory cytokine, and IL-6 and TNF- α , two pro-inflammatory cytokines, by CD38/CD24 B cell subsets. These cytokines have been extensively studied in aging, as they are involved in the control (IL-10) or maintenance (IL-6 and TNF- α) of inflammation, one of main characteristics of aged people (inflamm-aging) (Franceschi et al. 2000a; Lio et al. 2003; Sanjabi et al. 2009). Our analysis revealed that in all the subjects studied, 5 h after stimulation, the main IL-10-producing cells were of the CD19⁺CD38^{hi}CD24^{hi} phenotype (Table 1). In contrast, CD19⁺CD38⁻CD24⁺ and CD19⁺CD38⁻CD24⁻ cells are the more responsive B cell populations involved in TNF- α production (Table 2). As no differences were detected (not shown) regarding the age classes, the tables show the mean fluorescent intensity (MFI) values (mean \pm SEM) of all the subjects studied. On the other hand, this kind of stimulation was not able to induce IL-6 production. Indeed, we did not detect

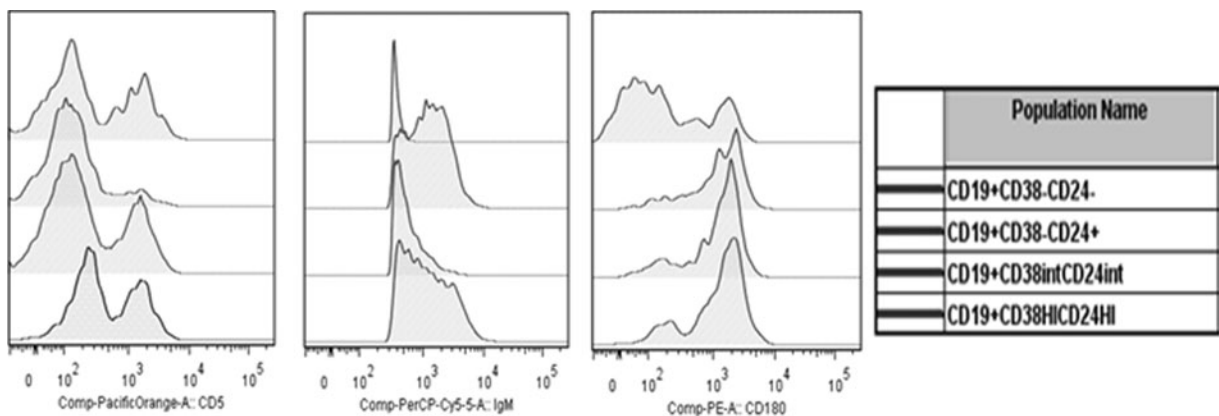


Fig. 2 Evaluation of IgM, CD5, and CD180 on CD38/CD24 B cells. A typical experiment showing the expression of the immunological markers on CD19⁺CD38^{hi}CD24^{hi}, CD19⁺CD38^{int}CD24^{int}, CD19⁺CD38⁻CD24⁺, and CD19⁺CD38⁻CD24⁻ B cell subsets. Histograms are a representative of all subjects (young, old, CO, and age matched) analyzed. As shown, CD5 is well expressed on CD19⁺CD38^{hi}CD24^{hi} and CD19⁺CD38⁻CD24⁻ (MFI 789 and 704, respectively); a lower level is expressed on CD19⁺CD38^{int}CD24^{int} (MFI 570), whereas CD19⁺CD38⁻CD24⁺ cells show the lowest level of expression of CD5 (MFI 278).

Concerning IgM, CD19⁺CD38^{hi}CD24^{hi} and CD19⁺CD38⁻CD24⁻ cells are IgM⁺ (MFI 1,601 and 1,489, respectively); CD19⁺CD38^{int}CD24^{int} cells are IgM[±] (MFI 682), whereas CD19⁺CD38⁻CD24⁺ cells show the lowest level of expression of IgM (MFI 391). The evaluation of CD180 on CD38/CD24 B cell subsets has revealed that CD19⁺CD38^{hi}CD24^{hi} (MFI 1,578), CD19⁺CD38^{int}CD24^{int} (MFI 1,413), and CD19⁺CD38⁻CD24⁺ (MFI 1,507) cells are CD180⁺, whereas CD19⁺CD38⁻CD24⁻ cells show the lowest level of expression of CD180 (MFI 484), resulting to CD180⁻.

Table 1 IL-10 production by CD38/CD24 B cell subsets with or without CpG/PMA/ionomycin stimulation

IL-10		CpG/PMA/ionomycin					
RPMI		CD38 ^{hi} CD24 ^{hi}	CD38 ^{int} CD24 ^{int}	CD38 ^{lo} CD24 ^{lo}	CD38 ^{hi} CD24 ^{hi}	CD38 ^{int} CD24 ^{int}	CD38 ^{lo} CD24 ^{lo}
	subsets MFI values (mean±SEM)	(1,126.4±208.3)	(611.7±127.6)	(481.4±45.1)	(2,150.2±316.7)	(1,039.4±276)	(784±75.9)
CD38 ^{hi} CD24 ^{hi}	–		p1=0.03	p2=0.02		p1=0.002	p2=0.001
CD38 ^{int} CD24 ^{int}	p1=0.03			p4=0.6			p4=0.1
CD38 ^{lo} CD24 ^{lo}	p2=0.02		p4=0.6			p4=0.1	p6=0.02
CD38 ^{hi} CD24 ^{hi}	p3=0.005		p5=0.03			p5=0.04	–
CD38 ^{int} CD24 ^{int}							
CD38 ^{lo} CD24 ^{lo}							

Statistically significant differences are marked in bold

A total of 35 healthy Sicilians were analyzed. CD19⁺CD38^{hi}CD24^{hi} is the main B cell subset involved in IL-10 production. Data are expressed as MFI values (mean±SEM). p1=CD19⁺CD38^{hi}CD24^{hi} vs CD19⁺CD38^{int}CD24^{int}; p2=CD19⁺CD38^{hi}CD24^{hi} vs CD19⁺CD38^{lo}CD24^{lo}; p3=CD19⁺CD38^{hi}CD24^{hi} vs CD19⁺CD38^{int}CD24^{int}; p4=CD19⁺CD38^{int}CD24^{int} vs CD19⁺CD38^{lo}CD24^{lo}; p5=CD19⁺CD38^{int}CD24^{int} vs CD19⁺CD38^{hi}CD24^{hi}; p6=CD19⁺CD38^{lo}CD24^{lo} vs CD19⁺CD38^{int}CD24^{int}

Table 2 TNF- α production by CD38^{hi}/CD24^{hi} B cell subsets with or without CpG/PMA/ionomycin stimulation

TNF- α		CpG/PMA/ionomycin					
RPMI		CD38 ^{hi} CD24 ^{hi}	CD38 ^{int} CD24 ^{int}	CD38 ^{lo} CD24 ^{lo}	CD38 ^{hi} CD24 ^{hi}	CD38 ^{int} CD24 ^{int}	CD38 ^{lo} CD24 ^{lo}
subsets MFI values (mean \pm SEM)		(202.1 \pm 19.2)	(94 \pm 14.2)	(97.3 \pm 15.8)	(297.4 \pm 26.2)	(341.9 \pm 51.1)	(653 \pm 152.7)
CD38 ^{hi} CD24 ^{hi}	–	p1=0.003	p2=0.0004	p3=0.0001	–	p1=0.8	p2=0.05
CD38 ^{int} CD24 ^{int}	p1=0.003	–	p4=0.7	p5=0.3	CD38 ^{hi} CD24 ^{hi}	–	p3=0.006
CD38 ^{lo} CD24 ^{lo}	p2=0.0004	p4=0.7	–	p6=0.4	CD38 ^{int} CD24 ^{int}	p1=0.8	p4=0.04
CD38 ^{hi} CD24 ^{hi}	p3=0.0001	p5=0.3	p6=0.4	–	CD38 ^{lo} CD24 ^{lo}	p2=0.05	p5=0.1
							p6=0.7

Statistically significant differences are marked in bold

A total of 35 healthy Sicilians were analyzed. The major contribution to the production of TNF is seen from CD38^{hi}CD24^{hi} and CD38^{lo}CD24^{lo} cells. Data are expressed as MFI values (mean \pm SEM). p1=CD19⁺CD38^{hi}CD24^{hi} vs CD19⁺CD38^{int}CD24^{int}; p2=CD19⁺CD38^{hi}CD24^{hi} vs CD19⁺CD38^{lo}CD24^{lo}; p3=CD19⁺CD38^{hi}CD24^{hi} vs CD19⁺CD38^{int}CD24^{int}; p4=CD19⁺CD38^{int}CD24^{int} vs CD19⁺CD38^{lo}CD24^{lo}; p5=CD19⁺CD38^{int}CD24^{int} vs CD19⁺CD38^{hi}CD24^{hi}; p6=CD19⁺CD38^{lo}CD24^{lo} vs CD19⁺CD38^{int}CD24^{int}

a significant increase in production of IL-6 compared to unstimulated PBMC (not shown).

Age-related changes result in altered distribution of naïve/memory subsets

We have determined whether the distribution of B cell phenotypes including the newly defined subset is different in the elderly compared to the young, and whether this is different in centenarian offspring compared to age-matched and elderly controls (Table 3). We did not observe any significant differences in the CD19⁺CD38^{hi}CD24^{hi} (Fig. 3a) or CD19⁺CD38^{int}CD24^{int} (Fig. 3b) populations between any groups of subjects tested. However, we observed a trend towards higher proportions of CD19⁺CD38⁻CD24⁺ cells in the elderly. Interestingly, the percentage of these cells in centenarian offspring are more similar to what is observed in young donors than the age-matched (AM) controls (Fig. 3c). Most strikingly, the CD19⁺CD38⁻CD24⁻ population was highly significantly enriched in the older subjects. Again, their distribution in CO fell between the young and the elderly in the general population (Fig. 3d).

CD180-negative B cells and CD180 expression on CD38/CD24 and IgD/CD27 B cell subsets in different age groups

It is known that immune system of elderly people shows some characteristics typical of chronically stimulated subjects (CMV-positive elderly subjects and HIV and SLE patients). Moreover, circulating B cells have been reported to lack the TLR-4 homologue CD180 (RP105) in chronically stimulated SLE patients (Koarada and Tada 2012). We therefore analyzed the percentage of

CD19⁺CD180⁻ B lymphocytes in PBMC of young, elderly, CO, and their AM controls. Figure 4a shows a significant increase of this population in the elderly relative to the young. However, no differences were seen between CO and AM controls, although the percentage of CD180⁻ B cells in CO and AM controls are higher than in the young but lower than in the old donors. Next, we have evaluated the level of expression of CD180 in the four subsets identified on the basis of the markers CD38 and CD24 in the four age groups (Fig. 4b). The phenotypic characterization of CD38/CD24 B cells has shown that most CD19⁺CD38⁻CD24⁻ B cells are CD180 negative (data shown in Fig. 2). Our comparative analysis between young, CO, AM, and old (O) subjects did not reveal age-associated changes in the level of CD180 expression. Moreover, as we have observed that CD19⁺CD38⁻CD24⁻ B cells mainly include both switched memory (IgD⁻CD27⁺) and DN (IgD⁻CD27⁻) B cells, we have also evaluated the expression of CD180 on B cell subsets identified on the basis of the expression of IgD and CD27 (Fig. 4c). Interestingly, in all the subjects, this analysis revealed a progressive decrease of CD180 expression through the different stages of maturation, from naïve (IgD⁺CD27⁻) to memory-switched (IgD⁻CD27⁺) and DN (IgD⁻CD27⁻) B cells and from CD19⁺CD38^{hi}CD24^{hi} to CD19⁺CD38⁻CD24⁻ B cells, although the comparative study did not show any changes between age classes.

Age-related differences in the distribution of IgM⁺CD27⁻ (naïve mature), IgD⁺IgM⁺CD27⁺ (natural effector), and IgD⁻IgM⁺CD27⁺ (IgM only) B cell phenotypes

The complexity of the memory B cell pool is further documented by the evidence of different IgM⁺ B cell

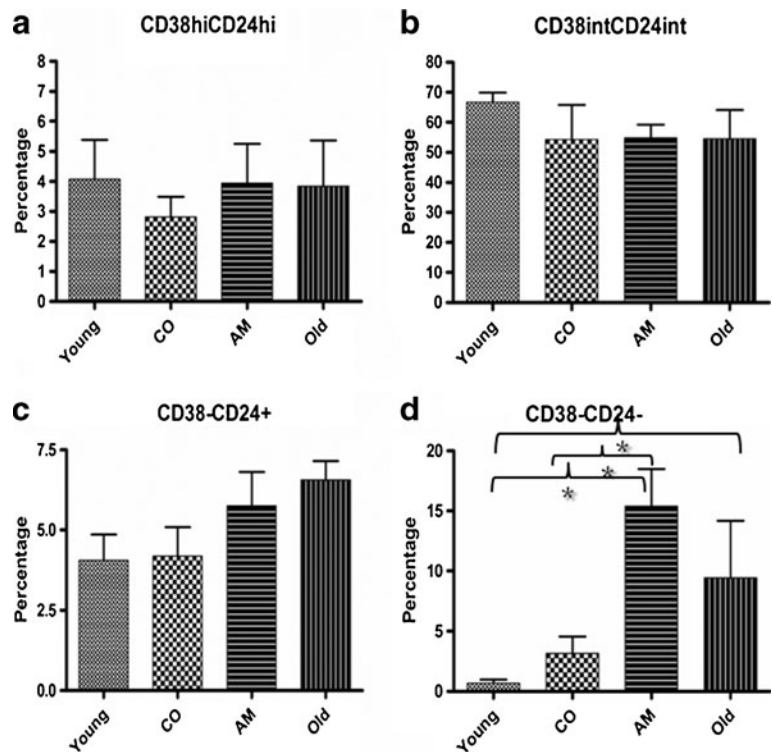
Table 3 Distribution of CD38/CD24 B cell phenotypes in young, centenarian offspring, age-matched controls, and old subjects

CD38/CD24 B cell subsets	Age groups				P value					
	Y	CO	AM	O	p1	p2	p3	p4	p5	p6
CD19 ⁺ CD38 ^{high} CD24 ^{high}	4.1±1.3	2.8±0.7	3.9±1.3	3.8±1.5	0.9	0.5	0.9	0.7	0.8	0.7
CD19 ⁺ CD38 ^{int} CD24 ^{int}	66.8±3.1	54.3±11.5	54.8±4.3	54.5±9.6	0.4	1	0.1	1	1	1
CD19 ⁺ CD38 ⁻ CD24 ⁺	4.1±0.8	4.2±0.9	5.7±1.1	6.6±0.6	0.06	0.2	0.4	1	0.2	0.7
CD19 ⁺ CD38 ⁻ CD24 ⁻	0.9±0.3	3.1±1.4	15.4±3.1	9.4±4.7	0.03	0.02	0.01	0.06	0.3	0.5

Statistically significant differences are marked in bold

Percentage values are expressed as mean±SEM. Differences between age groups were analyzed with the Mann–Whitney test. p1=Y vs O; p2=CO vs AM; p3=Y vs AM; p4=Y vs CO; p5=CO vs O; p6=AM vs O

Fig. 3 Distribution of B cell phenotypes in young, old, centenarian offspring, and age-matched controls. Percentage of $CD19^+CD38^{hi}CD24^{hi}$ (a) and $CD19^+CD38^{int}CD24^{int}$ (b) B cell populations do not change when analyzed in different age groups. c Trend towards an increase in the age-related $CD19^+CD38^-CD24^+$ B cell subset. d Significant increase of the $CD19^+CD38^-CD24^-$ B cell subset in elderly people (old and AM) when compared to young. CO, like young, show a lower percentage of this population when compared to their age-matched controls. Differences between age groups were analyzed with the Mann–Whitney test. * $P < 0.05$



phenotypes presumably representing different stages of B cell differentiation. We and others have demonstrated that IgM B lymphocytes are reduced in the elderly (Shi et al. 2005; Buffa et al. 2011). As the previous phenotypic characterization has not revealed differences in the IgM expression, we chose to analyze IgM⁺ B cell phenotypes on our samples according to the Berkowska gating strategy (Berkowska et al. 2011), based on the major subset of $CD38^{dim}CD24^{dim}$, excluding transitional B cells (Fig. 5). So, $CD38^{dim}CD24^{dim}$ B cells can be separated into three IgM populations: IgM⁺CD27⁻ (naïve mature), IgD⁺IgM⁺CD27⁺ (natural effector), and IgD⁻IgM⁺CD27⁺ (IgM only) (Fig. 5a). A comparison between our subjects confirmed (Shi et al. 2005; Buffa et al. 2011) a different distribution of B cells identified on the basis of these markers between young and old donors and revealed that CO possess a more “youthfully” distributed immune profile compared with age-matched controls. Indeed, the percentage of IgM⁺CD27⁻ (naïve mature) cells (Fig. 5b) are significantly greater in CO, a group of people presumably advantaged for longevity. The analysis of the other two IgM⁺CD27⁺ memory B cell phenotypes revealed significantly fewer of these memory cells in the CO (Fig. 5c, d).

Discussion

During aging, the immune system progressively loses its ability to fight off infections and to respond as quickly or as efficiently to different stimuli. Moreover, the inability of old individuals to effectively respond to vaccines results in less effective immunizations against viruses and bacteria, thus increasing the risk of infections and diseases (Miller and Cancro 2007; Cancro et al. 2009; Gibson et al. 2009; Dunn-Walters and Ademokun 2010; Frasca and Blomberg 2011). Indeed, it is well documented that the immune response to vaccination declines with age, although the reasons for this are poorly understood (Kumar and Burns 2008; Frasca et al. 2010; McElhaney et al. 2012). It is also known that in the elderly, there is an impairment of both innate and adaptive immune responses. As we age, the numbers of critical cells in the immune system change, and many may become less functional. However, innate immunity seems to be better preserved, while more severe age-dependent changes occur in the adaptive immune system (Franceschi et al. 2000b). There are clear changes in B cell generation and repertoire, and as a consequence of this and changes in the T cells, elderly people show defective humoral immunity (Cancro et al. 2009; Bulati

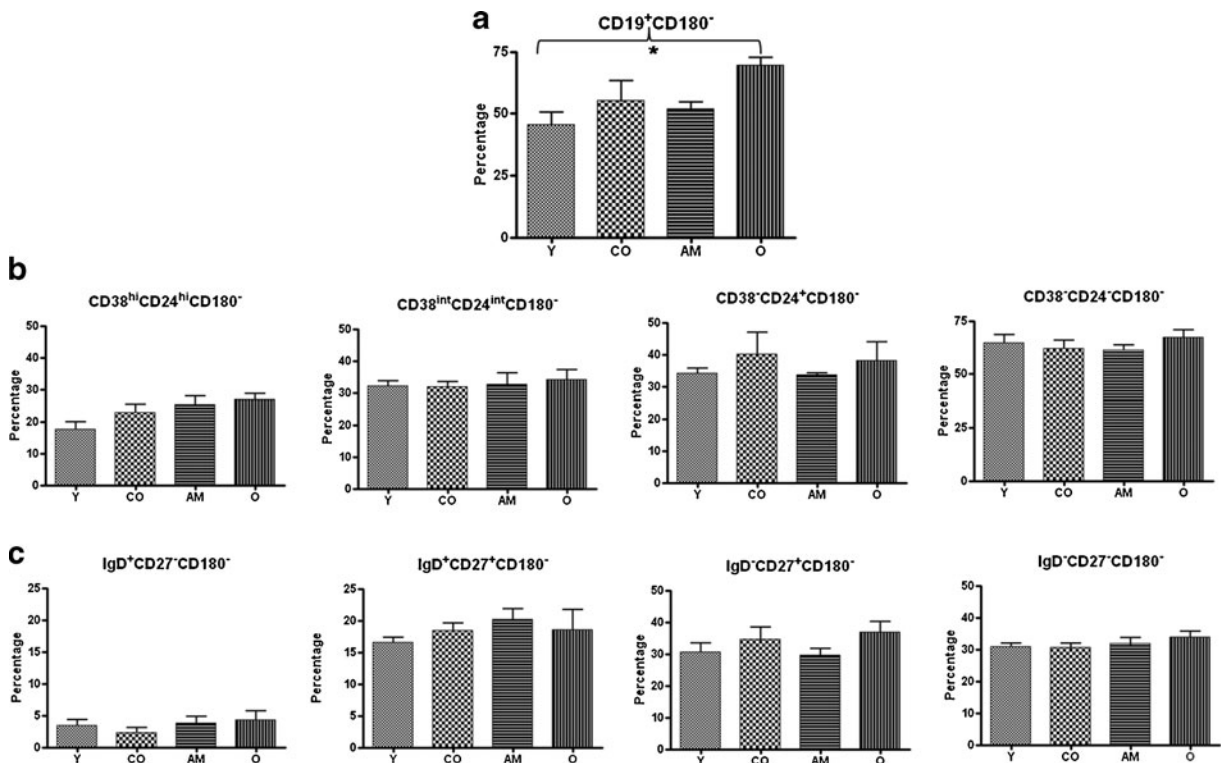


Fig. 4 CD180-negative B cells and CD180 expression on CD38/CD24 and IgD/CD27 B cell subsets. **a** Evaluation of CD19⁺CD180⁻ (RP105)⁻ B cell population among the different age groups analyzed. Old people show a significant increase in the percentage of the B cell subset lacking CD180 expression, when compared with young. **b** Histograms show CD180-negative B cells among CD38^{hi}CD24^{hi}, CD38^{int}CD24^{int}, CD38⁻CD24⁺, and CD38⁻CD24⁻ B cell compartments in young (Y), centenarian offspring (CO), age-matched (AM), and old (O) subjects. A progressive increase in percentage of CD180-

negative cells from CD38^{hi}CD24^{hi} to CD38⁻CD24⁻ cells has been demonstrated without age-related changes between groups studied. **c** Expression of CD180 on naïve memory B cells identified on the basis of the expression of IgD and CD27. A progressive down-modulation of CD180 from naïve (IgD⁺CD27⁻) to DN (IgD⁻CD27⁻) B cells has shown but, again, no change between age classes. Differences between age groups were analyzed with the Mann–Whitney test. **P*<0.05. *Bar graphs* indicate mean±SEM

et al. 2011; Frasca and Blomberg 2011). There is a decrease both in percentage and absolute number of total B lymphocytes (Pawelec et al. 2005; Shi et al. 2005; Frasca et al. 2008; Veneri et al. 2009; Bulati et al. 2011), and in previous papers, we (Colonna-Romano et al. 2009) and others (Gupta et al. 2005) have demonstrated that the percentage of naïve B cells are significantly reduced in aged individuals. However, this is still a somewhat controversial finding as some authors reported an increase, a decrease, or no changes in the naïve/memory B cell compartments, as we have reviewed (Bulati et al. 2011). The CD27⁺ memory B cells and, in particular, the IgM memory CD19⁺IgM⁺IgD⁻CD27⁺ cells, which are considered to be the recirculating equivalent of murine marginal zone cells, have been shown to decline in both proportion and numbers with age (Shi et

al. 2005; Buffà et al. 2011). In a previous study, we documented the expansion of CD19⁺IgD⁻CD27⁻ cells, a DN memory B cell population, in the elderly (Colonna-Romano et al. 2009). This population is also expanded in subjects chronically stimulated (Anolik et al. 2004; Wei et al. 2007; Sanz et al. 2008; Cagigi et al. 2009) and might be considered the result of long-enduring stimulation. However, not all subjects or population groups are equally susceptible to the effects of long-term chronic stimulation of the immune system. Indeed, recent evidence has shown a well-preserved immune profile of a group of healthy elderly centenarian offspring who seem to have genetic and functional advantages associated with the reduced risk of disability with age (Terry et al. 2003, 2004). We have demonstrated that the naïve B cells subset is well preserved in CO compared with the age-

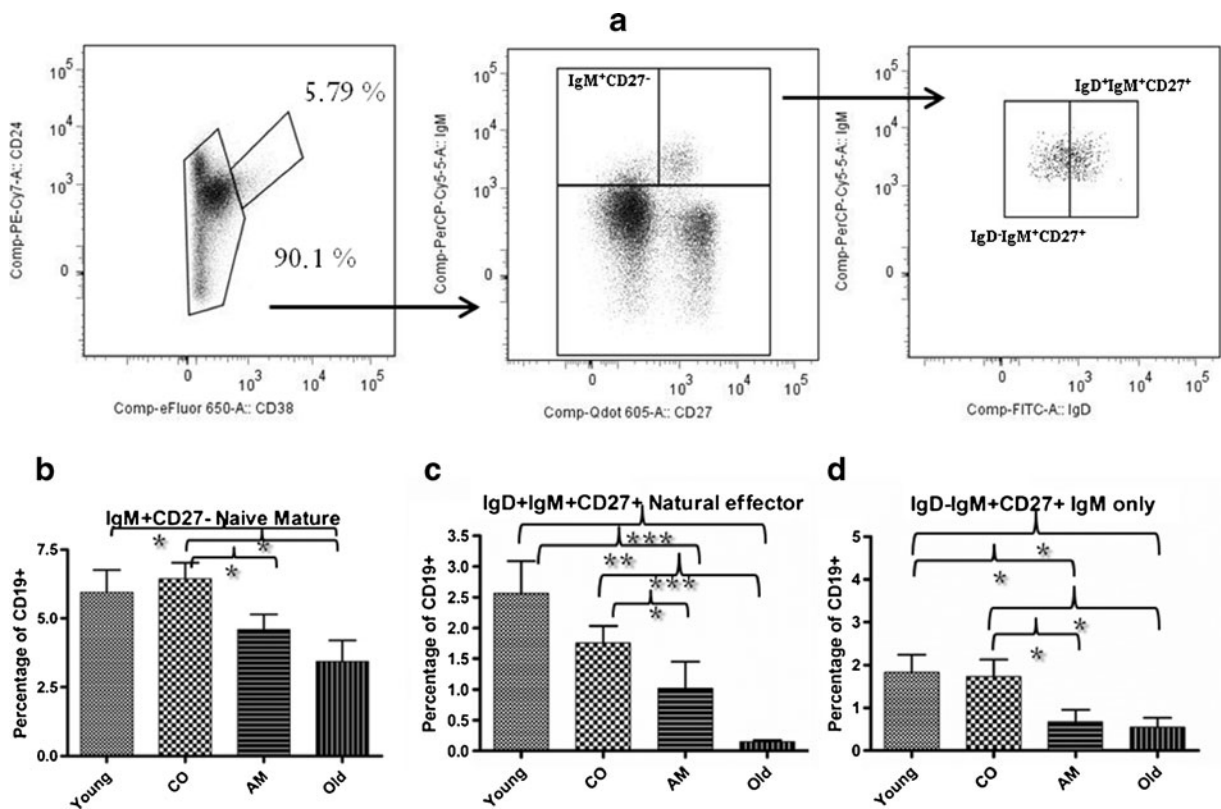


Fig. 5 Diversity in the human IgM^+ B cell compartment and distribution in young and old, centenarian offspring (CO), and their age-matched (AM) controls. **a** Within the CD19^+ B cell compartment, B lymphocytes are characterized on the basis of CD38 and CD24 expression. According to the Berkowska et al. (2011) gating strategy, $\text{CD38}^{\text{dim}}\text{CD24}^{\text{dim}}$ B lymphocytes were subdivided on the basis of IgM and CD27 expressions, defining $\text{IgM}^+\text{CD27}^-$ naïve mature. Subsequently, on the basis of IgD expression, $\text{IgM}^+\text{CD27}^+$ B cells were gated into $\text{IgD}^+\text{IgM}^+\text{CD27}^+$ natural effector and $\text{IgD}^-\text{IgM}^+\text{CD27}^+$ IgM only. **b** Young donors have a significant

higher percentage of naïve mature cells compared to the elderly. A direct comparison between CO and aged people (AM and old subjects) emphasizes that the former shows a significantly higher percentage of this IgM population. **c** A substantial and significant age-related decrease was observed in the natural effector subset. **d** Age-related decrease of the IgM-only B cell subset observed in old and age-matched controls compared with young and centenarian offspring subjects. Differences between age groups were analyzed with the Mann–Whitney test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

matched controls. The mirror image of this is a more restricted memory pool, which includes, in addition, a less expanded DN compartment (Colonna-Romano et al. 2010). Collectively, our data suggest age-related changes, but they are of course cross-sectional in the present study and therefore can only formally demonstrate differences between groups. Other investigations, such as a longitudinal follow-up studies, will be necessary to affirm a real age-related change.

Recently, several new B cell populations have been described, although their phenotype has not been always associated with established biological function. In the present work, we gained insight into the naïve/memory B cell compartment focusing, in particular, on the different age groups analyzed. According to the gating strategy

proposed by Carsetti et al. (2004), our study has focused on four distinct B cell populations: $\text{CD19}^+\text{CD38}^{\text{hi}}\text{CD24}^{\text{hi}}$ (classical transitional), $\text{CD19}^+\text{CD38}^{\text{int}}\text{CD24}^{\text{int}}$ (mature naïve), $\text{CD19}^+\text{CD38}^{\text{lo}}\text{CD24}^{\text{lo}}$ (primarily memory), and in addition, a not previously described $\text{CD19}^+\text{CD38}^{\text{lo}}\text{CD24}^{\text{lo}}$ population. A previous study reported that the frequency of transitional B cells is rapidly reduced during the first years of life, stabilizing after 5 years of age (Morbach et al. 2010). Here, we found that there are no differences in the percentage of transitional and mature naïve B cells, comparing young and the three different groups of old donors CO, AM, and elderly, but we did observe a higher proportion of $\text{CD19}^+\text{CD38}^{\text{lo}}\text{CD24}^{\text{lo}}$ primarily memory cells in young and CO compared to AM, although the difference did not achieve statistical significance. However, the

CD19⁺CD38⁻CD24⁻ putative new population was significantly increased in the elderly, and the percentage of this population in CO is more similar to that observed in young than in elderly or age-matched donors. This may therefore contribute to their immunological “youthfulness.” Apart from this, the other B cell populations identified on the basis of the expression of CD38/CD24 show the same distribution in the different groups studied. The evaluation of both IgD and CD27 on CD38/CD24 B cells also supports the developmental dynamic from transitional B cells that are mainly IgD⁺CD27⁻ (naïve), through mature naïve B cells, in which we observe the progressive appearance of CD27, which is, finally, most strongly expressed on the primarily memory CD19⁺CD38⁻CD24⁺, together with the progressive loss of IgD. CD19⁺CD38⁻CD24⁺ B lymphocytes, also known as primarily memory B cells, show a variety of different phenotypes. Although more than 70 % are CD27⁺, the simultaneous evaluation of IgD and CD27 has revealed that some of them (27 %) are IgD⁺CD27⁺ (unswitched memory); others (44 %) are IgD⁻CD27⁺ (switched memory); 25 % of them are naïve, as they express IgD, and are negative for CD27 expression, and those that have a very low percentage (4.3 %) are IgD⁻CD27⁻ (DN). Some authors have shown that this population should include only memory B cells, although their analyses have revealed a percentage of CD27-negative B cells gated inside the aforementioned subset (Carsetti et al. 2004; Blair et al. 2010). Unfortunately, those authors did not report the percentage of CD27⁻, so we cannot numerically compare our data with the data reported in their papers. Further analyses are necessary to elucidate this point. Focusing on CD19⁺CD38⁻CD24⁻ B lymphocytes, we did not observe any changes in distribution of IgD/CD27 phenotypes in the different age groups. Moreover, here, we show that about half of these cells are IgD⁻CD27⁺-switched memory, not different in the elderly (Colonna-Romano et al. 2009; Buffa et al. 2011), whereas almost 30 % of them are IgD⁻CD27⁻ DN B cells that are increased in the elderly but not in CO (Colonna-Romano et al. 2009, 2010). It is of note that a fraction of the IgD⁻CD27⁺ subset also includes IgD⁺IgM⁺CD27⁺ memory B cells that we and others have described as being decreased in the elderly (Shi et al. 2005; Buffa et al. 2011). Probably, their low percentage does not influence the percentage of CD19⁺CD38⁻CD24⁻ B cells in the different age groups. The evaluation of IgM has revealed that this marker is

strongly expressed on CD19⁺CD38^{hi}CD24^{hi} and on IgD⁺CD27⁻ cells (Colonna-Romano et al. 2009) and tends to disappear from both CD19⁺CD38⁻CD24⁻ and IgD⁻CD27⁻ cells. We have also evaluated CD5 expression on CD38/CD24 B cell phenotypes. Our analysis revealed that all different subpopulations had different levels of CD5. Our characterization of CD38/CD24 B cells has confirmed that CD19⁺CD38^{hi}CD24^{hi} cells were positive for CD5. Interestingly, CD19⁺CD38⁻CD24⁻ cells were also positive for the same marker. This molecule is present in adulthood on all B cell subsets, but at a low and variable frequency. CD5 expression is associated with the presence of the B cell receptor (BCR), and it appears in the bone marrow; it has been shown to be a negative regulator of BCR signaling, and its expression may be a marker of antigen exposure (Gary-Gouy et al. 2000, 2002; Carsetti et al. 2004). Additional characterization of these CD19⁺CD38⁻CD24⁻ B lymphocytes has demonstrated here that almost all are CD180 negative. This marker, also known as RP105, is a toll-like receptor-associated molecule, originally identified as a B cell surface molecule mediating activation and proliferation in mice. Data from the literature indicate that RP105-deficient mice are hypo-responsive to TLR-4 and TLR-2 stimulation (Ogata et al. 2000; Nagai et al. 2005). Recently, increasing interest in this molecule has led several investigators to evaluate CD180 in humans and, in particular, in subjects suffering from autoimmune diseases. It has been demonstrated that the percentage of CD180-negative B cells are significantly increased in systemic lupus erythematosus and, to a lesser extent, in rheumatoid arthritis patients, compared to healthy controls. Its expression declines during the inactive stages of disease, suggesting that the expansion of this population is correlated with disease activity (Koarada and Tada 2012). The phenotypic characterization of the above-described subset has defined a profile of activated B cells producing immunoglobulins and anti-dsDNA antibodies. Moreover, approximately 50 % of these CD180-negative B cells had intracellular IgG (Koarada et al. 1999), suggesting that they could be induced to differentiate into IgG-secreting B cells by certain stimuli. As autoimmune phenomena are a common feature in the elderly, we have evaluated the expression of this marker on total B lymphocytes in different age groups, observing a reduced expression with age. Indeed, CD180-negative B cells are significantly increased in the very elderly donors (age range 86.4±3.8) when compared to the young (age range 28.5±1.9), but not in CO and AM

(age range 70.1 ± 8.3 and 69.1 ± 9 , respectively). Furthermore, we assessed the level of expression of CD180 in the four subsets identified on the basis of the markers CD38 and CD24 in the four age groups. In any event, our comparative analysis between young, CO, AM, and O subjects did not reveal age-associated differences in the level of CD180 expression. Interestingly, the evaluation of $CD38^-CD24^-CD180^-$ B cells gated inside $CD19^+$ B cell population has shown a trend (not significant) towards higher proportions in elderly donors and age-matched controls when compared with young donors and centenarian offspring (data not shown). Moreover, as we have observed that $CD19^+CD38^-CD24^-$ B cells, which are increased in elderly people when compared to young donors and in age-matched controls more than in centenarian offspring, mainly include both switched memory (IgD^-CD27^+) and DN (IgD^-CD27^-) B cells, we have also evaluated the expression of CD180 on $IgD/CD27$ B cell subpopulations. As expected, we saw a lower expression of this marker from naïve to memory-switched and DN B cells, although the comparative analysis did not show any changes between age classes. Data obtained from CD180 evaluation on CD38/CD24 and $IgD/CD27$ phenotypes cannot be considered similar, although, in both gating systems, CD180-negative B cells are increased from naïve to memory B cells. Thus, reduced expression of the TLR-associated molecule CD180 on total B cells is observed in the elderly, and an age-related increase of DN and of $CD19^+CD38^-CD24^-$ B cells, at least defective for CD180, could be related to the increased susceptibility to Gram-negative bacterial diseases of elderly people. Indeed, it is known that elderly people are more susceptible than the young to pneumococcal infection. The decline of the IgM memory B cell pool in the elderly could also be involved in defective immune responses against infections by encapsulated bacteria.

On the basis of IgM , CD27, and IgD expression, IgM B lymphocytes can be subdivided into IgM^+CD27^- “naïve mature,” $IgD^-IgM^+CD27^+$ IgM -only, and $IgD^+IgM^+CD27^+$ “natural effector” B cells. As the previous phenotypic characterization of CD38/CD24 B cells has not revealed differences in the IgM expression, we chose to analyze IgM^+ B cell phenotypes on our samples according to the Berkowska gating strategy (Berkowska et al. 2011), based on the major subset of $CD38^{dim}CD24^{dim}$. We analyzed IgM B cell subsets, comparing data between subjects included in this study.

We have confirmed data from the literature demonstrating impairment decrease of IgM B lymphocytes in the elderly (Shi et al. 2005; Buffa et al. 2011). On the contrary, a further evaluation of CO B cell populations revealed that the percentage of these cells are not reduced to the same extent as their age-matched controls and remain more similar to that observed in young people. These data reinforce our previous hypothesis according to which CO have a younger B cell profile. This hypothesis is corroborated by the reduced number of $CD19^+CD38^-CD24^-$ B cells and a higher level of IgM B cell subsets in CO, compared to AM donors. This immunological advantage, likely due to a good bone marrow reservoir, could help CO both to fight the main age-related diseases and to maintain healthy aging.

Here, we have also performed a preliminary functional study on the CD38/CD24 B cells to better understand their biological role and function. We tested their ability to produce pro- and anti-inflammatory cytokines on strong stimulation with CpG/PMA/ionomycin *in vitro*. We know that cytokines are considered key players in maintaining lymphocyte homeostasis. Their function is to induce and modulate the nature of the response after an immune insult, or in contrast, they may cause non-responsiveness and active immune suppression (Sanjabi et al. 2009). Moreover, it is known that elderly people show a pro-inflammatory microenvironment that has been related to the increased risk of morbidity and mortality (Licastro et al. 2005; Vasto et al. 2007). Concerning the anti-inflammatory cytokine IL-10, also our stimulation system provides data consistent with a biological role of $CD19^+CD38^{hi}CD24^{hi}$ cells as regulatory cells, i.e., a B cell population involved in IL-10 production. Our comparative analysis did not reveal any differences between the four age groups analyzed. We have previously demonstrated that under the same conditions, IgD^+CD27^- (naïve) B cells produce high levels of IL-10 in the elderly (Buffa et al. 2011). Although we have confirmed that $CD19^+CD38^{hi}CD24^{hi}$ cells are mainly IgD^+CD27^- , our analysis of CD38/CD24 populations revealed a certain percentage of naïve $IgD/CD27$ in all four populations analyzed, extending the potential role of IL-10-producing cells. Finally, regarding the pro-inflammatory cytokine TNF- α , the $CD19^+CD38^-CD24^+$ and $CD19^+CD38^-CD24^-$ B cell subsets, which seem to be sensitive to biological and not chronological aging, are the main B cells involved in the *in vitro* production of this pro-inflammatory cytokine, with no differences between the groups studied. At the

present time, we cannot state which population is more responsible for the impaired cytokine production. Nonetheless, as TNF- α is a pro-inflammatory cytokine recently reported to be increased in a mouse model made of aged B cells (Frasca et al. 2012), we hypothesize that switched memory (IgD⁻CD27⁺) and/or DN (IgD⁻CD27⁻) B cells could be responsible for this cytokine production. We suggest that the expansion of these B cell subsets in the elderly might contribute to the increased inflammatory status designated “inflammaging.”

Taken together, our data document the phenotypic and biological characteristics of a CD19⁺CD38⁻CD24⁻ B cell population and suggest that these cells might act as memory B cells involved in inflammation. Analysis with additional markers, such as IgG, IgA, HLA, CD138, CD43, and CD70, may be useful for a deeper characterization of this subset, as well as investigating them under pathological conditions. We also need to evaluate if they might act as antibody (Ab)-producing cells or develop into Ab-secreting cells, and in particular, whether these cells could be responsible for autoantibody production. Moreover, our data emphasize that modifications to B cell immunity could play a pivotal role during processes associated with aging. A better understanding of B cell immunosenescence is crucial for the improvement of the quality of life of the growing elderly population worldwide. Hence, B cell immune profiling must be included in any attempts to develop useful biomarkers of human immunosenescence. The study of B cell phenotypes in the peripheral blood of centenarian offspring revealed that many parameters are well preserved and more closely resemble that of the young than of either their age-matched controls or old subjects. In any event, other immune parameters, such as CD180 expression and cytokine production, seem not suggest a complete preservation of the B cell branch in the offspring of long-lived subjects. Further analyses are necessary to correlate the environmental and genetic background with a well-preserved immune system of centenarian offspring that could support the hypothesis of a “familial youth” of the immune system.

Acknowledgments This work was supported by the Italian Ministry of Education, University and Research grant (in vitro and ex vivo assessment of immune-inflammatory responses in AD patients) to G. C.-R. This work was also supported by the Deutsche Forschungsgemeinschaft (DFG PA 361/14-1), the Bundesministerium für Bildung und Forschung (BMBF Gerontoshield 0315890F), and the European Commission (EU-FP7

IDEAL 259679) (to G. P.). A. M. is a PhD student of Pathobiology PhD course (directed by C. C.) at the Palermo University, and this work was submitted in partial fulfillment of the requirement for her PhD degree.

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