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RESEARCH PAPER

B cell immunosenescence: different features of naive and memory B cells in elderly

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Abstract Elderly people show a reduced protection against new infections and a decreased response to vaccines as a consequence of impairment of both cellular and humoral immunity. In this paper we have studied memory/naïve B cells in the elderly, evaluating surface immunoglobulin expression, production of the pro- and anti-inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-10, and presence of somatic hypermutation, focusing on the $IgG^{+}IgD^{-}CD27^{-}$ double negative (DN) B cells that are expanded in the elderly. Our results show that naïve B cells from young donors need a sufficiently strong stimulus to be activated "in vitro", while naïve B cells from old subjects are able to produce IL-10 and TNF-α when stimulated "physiologically" (α-CD40/IL-4), suggesting that these cells might play a role in the control of the immuno-inflammatory environment in the elderly. In addition, in the elderly there is an accumulation of DN B cells with a reduced rate of somatic hypermutation. Thus, DN B lymphocytes may be exhausted cells that are expanded and accumulate as a by-product of persistent stimulation or impaired germinal center formation.

Keywords Cytokines · Elderly · Hypermutation · Inflammation · Memory B cells

Introduction

During ageing the humoral immune response is both quantitatively and qualitatively diminished compared with the immune response in young people. Many authors have reported a reduced antibody specificity, affinity, and isotype switch in the elderly (Weksler 2000; Frasca et al. 2004; Schenkein et al. 2008; Cancro et al. 2009; Frasca et al. 2011). As a consequence, old people show reduced protection against new infectious agents and a decreased response to vaccines (Frasca et al. 2010; Frasca and Blomberg 2011). The generation and maintenance of memory lymphocytes is a crucial event in the immune response; in fact, these cells are essential for effective vaccination and facilitate a recall

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(anamnestic) response to previously encountered antigens (Weinberger et al. 2008). Studies on T cell memory have suggested that the filling of the immunological space with memory T cells renders the immune system less able to respond to new antigens (Franceschi et al. 2000a, b) contributing to immunosenescence.

Memory B lymphocytes may also be important in the older immune system, they can be distinguished from naïve B cells by the expression of switched immunoglobulins and by the expression of the CD27 marker, although these markers don't discriminate unequivocally between naïve and memory B cells (Tangye and Hodgkin 2004; Fecteau et al. 2006; Bulati et al. 2011). Subsets of memory B cells can be easily, but not exhaustively, identified by the different expression of surface IgD and CD27 (Shi et al. 2003). In addition, memory B cells are characterized by the presence of somatic hypermutation in the variable gene sequences of the immunoglobulins (Klein et al. 1998).

In a previous paper we have reported the increase in a population of double negative, $IgD^{-}CD27^{-}$, (DN) B cells in the elderly that might be an exhausted pool of memory B cells that fill the immunological B cell space (Colonna-Romano et al. 2009), thus reducing the availability of naïve B cells that is crucial for a response to new antigens. Moreover, naïve and memory B cells produce different pro- and anti-inflammatory cytokines (Duddy et al. 2004; Duddy et al. 2007; Sanz et al. 2007, 2008; Lund 2008) and might play a role in the generation of the inflammatory environment typical of the elderly (Licastro et al. 2005; Vasto et al. 2007).

In aged people a change in the B cell repertoire has also been described, particularly in the heavy chain of BCR. Indeed, an increased oligoclonality and a reduced frequency of somatic hypermutation in the elderly response to pneumococcal vaccination has been reported (Kolibab et al. 2005). The consequence is a collapse in B cell diversity in elderly people which is correlated with poor health status in these subjects (Gibson et al. 2009).

In this report, we have analyzed some characteristics of DN B cells to evaluate whether these cells, that are expanded in the elderly, play any role in the ageing of the immune system and in the generation of the immune-inflammatory environment of the elderly.

Materials and methods

Subjects

Fifty-four healthy Sicilian subjects were studied, 25 young (age range 20–45 years) and 29 elderly (age range 70–86). None of the selected subjects had neoplastic, infectious, autoimmune diseases, or received any medications influencing immune function at the time of the study. All subjects gave informed consent according to Italian law. We have performed the evaluation of surface immunoglobulin expression on separated B cells of all subjects in the study, but we have selected ten young and ten elderly donors for B cell activation and cytokine detection, and three samples of each age class for assessment of somatic hypermutation.

Cell preparation and B cell enrichment

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised venous blood by density gradient centrifugation on Ficoll-Lympholyte (Cedarlane Laboratories Limited, Ontario, Canada). PBMCs were adjusted to 1×10^6/ml in RPMI 1640 medium (Euroclone, Devon, UK) supplemented with 10% heat-inactivated fetal calf serum (Euroclone), 1% penicillin/streptomicin, 10 mM HEPES, and 1 mM L-Glutamin. B lymphocytes were separated from PBMCs by immunomagnetic sorting, as described by Miltenyi et al. (1990) using anti-CD19 magnetic microbeads (MACS CD19 Multisort Microbeads; Miltenyi Biotec, Aubum, CA, USA). Cells obtained from immunomagnetic sorting were >98% $CD19^{+}$ lymphocytes, as determined by flow cytometry analysis.

Immunoglobulin expression on B cell surface

Purified B cells were stained with different combinations of the following monoclonal antibodies (BD, Pharmingen): anti-IgD_{FITC} or anti-IgD_{PE}, anti-IgG_{FITC} or anti-IgG_{PECy7}, anti-IgM_{PE}, anti-IgA_{APC}, anti-$CD27_{PE}$ or anti-$CD27_{APC}$. Cells were washed twice and analyzed. All measurements were made with a FACSCalibur flow cytometer (Becton–Dickinson, San Jose, CA, USA) with the same instrument setting. At least 10^4 cells were analyzed using CellQuestPro (Becton–Dickinson, San Jose, CA, USA) software.

B cell activation and intracellular cytokine detection

Stimulation of B cells with anti-CD40 and interleukin (IL)-4 for intracellular IL-10 and tumor necrosis factor (TNF)-α cytokine detection

Separated B cells (1×10^5/200 µl) were cultured in 96-well round-bottom plates and activated with 1 µg/ml of anti-human CD40 (BD, Pharmingen) and 10 ng/ml of human recombinant IL-4 (BD, Pharmingen) with or without 2 µg/ml of anti-BCR [F(ab')$_2$] (Jackson ImmunoResearch Laboratories, Inc, Philadelphia) for 48 h, at 37°C (CO_2 5%). Cells were harvested, washed and stained with anti-IgD$_{FITC}$ or anti-IgD$_{PE}$, (BD, Pharmingen) and anti-CD27$_{APC}$ (BD, Pharmingen). After two washes, cells were fixed with 100 µl of Perm & Fix Solution A (Caltag Burlingame), washed and permeabilized with 100 µl of Perm & Fix Solution B (Caltag Burlingame). Finally cells were stained with anti TNF-α$_{FITC}$ (BD, Pharmingen) or anti IL-10$_{PE}$ (BD, Pharmingen), washed and analyzed.

Stimulation of PBMCs with CpG/phorbol myristate acetate (PMA)/ionomycin for B cell IL-10 production

For intracellular IL-10 analysis, PBMCs (10^6 cells/ml) were suspended in complete medium with or without CpG-B 2006 (3 µg/ml, Tib Molbiol), PMA (50 ng/mL), ionomycin (1 mM) and monensin (2 mM; eBioscience) in 24-well flat-bottom plates for 5 h, at 37°C (CO_2 5%) (Bouaziz et al. 2010). Cells were harvested, washed and stained with anti-IgD$_{FITC}$, anti-CD19$_{PerCP}$ and anti-CD27$_{APC}$ (BD, Pharmingen). Then cells were fixed with 100 µl of Perm & Fix Solution A (Caltag Burlingame), washed twice and permeabilized with 100 µl of Perm & Fix Solution B (Caltag Burlingame). Finally cells were stained with anti IL-10$_{PE}$ (BD, Pharmingen), washed and analyzed.

Somatic hypermutation assay

B cells, separated by MACS (1×10^7), were stained with 20 µl of anti IgG$_{FITC}$, anti-IgD$_{PE}$ and anti-CD27$_{APC}$ (BD, Pharmingen), then washed and resuspended in 1 ml of PBS/BSA (0,5%). After defining the sorting region gate ($IgG^+IgD^-CD27^-$ or $IgG^+IgD^-CD27^+$) we optimized the sample concentration, verifying the event rate and the sort rate to maximise the efficiency of cell separation. cDNA was synthesised from $IgG^+IgD^-CD27^+$ and $IgG^+IgD^-CD27^-$ cells. Immunoglobulin IgG genes were isolated by semi-nested PCR and sequenced using the Roche Titanium platform as previously reported (Wu et al. 2010). Briefly: a 25-µl PCR1 reaction mix contained 6.25 µl of cDNA, 0.625 U Phusion DNA polymerase (NEB), 200 µM each dNTPs, 41.75 nM 5′ primer (mix of IGHV gene family 1–6 primers) and 250 nM constant region Cgamma primer. A 20 µl of PCR2 reaction mix comprising 0.5U Phusion DNA polymerase, 200 µM each dNTPs, 41.75 nM each 5′ primer mix (multiplex-identifier (MID)-linked IGHV family 1–6 primers) and 250 nM MID-linked Cgamma primer was used to amplify 2 µl of PCR1 products. PCR thermalcycling conditions are as follows: 98°C for (30 s), 15 (PCR1) or 20 (PCR2) cycles of 98°C (10 s); 58°C (15 s); 72°C (30 s), and 1 cycle of 72°C (5 min). Purification of PCR products in sufficient quantity for sequencing and the downstream data processing pipeline are as previously published (Wu et al. 2010). Mutational analysis of VH IgG transcripts was done by comparison with germline sequences from the ImMuno-GeneTics (IMGT) database (available at http://imgt.cines.fr_). The number of mutated nucleotides was determined for each transcript after their alignment with the germline gene.

Statistical analysis

Values (percentage or MFI), given as the mean ± SD or SE, were compared using one-way analysis of variance (ANOVA). Differences were considered significant when a P value < 0.05 was obtained by comparison between the different groups.

Results

Surface immunoglobulin of DN cells

As we are interested in B cell memory, and particularly in DN (IgD^-CD27^-) B cells, we have evaluated the immunoglobulin expression (IgM, IgG or IgA) on B cells, gated on the basis of the presence/absence of IgD and CD27. Concerning naïve (IgD^+CD27^-) and memory unswitched (IgD^+

$CD27^+$) B cells, we found that all these cells are IgM^+ (not shown). Most of the DN cells are IgG^+ (more than 60%), others (more than 20%) are IgA and few of them (less than 10%) are IgM^+ (Fig. 1a). We did not find any age-related differences in the relative expression of IgM, IgG or IgA isotypes in memory DN B cells (IgD^-CD27^-) (Table 1). In contrast, the proportion of IgG, IgA and IgM memory B cells in the classical switched memory compartment (IgD^-CD27^+) is different between young and old (Fig. 1b), with a significant decrease of IgM^+ IgD^-CD27^+ memory B cells in old subjects (Table 2). This is in agreement with previous data that have demonstrated a reduction of IgM "only" memory B cells in the elderly (Shi et al. 2005). We show a slight increase in the expression of IgG and IgA on switched memory B cells but this did not reach significance, although the relative proportions of the different isotypes of memory cells mirrors the relative proportion of serum immunoglobulin of the different isotypes (Paganelli et al. 1992; Listì et al. 2006).

Intracellular cytokine production

In order to assess whether the modifications of the B cell subpopulations described in the elderly might in turn affect the cytokine environment of the aged we have evaluated TNF-α (pro-inflammatory) and IL-10 (anti-inflammatory) production by naïve and memory B cells. To this purpose magnetically sorted B lymphocytes from the two age groups of donors were stimulated by anti-CD40/IL-4 (Frasca et al. 2008). We analyzed the production of IL-10 and TNF-α by $CD27^+$ or $CD27^-$ B cells from young and old donors. As shown in Fig. 2, there is a different pattern of cytokine production between the two age groups: in fact, $CD27^-$ B cells from the elderly produce significantly higher levels of both IL-10 and TNF-α when compared to $CD27^-$ B cells from the young, whereas in the $CD27^+$ B cell subset the young donors produce the higher levels of these cytokines.

Evaluating the specific contribution of each B cell subset to IL-10 and TNF-α production (Table 3), most IL-10 (a) and TNF-α (b) production is from unswitched memory B cells in both young and elderly subjects. Interestingly, naïve B cells from old donors produce a large amount of these cytokines, significantly higher than those produced by naïve B cells from young donors.

In a recent paper by Bouaziz et al. (2010), the authors tested the ability of blood B cells to produce IL-10 after a short stimulation with CpG, PMA and Ionomycin, showing that the combined action of these stimuli was the most potent inducer of IL-10 production. We have performed this kind of analysis in our young and elderly subjects and we show that, unlike the CD40 stimulation, the main IL-10 producing cells are IgD^+CD27^+ memory unswitched B cells and IgD^+CD27^- naïve B cells in both young and elderly people with no significant differences between the two groups (Table 4). So the non-physiological stimulus activates more naïve cells than the physiological stimulus in the young donors. Neither the classical switched memory nor the double negative B cells are activated by the non-physiological "strong stimulus" at any age.

Somatic hypermutation in DN ($CD19^+IgG^+$ IgD^-CD27^-) cells

In order to evaluate the effect of ageing on B cell receptor hypermutation in memory B cell subsets, we have evaluated the number of somatic mutations in the IgG VH regions of $CD27^+$ and $CD27^-$ B cells in young and elderly donors. Our results show that, both in young and in elderly donors, DN memory B cells have significantly fewer mutations than $CD19^+IgG^+IgD^-CD27^+$ classical switched memory B lymphocytes (Fig. 3a), although the rate of mutations observed in the DN $CD19^+IgG^+$ IgD^-CD27^- B cell subset of old people is significantly reduced when compared to the rate of mutations observed in DN cells from young subjects (Fig. 3b). Moreover, there was no significant difference between the two age groups in the $CD27^+$ memory B cell compartment.

Discussion

The ageing of the immune system involves both cell-mediated and humoral immunity as well as aspects of innate immunity. Most researchers have studied the impairment of cell-mediated immune response in the elderly, although B cell function is also modified with age (Cancro et al. 2009; Bulati et al. 2011; Frasca and Blomberg 2011).

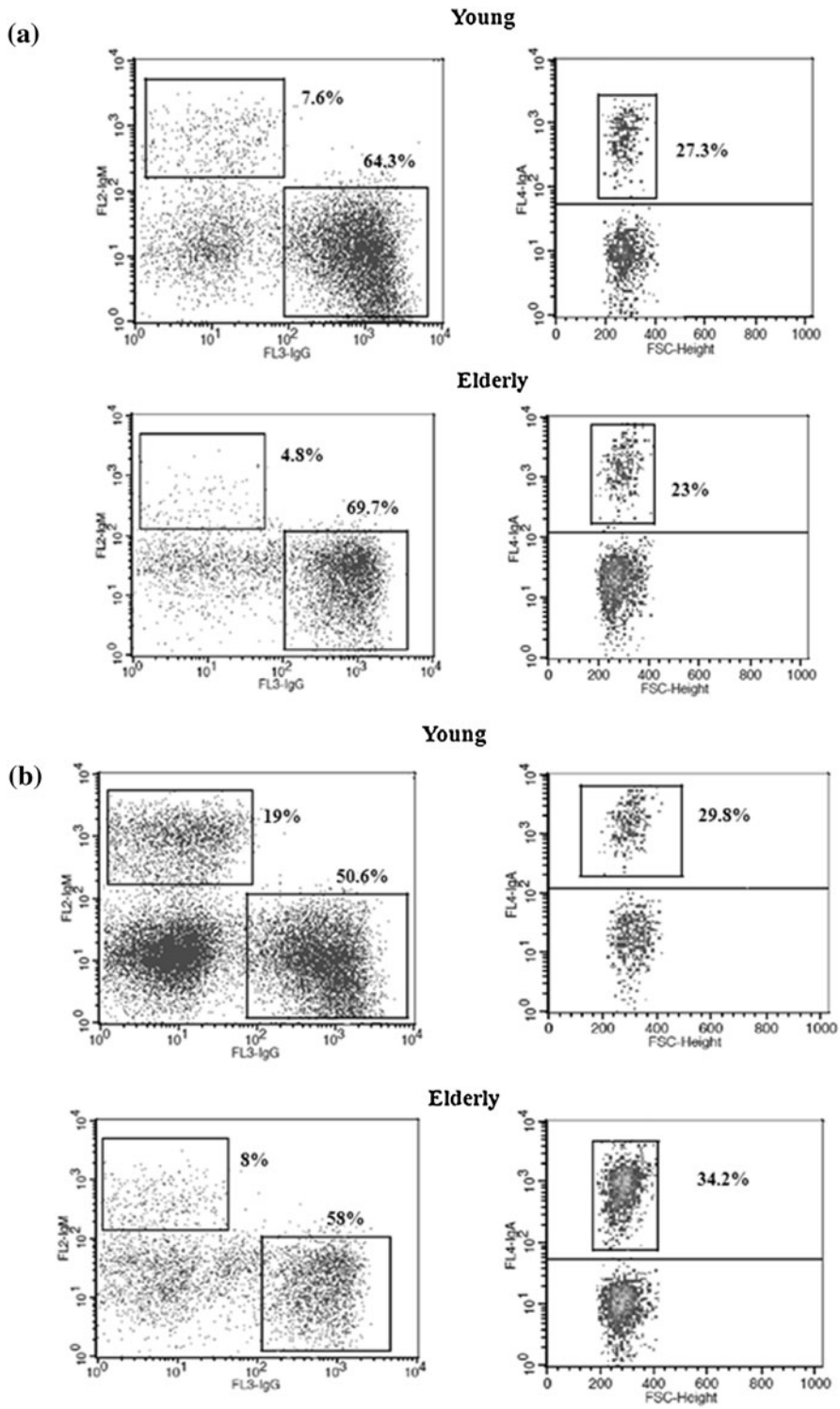


Fig. 1 **a** A typical experiment showing IgM, IgG or IgA expression on gated $IgD^{-}CD27^{-}$ (DN) B cells in young and elderly donors. No difference in expression was observed between the two groups analyzed. **b** A typical experiment showing IgM, IgG or IgA expression on gated $IgD^{-}CD27^{+}$ (memory switched) B cells in young and elderly donors. We show significant decrease of $IgM^{+}IgD^{-}CD27^{+}$ memory B cells in old subjects studied when compared to young

Table 1 IgM, IgG and IgA expression on gated $IgD^{-}CD27^{-}$ (DN) B cells from young and old donors

DN ($IgD^{-}CD27^{-}$) B cells	Young (n = 25) (% Mean±SD)	Elderly (n = 29) (% Mean±SD)	P value
IgM^{+}	6.64 ± 2.6	5.8 ± 3.1	0.7
IgG^{+}	63.7 ± 16.6	68.2 ± 17.3	0.9
IgA^{+}	23.8 ± 9.6	20.8 ± 10.9	0.8

No significant differences have been observed between the two groups studied

Table 2 IgM, IgG and IgA expression on gated $IgD^{-}CD27^{+}$ (memory switched) B cells from young and old donors

Memory switched ($IgD^{-}CD27^{-}$) B cells	Young (n = 25) (% Mean±SD)	Elderly (n = 29) (% Mean±SD)	P value
IgM^{+}	18.6 ± 4.7	8.1 ± 3.8	0.03
IgG^{+}	49.6 ± 8.1	57.2 ± 6.7	0.6
IgA^{+}	37.3 ± 7.7	41.5 ± 8.9	0.8

P value < 0.05, obtained by one way ANOVA nonparametric test, is considered significant

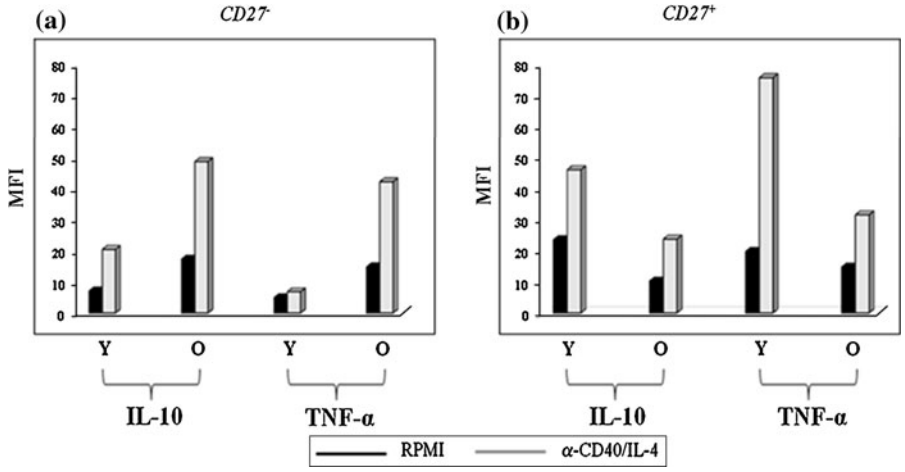


Fig. 2 Evaluation of pro- and anti-inflammatory cytokines production by $CD27^{+}$ and $CD27^{-}$ B lymphocytes in 10 young (Y) and 10 old (O) donors with or without IL-4/CD40 activation. **a** $CD27^{-}$ B cells from elderly subjects produce significantly higher levels of TNF- α and IL-10, both at basal level (TNF-α P value = 0.003, IL-10 P value = 0.02) and after activation (TNF-α P value = 0.002, IL-10 P value = 0.002), when compared with young donors. **b** *Viceversa*, $CD27^{+}$ B cells from young subjects produce higher levels of studied cytokines, when compared with old donors: we found, at basal level, significant differences between two age groups only for IL-10 (P value = 0.01), while, after activation, both cytokines have significant differences (TNF-α P value = 0.04; IL-10 P value = 0.01). (P value < 0.05, obtained by one way ANOVA nonparametric test, is considered significant)

In a previous paper (Colonna-Romano et al. 2009) we have demonstrated the expansion of a B memory subpopulation lacking the classic memory marker CD27 in elderly people, namely double negative (DN, $IgG^{+}IgD^{-}CD27^{-}$) $CD19^{+}$ cells. This kind of memory B cell has also been described by Fecteau et al. (2006) in healthy donors, Wei et al. (2007) in active Lupus patients and by Sanz et al. (2008) in healthy subjects challenged with respiratory syncitial virus (RSV). These cells are probably the consequence of a persistent stimulation of the immune system, for example in patients with lupus (Anolik et al. 2004) where the expansion of these cells correlates positively with the clinical manifestations

Table 3 IL-10 and TNF-α production among B cell subsets in 10 young (Y) vs 10 old (O) donors after CD40/IL-4 activation

	Percentage of IL-10 producing B cells		
	CD40/IL-4		*P*
	Y	O	
(a)			
Naive	15.1 ± 7	40.8 ± 5.1	**0.04**
Memory unswitched	79.6 ± 9.8	58.4 ± 4.9	0.2
Memory switched	3.1 ± 1.8	0.5 ± 0.2	0.2
DN	2 ± 1	0.2 ± 0.1	0.1
	Percentage of TNF-α producing B cells		
	CD40/IL-4		*P*
	Y	O	
(b)			
Naive	7 ± 3	24.9 ± 1.9	**0.01**
Memory unswitched	87.8 ± 4.8	72.3 ± 9	0.2
Memory switched	4.6 ± 3	1.6 ± 0.9	0.4
DN	0.5 ± 0.1	1.1 ± 0.8	0.5

In table (a) and (b) it is shown as the major contribute on the production of the two studied cytokines is given by unswitched memory B cells in both young and elderly subjects. Moreover, among the IL-10 and TNF-α producing cells from elderly donors, we show a significant amount of naïve B cells. Data are expressed as percentage (Mean ± SD). (*P* value < 0.05, obtained by one way ANOVA nonparametric test, is considered significant)

Table 4 IL-10 producing cells among B cell subsets in 10 young (Y) vs 10 old (O) donors after CpG/PMA/IONOMYCIN activation

	Distribution of B populations inside IL-10 producing B cells		
	CpG/PMA/IONOMYCIN		*P*
	Y	O	
Naive	28.6 ± 4.6	37.7 ± 15.4	0.6
Memory unswitched	64.3 ± 9.2	59.3 ± 16	0.8
Memory switched	5.6 ± 4	1.4 ± 0.2	0.3
DN	1.4 ± 0.6	1.6 ± 0.7	0.9

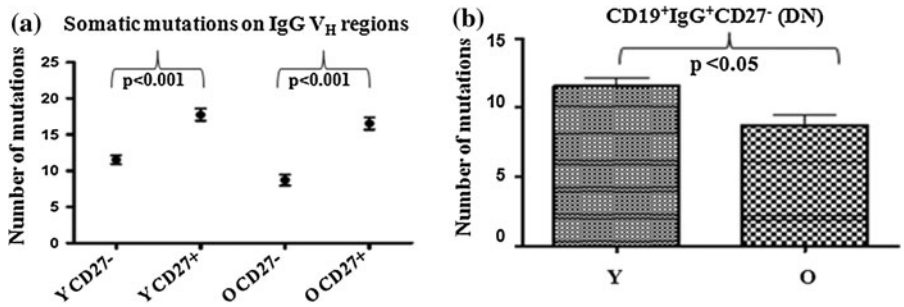
Data are expressed as percentage (Mean ± SEM). (*P* value < 0.05, obtained by one way ANOVA nonparametric test, is considered significant)

of the disease (Wei et al. 2007). Intriguingly we have reported (Colonna-Romano et al. 2010) that DN B cells are not increased in a "genetically advantaged" cohort of centenarian's offspring that also show a higher level of naïve B cells compared to their age-matched controls (70–80 years old).

In the present paper we have further studied memory/naïve B cells in the elderly, evaluating surface Immunoglobulin (sIg), production of pro-and anti-inflammatory cytokines and somatic hypermutation.

Our data confirms a previous report of reduced IgM only ($IgM^+IgD^-CD27^+$) memory B cells in old subjects (Shi et al. 2005), although the compensatory increase in the expression of IgG and IgA on switched memory $CD27^+$ B cells did not reach significance. No significant differences were observed in the expression of the different immunoglobulin classes between old and young donors in DN B cells.

It has been described (Duddy et al. 2004; Duddy et al. 2007; Sanz et al. 2007, 2008; Lund 2008) that



Subjects studied	Number of sequences analyzed	Comparison between-p value
Young (Y) = 3	Y CD27+ = 79	Y CD27- Vs Y CD27+ : **p < 0.001**
	Y CD27- = 123	O CD27+ Vs O CD27- : **p < 0.001**
Old (O) = 3	O CD27+ = 123	Y CD27+ Vs O CD27+ : p > 0.05
	O CD27- = 117	Y CD27- Vs O CD27- : **p < 0.05**

Fig. 3 Evaluation of somatic mutations on IgG V_H regions in 3 young (Y) and 3 old (O) subjects. **a** Both in young and in the elderly donors, $CD19^+IgG^+IgD^-CD27^-$ ($CD27^-$) B cells have shown significantly fewer mutations than $CD19^+IgG^+IgD^-CD27^+$ ($CD27^+$) B lymphocytes. **b** Frequency of somatic mutations on $CD19^+IgG^+CD27^-IgD^-$ (DN) B cells evaluated in young and old subjects. The latter show a significantly reduced rate of mutations when compared to the former (P value < 0.05, obtained by one way ANOVA nonparametric test, is considered significant). The number of sequences analyzed are summarized in the bottom of the figure

naïve and memory B cells are able to produce different levels of pro- and anti-inflammatory cytokines (TNF-α and IL-10). Moreover it is known that elderly people show a pro-inflammatory micro-environment that has been related to the increased risk of morbidity and mortality (Licastro et al., 2005; Vasto et al. 2007). With this in mind we have evaluated whether changes in the relative proportions of different B cell populations could affect the cytokine environment. Here we show that in both young and old donors the physiological (α-CD40 and IL4) stimulation of B cells induces a good IL-10 and TNF-α production by unswitched (IgD^+CD27^+) memory B cells. Interestingly, in the elderly, naïve B cells are also highly activated to produce cytokines under these conditions. Not all studies are in agreement with respect to the identification of B cell subpopulations responsible for IL-10 (a very relevant anti-inflammatory cytokine) production in humans. In fact this feature has been attributed to both memory ($CD27^+$, principally IgD^+CD27^+) (Amu et al. 2007) and immature transitional ($CD38^{high}CD24^{high}$) B cell pools (Blair et al. 2010). On the other hand, Bouaziz et al. (2010), have shown that both IgD^+CD27^- (classically naïve) and IgD^+CD27^+ (memory unswitched) B cells participate in IL-10 production by evaluating the differential expression of IgD and CD27, or CD38 and CD24 on IL-10-producing B cells after a "strong" (CpG/PMA/Ionomycin) activation. In our study we confirm that the "strong" activation induces IL-10 production by IgD^+CD27^- naïve and IgD^+CD27^+ unswitched memory B cells in the control (young donors), we also show that the old donors behave similarly in that both naïve and unswitched memory B cells from old subjects are activated to produce IL-10. Taken together our IL-10 production data might suggest that naïve B cells from young donors need an adequate stimulus (e.g. TLR engagement) to be activated "in vitro" but B cells from old subjects have higher "in vitro" basal levels of IL-10 and TNF-α production (not shown) and therefore may already be basally activated, possibly by bacteria (which can be harbored in places such as the urinary tract) or viruses (such as CMV). It has been demonstrated (Lampropoulou et al. 2008) that TLR2, TLR4 and TLR9 engagement induces IL-10 production by splenic B cells in the mouse. In addition, as proposed by Rieger and Bar-Or (2008), IL10 production by naïve B cells may act as a control mechanism to prevent the exacerbation of inflammation in an autoimmune context, whereas IL10 production by memory B cells might be active in

the resolution of the disease. In the evaluation of these results we have kept in mind that transitional ($CD24^{high}CD38^{high}$) B cells are also present in the $IgD^{+}CD27^{-}$ (naïve) gate. These are known to have regulatory properties by production of the anti-inflammatory cytokine IL-10 (Blair et al. 2010), so it could be hypothesized that in the elderly there is an increased number of IL-10-producing transitional B cells with regulatory function. We suggest that the cytokines produced in young donors are active in modulating the size of immune response, whereas in the elderly the higher production of cytokines by naïve B cells may be due to a basal overstimulation of the immune-inflammatory system.

To gain insight into the biological significance of the different naive and memory B cell subsets, we have studied the level of mutation in the V region of IgG and showed that $CD27^{+}$ B cells have a significant higher number of somatic mutations both in young and old donors compared with the $CD27^{-}$ B cells. No significant differences in the level of hypermutation in $CD27^{+}$ cells are observed between the two age groups. In contrast, the rate of mutations in the DN ($IgG^{+}IgD^{-}CD27^{-}$) B cell population is lower in the elderly. It is well known that memory B cells are characterized by the high rate of somatic hypermutation, and that this event occurs in the germinal center and that it involves T-B cell interaction. So our results might be due to different circumstances. As previously published (Bulati et al. 2011), and as shown here, $IgG^{+}IgD^{-}CD27^{-}$ DN B cells show a low frequency of somatic mutation, and this supports the theory that these cells might emerge independently from T cell help, or from CD40-CD154 interaction. Alternatively, the reduced amount of somatic mutation in the old group might be due to reduced co-stimulation of B cells in the elderly (Weiskopf et al. 2009) as a result of intrinsic T cell defects, although this would also affect the rate of mutation in the classical switched memory ($IgG^{+}IgD^{-}CD27^{+}$) B cells which is not seen here. This leads us to the hypothesis that there is a large population of DN cells that are unrelated to classical memory B cells. The increase of the double negative memory B cells in the elderly (Colonna-Romano et al. 2009; Bulati et al. 2011), together with the reduced rate of mutation shown here, might be due to the disconnected generation of these cells from germinal centers, as it has been demonstrated that ageing negatively affects the germinal center formation in secondary lymphoid tissues (William et al. 2002; Frasca et al. 2005).From this and our previous papers (Colonna-Romano et al. 2009, 2010) we can conclude that DN B lymphocytes are exhausted cells. In fact they are not activated by anti-CD40/IL4, or by CpG/PMA/Ionomycin and behave differently as CMV-specific effector- memory CD8 lymphocytes. These are T cells usually supposed to be terminally differentiated and unable to proliferate, although they can be activated when appropriately stimulated "in vitro" (Waller et al. 2007). It might be interesting to know whether these cells are a by product of time-enduring stimulation by an unknown, infectious agent. One possible candidate would be CMV, since Chidrawar et al. (2009) have demonstrated that CMV infection in the elderly influences not only T lymphocytes (Pita-Lopez et al. 2009; Pawelec et al. 2009, 2010), but also negatively affects B cells.

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