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Double negative (CD19⁺IgG⁺IgD⁻CD27⁻) B lymphocytes: A new insight from telomerase in healthy elderly, in centenarian offspring and in Alzheimer's disease patients

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ABSTRACT

Immunosenescence is characterized by the impairment of humoral immunity with changes in the memory/naive B cell compartment. In particular we have previously reported the percentage increase of a Memory IgD⁻CD27⁻ (Double Negative, DN) B cell population in aged people. In this study, we have further characterized DN B cells with the aim to better understand their contribution to immunosenescence. As DN B cells show a poor ability to proliferate in vitro, we have evaluated the expression of the inhibitory receptors CD307d and CD22 on these cells from young and old individuals. In addition we have evaluated the ability to activate DN B cells by the simultaneous use of innate (CpG) and adaptive (α -Ig/CD40) ligands. Our data demonstrate that the refractoriness to proliferate of DN B cells does not depend on the expression of inhibitory receptors, but it is due to the kind of stimulation. Indeed, when DN B cells are stimulated engaging both BCR and TLR9, they become able to proliferate and reactivate the telomerase enzyme. In the present study, we have also compared the telomerase activity in a group of people genetically advantaged for longevity as centenarian offspring (CO) and in a group of moderate–severe Alzheimer's disease (AD) patients, who represent a model of unsuccessful aging. Our study suggests that telomerase reactivation of DN B cells, as well as their number and ability in activating, depend essentially by the biological age of the subjects studied, so the evaluation of DN B cells might allow to gain insight to healthy and unsuccessful aging.

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1. Introduction

Advancing age yields numerous immune system changes in both innate and adaptive immune system, responsible of blunted primary and recall response, feeble vaccine efficacy and increased prevalence of inflammatory pathologies [1–5]. In particular, the humoral branch of the immune system is characterized, in aged individuals, by reduction of circulating B cells, lack of B clonotypic immune response to new extracellular pathogens, impaired class switch recombination and affinity maturation [6–8]. Moreover, the proportion of different subsets of B cells is also altered as reviewed

by Bulati et al. [9]. We and others [10,11] have shown that, in the elderly, there is a significant decrease of naive (IgD⁺CD27⁻) B cells and no significant reciprocal increase of CD27⁺ memory B lymphocytes (IgD⁺CD27⁺, IgD⁻CD27⁺) [12,13]. In contrast, others [7,14] report a different scenario with an increase of naive and a decrease of memory B cells. Besides, in the elderly, we have also demonstrated the increase of a B cell population identified as IgD⁻CD27⁻ (DN B cells) [9,10]. These cells are also increased in patients affected by chronic immune inflammation, such as chronic HIV infection [15], systemic lupus erythematosus (SLE) [16,17] and in healthy subjects challenged with respiratory syncytial virus (RSV) [18]. As the “classical memory” IgD⁻CD27⁺ B cells, DN B cells also show features of memory lymphocytes, as most of them are IgG⁺ (and less IgA⁺), have low levels of ABCB1 and short telomeres [10,19]. Furthermore, DN B cells have characteristics of cell senescence, including incapacity to respond to CpG stimulation, even if they can be weakly activated with F(ab')₂ (anti-IgG) [10]. Taken together all

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these data suggest that the amount of circulating naive and DN B cells is strictly related to the “biological age” of the immune system i.e. the long term stimulation due to chronic stimuli or age. Moreover it seems that also genetic background plays a role as supported by our previous studies on centenarian offspring (CO), “genetically advantaged” for longevity, who have percentages of DN and naive B cells more similar to those observed in young donors, than those observed in coeval healthy elderly donors [20]. Besides, Alzheimer’s Disease patients (AD), who represent a model of “unsuccessful ageing”, show, when compared with their age matched healthy control donors, a dramatic reduction of total B cells [21,22] and, in particular, a reduction of naive B cells and high levels of DN B cells, (*manuscript in preparation*).

On these basis, the aim of the present study was to further characterize DN B cells evaluating some phenotypic and functional activities. In particular, we investigated whether the low ability of DN B cells to proliferate, after the *in vitro* stimulation, depends on the expression levels of the CD307d and CD22 inhibitory receptors, as demonstrated in exhausted tissue-like memory B lymphocytes from HIV-viraemic individuals [23]. In addition, we evaluated whether DN B cells can be stimulated and proliferate under particular conditions, as demonstrated in a murine model [24], even if they have short telomeres. Reduced telomere length has been associated with increased morbidity and mortality [25]. Moreover, in AD patients the telomere length is reduced and the telomere erosion is related to mortality and increased risk for dementia [26]. It is well-known that telomere elongation is mediated by telomerase enzyme, whose activity is under dynamic control. Indeed, acute stress, e.g. cortisol [27], may compromise telomerase activity, while mitogenic stimulation, e.g. antigen stimulation for B lymphocytes [28], may induce it [29,30]. Thus, we detected the ability of DN B cells to reactivate telomerase by the engagement of both innate and adaptive immune receptors. Besides, we also extended this analysis to DN B cells obtained from CO and moderate–severe AD patients, in order to evaluate whether the deep study of DN B cells might allow to gain insight to healthy and unsuccessful aging.

2. Materials and methods

2.1. Subjects

Sixty-three Sicilian subjects, 20 young (age range 25–40 years), 20 elderly (age range 78–90 years), 8 CO (age range 60–70), 7 age-matched controls (AM) (age range 63–74) and 8 moderate–severe AD patients (age range 69–76) were enrolled. In particular, CO have almost one of their parents centenarian (>99 years). AD subjects included in the study were assessed with a multidimensional protocol including: demographic characteristics, medical history, pharmacological treatments, clinical, neuropsychological and neurological examination, standard laboratory blood tests and neuro-imaging study with CT and/or MRI scan. The exclusion criteria were used, including a diagnosis of severe systemic disorder, the presence of psychosis, a history of significant head injury or substance abuse. Diagnosis of probable AD was performed according to standard clinical procedures and the DSM-IV criteria [31] and the criteria of the National Institute for Neurological and Communicative Disorders and Stroke–Alzheimer Disease and Related Disorders Association (NINCDS-ADRDA) [32]. Cognitive performance and alterations were measured according to the Mini Mental State Examination (MMSE) and the Global Deterioration Scale. According to MMSE [33] AD patients were affected by moderate–severe dementia (score ≤ 17).

Whole blood samples were collected by venopuncture in vacuum tubes containing ethylenediamine tetraacetic acid. The

samples were kept at room temperature and used within 2 h for the various experiments.

The University Hospital Ethics Committee approved the study, and informed consent was obtained from all care givers of patients and controls according to Italian law.

2.2. B lymphocytes immune-magnetic separation, *in vitro* B cells stimulation, DN B (CD19⁺IgG⁺CD27⁻) lymphocytes FACS-sorting

Peripheral blood mononuclear cells (PBMCs) were isolated 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 Bovin Serum (Euroclone), 1% penicillin/streptomycin, 10 mM HEPES, and 1 mM L-Glutamin. B lymphocytes were separated from PBMCs by immunomagnetic sorting, as described by Miltenyi et al. [34], using anti-CD19 magnetic microbeads (MACS CD19 Multisort Microbeads; Miltenyi Biotec, Auburn, CA, USA). Cells obtained from immunomagnetic sorting were >98% CD19⁺ lymphocytes, as determined by flow cytometry analysis.

Purified B cells (1×10^5 /200 μ l) were cultured in 96-well round-bottom plates, in complete RPMI medium with 10% Fetal Bovin Serum in absence or presence of 2 μ g/ml of anti-IgG [F(ab')₂] (Jackson ImmunoResearch Laboratories, Inc, Philadelphia), 3 μ g/ml of CpG-B 2006 oligodeoxynucleotide (TIB Molbiol, Genova, Italy), and 500 ng/ml of anti-human CD40 purified (BD, Pharmingen) for 72 h, at 37 °C in 5% CO₂.

To obtain DN B cells for TRAP assay, immunomagnetically sorted B cells were treated as follows: after 72 h of culture, B cells, stimulated or not, as above mentioned, were stained with 20 μ l of anti-IgG_{FITC}, anti-CD27_{PE} and anti-CD19_{APC} (Pharmingen, BD Bioscience, Mountain View, CA, USA) for 30 min at 4 °C. Next, cells were washed and 1 ml of PBS/BSA (4%) was added. After defining the sorting region gate of CD19⁺IgG⁺CD27⁻ (Double Negative, DN B cells) population, we optimized the sample concentration, verifying the event rate and the sort rate to maximize the efficiency of cell separation. Finally, DN B lymphocytes were sorted by a FACS-Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and collected in cytometry tubes and used for telomerase activity measurements.

2.3. Flow cytometry analysis

2.3.1. Phenotypic analysis

For phenotypic analysis, immunomagnetically purified B cells were stained with different combinations of the following monoclonal antibodies: anti-IgG_{FITC}, anti-CD27_{PE} or anti-CD27_{APC}, anti-CD22_{PE-Cy5} (BD, Pharmingen) and anti-CD307_{dPE} (FcRL4) (BioLegend). Cells were washed twice and analyzed.

Cell proliferation of immunomagnetically sorted B cells was performed by Ki67 evaluation as described [10] using the following combination of MoAb: anti-IgD_{PE} or anti anti-IgG_{PE}, anti-CD19_{PECy5} and anti-CD27_{APC} and anti-Ki67_{FITC} (Becton Dickinson). All measurements were made with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with the same instrument setting. At least 10⁴ Cells were analyzed using CellQuestPro (Becton Dickinson, San Jose, CA, USA) software. To evaluate MFI values, before each detection, spectral overlap of fluorescent signals was minimized by electronic compensation with Calibrite beads. Isotype-matched negative controls were used to determine the background of fluorescence.

2.4. Detection of telomerase activity by TRAP assay

For quantitative analysis of telomerase activity, a Telomeric Repeat Amplification Protocol (TRAP) [35] and a photometric

enzyme immunoassay were performed using a TeloTAGGG Telomerase PCR Elisa-Plus kit (Roche Diagnostics, Indianapolis, USA), according to the manufacturer's protocol. This precisely involved elongation and amplification of telomerase reaction products to allow highly sensitive detection of telomerase activity using a photometric enzyme immunoassay. In addition, cellular extracts from human CD19⁺ or DN B lymphocyte cultures under baseline conditions or activated, incubated for 72 h and sorted (as above described) were utilized.

Briefly, we firstly obtained pellets of sorted CD19⁺IgG⁺CD27⁻ cells through centrifugation at 3000 × g for 5 min at 2–8 °C. They were lysed directly in sterile reaction tubes using the lysis buffer provided in the kit. Protein lysate was kept in ice for 30 min and centrifuged at 16,000 × g for 20 min at 2–8 °C. Protein concentration was measured by standard methods. Subsequently, the supernatants obtained were utilized in quantity of 0.5–10 µg of total protein for the TRAP reaction, having the assurance to prepare, for each sample, a negative control by heating at 85 °C for 20 min. In performing the TRAP reaction, high control template (concentration 0.1 amol/µl; quantity used for each reaction 1 µl), a reaction mixture (25 µl for each sample), an Internal standard (IS; 5 µl for each sample) provided in the kit were also utilized. Thus, sterile tubes (each containing a total of 30 µl of the master mix-25 µl of reaction mixture and 5 µl of IS- and a suitable volume of each negative or positive sample or 1 µl of control template) were transferred to thermal cycler (MyCycler, Biorad). A combined primer elongation/amplification reaction was performed according to the manufacturer's protocol. During the reaction, telomerase adds telomeric repeats (TTAGGG) to the 3' end of the biotin-labeled primer. The elongation products, as well as the IS included in the same reaction tube, were then amplified. The PCR products were split into two aliquots, denatured, bound to a streptavidin-coated 96-well plate and hybridized to a digoxigenin (DIG)-labeled telomeric repeat-specific probes, specific for the telomeric repeats and IS. The resulting products were immobilized via the biotin label to streptavidin-coated 96-well microplate. Immobilized amplicons were then detected with an antibody against digoxigen that is conjugated to horseradish peroxidase (anti-DIG-HRP) and the sensitive peroxidase substrate TMB. Sample absorbance was measured at 450 nm (reference wavelength 690 nm) using an ELISA plate reader within 30 min after the addition of the stop reagent. Absorbance values were then utilized to calculate the relative telomerase activity of each sample using an appropriate formula provided in the kit protocol.

2.5. Statistical analysis

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 ± standard error of the mean (SEM).

3. Results

3.1. Expression of the BCR-inhibitory receptors CD307d and CD22

We evaluated the expression of two BCR-inhibitory receptors, CD307d (FcRL4) and CD22, on B cells from young and old donors. As shown in Table 1, no significant differences were observed, although B cells from elderly showed a higher (marginally significant) expression of CD307d. No significant differences, between the two age groups, were also detected analysing B cells on the bases of CD27. In particular, DN B cells expressed very low levels

of CD307d whereas CD22 was well expressed both in young and elderly donors. These data evidence that age does not influence the modulation of these inhibitory receptors. Thus, the proliferation ability of total and DN B cells from young and elderly does not depend on the expression of these two inhibitory receptors.

3.2. Activation of total, naive and memory B cells by CpG/α-IgG/α-CD40

We also assessed, in young and elderly subjects, the ability of total, naive and memory (unswitched, switched and DN) B cells to proliferate in response to different stimuli.

The proliferation data, evaluated as intracellular expression of Ki67 in B cells obtained from young and elderly donors, are reported in (Fig. 1A). As shown, CpG and α-IgG/α-CD40 activated B cells from young donors. On the contrary, B cells from old subjects had very low levels of activation (Fig. 1A). Moreover, the engagement of both the innate and the adaptive receptors of total B cells caused a good proliferation in young and, interestingly, a very high response by B cells from elderly subjects. Besides, as shown in Fig. 1B, the DN B cell population, that is increased in the elderly, is strongly activated both in young and in elderly donors by CpG/α-IgG/α-CD40, whereas the stimulation with CpG or α-IgG/α-CD40 are able to activate only DN B cells from young donors. As DN B cells are a very low fraction of total B lymphocytes, to further evaluate which B cell subpopulation contributes to the total B cell proliferation in the elderly, we have evaluated Ki67 expression in the four B cell subpopulation, identified by IgD and CD27 expression. As shown (Fig. 2) all the subpopulations from both young (Fig. 2A) and elderly donors (Fig. 2B) are activated by the use of CpG/α-IgG/α-CD40. Therefore, we conclude that all B cell subsets, including DN B cells from elderly donors, can be strongly activated by the use of CpG/α-IgG/α-CD40.

3.3. Relative telomerase activity (RTA) in IgD⁻CD27⁻ DN B cell subset upon in vitro stimulation

As mentioned in section 1, DN memory B cells had very short telomeres both in young and elderly subjects. As result, their proliferation rate might depend on the ability to reactivate telomerase. To verify whether the stimulation with the combined stimuli (CpG/α-IgG/α-CD40) also induces telomerase reactivation, we assessed the Relative Telomerase Activity (RTA) mean values in DN B cells stimulated in vitro for 3 days. Fig. 3 shows that this kind of stimulation induces a strong activation of telomerase in DN B cells from young donors (RTA 40 ± 3.1), and a significant lower level of activation is shown in all the groups of elderly compared with the young group. Moreover, comparing one another the data obtained from the in vitro stimulation of DN B cells from healthy elderly, CO, moderate–severe AD patients and their age-matched controls (AM), we show interesting differences among the groups studied. In particular, we prove that AD patients have the lowest significantly level of RTA (4.6 ± 0.4), whereas healthy elderly and AM controls show super imposable levels of RTA (8.8 ± 1.2 and 8.8 ± 1.4, respectively) although they differ by about ten years of age. On the other side CO show levels of RTA (14.4 ± 2.5) that are increased (marginally significant) when compared with the data obtained from the elderly studied (both healthy elderly and AM), whereas the result reach significance when compared with AD patients.

4. Discussion

Humoral immunity in aged people is known to be both qualitatively and quantitatively impaired [3,5,9,36]. Indeed, it has been widely demonstrated that, in the elderly, there is a decrease in percentage and absolute number of total B lymphocytes and an impairment of specific Ig production [37–39].

Table 1
Evaluation of BCR-inhibitory receptors (CD307d and CD22) on total (CD19⁺), CD27⁻, CD27⁺ and DN (IgD⁻CD27⁻) B cells from 20 young and 20 elderly donors. Data are expressed as median, P25 and P75 of Mean Fluorescence Intensity (MFI).

Inhibitory receptor	Median (P25-P75)	Total (CD19 ⁺) B cells	CD27 ⁻ B cells	CD27 ⁺ B cells	Double Negative (DN) (IgD ⁻ CD27 ⁻)	
Young		26.2 (22.6–29.8)	43.5 (42.9–44.1)	87 (58.9–115.2)	15.7 (14.6–16.7)	CD307d
Elderly		40 (36.9–48.5)	58.7 (54.8–73)	101.9 (87.5–122.1)	21.2 (20–27.8)	
<i>p</i> Young vs Elderly		0.07	0.4	0.2	0.1	
Young		356.8 (329.6–382)	406.3 (365.3–431.2)	335.6 (325–377)	353.3 (299.7–437.5)	CD22
Elderly		356.6 (356.3–493.6)	410.6 (356.2–535.6)	365 (278.4–380.2)	359.7 (232.9–378)	
<i>p</i> Young vs Elderly		0.3	0.5	0.4	0.3	

Focusing on B cell subsets, there are controversial findings about the naive/memory B cells distribution during the aging process [9]. We and others [10,11] have demonstrated the reduction of naive (IgD⁺CD27⁻) B lymphocytes, that are crucial for the response to new antigens. At the same time, we have demonstrated the increase in percentage, but not in absolute number, of a “Double negative” (IgG⁺IgD⁻CD27⁻) memory B cell population in elderly people. As previously shown [10] these cells can be depicted as a senescent pool of memory B lymphocytes in as much as they have short telomeres, low levels of ABCB1 and low replicative ability after “*in vitro*” incubation with physiological (CpG) stimulation. Other evidences have reported the expansion of this B cell subset in patients chronically stimulated (i.e. HIV, SLE and RSV challenged subjects) [15,18,40]. Interestingly, in SLE patients the increase of

DN B cells is correlated with the disease activity index [17], so the increase of DN B lymphocytes could be the consequence of a time-enduring stimulation related to age-associated physiologic modifications, or to pathologic deregulation of the immune system [41–44]. Accordingly, it has been extensively demonstrated that chronic antigenic stimulation leads to the accumulation of late differentiated, antigen specific CD8⁺ T cells, characterized by critically shortened telomeres, loss of CD28 and gain of CD57, typical markers of senescent cells [45]. Moreover, there are evidences that this T cell population plays a significant role in various diseases associated with chronic inflammation, and has a great influence on age-related changes of the immune system [46–49].

We have previously demonstrated that DN B cells from young and elderly donors are differently activated both in terms of

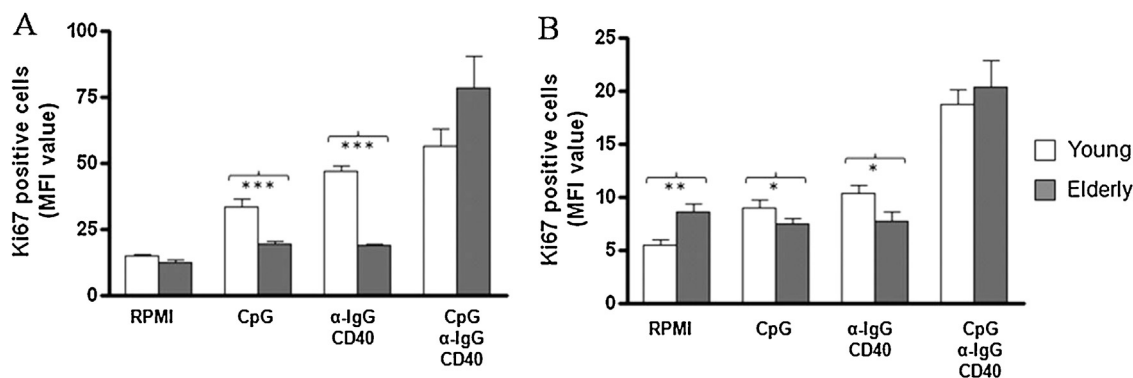


Fig. 1. Ki67 evaluation on CD19⁺ total (A) and Double Negative (DN) (IgD⁻CD27⁻) B cells (B) before and after stimulation with CpG or α-IgG/CD40 or CpG/α-IgG/CD40. Bar charts illustrate the mean MFI and SEM of Ki67 expression in 10 young (age range 25–40 years) and 10 elderly (age range 78–90 years). Significant differences are evaluated by Mann–Whitney nonparametric *U* testing and are indicated by **p* < .05, ***p* < .01, ****p* < .001.

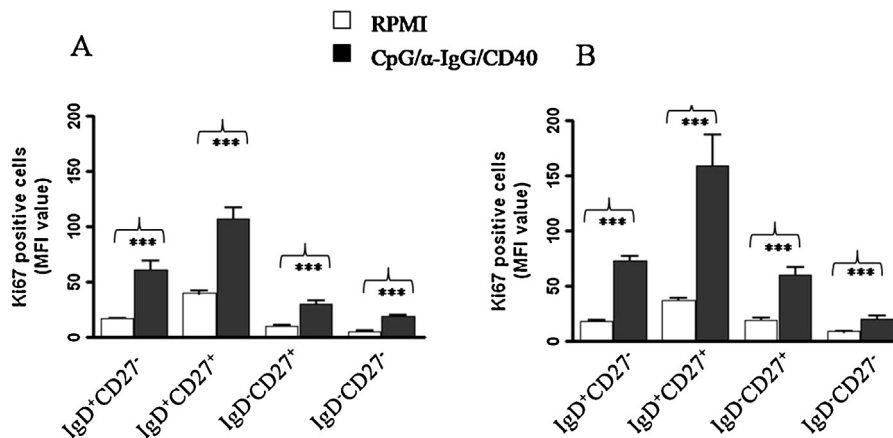


Fig. 2. Ki67 evaluation on IgD⁺CD27⁻, IgD⁺CD27⁺, IgD⁻CD27⁺ and IgD⁻CD27⁻ from young (A) and elderly donors (B). Bar charts illustrate the mean MFI and SEM of Ki67 expression in 10 young (age range 25–40 years) and 10 elderly (age range 78–90 years). Significant differences are evaluated by Mann–Whitney nonparametric *U* testing and are indicated by **p* < .05, ***p* < .01, ****p* < .001.

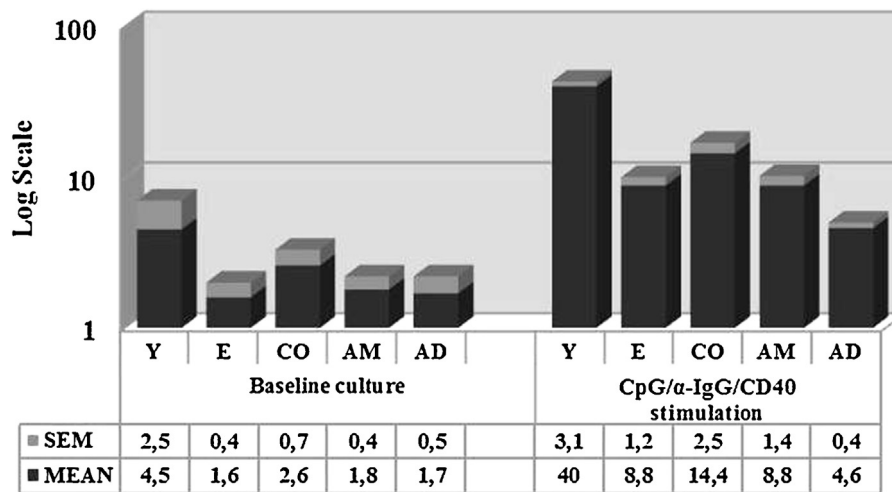


Fig. 3. The relative telomerase activity (RTA) in Double Negative (DN) (IgD⁻CD27⁻) B cells was measured before and after stimulation with CpG/α-IgG/CD40 in 7 young controls (Y) (age-range 25–40), 8 elderly subjects (E) (age-range 78–90), 8 centenarian offspring (CO) (age range 60–70), 7 age-matched controls (AM) (age range 63–74) and 8 Alzheimer’s disease patients (AD) (age range 63–74). Bar charts illustrate the mean and SEM of RTA (values are in a logarithmic scale). Significant differences are evaluated by Mann–Whitney nonparametric *U* testing and are indicated by **p* ≤ .05, ***p* ≤ .01, ****p* ≤ .001. Y vs all different aged groups (E, CO, AM, AD) = ***; E vs AD = *; CO vs AD = **; AD vs AM = *.

proliferation [10] and cytokine production [50,51]. In recent years, Moir et al. [23] have described the increase of an exhausted B cell population, named “tissue-like” B cells, in HIV-viraemic patients. This B cell population (CD20⁺CD27⁻CD21⁻) is characterized by the high expression of some inhibitory receptors, among which CD307d and CD22 are probably responsible of their low ability in proliferating.

As IgD⁻CD27⁻ DN B cells, especially in elderly people, seem to be refractory to activation, we evaluated the expression of the aforementioned BCR inhibitory receptors on these cells. Our results revealed that, differently to “tissue-like” B cells, DN B cells express very low levels of CD307d. On the other hand, the inhibitory receptor CD22 is well expressed in all B cell populations without any difference. Moreover we did not detect any difference between the two age groups studied, with the exception of CD307d slightly increased (not significantly) on total B cells from elderly donors. In this way, the reduced ability of B lymphocytes from old donors to respond to immune challenge cannot be fully attributed to a mechanism lying on the expression of these molecules. This result might suggest that “tissue-like” B cells and DN B cells are differently controlled in their ability to proliferate after BCR engagement and supports the hypothesis that the chronic stimulation, due to the time-enduring exposure to different antigens, might render these cells less able to proliferate [10].

The data obtained in the present study demonstrate that DN B cells, from young and elderly donors, strongly respond to the simultaneous engagement of both the innate receptor (stimulated by CpG) and the adaptive (stimulated by α-IgG/CD40) one. These evidences lead to suppose that DN B cells are analogous to a mature B cell subset that accumulates with age, as described by Hao’s group [24] in old mice. These cells are also IgD negative, proliferate actively to combined TLR and BCR stimuli and seem to be refractory to activation by adaptive immune receptors, although they respond to TLR9 or TLR7 ligation. Unlikely from what was observed in young, stimulation with CpG/α-IgG/CD40 seems to be the sole way to properly activate total B cells from the elderly, without any differences between the different subsets of B cells identified by IgD and CD27.

The hypothesis that DN are senescent cells is also supported by our previous observation in which we described them as cells with short telomeres [10]. Moreover the activation by CpG/α-IgG/CD40 renders these cells similar to CD8⁺CD28⁻CD45RA^{high} senescent T

cells that were considered unable to proliferate under antigenic stimulation although, as demonstrated, that they proliferate under particular conditions [52], despite their short telomeres and the loss of telomerase activity [53]. One possible explanation is that they need specific milieu consisting of particular co-stimuli and/or cytokines, which are able to induce the up-regulation of telomerase activity and enable these cells to divide [45]. In this perspective, we investigated whether the kind of stimulus that influences the proliferative response could re-activate telomerase in senescent DN B lymphocytes whose increase seems to be related to chronic antigenic stimulation as for CD8 terminal differentiated senescent cells.

During cell division, the number of telomere repeats decreases leading to cell senescence or apoptosis. This phenomenon is counteracted by telomerase activity. This enzyme adds telomeric repeats to the G-rich strand and its activity decreases with age [54–56]. Furthermore, it is also known that telomerase is constitutively expressed in germ-line cells [54,57,58] and is absent or low expressed in adult differentiated cells. It is believed that the up-regulation of telomerase might play a role in maintaining lymphocyte replicative potential and function [59]. For this reason, we examined the relative telomerase activity (RTA) after stimulation with CpG/α-IgG/CD40 in DN B lymphocytes from young and elderly subjects. The data obtained in this study demonstrate that the stimulation with CpG/α-IgG/CD40 induces the activation of telomerase in DN B cells from young and elderly subjects. In our preliminary tests, RTA increases to a great extent when B cells are stimulated with CpG/α-IgG/CD40 and shows a low level of activation when cultured with low doses of CpG or with a single stimulation (not shown). Furthermore, to evaluate whether RTA could be considered a tool to monitor the aging process, we performed this analysis in DN B cells from centenarian offspring and subjects affected by a moderate–severe form of Alzheimer’s disease, as it has been demonstrated that healthy elderly, CO [9,10,20] and AD patients [manuscript in preparation] show different amount of total and DN B cells which seems to depend on the health status of aged people.

Here, we have observed that, after stimulation with CpG/α-IgG/CD40, DN B cells telomerase activity in young, elderly, CO, AD patients and AM controls, mirrors the age and health status of the subjects studied. Indeed young donors show the highest levels of RTA, whereas AD patients show the lowest level of telomerase activity. Healthy elderly and CO show lower levels than

young with a marginally significant increase of activity in CO vs elderly. Although healthy elderly and AM controls have the same RTA values, we split them in two distinct groups to compare CO and AD subjects with a coeval elderly group without familiar longevity nor dementia. Indeed, in our studies, CO, AD and AM controls are in their 60s–70s, whereas old subjects are usually in their 80s–90s.

Taken together, our data suggest that under appropriate stimuli the DN B cells acquire the ability to begin new cellular cycles as demonstrated by the mean RTA values. Age is not per se a limiting factor for the telomerase function, although it influences the levels of expression of this enzyme. Furthermore, telomerase reactivation in adult people (healthy elderly, CO and AD subjects) might counteract telomere shortening associated with aging, so maintaining telomere length in actively dividing DN B cells, and increasing both health span and longevity.

In conclusion our present data add new information to our knowledge on DN B cells, and suggest that these cells might allow to gain insight to healthy and unsuccessful aging. Indeed, the number, the ability to be activated and the telomerase reactivation, seem to be dependent not only on the age, but also on the health status of the subjects studied. They seem to be basically activated in the elderly and probably this condition renders them refractory to other physiological stimulations (via TLR or BCR), although the stimulation of both innate and adaptive receptors overcomes this condition. Indeed although DN B cells are less responsive, some particular conditions (i.e. IL-21 + α -IgG) can activate them [60] as demonstrated by their ability to produce Granzyme B that act against viruses or cancer cells [61,62]. Finally, the data on the expression of the inhibitory receptors CD307d and CD22 suggest that DN cells are not fully comparable to other B cell populations described in old or chronically stimulated mice and humans [23,24].

A future goal is to understand whether the activation of DN B cells by TLR/BCR renders them able to produce effector molecules so playing a protective role “*in vivo*”.

Authors' contributions

A.M. and G.C.-R. designed the experiments; A.M. and C.R.B. performed experiments; A.M., S.B. and M.B. analyzed the data; D.M.A., C.C., R.M., C.C. and G.C.-R. provided samples and material necessary for performing experiments; A.M., M.B., S.B. and G.C.-R. wrote the manuscript; all authors have seen and approved the final draft of the manuscript.

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