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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.

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 vacutainer 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/streptomicin, 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, Aubum, CA, USA). Cells obtained from immunomagnetic sorting were >98% $CD19^+$ lymphocytes, as determined by flow cytometry analysis.

Purified B cells (1×10^5/200 μl) were cultured in 96-well round-bottom plates, in complete RPMI medium with 10% Fetal Bovin Serum in absence or presence of 2 μg/ml of anti-IgG [$F(ab')_2$] (Jackson ImmunoResearch Laboratories, Inc, Philadelphia), 3 μg/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% CO_2.

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 μl 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-IgD_{FITC}, anti-$CD27_{PE}$ or anti-$CD27_{APC}$, anti-$CD22_{PE\text{-}Cy5}$ (BD, Pharmingen) and anti-$CD307d_{PE}$ (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 10^4 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