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A double-negative (IgD^-CD27^-) B cell population is increased in the peripheral blood of elderly people

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SUMMARY

The T cell branch of the immune system has been extensively studied in the elderly and it is known that the elderly have impaired immune function, mainly due to the chronic antigenic load that ultimately causes shrinkage of the T cell repertoire and filling of the immunologic space with memory T cells. In the present paper, we describe the IgD^-CD27^- double-negative B cell population which (as we have recently described) is higher in the elderly. Most of these cells were IgG^+. Evaluation of the telomere length and expression of the ABCB1 transporter and anti-apoptotic molecule, Bcl2, shows that they have the markers of memory B cells. We also show that these cells do not act as antigen presenting cells, as indicated by the low levels of CD80 and DR, nor do they express significant levels of the CD40 molecule necessary to interact with T lymphocytes through the ligand, CD154. Hence, we hypothesize that these expanded cells are late memory or exhausted cells that have down-modulated the expression of CD27 and filled the immunologic space in the elderly. These cells might be the age-related manifestation of time-enduring stimulation or dysregulation of the immune system.

1. Introduction

The immune system of the elderly has been extensively studied, for the most part involving the T cell branch (Miller, 2000; Pawelec et al., 2002a). As a result, we know that the elderly have decreased immune function due mainly to the chronic antigenic load that ultimately causes shrinkage of the T cell repertoire and filling of the immunologic space with memory T cells (Franceschi et al., 2000; Pawelec et al., 2005). The direct consequence of these changes, together with the reduced output of naïve cells from the thymus (Pawelec et al., 2002a), is a reduction of antigen-inexperienced T lymphocytes available for adequate immune response against newly encountered antigens, in particular, infectious agents (Pawelec et al., 2002b, 2004, 2005).

In contrast to T cells, B lymphocytes have not been extensively studied in the elderly (Weksler, 2000; Weksler and Szabo, 2000; Colonna-Romano et al., 2003), although the B cell branch is involved in some hematological cancers common in the elderly, such as chronic lymphatic leukemia (Chiorazzi et al., 2005), and in the increased autoimmune responses observed in the elderly (Candore et al., 1997). On the other hand, although B cells are numerically reduced in the elderly (Globerson and Effros, 2000; Colonna-Romano et al., 2002), total serum immunoglobulin levels do not actually change (Le Maoult et al., 1997; Weksler and Szabo, 2000). However, we have recently reported a reduced ability of the elderly to produce IgD that is expressed together with IgM on mature B cells newly produced by the bone marrow, as demonstrated by low serum levels of this isotype (Listì et al., 2006). Interestingly, we have also shown an age-related increase in IgG and IgA levels, whereas there is an age-related decrease in IgM levels (Listì et al., 2006). We and others (Colonna-Romano et al., 2003; Gupta et al., 2005) have also shown that in the elderly there is a significant decrease in naïve $CD27^-$ B lymphocytes and no significant reciprocal increase in $CD27^+$ memory B cells (Klein et al., 1998; Agematsu et al., 2000). On the other hand, Shi et al. (2005) have shown that $CD27^+$ memory B cells, particularly $IgD^+IgM^+CD27^+$ IgM memory B cells, decline dramatically in elderly subjects.

Different subsets of memory $CD27^+$ B cells and $CD27^-$ naïve B cells have been described (Shi et al., 2003; Fecteau et al., 2006; Wei et al., 2007), providing the immunity and ageing fields with new opportunities to gain insight into B cell immunosenescence. Accordingly, we have recently focused our attention on B cell subpopulations, demonstrating that a double-negative (DN) IgD^-CD27^- B cell subset is significantly higher in the elderly (Colonna-Romano et al., 2008). In the present report, we describe

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the characteristics of this subset of B cells present in both young and old donors. We suggest that these cells, which are expanded in the elderly, might be the age-related manifestation of a time-enduring stimulation or dysregulation of the immune system.

2. Materials and methods

2.1. Subjects

Eighty-eight healthy Sicilian subjects were studied (thirty-nine young, age range 20–55 years, mean 37.5 ± 17.5, median 35 years) and forty-nine elderly (age range 75–102 years, mean 89.0 ± 13, median 91 years). 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.

2.2. Cell preparation, activation, B lymphocyte separation, and flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized 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-glutamine.

In some experiments PBMCs have been activated with unmethylated single-stranded DNA motif (CpG oligonucleotides), or with anti-BCR [$F(ab')_2$] (Jackson ImmunoResearch Laboratories, Inc, Philadelphia) as follows: PBMCs were isolated from heparinized venous blood by density gradient centrifugation on Ficoll-Lympholyte (Cedarlane Laboratories Limited, Ontario, Canada), then were adjusted to 1 × 106/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-glutamine. Cells were then cultured in 24-well plate bottom in medium or were activated with 10 μg/ml of CpG oligodeoxynucleotide (TIB Molbiol, Genova, Italy), or with 2 μg/ml of F ($ab')_2$), for 5 days. Then Ki67 expression was evaluated (see below).

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). The cells obtained from immunomagnetic sorting were >98% $CD19^+$ lymphocytes, as determined by flow cytometry analysis.

Total PBMCs or purified B cells were stained with different combinations of the following monoclonal antibodies: anti-IgD_{FITC}, anti-IgG_{FITC}, anti-$CD40_{PE}$, anti-$HLA\text{-}DR_{PE}$, anti-$CD19_{PE}$, anti-IgD_{PE}, and anti-$CD27_{APC}$ (Pharmingen, BD Bioscience, Mountain View, CA, USA), anti-$CD80_{TC}^{TM}$ (Caltag, Burlingame, CA, USA).

For Ki67 evaluation (Lopez et al., 1991), activated PBMCs were first stained with anti-IgD_{PE} or anti $IgG_{PE,}$ anti-$CD19_{PECy5}$, anti-$CD27_{APC}$. Then the cells were fixed with 100 μl of Perm & Fix Solution A (Caltag Burlingame) for 20 min at 4 °C, washed twice with PBS-BSA (0.2%) and permeabilized with 500 μl of Perm & Fix Solution B (Caltag Burlingame) for 10 min at RT. Then cells were stained for 40 min on ice with 20 μl of anti-Ki67FITC (Pharmingen). After two washings the Ki67 positive cells were 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.

2.3. Bromodeoxyuridine (BrdU) incorporation

To evaluate bromodeoxyuridine (BrdU) incorporation, separated PBMCs were cultured in 24-well plate bottom in medium with 10 μM of BrdU (kit BD-Bioscience) and then they were activated with 10 μg/ml of CpG oligodeoxynucleotide (TIB Molbiol, Genova, Italy) or 2 μg/ml of F ($ab')_2$ (Jackson Immunoresearch) for 5 days. Activated PBMC were first stained with anti-CD27FITC, anti-IgDPE, anti-CD19PECy5. Then the cells were fixed with 100 μl of Perm & Fix Solution A (Caltag Burlingame) for 20 min at 4 °C, washed twice with PBS-BSA (0.2%) and permeabilized with 500 μl of Perm & Fix Solution B (Caltag Burlingame) for 20 min at RT, washed twice and incubated with 300 μg/ml of DNase (kit BD-Bioscience) for 60 min at 37 °C. Then cells were re-suspended in 50 μl of PBS and stained with 1 μl of anti-$BrdU_{APC}$ (kit BD-Bioscience) for 20 min at RT, washed and analyzed.

2.4. Evaluation of the ATP-binding cassette-B1 transporter (ABCB1)

To evaluate the ABCB1 transporter, $CD19^+$ cells were incubated with 25 μM of cyclosporin A, which allows the exposure of the epitope of the active conformation of the ABCB1 protein (Wirths and Lanzavecchia, 2005), and 20 μl of anti-$ABCB1_{PE}$ (clone UIC2 MsIgG2a; Coulter Immunotech, Marseille Cedex, France), for 1 h at 37 °C in a water bath. Then the cells were incubated for 20 min at room temperature with anti-$CD27_{APC}$ and anti-IgD_{FITC}. The fluorescence was then measured with a FACSCalibur flow cytometer. The results are expressed as the mean fluorescence intensity (MFI) mean ± SE.

2.5. Evaluation of telomere length

Telomere length was evaluated by the flow-fish technique, as described by Rufer et al. (1998). Magnetically sorted $CD19^+$ B cells were labelled with antibodies specific for the surface markers (i.e. anti-IgD_{FITC} and anti-$CD27_{APC}$) and washed with phosphate buffered saline (PBS) with 0.2% weight/volume of bovine serum albumin (BSA; Sigma Chemical Co, St. Louis, MO, USA Then the cells were fixed and permeabilizated with Fix and Perm (Caltag, Burlingame, CA, USA), according to the manufacturer's instructions. Briefly, cells were first incubated with 100 μl of solution A for 15 min in the dark at room temperature, washed with PBS 0.2% BSA, then incubated for 15 min with solution B under the same conditions, and washed twice with PBS-BSA (0.2%). PBMCs were then washed twice with hybridization solution containing 70% de-ionized formamide, 20 mM Tris (pH 7.1), 1% BSA, and 15 mM NaCl (Sigma Chemical). The cell pellet was gently re-suspended with 200 μl of hybridization solution and incubated with 0.3 mg/ml of $(C_3TA_2)_3$ PNA probe Cy5-conjugated (Applied Biosystems, Bedford, MA, USA). The cell suspension was maintained at room temperature for 10 min prior to denaturation in a circulating water bath at 87 °C for 15 min. Hybridization was performed for 90 min at room temperature in the dark. Four washes were performed at room temperature by adding 1 ml of 70% de-ionized formamide, 10 mM Tris (pH 7.1), 0.1% BSA, and 0.1% Tween 20 (Sigma Chemical), followed by centrifugation of the tubes at 1500 × g for 5 min at 16 °C and aspiration of the supernatant, leaving 100 μl in which the cell pellet was gently re-suspended before addition and mixing with 1 ml of fresh wash fluid. The last wash step was performed with 1 ml of 5% glucose, containing 10 mM Hepes, 0.1% BSA, and 0.1% Tween 20 (Sigma Chemical), and centrifugation was at 900 *g* for 5 min at 16 °C. The cell pellet was shaken and the cells were counterstained with FACSFlow (Becton Dickinson) containing 0.1% BSA.

The fluorescence was then measured with a FACSCalibur flow cytometer. The results are expressed as the MFI mean ± SE of the PNA probe incorporation.

2.6. Intracellular staining of Bcl2

$CD19^+$ lymphocytes were firstly stained with anti-IgD_{FITC} and anti-$CD27_{APC}$. Then the cells were permeabilizated with 150 μl of permeabilization buffer (Pharmingen, BD Bioscience, Mountain View, CA, USA) for 20 min at room temperature, washed twice with PBS-BSA (0.2%) and stained for 30 min at room temperature with 20 μl of anti-$Bcl2_{PE}$ (Pharmingen). After two washings, the Bcl2-positive cells were analyzed. The results are expressed as the MFI mean ± SE.

2.7. 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.

3. Results

3.1. Analyses of $CD19^+$ cell subpopulations on the basis of IgD and CD27 expression

We analyzed circulating $CD19^+$ lymphocytes obtained from the peripheral blood of young and elderly subjects. The analyses were performed after staining B cells identified by anti-CD19 antibody or sorted by immunomagnetic beads with a combination of anti-IgD and anti-CD27 fluorochrome-conjugated antibodies. In both cases, the results were similar (data not shown). As shown in Fig. 1, we identified IgD^+CD27^-, IgD^+CD27^+, IgD^-CD27^+, and IgD^-CD27^- (i.e. DN) B cells. Remarkably, in the elderly we observed a significant reduction of IgD^+CD27^- naïve B cells (young, 49.5 ± 2.9; elderly, 40.1 ± 3.9; $p = 0.005$) and a highly significant increase of IgD^-CD27^- (DN) B cells (young, 5.8 ± 0.5; elderly, 14.9 ± 2.1; $p = 0.0001$). No differences in the percentage of the other two subpopulations of B cells were found.

3.2. Immunoglobulin expression of DN B cells

We evaluated the expression of membrane immunoglobulin on DN B cells. Initial presumptive evidence suggested to us that these cells were IgG-positive. In fact, the percentages of DN cells and IgG^+CD27^- were nearly the same in the subjects studied (data not shown). Further analysis of IgD^-CD27^- DN B cells with 4-color immunofluorescence demonstrated this association (Fig. 2). Most, but not all, of these cells were IgG-positive. All the IgG^+ cells were IgD^-, although not all the IgD^- cells were IgG^+, suggesting that they