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Trafficking phenotype and production of granzyme B by double negative B cells ($IgG^+IgD^-CD27^-$) in the elderly

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ABSTRACT

The impairment of humoral immune response in elderly humans has been extensively demonstrated. We have reported the increase of memory B cells ($IgG^+IgD^-CD27^-$, double negative, DN) population in the elderly, in which there is also a typical inflammatory micro-environment. In order to evaluate whether this pro-inflammatory status could influence the trafficking phenotype of naïve/memory B cells, we have assessed the expression of CCR7, CCR6, CXCR3, CXCR4, CXCR5 and CD62L on naïve/memory B cell subpopulations in young and elderly subjects. Moreover, the combination of pro-inflammatory interleukin-21 (IL-21) and B cell receptor (BCR) stimulation enables B cells to produce and secrete granzyme B (GrB), which plays a critical role in early anti-viral immune responses, in the regulation of autoimmune mechanisms and in cancer immunosurveillance.

Our data demonstrate that in the elderly, naïve/memory B cell populations present a different expression of the studied receptors that could be discussed in terms of "inflamm-aging". In particular $IgG^+IgD^-CD27^-$ DN B cells show a tissue trafficking phenotype and they can be stimulated to produce GrB.

1. Introduction

B lymphocytes represent the humoral arm of adaptive immune response. The defects in B cell production/development cause a variety of disorders that are the basis of immune deficiencies and/or autoimmune diseases (Blair et al., 2010; Mauri, 2010; Vitale et al., 2010). For this reason the deep knowledge of B cell subsets and functions provides crucial information on immune assessment. Moreover B lymphocytes, due to their ability to present antigen to T lymphocytes, produce cytokines and synthesize granzymes, are now recognized as eclectic and essential cells for an exhaustive immune response (Blair et al., 2010; Bouaziz et al., 2010; Buffa et al., 2011; Hagn and Jahrsdörfer, 2012; Hagn et al., 2009, 2012; Mauri, 2010; Vitale et al., 2010). The different B cell subsets have been identified using many cellular markers by which many functional subsets, as transitional, naïve, memory and plasmablasts may be recognized. In particular IgD, CD27, CD24 and CD38, other than other molecules, may be used to study peripheral B cells in humans. Nevertheless "core" subsets may be identified by using IgD and CD27 expression on CD19 B cells and this kind of classification has been suggested to be useful as potential biomarkers in some autoimmune diseases (Kaminski et al., 2012).

It is well known that the impairment of the immune system in the elderly (immunosenescence) has been related to the increased susceptibility to infectious diseases, cancer and autoimmunity; moreover, immunosenescence also involves the B cell branch (Bulati et al., 2011; Cancro et al., 2009; Frasca and Blomberg, 2011; Frasca et al., 2004, 2010, 2011; Schenkein et al., 2008), although most of the studies consider the T lymphocytes (Ouyang et al., 2003; Pawelec and Larbi, 2008; Pawelec et al., 2005). In particular, in the elderly, we have demonstrated the reduction, in percentage but not in absolute number, of naïve B lymphocytes (IgD^+CD27^-) and the increase in percentage of a "Double Negative" (DN, $IgD^-CD27^-IgG^+$) memory B cell population (Colonna-Romano et al. 2009). DN B cells have also been reported to be expanded in patients affected by SLE, HIV and challenged with RSV (Cagigi et al., 2009; Fecteau et al., 2006; Sanz et al., 2008; Wei et al., 2007).

The increase in percentage of the DN B cell population in the elderly might be related to the typical inflammatory micro-environment, characterized by a general increase in plasma levels of pro-inflammatory cytokines and other inflammatory mediators (inflamm-aging) (Franceschi et al., 2007; Licastro et al., 2005; Singh and Newman, 2011; Vasto et al., 2007). As it is known that the absolute number of B cells is significantly reduced in the elderly, the proportional increase of the DN B cell population might be due to the exhaustion of memory B lymphocytes chronically stimulated in the elderly (Colonna-Romano et al., 2009). On the other hand, it has also been reported that these cells can be stimulated "in vitro" to secrete immunoglobulins against tetanus toxoid and influenza virus (Wirths and Lanzavecchia, 2005), although their ability to be

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activated by different stimuli is very low (Buffa et al., 2011; Colonna-Romano et al., 2009; Hao et al., 2011).

In the present paper in order to evaluate whether the inflammatory milieu influences the B cell trafficking, we have assessed the expression of some chemokine receptors on the four subsets of B cells. Indeed certain combination of chemokines and their receptors guides all the immune cells to specific tissues (Kunkel and Butcher, 2003). Concerning B cells it has been demonstrated that CXCR4, CXCR5, CCR6 and CCR7 drive them to lymph node, while CXCR3 leads B cells to sites of inflammation (Kunkel and Butcher, 2003). More recently (Kaminski et al., 2012), the chemokine receptor CXCR3 has been found to be expressed on DN B cells as additional marker, and the expression of CXCR3 might be consistent with migration of cells to chronically inflamed tissues (Moir et al., 2008). We have also evaluated the expression of the homing molecule CD62L that is involved in the homing of naïve lymphocytes to peripheral lymph nodes and Peyer's patches. CD62L mediates the tethering and rolling of leukocytes on endothelial surfaces, contributing to the recruitment of leukocytes from the blood to areas of inflammation.

It has been recently shown that interleukin-21 (IL-21), produced by various subsets of activated $CD4^+$ T cells, NKT and Th17 cells (Spolski and Leonard, 2008), other than regulating multiple innate and adaptive immune responses can stimulate immune cells to synthesize various inflammatory molecules. Moreover, excessive production of IL-21 has been described in many human chronic inflammatory disorders and there is evidence supporting the pathogenic role of IL-21 in immune-inflammatory pathologies (Sarra et al., 2013). It has also been reported that IL-21 levels are increased in healthy elderly (Agrawal et al., 2012).

In the present paper, we show a different expression of chemokine receptors on DN B cells from the elderly and we also show that the ability to produce granzyme B under the control of IL-21 (Hagn and Jahrsdörfer, 2012; Hagn et al., 2009; Hagn et al., 2012) is not impaired in B cells obtained from old subjects; moreover, DN B cells seem to be sensitive to the action of IL-21 that, as mentioned (Agrawal et al., 2012), is increased in the elderly.

2. Materials and methods

2.1. Subjects

Forty healthy Sicilian subjects were studied, 20 young (age range 25–40 years) and 20 elderly (age range 78–90 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 the Italian law.

2.2. Cell preparation and B cell enrichment

Peripheral blood mononuclear cells (PBMCs) were isolated from 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 bovine serum (FBS) (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.

2.3. Antibodies and flow cytometry panels

Purified B cells were stained with different combinations of the following monoclonal antibodies: anti-IgD_{FITC} or anti-IgD_{APC}, anti-$CD27_{PE}$ or anti-$CD27_{APC}$, anti-$CD196_{PE}$ (CCR6), anti-$CD197_{PE}$ (CCR7), anti-$CD62L_{PE}$, anti-$CD183_{APC}$ (CXCR3), anti-$CD184_{PE}$ (CXCR4, Fusin), anti-GrB_{FITC}, anti-$CD185_{PE\text{-}Cy7}$ (CXCR5) (BD, Pharmingen). 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) or FlowJo (Tree Star) software.

2.4. Reagents for functional assays

For flow cytometric intracellular GrB detection, magnetically sorted B cells were cultured in AIM-V medium (Invitrogen) at 1×10^6/ml for 16 h and incubated at 37 °C and 5% CO_2 atmosphere in the presence/absence of both recombinant human IL-21 (Gibco®, Life Technologies), used at a final concentration of 50 ng/ml, and, for Ag-independent BCR stimulation (anti-BCR), affiniPure $F(ab')_2$ fragment goat anti-human IgG, $F(ab')_2$ fragment specific (Jackson ImmunoResearch Laboratories) at 6.5 μg/ml. Brefeldin A (Biolegend) was added to a final concentration of 1 μg/ml, and cells were cultured for four more hours (Hagn et al., 2009). At the indicated time point, cells were harvested, washed and stained with anti-$CD27_{PE}$ and anti-IgD_{APC}. Intracellular staining was performed using a fixation and permeabilization buffer (Fix & Perm cell permeabilization kit, Invitrogen). Briefly, cells were washed and resuspended in fixation buffer, incubated for 15 min at room temperature, and washed with PBS/FCS 5%. Cells were then resuspended in permeabilization buffer and anti-GrB_{FITC} was added. After another 20 min of incubation at room temperature, cells were washed with PBS/FCS 5%. Flow cytometric analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and data were analyzed using FlowJo software (Tree Star).

2.5. Statistical analysis

Values are given as median and range of mean fluorescence intensities (MFI) and are compared using Mann–Whitney nonparametric U test. Differences are considered significant when a p value < 0.05 was obtained by comparison between the different groups.

3. Results

3.1. Profile of trafficking receptors in B cell subpopulations

In order to evaluate the trafficking phenotype of naïve/memory B cells in the different age groups, we have assessed the expression of CCR7, CCR6, CXCR3, CXCR4, CXCR5, and CD62L on B cell populations identified on the basis of the different expression of CD27 and IgD in young (Y) and elderly (O) subjects (Table 1).

Concerning the expression of CCR7 on DN cells, we report that DN B cells obtained from elderly donors show significant increase of this chemokine receptors when compared with the expression evaluated in the same cells obtained from the young group. Besides CCR7 expression is differently modulated in young and in elderly donors as shown by the different median values in the four populations in both age groups.

As reported by others (Kunkel and Butcher, 2003), CCR6 is mainly expressed on naïve B cells from healthy adult subjects. We confirm these results in our young donors, moreover we demonstrate a high expression of this receptor on memory unswitched cells too, whereas memory switched and DN B cells express very low levels of CCR6. In the elderly group, CCR6 is differently modulated and, as a median value, it is expressed at significantly higher levels on memory switched and DN B cells.

Concerning CXCR3, in young donors this is expressed at higher significant levels on memory unswitched and on DN B cells comparing to the other populations of the same group. Differently, in the elderly higher levels of CXCR3 are observed only on memory unswitched and not in DN B cells.

As reported (Kunkel and Butcher, 2003), CXCR4 is mainly expressed on naïve B cells. Here we show that it is also expressed on memory