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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

Table 1
Expression of trafficking receptors on B cell subpopulation, identified by the "core" markers IgD and CD27, of young and elderly donors. Values are expressed as median and range (P25–P75) of mean fluorescence intensities (MFI). NS = not significant. In bold are expressed the significant p values.

Subjects	Naïve (IgD^+CD27^-)	Memory unswitched (IgD^+CD27^+)	Memory switched (IgD^-CD27^+)	Double negative (IgD^-CD27^-)	
Young	99.1 (82.8–126.7)	69.1 (48.5–78.2)	9.9 (8.8–11.5)	10.3 (9–14.5)	CCR7
Old	45.5 (30–71.3)	79 (33.9–104)	14 (13.1–18.4)	38 (24.7–55.3)	
p Young vs Old	NS	NS	NS	**0.005**	
Young	163.4 (51.1–278.8)	113.8 (60.6–167.2)	24 (20.4–26)	22.7 (15.4–33.7)	CCR6
Old	60.2 (51.9–76.5)	51.1 (50.7–64.3)	43.3 (20–67.4)	86.1 (21.7–176.8)	
p Young vs Old	**0.02**	**0.03**	**0.01**	**0.02**	
Young	62.2 (50.2–68.8)	141.6 (135.2–147.2)	67.8 (49.7–86.7)	136.2 (78.9–241)	CXCR3
Old	50.4 (44.1–52.8)	245.9 (112.6–278.3)	61.8 (56.2–67.2)	67.6 (60.2–71.3)	
p Young vs Old	NS	**0.02**	NS	**0.01**	
Young	39.9 (31.4–46.8)	49 (32.9–93.2)	20.1 (13.9–27.1)	16.9 (15.1–18.9)	CXCR4
Old	73.4 (49.6–102.8)	78.2 (31.3–140.4)	26.5 (23.2–29.5)	33.6 (24.3–36.5)	
p Young vs Old	**0.03**	NS	NS	NS	
Young	34.9 (29.2–73.2)	230.3 (119.4–241.1)	49 (47.8–51.5)	26.1 (19–37.7)	CXCR5
Old	26.6 (23.5–55.6)	220.5 (78.9–231.1)	46.2 (40.5–60.3)	24 (22.7–33)	
p Young vs Old	NS	NS	NS	NS	
Young	69.3 (52.6–90.1)	93.4 (76.5–117.4)	115.7 (91.2–145.1)	77.5 (69.4–82.6)	CD62L
Old	145 (82–201.7)	278 (127.3–343)	389.5 (133.6–417.8)	133.6 (75.2–149)	
p Young vs Old	**0.05**	**0.05**	**0.05**	**0.05**	

unswitched cells, whereas it is expressed at significantly low levels on memory switched and DN B cells. No significant differences are observed between young and old subjects except for the naïve cells (O > Y).

CXCR5 is mainly expressed on memory unswitched B cells, whereas we observe a significant reduction of CXCR5 expression on memory switched and a very low expression on naïve and DN B populations obtained from both age class donors. Moir et al. (2008), show the higher level of CXCR5 expression on naïve B cells, but they identify this population using different phenotypical markers ($CD21^{High}CD27^-$).

Concerning the expression of CD62L on the four different B cell populations (from young donors) we observe the higher expression on switched memory. The same feature is observed on B cells from elderly donors, although the median value of MFI is significantly higher on cells from the elderly compared to that from young subjects.

Table 2 shows, on the whole, the trafficking receptor phenotype of the four B cell populations in young and elderly donors. As expected, naïve and memory unswitched B cells have a "lymph node phenotype", whereas memory switched express molecules useful to leave the lymphoid organs. Memory unswitched and DN B cells from young subjects show also high levels of CXCR3, a chemokine receptor that leads cells to site of inflammation (Henneken et al., 2005; Kunkel and Butcher, 2003; Stein and Nombela-Arrieta, 2005). In B cells from elderly donors this receptor is well expressed only on memory unswitched subpopulation. Notably, memory switched and DN B cells of elderly donors express CCR6 which is also involved for the recruitment of cells in the site of inflammation (Comerford et al., 2010; Othani et al., 2011; Welsh-Bacic et al., 2011; Williams, 2006). DN B cells from elderly donors also express CCR7.

3.2. DN B cells produce GrB after IL-21-stimulation and BCR engagement in the absence of CD40 ligation

In order to evaluate whether total B cells and DN memory B lymphocytes are able to act as GrB producing cells, we have investigated their ability to respond to the simultaneous in vitro stimulation with IL-21 and the triggering of BCR with anti-human IgG, in young and elderly subjects. As shown in Fig. 1, after stimulation, both in young and elderly people, total B cells (Fig. 1A) produce GrB when compared to the not stimulated cells without differences between the two groups. Next (Fig. 1B), we focused on DN B cells, observing that also this particular memory population, under the same condition, shows GrB production ability without differences between the two age groups studied. In Fig. 1C and D we report a typical experiment in which we show the capacity of total B cells and IgD^-CD27^- (DN) B lymphocytes, obtained from an elderly donor, to produce GrB after IL-21 and α-IgG stimulation.

4. Discussion

It is well known that with aging, the levels of inflammatory mediators increase even in the absence of acute infection or other stressors (Singh and Newman, 2011). This situation, characterized by a general increase in plasma levels of pro-inflammatory cytokines, leads to a chronic, low-grade, pro-inflammatory status known as "Inflamm-aging" (Franceschi et al. 2007; Salvioli et al., 2013). There is a common consensus in the scientific community that ascribes the cause and/or the consequence of many aspects of senescence to the increased baseline inflammatory status in elderly people. It is also known that the impairment of the adaptive immune system in the elderly involves not only the widely studied T cell branch (Ouyang et al., 2003; Pawelec and Larbi, 2008; Pawelec et al., 2005), but also the humoral arm (Bulati et al., 2011; Cancro et al., 2009; Frasca and Blomberg, 2011; Frasca et al., 2004; Schenkein et al., 2008). These changes include shifts in the magnitude of all B cell compartments, specificity repertoire changes, modified peripheral B cell dynamics, and weakened humoral responses (Aberle et al., 2013; Bulati et al., 2011; Faria et al., 2008; Frasca et al., 2008). Using the "core markers" IgD and CD27 (Kaminski et al., 2012), we have also demonstrated the increase of a DN ($IgD^-CD27^-IgG^+$) B cell population (Colonna-Romano et al. 2009) that has also been shown to be increased in patients affected by SLE, HIV or in healthy subjects challenged with RSV (Cagigi et al., 2009; Fecteau et al., 2006;

Table 2
Homing/trafficking receptor phenotype of the four B cell subpopulation in young and elderly donors.

B cell subpopulation	Phenotype of trafficking receptors	
	Young	Elderly
Naïve (IgD^+CD27^-)	$CCR7^+CCR6^+CXCR4^+$	$CCR7^+CCR6^+CXCR4^+$
Memory unswitched (IgD^+CD27^+)	$CCR7^+CCR6^+CXCR3^+CXCR4^+CXCR5^+CD62L^+$	$CCR7^+CCR6^+CXCR3^+CXCR4^+CXCR5^+CD62L^+$
Memory switched (IgD^-CD27^+)	$CXCR5^{dim}CD62L^+$	$CCR6^+CXCR5^{dim}CD62L^+$
Double negative (IgD^-CD27^-)	$CXCR3^+$	$CCR7^+CCR6^+$

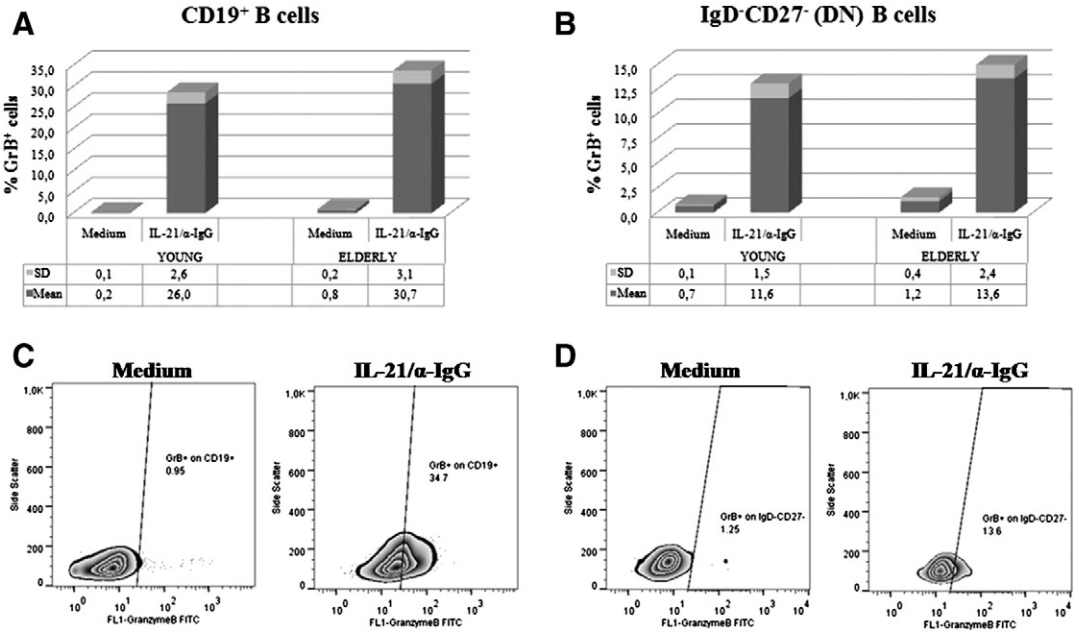

Fig. 1. IL-21 and BCR engagement induce GrB in total human CD19$^+$ B cells and in IgD$^-$CD27$^-$ (DN) B lymphocytes. Magnetically sorted B cells or IgD$^-$CD27$^-$ (DN) B lymphocytes were cultured in AIM-V medium for 16 h and incubated in the presence/absence of both recombinant human IL-21 (50 ng/ml) and affiniPure F(ab′)$_2$ fragment goat anti-human IgG, F(ab′)$_2$ fragment specific (6.5 μg/ml). Brefeldin A (1 μg/ml) was added and cells cultured for four more hours. (A) Production of GrB before (medium) and after stimulation (IL-21/α-IgG) by CD19$^+$ B cells in young and elderly donors. No significant differences were observed between the two age-groups studied. (B) Production of GrB before (medium) and after stimulation (IL-21/α-IgG) by IgD$^-$CD27$^-$ (DN) B lymphocytes in young and elderly donors. No significant differences were observed between the two age-groups studied. (C) The figure illustrates an example of Zebra plot that shows the percentage of GrB$^+$ total B cells in old subjects before (left panel) and after stimulation (right panel). (D) The figure illustrates an example of Zebra plot that shows the percentage of GrB$^+$ IgD$^-$CD27$^-$ (DN) B lymphocytes in old subjects before (left panel) and after stimulation (right panel). We have performed these analyses on 10 young and 10 elderly subjects.

Sanz et al., 2008; Wei et al., 2007). This DN population seems to be an "exhausted" memory population (Buffa et al., 2011; Bulati et al., 2011; Colonna-Romano et al., 2009), although it has also been demonstrated that these cells may be stimulated to secrete immunoglobulins (Wirths and Lanzavecchia, 2005). A similar population has recently been identified by Hao et al. (2011) in elderly mice.

A link between "Inflamm-aging" and adaptive immune responses may be identified in the expression of chemokine receptors. The relevance of chemokine receptors is doubtless suggested by the knowledge that, although there is much promiscuity within the chemokine network, certain combinations of chemokines and their receptors guide all the immune cells, and also B cells, to specific tissues (Kunkel and Butcher, 2003). Accordingly, CXCR4, CXCR5, CCR6 and CCR7 have been identified as receptors that drive B cells to lymph node attracted by a combination of CXCL12, CXCL13, CCL20 and CCL19 respectively, while CXCR3 leads B cells to sites of inflammation (Kunkel and Butcher, 2003). Indeed, as known, the CXCR3 ligands, monokine-induced by Interferon-γ (CXCL9) and IP10 (CXCL10), are widely expressed by the endothelium and other cells in inflamed tissues (Farber, 1997), indicating that B cells that express CXCR3 can directly enter these inflamed sites. Expression of CXCR4, CXCR5, CCR6 and CCR7 has been reported on circulating naïve and memory B cells (Caraux et al., 2010), although many authors report the involvement of CCR6 in the recruitment in sites of inflammation (Comerford et al., 2010; Schutyser et al., 2003; Welsh-Bacic et al., 2011).

Our data show a different expression of these chemokine receptors on peripheral naïve and memory B cells from young and old donors. Indeed, as expected, in young donors naïve B cells express CCR7, CCR6 and CXCR4 allowing B cells to circulate. Thereafter they (both memory unswitched and memory switched) modulate the expression of CD62L and CXCR5 necessary to cooperate with T cells in lymphoid organs. Memory unswitched cells also express CXCR3, the chemokine receptor that consents cells to reach the inflammatory sites. This chemokine receptor is the sole expressed in the DN B population in young people. So, it seems that both memory unswitched and DN B cells are able to migrate into the inflamed sites in a common flogistic reaction. With aging, naïve/memory B cell populations show some modifications on the expression of the studied receptors. Indeed, due to their expression of CXCR3, memory unswitched B cells retain the capacity to migrate to the sites of inflammation, while double negative B cells lose the expression of this molecule, but express CCR6 and CCR7, i.e. chemokine receptors, which are also implicated in the migration to the inflammatory sites (Comerford et al., 2010; McNamee et al., 2013; Welsh-Bacic et al., 2011), as described below.

These data are interesting and can be discussed in terms of "inflamm-aging". Our hypothesis is that the inflammatory environment, typical of aging, in some way changes the trafficking ability of B cells and renders them more sensitive both to the cytokines and the pro-inflammatory molecules which are over-produced in the elderly (Salvioli et al., 2013; Singh and Newman, 2011). Concerning CCR6, this chemokine receptor only binds CCL20, which is produced by a variety of epithelial cell types, as keratinocytes, pulmonary and intestinal epithelial cells (Iwasaki and Kelsall, 2000; Nakayama et al., 2001; Reibman et al., 2003) and can be strongly induced by pro-inflammatory signals such as cytokine (TNF-α) and Toll-like receptor ligands (Schutyser et al., 2003). The ligand/receptor pair CCL20/CCR6 is responsible for the chemoattraction of immature dendritic cells (DC), effector/memory T and B cells and plays a role in various human pathologies, including cancer, rheumatoid arthritis (Schutyser et al., 2003) and other autoimmune diseases (Comerford et al., 2010). Given that CCL20 is expressed in different tissues in resting condition and that it mediates the migration of a variety of leukocyte subsets in vitro (Casamayor-Pallejà et al., 2002; Liao et al., 2002; Vanbervliet et al., 2002), it is now clear that CCR6 and CCL20 only play a limited role in homeostatic lymphocyte trafficking in the periphery, but contribute more to homing of leukocytes to the intestinal epithelium, a tissue that displays characteristics of chronic inflammation (Comerford et al., 2010). New evidences suggest that the CCR6/CCL20 axis provides key homing signals for adaptive immune system cells, such as Th17 and Treg cells. Inflammatory T and B cells, neutrophils and monocytes may also be recruited by virtue of CCR6 expression, leading to the

development of inflammatory responses (Comerford et al., 2010). Furthermore, CCR6 is also involved in the recruitment of CCR6-expressing B cells to the follicle-associated epithelium, Peyer's Patches and isolated lymphoid follicles (Williams, 2006). Moreover, other authors (Welsh-Bacic et al., 2011) show that CCR6 and the corresponding ligand CCL20 might be involved in the recruitment of T and B cells to form organized nodular infiltrates in chronic renal inflammation.

DN B cells from old donors also express CCR7 that is involved in the cognate interaction between B and T cells (Reif et al., 2002). At now, we might only speculate on this result as a role of the CCR7/CCL19/CCL21 chemokine axis in the development of tertiary lymphoid tissue (TLT), has been recently demonstrated in the chronically inflamed intestine of a mouse model of Crohn's-like ileitis (McNamee et al., 2013). Moreover the involvement of CCR7 and its ligands has been also shown in other autoimmune and infectious diseases, such as rheumatoid arthritis, *Helicobacter pylori*-induced gastritis, and Sjögren's syndrome (Müller and Lipp, 2003).

These data suggest that that DN B cells, which are increased in old subjects, are in some way involved in the inflamm-aging and that they might be either a by-product of systemic inflammation or might be directly involved in the immune response against pathogens. In this perspective, we have searched for a functional role of these cells in the inflammatory processes. Recently, it has been demonstrated that the combination of the pro-inflammatory interleukin-21 (IL-21) and B cell receptor (BCR) stimulation enables B cells to produce and secrete the active form of the cytotoxic serine protease granzyme B (GrB), that, even if it is not accompanied by the production of perforin, as in Natural Killer (NK) and Cytotoxic T lymphocytes, plays a critical role in early anti-viral immune responses, in the regulation of autoimmune mechanisms and in cancer immunosurveillance (Hagn and Jahrsdörfer, 2012). Moreover, recent studies have revealed an increase of IL-21 levels in the elderly (Agrawal et al., 2012) and in SLE patients (Dolff et al., 2011) in which we and other groups (Colonna-Romano et al., 2009; Sanz et al., 2008; Wei et al., 2007) have shown the increase of DN B cell population. In order to evaluate whether DN B cells are also involved in IL-21-mediated immune response, we tested the capacity of these cells to produce GrB. In our experiments, we show no differences of total B cells to produce GrB between young and elderly subjects, after stimulation with human recombinant IL-21 and anti-human IgG. Interestingly, we also observed that this kind of stimulation renders DN B lymphocytes able to produce GrB, although, again, without any difference between the two age groups. These are intriguing data especially if we consider what we have previously discussed about the chemokine receptor profile of DN cells that suggest their ability to migrate into the inflamed tissues in different ways in young (by CXCR3 expression) and in elderly donors (expression of CCR6 and CCR7). Although GrB production and secretion are not prerogative of only a specific B cell population, and naïve/memory B cells participate in all the phases of the inflammatory responses, in this work our attention was caught by $CD19^{+}IgG^{+}IgD^{-}CD27^{-}$ memory population. Indeed, DN B lymphocytes, that show different pro-inflammatory trafficking profiles, in young and elderly subjects, are able, if properly stimulated, to migrate into inflammatory sites, and, cooperating with other immune cells (e.g. memory unswitched B cells), to produce GrB. With aging, there is, other than an increase in percentage of DN B cells, also a remodeling of these cells, probably due to the typical pro-inflammatory milieu of the aged people. Indeed, in the elderly, DN B lymphocytes express a different chemokine receptor profile that, however, renders them able to reach chronic inflamed tissues or tertiary lymph node. Moreover, as their capacity to produce GrB is not impaired, they behave as in the young (Fig. 2) exerting a biological function. Finally, DN B cells, in the same or in different behavior conditions, could produce other kinds of pro- or anti-inflammatory molecules, as cytokines. So, it is important to improve the study on DN B cells to better understand their active role in immunosenescence and in the age-related diseases.

Conflict of interest

The authors declare no competing financial interests.

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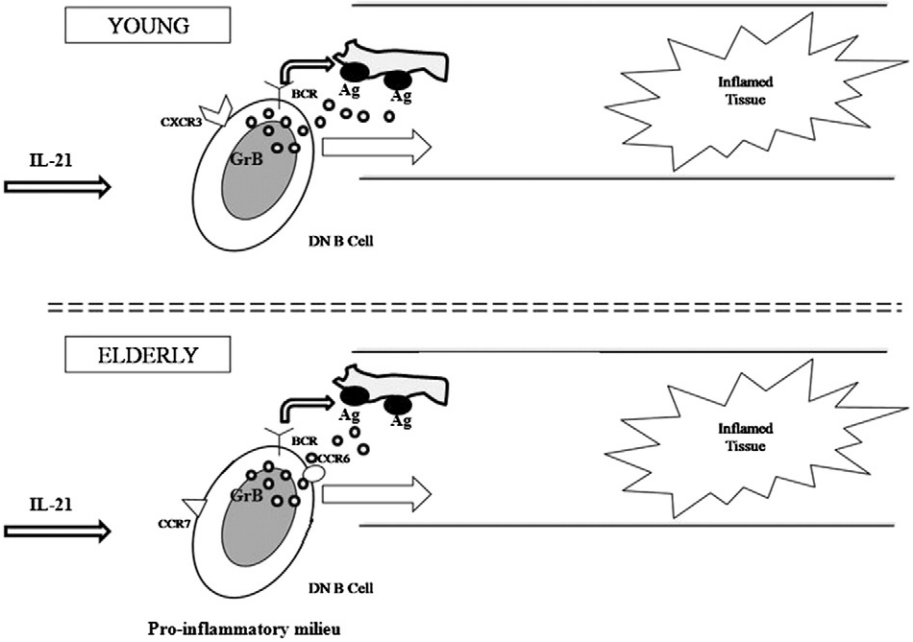

Fig. 2. The "intriguing scenario" of the DN B cells in the inflamed tissue. Properly stimulated, DN B lymphocytes of young donors are able not only to migrate into inflammatory sites (CXCR3 expression), but herein they exert their function producing GrB (upper side of the figure). In the elderly, the inflammatory milieu provides adequate stimuli for the migration of DN to the inflammatory sites by the expression of other chemokine receptors (CCR6 and CCR7) involved in the aforesaid process (lower side of the figure).

the final draft of the manuscript. AM is a PhD student of Pathobiology PhD course (directed by C.C.) at Palermo University and this work is submitted in partial fulfillment of the requirement for her PhD degree.

References

Aberle, J.H., Stiasny, K., Kundi, M., Heinz, F.X., 2013. Mechanistic insights into the impairment of memory B cells and antibody production in the elderly. Age 35 (2), 371–381.

Agrawal, A., Su, H., Chen, J., Osann, K., Agrawal, S., Gupta, S., 2012. Increased IL-21 secretion by aged CD4$^+$ T cells is associated with prolonged STAT-4 activation and CMV seropositivity. Aging 4 (9), 648–659.

Blair, P.A., Noreña, L.Y., Flores-Borja, F., Rawlings, D.J., Isenberg, D.A., Ehrenstein, M.R., Mauri, C., 2010. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. Immunity 32 (1), 129–140.

Bouaziz, J.D., Calbo, S., Maho-Vaillant, M., Saussine, A., Bagot, M., Bensussan, A., Musette, P., 2010. IL-10 produced by activated human B cells regulates CD4$^+$ T-cell activation in vitro. Eur. J. Immunol. 40, 2686–2691.

Buffa, S., Bulati, M., Pellicanò, M., Dunn-Walters, D.K., Wu, Y.C., Candore, G., Vitello, S., Caruso, C., Colonna-Romano, G., 2011. B cell immunosenescence: different features of naive and memory B cells in elderly. Biogerontology 12 (5), 473–483.

Bulati, M., Buffa, S., Candore, G., Caruso, C., Dunn-Walters, D.K., Pellicanò, M., Wu, Y.C., Colonna-Romano, G., 2011. B cells and immunosenescence: a focus on IgG$^+$IgD$^-$CD27$^-$ (DN) B cells in aged humans. Ageing Res. Rev. 10 (2), 274–284.

Cagigi, A., Du, L., Dang, L.V., Grutzmeier, S., Atlas, A., Chiodi, F., Pan-Hammarström, Q., Nilsson, A., 2009. CD27(−) B-cells produce class switched and somatically hypermutated antibodies during chronic HIV-1 infection. PLoS One 4 (5), e5427.

Cancro, M.P., Hao, Y., Scholz, J.L., Riley, R.L., Frasca, D., Dunn-Walters, D.K., Blomberg, B.B., 2009. B cells and ageing: molecules and mechanisms. Trends Immunol. 30, 313–318.

Caraux, A., Klein, B., Paiva, B., Bret, C., Schmitz, A., Fuhler, G.M., Almeida, J., Bos, N., Johnsen, H.E., Orfao, A., Perez-Andres, M., 2010. Circulating human B and plasma cells. Age associated changes in count and detailed characterization of circulating normal CD138$^-$ and CD138$^+$ plasma cells. Haematologica 95, 1015–1020.

Casamayor-Pallejà, M., Mondière, P., Verschelde, C., Bella, C., Defrance, T., 2002. BCR ligation reprograms B cells for migration to the T zone and B-cell follicle sequentially. Blood 99 (6), 1913–1921.

Colonna-Romano, G., Bulati, M., Aquino, A., Pellicanò, M., Vitello, S., Lio, D., Candore, G., Caruso, C., 2009. A double-negative (IgD$^-$CD27$^-$) B cell population is increased in the peripheral blood of elderly people. Mech. Ageing Dev. 130, 681–690.

Comerford, I., Bunting, M., Fenix, K., Haylock-Jacobs, S., Litchfield, W., Harata-Lee, Y., Turvey, M., Brazzatti, J., Gregor, C., Nguyen, P., Kara, E., McColl, S.R., 2010. An immune paradox: how can the same chemokine axis regulate both immune tolerance and activation? CCR6/CCL20: a chemokine axis balancing immunological tolerance and inflammation in autoimmune disease. Bioessays 32 (12), 1067–1076.

Dolff, S., Abdulahad, W.H., Westra, J., Doornbos-van der Meer, B., Limburg, P.C., Kallenberg, C.G., Bijl, M., 2011. Increase in IL-21 producing T-cells in patients with systemic lupus erythematosus. Arthritis Res. Ther. 13 (5), R157.

Farber, J.M., 1997. Mig and IP-10: CXC chemokines that target lymphocytes. J. Leukoc. Biol. 61 (3), 246–257.

Faria, A.M., de Moraes, S.M., de Freitas, L.H., Speziali, E., Soares, T.F., Figueiredo-Neves, S.P., Vitelli-Avelar, D.M., Martins, M.A., Barbosa, K.V., Soares, E.B., Sathler-Avelar, R., Peruhype-Magalhaes, V., Cardoso, G.M., Comin, F., Teixeira, R., Eloi-Santos, S.M., Queiroz, D.M., Correa-Oliveira, R., Bauer, M.E., Teixeira-Carvalho, A., Martins-Filho, O.A., 2008. Variation rhythms of lymphocyte subsets during healthy ageing. Neuroimmunomodulation 15, 365–379.

Fecteau, J.F., Coté, G., Néron, S., 2006. A new memory CD27$^-$IgG$^+$B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation. J. Immunol. 177, 3728–3736.

Franceschi, C., Capri, M., Monti, D., Giunta, S., Oliveri, F., Sevini, F., Panourgia, M.P., Invidia, L., Celani, L., Scurti, M., Cevenini, E., Castellani, G.C., Salvioli, S., 2007. Inflamm-ageing and anti-inflamm-ageing: a systemic perspective of ageing and longevity emerged from studies in humans. Mech. Ageing Dev. 128, 92–105.

Frasca, D., Blomberg, B.B., 2011. Aging affects human B cell responses. J. Clin. Immunol. 31 (3), 430–435.

Frasca, D., Riley, R.L., Blomberg, B.B., 2004. Effect of age on the immunoglobulin class switch. Crit. Rev. Immunol. 24, 297–320.

Frasca, D., Landin, A.M., Lechner, S.C., Ryan, J.G., Schwartz, R., Riley, R.L., Blomberg, B.B., 2008. Ageing down-regulates the transcription factor E2A, activation induced cytidine deaminase, and Ig class switch in human B cells. J. Immunol. 180, 5283–5290.

Frasca, D., Diaz, A., Romero, M., Landin, A.M., Phillips, M., Lechner, S.C., Ryan, J.G., Blomberg, B.B., 2010. Intrinsic defects in B cell response to seasonal influenza vaccination in elderly humans. Vaccine 28 (51), 8077–8084.

Frasca, D., Diaz, A., Romero, M., Landin, A.M., Blomberg, B.B., 2011. Age effects on B cells and humoral immunity in humans. Ageing Res. Rev. 10, 330–335.

Hagn, M., Jahrsdörfer, B., 2012. Why do human B cells secrete granzyme B? Insights into a novel B-cell differentiation pathway. Oncoimmunology 1 (8), 1368–1375.

Hagn, M., Schwesinger, E., Ebel, V., Sontheimer, K., Maier, J., Beyer, T., Syrovets, T., Laumonnier, Y., Fabricius, D., Simmet, T., Jahrsdörfer, B., 2009. Human B cells secrete granzyme B when recognizing viral antigens in the context of the acute phase cytokine IL-21. J. Immunol. 183 (3), 1838–1845.

Hagn, M., Sontheimer, K., Dahlke, K., Brueggemann, S., Kaltenmeier, C., Beyer, T., Hofmann, S., Lunov, O., Barth, T.F., Fabricius, D., Tron, K., Nienhaus, G.U., Simmet, T., Schrezenmeier, H., Jahrsdörfer, B., 2012. Human B cells differentiate into granzyme B-secreting cytotoxic B lymphocytes upon incomplete T-cell help. Immunol. Cell Biol. 90 (4), 457–467.

Hao, Y., O'Neill, P.J., Naradikian, M.S., Scholz, J.L., Cancro, M.P., 2011. A B-cell subset uniquely responsive to innate stimuli accumulates in aged mice. Blood 118 (5), 1294–1304.

Henneken, M., Dörner, T., Burmester, G.R., Berek, C., 2005. Differential expression of chemokine receptors on peripheral blood B cells from patients with rheumatoid arthritis and systemic lupus erythematosus. Arthritis Res. Ther. 7 (5), R1001–R1013.

Iwasaki, A., Kelsall, B.L., 2000. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. J. Exp. Med. 191 (8), 1381–1394.

Kaminski, D.A., Wei, C., Qian, Y., Rosenberg, A.F., Sanz, I., 2012. Advances in human B cell phenotypic profiling. Front. Immunol. 3, 302–316.

Kunkel, E.J., Butcher, E.C., 2003. Plasma-cell homing. Nat. Rev. Immunol. 3, 822–829.

Liao, F., Shirakawa, A.K., Foley, J.F., Rabin, R.L., Farber, J.M., 2002. Human B cells become highly responsive to macrophage-inflammatory protein-3 alpha/CC chemokines ligand-20 after cellular activation without changes in CCR6 expression or ligand binding. J. Immunol. 168 (10), 4871–4880.

Licastro, F., Candore, G., Lio, D., Porcellini, E., Colonna-Romano, G., Franceschi, C., Caruso, C., 2005. Innate immunity and inflammation in ageing: a key for understanding age-related diseases. Immun. Ageing 18, 2–8.

Mauri, C., 2010. Regulation of immunity and autoimmunity by B cells. Curr. Opin. Immunol. 22 (6), 761–767.

McNamee, E.N., Masterson, J.C., Jedlicka, P., Collins, C.B., Williams, I.R., Rivera-Nieves, J., 2013. Ectopic lymphoid tissue alters the chemokine gradient, increases lymphocyte retention and exacerbates murine ileitis. Gut 62 (1), 53–62.

Miltenyi, S., Muller, W., Weichel, W., Radbruch, A., 1990. A high gradient magnetic all separation with Macs. Cytometry 11, 231–238.

Moir, S., Ho, J., Malaspina, A., Wang, W., DiPoto, A.C., O'Shea, M.A., Roby, G., Kottilil, S., Arthos, J., Proschan, M.A., Chun, T.W., Fauci, A.S., 2008. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. J. Exp. Med. 205 (8), 1797–1805.

Müller, G., Lipp, M., 2003. Shaping up adaptive immunity: the impact of CCR7 and CXCR5 on lymphocyte trafficking. Microcirculation 10 (3–4), 325–334.

Nakayama, T., Fujisawa, R., Yamada, H., Horikawa, T., Kawasaki, H., Hieshima, K., Izawa, D., Fujiie, S., Tezuka, T., Yoshie, O., 2001. Inducible expression of a CC chemokine liver- and activation-regulated chemokine (LARC)/macrophage inflammatory protein (MIP)-3 alpha/CCL20 by epidermal keratinocytes and its role in atopic dermatitis. Int. Immunol. 13 (1), 95–103.

Othani, H., Nakayama, T., Yoshie, O., 2011. In situ expression of the CCL20–CCR6 axis in lymphocytes-rich gastric cancer and its potential role in the formation of lymphoid stroma. Pathol. Int. 61, 645–651.

Ouyang, Q., Wagner, W.M., Voehringer, D., Wikby, A., Klatt, T., Walter, S., Müller, C.A., Pircher, H., Pawelec, G., 2003. Age-associated accumulation of CMV-specific CD8$^+$ T cells expressing the inhibitory killer cell lectin-like receptor G1 (KLRG1). Exp. Gerontol. 38, 911–920.

Pawelec, G., Larbi, A., 2008. Immunity and ageing in man: annual review 2006/2007. Exp. Gerontol. 43, 34–38.

Pawelec, G., Akbar, A.N., Caruso, C., Solana, R., Grubeck-Loebenstein, B., Wikby, A., 2005. Human immunosenescence: is it infectious? Immunol. Rev. 20, 257–268.

Reibman, J., Hsu, Y., Chen, L.C., Bleck, B., Gordon, T., 2003. Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. Am. J. Respir. Cell Mol. Biol. 28 (6), 648–654.

Reif, K., Ekland, E.H., Ohl, L., Nakano, H., Lipp, M., Förster, R., Cyster, J.G., 2002. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. Nature 416 (6876), 94–99.

Salvioli, S., Monti, D., Lanzarini, C., Conte, M., Pirazzini, C., Bacalini, M.G., Garagnani, P., Giuliani, C., Fontanesi, E., Ostan, R., Bucci, L., Sevini, F., Yani, S.L., Barbieri, A., Lomartire, L., Borelli, V., Vianello, D., Bellavista, E., Martucci, M., Cevenini, E., Pini, E., Scurti, M., Biondi, F., Santoro, A., Capri, M., Franceschi, C., 2013. Immune system, cell senescence, aging and longevity–inflamm-aging reappraised. Curr. Pharm. Des. 19 (9), 1675–1679.

Sanz, I., Wei, C., Lee, F.E., Anolik, J., 2008. Phenotypic and functional heterogeneity of human memory B cells. Semin. Immunol. 20 (1), 67–82.

Sarra, M., Pallone, F., Monteleone, G., 2013. Interleukin-21 in chronic inflammatory diseases. Biofactors 39 (4), 368–373.

Schenkein, J.G., Park, S., Nahm, M.H., 2008. Pneumococcal vaccination in older adults induces antibodies with low opsonic capacity and reduced antibody potency. Vaccine 26, 5521–5526.

Schutyser, E., Struyf, S., Van Damme, J., 2003. The CC chemokine CCL20 and its receptor CCR6. Cytokine Growth Factor Rev. 14 (5), 409–426.

Singh, T., Newman, A.B., 2011. Inflammatory markers in population studies of aging. Ageing Res. Rev. 10 (3), 319–329.

Spolski, R., Leonard, W.J., 2008. Interleukin-21: basic biology and implications for cancer and autoimmunity. Annu. Rev. Immunol. 26, 57–79.

Stein, J.V., Nombela-Arrieta, C., 2005. Chemokine control of lymphocytes trafficking: a general overview. Immunology 116, 1–12.

Vanbervliet, B., Homey, B., Durand, I., Massacrier, C., Aït-Yahia, S., de Bouteiller, O., Vicari, A., Caux, C., 2002. Sequential involvement of CCR2 and CCR6 ligands for immature dendritic cell recruitment: possible role at inflamed epithelial surfaces. Eur. J. Immunol. 32 (1), 231–242.

Vasto, S., Candore, G., Balistreri, C.R., Caruso, M., Colonna-Romano, G., Grimaldi, M.P., Listi, F., Nuzzo, D., Lio, D., Caruso, C., 2007. Inflammatory networks in ageing, age-related diseases and longevity. Mech. Ageing Dev. 128 (1), 83–91.

Vitale, G., Mion, F., Pucillo, C., 2010. Regulatory B cells: evidence, developmental origin and population diversity. Mol. Immunol. 48 (1–3), 1–8.

Wei, C., Anolik, J., Cappione, A., Zheng, B., Pugh-Bernard, A., Brooks, J., Lee, E.H., Milner, E.C.B., Sanz, I., 2007. A new population of cells lacking expression of CD27 represents

a notable component of the B cell memory compartment in systemic lupus erythematosus. J. Immunol. 178, 6624–6633.
Welsh-Bacic, D., Lindenmeyer, M., Cohen, C.D., Draganovici, D., Mandelbaum, J., Edenhofer, I., Ziegler, U., Regele, H., Wüthrich, R.P., Segerer, S., 2011. Expression of the chemokine receptor CCR6 in human renal inflammation. Nephrol. Dial. Transplant. 26 (4), 1211–1220.
Williams, I.R., 2006. CCR6 and CCL20. Partners in intestinal immunity and lymphorganogenesis. Ann. N.Y. Acad. Sci. 1072, 52–61.
Wirths, S., Lanzavecchia, A., 2005. ABCB1 transporter discriminates human resting naive B cells from cycling transitional and memory B cells. Eur. J. Immunol. 35 (12), 3433–3441.