



## Review

# From lymphopoiesis to plasma cells differentiation, the age-related modifications of B cell compartment are influenced by “inflamm-ageing”



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## ABSTRACT

Ageing is a complex process characterized by a general decline in physiological functions with increasing morbidity and mortality. The most important aspect of ageing is the chronic inflammatory status, named “inflamm-ageing”, strictly associated with the deterioration of the immune function, termed “immunosenescence”. Both are causes of increased susceptibility of elderly to infectious diseases, cancer, dementia, cardiovascular diseases and autoimmunity, and of a decreased response to vaccination. It has been widely demonstrated that ageing has a strong impact on the remodelling of the B cell branch of immune system. The first evident effect is the significant decrease in circulating B cells, primarily due to the reduction of new B cell coming from bone marrow (BM) progenitors, as inflammation directly impacts on B lymphopoiesis. Besides, in aged individuals, there is a shift from naïve to memory immunoglobulins production, accompanied by the impaired ability to produce high affinity protective antibodies against newly encountered antigens. This is accompanied by the increase of expanded clones of B cells, which correlates with poor health status. Age-related modifications also occur in naïve/memory B cells subsets. Indeed, in the elderly, there is a reduction of naïve B cells, accompanied by the expansion of memory B cells that show a senescence-associated phenotype. Finally, elderly show the impaired ability of memory B cells to differentiate into plasma cells. It can be concluded that inflammation is the leading cause of the age-related impairment of B cell compartment, which play certainly a key role in the development of age-related diseases. This makes study of B cells in the aged an important tool for monitoring immunosenescence, chronic inflammatory disorders and the effectiveness of vaccines or pharmacological therapies.

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## 1. Introduction

In modern society, the increase of life expectancy of global population, coupled to a decline in birth rates in all developed countries, has led to an extraordinary demographic revolution characterized by an explosive growth in the number and proportion of elderly people. Indeed, it has been estimated the doubling of the number of people over sixty years in 2050, and the octogenarians are expected to increase from 125 million in 2015–434 million in 2050 (United Nations: World Population Prospects; <http://esa.un.org/unpd/wpp/>). This long human life span underscores the importance of studies on ageing, longevity and health status in the ageing population, as these strongly influence the Government's economic strategies because of the increase of the subjects, which are not autonomous and are affected by invalidating pathologies (Christensen et al., 2008).

Ageing is a complex process characterized by a general decline in physiological functions with an increasing morbidity and mortality (Avery et al., 2014). The goal of ageing research is to identify the processes involved in loss of physiological functions, which lead older individuals to develop multiple pathologies, with the aim of developing effective strategies of interventions. On this regard, many experimental evidences, from a wide variety of organisms, suggest some evolutionarily conserved hallmarks of ageing, as altered intercellular communication, genomic instability, epigenetic alterations, telomere attrition, impaired protein homeostasis, altered nutrient and growth factor responsiveness, mitochondrial dysfunction, stem cell exhaustion and cellular senescence (López-Otín et al., 2013).

However, there is a common consensus in the scientific community that attributes the cause and the consequence of many aspects of ageing to the increased inflammatory status in elderly. It is well known that in aged people, the levels of inflammatory mediators increase even in the absence of acute infections or other stressors (Singh and Newman, 2011). This condition, characterized by a general increase in plasma levels of pro-inflammatory mediators (Bruunsgaard, 2006; Krabbe et al., 2004; Puzianowska-Kuźnicka et al., 2016), leads to a chronic, low-grade, pro-inflammatory status known as "Inflamm-ageing" (Cevenini et al., 2010; Salvioli et al., 2013; Vasto et al., 2007). The origin of inflamm-ageing is not still completely understood, several concurrent contributing factors have been suggested, such as age-related remodelling of innate and acquired immune system cells and molecules, chronic antigenic stimulation, including latent virus infection, the increase in the amount of self-debris, due to the continuous turnover of cells and tissues, such as circulating mitochondrial DNA and modified macromolecules (DNA or proteins modified by oxidation, acylation or glycosylation) and the presence of senescent cells exhibiting a senescence-associated secretory phenotype, as well as age-related changes in microbiota (Franceschi and Campisi, 2014; Fulop et al., 2016; Magrone and Jirillo, 2013).

Inflamm-ageing is, therefore, directly linked to the immune system (Salvioli et al., 2013). Indeed, it has been widely demonstrated a strong association between changes in immune functions and longevity, that indicates as the deterioration of the immune function, termed "immunosenescence", could be the cause of the increased susceptibility to infectious diseases, cancer, dementia, cardiovascular diseases and autoimmunity, and a decrease response to vaccination which characterize elderly people (Bucci et al., 2014; Fulop et al., 2016; Grasse et al., 2016; Salvioli et al.,

2013). The age-associated deregulation of the immune system is due to changes in both innate and acquired immunity, strictly associated with a systemic chronic inflammation (Salvioli et al., 2013), as demonstrated in several longitudinal studies in which it was examined the relationship occurring between immune parameters and survival of very old subjects (Wikby et al., 2005). The Swedish longitudinal OCTO and NONA studies have shown the immune characteristics associated with mortality in advanced age, defined "Immune Risk Phenotype" (IRP), that involves the main lymphocyte subsets such as, high CD8<sup>+</sup> T cells percentage, low CD4<sup>+</sup> T helper and CD19<sup>+</sup> B cells percentages, the inverted CD4/CD8 ratio, impaired T cell proliferative responses and cytomegalovirus (CMV)-seropositivity (Strindhall et al., 2007; Wikby et al., 2005).

It has been well established that T cell compartment of elderly shows shrinkage of cellular repertoire and filling of the immunological space by a population of late-differentiated cytotoxic T cells, caused by the chronic antigenic load (Derhovanessian et al., 2010, 2013). The direct consequence of these changes, together with the reduced output of naïve T cells from the thymus, is the reduction of antigen-inexperienced T lymphocytes available for adequate immune response against newly encountered antigens (Aberle et al., 2013; Pawelec 2014a,b).

B cell branch of immune system also behaves as T cell compartment, with a shift in the magnitude of all B cell populations, a collapse in B cell receptor repertoire diversity, correlated with a poor health status, a modified peripheral B cell dynamics, and a weakened humoral response due to the impairment of antibodies production (Aberle et al., 2013; Bulati et al., 2011; Cancro et al., 2009; Dunn-Walters and Ademokun 2010; Frasca and Blomberg, 2016; Gibson et al., 2009; Naradikian et al., 2016).

From these considerations, it could be stated that ageing has a strong impact on the remodelling of the acquired immune system cells and that there is an age-related accumulation of immune cells with "exhausted/senescent" phenotype with altered functions, at the expense of naïve antigen-inexperienced cells (Bulati et al., 2011, 2014; Fulop et al., 2016; Larbi and Fulop, 2014; Naradikian et al., 2016; Pawelec, 2014a,b; Pinti et al., 2016; Rubtsova et al., 2015).

In this paper, we review data from literature on the age-related changes at systemic, molecular and cellular levels of B cells compartment, focusing on the important role played by B lymphocytes impairment as cause/effect of inflamm-ageing.

## 2. B cell development and peripheral maturation and differentiation

B cells originate in the bone marrow (BM) from hematopoietic stem cells (HSC) committed for specific B cell lineage. Pro- and pre-B cell precursor express two specific surface markers, CD10 and interleukin(IL)-7 receptor  $\alpha$ . In the presence of IL-17, produced into the BM, B cells rearrange the variable (V), diversity (D) and joining (J) regions of the immunoglobulin (Ig) heavy (H) chain genes in a stage defined pro-B cell. In this process are involved the products of RAG-1 and RAG-2 genes. Successful rearrangement initiates pre-B cell stage, where cells first express a pre-B cell receptor (BCR), together with two trans-membrane signaling molecules (Ig $\alpha$  and Ig $\beta$ ), then rearrange the Ig light chain gene (V and J gene) of the immunoglobulin. After that Ig genes rearrangement is complete, RAGs gene expression is down-regulated and CD10<sup>+</sup> immature B cells in the BM lose the expression of IL-7R $\alpha$ , express CD5 and start

**Table 1**  
Cellular age-related modification of B cell compartment.

	Name/Type	Location	Effect of ageing	References
Precursors	HSC	BM	Reduced	Pang et al. (2011), Rossi et al. (2005)
	B lymphopoiesis	BM	Reduced	Riley et al. (2013), Henry et al. (2015)
Mature B cells and Plasma Cells	CD19 <sup>+</sup> Total B cells	Periphery	Reduced	Bulati et al. (2011), Colonna-Romano et al. (2009), (2000), Lin et al., (2016), Pinti et al., (2016), Strindhall et al. (2007)
	Naïve [IgD <sup>High</sup> IgM <sup>High</sup> CD27 <sup>-</sup> ]	Periphery/LNs	Reduced	Bulati et al. (2011), Colonna-Romano et al. (2009), Gupta et al. (2005)
	Memory Unswitched [IgD <sup>High</sup> IgM <sup>High</sup> CD27 <sup>+</sup> ]	Periphery/LNs	Increased No Change	Frasca et al. (2008) Bulati et al. (2011), Colonna-Romano et al. (2009)
	Memory switched [IgD <sup>-</sup> (Switched Igs, IgG <sup>+</sup> /IgA <sup>+</sup> /IgE <sup>+</sup> ) CD27 <sup>+</sup> ]	Periphery/LNs	No Change	Bulati et al. (2011), Colonna-Romano et al. (2009)
	IgM-only memory [IgD <sup>-</sup> IgM <sup>+</sup> CD27 <sup>+</sup> ]	Periphery/LNs/Spleen	Reduced	Frasca et al. (2008, 2012b)
	Double Negative (DN) [IgD <sup>-</sup> (Switched Igs, IgG <sup>+</sup> /IgA <sup>+</sup> /IgE <sup>+</sup> ) CD27 <sup>-</sup> ]	Periphery/LNs/Inflamed Tissues	Reduced Increased	Buffa et al. (2011), Shi et al. (2005) Bulati et al. (2011), Colonna-Romano et al. (2009), Frasca et al. (2011)
	CD24 <sup>-</sup> CD38 <sup>-</sup> B cells	Periphery/LNs	Increased	Buffa et al. (2013), Palma et al. (2014)
	Age-associated B cells (ABC) [CD19 <sup>+</sup> CD11b <sup>+</sup> CD11c <sup>+</sup> CD21 <sup>-</sup> IgD <sup>-</sup> ]	Periphery/LNs	Increased	Naradikian et al. (2016), Rubtsova et al. (2015)
TNF- $\alpha$ -producing B cells	Periphery/LNs	Increased	Frasca et al. (2014)	
Plasma Cells	Periphery/BM	Reduced	Pritz et al. (2015)	

to express IgM as part of a not yet functional BCR (Sagaert and De Wolf-Peeters, 2003).

The most part of immature B cells leave BM and complete their differentiation in the periphery in a series of stages that lead to the formation of transitional B cells. These cells continue to express CD10, co-express IgD and IgM and present high expression of CD24 and CD38 (Carsetti et al., 2004). A little percentage of these CD24<sup>high</sup>CD38<sup>high</sup> transitional B cells are founded in peripheral blood and are able to produce IL-10, behaving as B regulatory cells (Blair et al., 2010; Carsetti et al., 2004). The fate of these cells depends on BCR signalling strength (Pillai and Cariappa, 2009). Indeed, some of them join the marginal zone of the spleen, where they differentiate into CD27<sup>+</sup>IgM<sup>+</sup> B cells, which have the role to provide a first line defence against encapsulated bacteria (Shi et al., 2005). While, the large majority of transitional B cells become mature follicular B cells, which include precursors of naïve and memory B cells, and represent about 80–90% of total B lymphocytes. Mature B cell survival and peripheral activation is influenced by B-cell maturation protein (BCMA), a member of the tumor necrosis factor (TNF) receptor family that is expressed in B lymphocytes. BCMA binds two proteins belonging to TNF family, B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), produced by monocytes, macrophages and dendritic cells, that stimulate cellular proliferation and survival (Reber et al., 2012). Mature B cells, can be found in the periphery as well as in secondary lymphoid organs, lose the expression of CD10, become able to recognize antigens and express different surface markers. In particular it is possible to identify circulating naïve and memory B cells on the basis of the differential expression of IgD and CD27 surface markers as follows: IgD<sup>+</sup>CD27<sup>-</sup> naïve B cells, IgD<sup>+</sup>CD27<sup>+</sup> memory unswitched B cells, IgD<sup>-</sup>CD27<sup>+</sup> memory switched B cells and IgD<sup>-</sup>CD27<sup>-</sup> double negative (DN) memory B cells (Buffa et al., 2013; Bulati et al., 2011, 2014; Colonna-Romano et al., 2009; Frasca et al., 2011; Kaminski et al., 2012; Shi et al., 2005). Moreover, mature B cells are able to recirculate from periphery to lymphoid organs or other tissues, due to the expression of different combination of

chemokine receptors on their surface (Perez-Andres et al., 2010; Stein and Nombela-Arrieta, 2005).

The ability of mature B cells to undergo germinal centre (GC) reaction, producing highly specific Igs, depends on immunoglobulin affinity maturation, a process governed by both cytokines production in GCs and by the T-B cells cognate interaction, through the triggering of CD40, on activated B cells, with CD154 (or CD40 Ligand), expressed on the T cell membrane. Ligation of cytokines, as IL-4, IL-10, IL-21, with their receptors on B cells, causes the activation of STAT6, NF- $\kappa$ B and of activation-induced deaminase (AID) transcription factors (Stavnezer et al., 2008). The action of AID, that introduces double strand breaks in the Ig germline, leads to the introduction of point mutations in the V region of memory B cells, called somatic hyper-mutations (SHMs), that modulate the antibody-antigen (Ab-Ag) affinity, while in the C region of naïve B cells, AID action, leads to class switch recombination (CSR), that allows the transcription of the selected V region in association with IgG, IgA, or IgE, having better effector functions than IgM.

Finally, serological memory is maintained by long lived plasma cells, that recirculate between the periphery and the BM, and by memory B cells being continuously re-stimulated by bystander cytokines producing-T cells as well as by microbial products triggering Toll-like Receptors (TLRs) expressed on B cells, even in the absence of specific antigens (Bernasconi et al., 2002).

### 3. The reduced ability of elderly people to respond to newly encountered antigens and vaccinations reflects the age-related impairment of humoral immune response

#### 3.1. Effect of ageing on B cell lymphopoiesis

As discussed, the ageing process is associated with functional impairment at the tissue, cellular and molecular levels (López-Otín et al., 2013). These age-associated changes are particularly evident in the BM. Indeed, in the elderly, it has been shown a reduction in the BM density and changes in the vascularization and in the com-

**Table 2**  
Serological and molecular age-related modification of B cell compartment.

Molecules	Age-related modification	Effect of modification	References
IL-7-enriched niches in the BM	Reduced	Impaired B lymphopoiesis	Henry et al. (2015), Ueda et al. (2004)
BAFF plasma levels	Reduced	Impaired survival and maturation of peripheral B cells	Jin et al. (2008)
APRIL plasma levels	Reduced		Jin et al. (2008)
Total Serum Igs	No Change	Impaired ability to respond to new antigenic challenge	Aberle et al. (2013), Listi et al. (2006), Naradikian et al. (2016), Weksler and Szabo (2000)
Serum IgD/IgM	Reduced		Dunn-Walters, (2016), Gibson et al. (2009), Weksler and Szabo (2000)
Serum IgG/IgA	Increased		
B cell receptor repertoire	Reduced	Impaired response against foreign antigens, expansion of oligoclonal populations, reduced diversity of BCR repertoire, reduced antibody specificity and affinity, increased reactivity against autologous molecules	
E47 expression	Reduced	Reduced ability to generate higher affinity protective antibodies	Frasca et al. (2013, 2014, 2015a,b)
AID expression	Reduced		
miR-155 expression	Increased	Induce AID mRNA degradation	Frasca et al. (2016)
miR-16 expression	Increased	Induce E47 mRNA degradation	
BTLA expression	Reduced	Decreased induction of long-lived plasma cells, memory B cells and antibody titres	Kannan et al. (2015)
Blimp-1 Expression	Reduced	Impaired ability of B cells to differentiate into plasma cells	Frasca et al. (2016)

position of the resident cells (Chan and Duque, 2002). It has been widely demonstrated that the function of HSC significantly declines with age (Pang et al., 2011; Rossi et al., 2005). Hematopoietic defects are particularly evident in the lymphoid lineages, and can be attributed to the skewing of the HSC repertoire toward myeloid lineages (Beerman et al., 2010; Guerretaz et al., 2008). Moreover, the aged BM microenvironment has been suggested to contribute to the reduction of B lymphopoiesis (Riley, 2013). Recently, it has been demonstrated that TNF- $\alpha$  and high concentration of transforming growth factor (TGF)- $\beta$ , suppress HSC activity (Challen et al., 2010; Pronk et al., 2011), while IFN- $\alpha$ , IFN- $\gamma$  and low concentration of TGF- $\beta$ , activate HSC proliferation (Baldridge et al., 2010; Essers et al., 2009). In addition, inflammation has been shown to directly impair B lymphopoiesis (Table 1) by preventing B progenitors localization to the IL-7-enriched niches required for B cell development (Henry et al., 2015; Ueda et al., 2004). So, given the strong correlation between advanced age and chronic systemic inflammation previously stated, inflamm-ageing has an important impact on B cell progenitors population in elderly, not only reducing the output of new B cells, but also influencing the evolution of leukaemia, typical cancer of elderly people (Henry et al., 2015). Indeed, preventing the age-associated reduction of B cell progenitors, by using transgenic expression of two different anti-inflammatory proteins, as  $\alpha$ -1-antitrypsin and IL-37, in old mice, it has been demonstrated that selection for oncogene-initiated progenitors were abrogated (Henry et al., 2015).

### 3.2. Serum immunoglobulins production in elderly

The effect of ageing on B cell compartment is a dramatic decrease in circulating B lymphocytes (Table 1), also observed in very old and centenarian subjects (Bulati et al., 2011; Colonna-Romano et al., 2009; Globerson and Effros, 2000; Lin et al., 2016; Pinti et al., 2016; Strindhall et al., 2007). As previously stated, reduced numbers and percentages of CD19<sup>+</sup>B cells are part of the cluster of immune parameters, collectively known as “IRP”, associated with 2-, 4-, and 6-year mortality of the very elderly in the Swedish OCTO/NONA longitudinal studies (Strindhall et al., 2007; Wikby et al., 2005). This dramatic age-related reduction of mature B cells is influenced, not only by the reduced output of new B cells, but also by changes in the microenvironment that supports the survival of mature B cells in the periphery. The two TNF ligand superfamily proteins, BAFF and APRIL, are critical survival factors for peripheral mature B cells and it has been demonstrated that, in elderly people, the blood plasma level of these factors is reduced (Table 2) (Jin et al., 2008).

Furthermore, total serum immunoglobulin levels do not change with age (Aberle et al., 2013; Naradikian et al., 2016; Weksler and Szabo, 2000). Switched immunoglobulins (IgG and IgA) levels are positively age-related, whereas a reduced ability of the elderly and centenarians to produce IgD and IgM is reported (Listi et al., 2006). These Igs are together expressed on mature B cells newly produced by the bone marrow, hence suggesting that in the elderly and in centenarians, the B cell repertoire available to respond to new antigenic challenge is impaired (Table 2). Moreover, functional studies reported the impaired ability of memory B cells to produce Igs, after *in vitro* stimulation, and the reduction of plasma cell differentiation in elderly people (Naradikian et al., 2016; Pritz et al., 2015; Shi et al., 2005). These results are in agreement with the reduction of clonotypic response to newly encountered antigens in the elderly, together with an increased expansion of monoclonal B cells, that always lead to lymphoid malignancy that emerge with a relative frequency in advanced age (Henry et al., 2015).

### 3.3. Age related intrinsic defects and the shrinkage of repertoire diversity in elderly people

As above discussed, in aged individuals there is a shift from IgM, mainly produced by naïve B cells, to IgG or IgA, produced by memory cells, production (Listi et al., 2006), accompanied by an impaired ability to produce high affinity protective antibodies against infectious agents. This feature is due to both extrinsic, or intrinsic defects, as impaired T-B cell signalling (Haynes et al., 2003; Pinti et al., 2016), or decreased SHM and CSR in germinal center B cells (Frasca and Blomberg, 2016; Zheng et al., 1997), respectively, which cause a reduced production of switched and high affinity Igs.

In particular, it has been demonstrated a pivotal role of the age-related intrinsic defects as the cause of the impaired ability of elderly people to respond to new infections or vaccines (Frasca et al., 2013, 2014, 2015a,b). These defects include the decreased expression and production of the E47 transcription factor, which controls the expression of AID, the enzyme of CSR and SHM, which, in turn, results reduced (Table 2). As AID is considered a measure of optimal B cell responses, its decreased expression on B cells from elderly individuals, explains, in part, their reduced ability to generate higher affinity protective antibodies (Frasca and Blomberg, 2014, 2016; Khurana et al., 2012). In addition, it has been also shown an age-related reduction of E47 and AID mRNAs expressions, caused by the reduced mRNA stability. This phenomenon is strictly related to inflamm-ageing (Frasca and Blomberg, 2008), indeed, in the elderly, it has been observed the increased expression of the



two pro-inflammatory micro-RNAs (miRNAs) (Table 2), miR-155 and miR-16, that respectively bind the 3'-untranslated region (UTR) of AID mRNA and the 3'-UTR of E47 mRNA, inducing their degradation (Frasca et al., 2015a). The increase of these two miRNAs in the elderly is also related to the stage of differentiation of B cells. Indeed, it has been recently observed that only memory B cells and, in particular, DN B cells, express high levels of these two molecules (Frasca et al., 2017).

Mi-RNAs are a class of molecule involved in gene expression regulation. Many studies have been demonstrated that they act as modulators of some pathways (NF- $\kappa$ B, mTOR, TGF- $\beta$ ) related to inflammation, cellular senescence and age-related diseases, including cancer (Reviewed by Oliveri et al., 2013).

Concerning miR-155, it has been shown that it is an onco-microRNA involved in tumor initiation and development of several B cell malignancies, typical of elderly (Lawrie, 2013). It has been recently shown that miR-155 was up-regulated in several malignancies, as diffuse large B cell lymphoma and chronic lymphocytic leukaemia, and its over-expression is also associated with poor prognosis, rendering miR-155 a good diagnostic and prognostic biomarker in B cell cancer (Due et al., 2016). Moreover, both miR-155 and miR-16, were demonstrated to be over-expressed, at the systemic level, during the natural course of Rheumatoid Arthritis (Churov et al., 2015), demonstrating the pro-inflammatory role of these two molecules.

At molecular level, the specificity of a single B cell is dependent on the variable region of Ig genes. For this reason the study of Ig genes can determine the extent of the repertoire in terms of BCR specificity. Age-related changes in the B cell repertoire (Table 2) involve impaired response against foreign antigens, expansion of oligoclonal populations, reduced diversity of the B cell repertoire, the increase of the reactivity against autologous molecules and, probably, reduced antibody specificity and affinity (Dunn-Walters, 2016; Gibson et al., 2009; Weksler and Szabo, 2000). Reduced B cell diversity could be due to both the impaired B cell lymphopoiesis in the BM and to the reduced diversification in the GC (Weksler and Szabo, 2000). The diversity of the B cell repertoire can be measured using spectratype analysis of the 3rd complementary-determining region (CDR3) of the immunoglobulin (Dunn-Walters, 2016; Gibson et al., 2009). The diversity of the Ig heavy chain (IgH) CDR3 region is due to the addition and/or deletion of nucleotides during V, D and J genes rearrangement (VanDyk and Meek, 1992). In healthy young people, the mRNA CDR3 size diversity, in spectratype analysis, is represented by a bell-shaped profile of peaks. While, elderly people show a distorted profile in their CDR3 spectratype from the normal distribution, indicating a collapse in B cell diversity and an oligoclonal expansion in the B cell repertoire which correlate with poor health survival in the very elderly (Gibson et al., 2009).

The advent of high-throughput sequencing has enabled a more detailed and comprehensive study of changes in the Ig gene repertoire. Indeed, it has been shown that older people have switched memory cells with a higher load of mutation also before vaccination; this observation confirms in detail that some people have pre-expanded repertoire of B cells (Ademokun et al., 2011; Dunn-Walters, 2016), which ultimately leads to a less efficient response to new encounter antigens or vaccines (Jiang et al., 2013).

So, the reduced antibody specificity and affinity may be true for some very specific case, but whether such change are directly caused by advanced age requires more studies on BCR sequencing and a better knowledge of their functions at the repertoire level. The age-related reduced AID activity and the oligoclonal expansion of the B cell repertoire, alone, are not enough to explain the altered Igs production and the impaired ability to respond to new antigens of elderly people. To confirm whether these features have an important role in the quality and quantity changes of immunoglobulin,

longitudinal analysis, in combination with deep sequencing of Igs, are indispensable.

### 3.4. Effect of chronic viral infections in humoral immune response

An important role in the ageing of B cell compartment, as well as in the age-related T cell impairment (Oxford et al., 2015; Pawelec et al., 2014a,b), is played by chronic viral infections such as CMV and Epstein-Barr virus (EBV). These two herpes viruses have a selective effect on the B cell repertoire, affecting the clonal expansion (EBV) or the levels of mutation of antibodies (CMV) (Wang et al., 2014). The consequence is that, with ageing, the CMV- or EBV-seropositivity and/or their reactivation, result to be a selective pressure, for the impairment of the humoral arm of the immune system in people of advanced age (Dunn-Walters, 2016). At this regard, it has been recently demonstrated (Pritz et al., 2015) that the frequency of plasma cell decreases with age, except for influenza A- and CMV-specific plasma cells, that are not affected by age. Moreover, with the exception of CMV, peripheral antibodies concentration is correlated with BM plasma cells of the same specificity, but is independent on antigen-specific memory B cells in the periphery. These results demonstrate that the frequency of specific plasma cells may reflect the number and time point of previous antigen encounters together with the intrinsic age-related changes in the BM (Pritz et al., 2015).

Inflamm-aging and, in particular, CMV-seropositivity, is also the cause of the age-related increase of B cells that spontaneously produce higher amounts of TNF- $\alpha$  (Table 1). These TNF- $\alpha$ -expressing B cells are positively correlated with serum TNF- $\alpha$  and negatively correlated with the ability of B cells to respond to in vitro stimulation, measured by AID (Frasca et al., 2012a, 2014). Additionally, high serum TNF- $\alpha$  levels and increased TNF- $\alpha$ -producing B cell, are also negatively correlated with the in vivo serum response to the influenza vaccine (Frasca et al., 2014), suggesting that TNF- $\alpha$ , in addition to micro-RNAs (miR-155 and miR-16), is a marker of intrinsic B cell inflammation, which contributes to the down-regulation of AID in B cells from aged individuals. The higher levels of both serum TNF- $\alpha$  and of TNF- $\alpha$ -producing B cells in the elderly are strictly associated with the age-related increase of CMV seropositivity (Pinti et al., 2016), which has been shown to have a negative effect on influenza vaccine-specific antibody responses (Frasca et al., 2015b).

### 3.5. The age-related impairment of B cell functions is associated with low responsiveness to influenza vaccination

Even though the vaccine efficacy tends to be less than optimal, influenza vaccination continues to be highly recommended in the elderly, as influenza is strictly associated with morbidity and mortality in very old people, because of the complications due to the infection as primary influenza pneumonia or secondary bacterial infections, together to the exacerbations of pre-existing medical conditions (Frasca and Blomberg, 2014). The immune response to influenza virus antigens is unique in humans, as they are repeatedly exposed to the virus, through natural infection or vaccination. In adults, influenza vaccine elicits both recall responses to epitopes that are shared with previously encountered viruses and primary responses to new antigenic epitopes (Kannan et al., 2015). For these reasons, recall responses are expected to be particularly predominant in the elderly due to their several exposures to influenza viruses or vaccines, but, as previously discussed, there is an age-related decrease of the influenza vaccine-specific antibody response. In a recent paper (Kannan et al., 2015), it has been demonstrated that B cells from elderly show a significant reduced expression of the co-inhibitor B and T lymphocyte attenuator (BTLA) prior to vaccination compared to young subjects. The

authors demonstrated that BTLA expression on mature B cells is linked to the increase of H1N1-specific IgG antibody titres and is involved in the shifting of the response from IgM to IgG. So, the decline of BTLA expression on B cells (Table 2), may be related to decreased induction of long-lived plasma cells, memory B cells and antibody titres in aged people (Kannan et al., 2015).

Moreover, in a recent paper (Frasca et al., 2016), it has been demonstrated that, with ageing, the *in vivo* antibody response to the new influenza vaccine, the *in vitro* B cell activation and the expression of the transcription factor E47, are significantly decreased. But, surprisingly, memory B cells are maintained, and this is probably due to their amplification in response to repeated vaccinations. Indeed, the authors have shown that, nevertheless the serum Igs titres after vaccination were lower in the elderly, the frequencies of influenza vaccine-specific memory B cells and plasma blasts were not dissimilar between young and elderly individuals. Moreover, they have shown an age-related reduction of the transcription factor Blimp-1 (Table 2), which regulates plasma cells differentiation, suggesting that, even if the frequency of memory B cells is maintained during ageing, they have an impaired ability to differentiate to plasma cells (Frasca et al., 2016). Finally, as discussed, chronic CMV infection, is able to down-regulate the antibody response to the influenza vaccine both directly, inducing TNF- $\alpha$  production by B cell, and indirectly through the induction of terminally differentiated T cells and accumulation of senescent T cells, which could lead to reduced generation of memory T cells (Derhovanessian et al., 2013; McElhane et al., 2010).

#### 4. Age-related remodeling of B cell subpopulations and the appearance of senescent/exhausted B cells involved in inflamm-ageing

##### 4.1. Age-related remodelling of naïve/memory B cell subsets

The age-related impairment of antibodies production and the shrinkage of the repertoire diversity are obviously reflected in changes occurring in naïve/memory B cells subsets. This is an important feature, as it is known that B cells also have regulatory and effector functions, such as T cell and dendritic cell regulation, cytokine, chemokine and other effector molecules, as granzyme B, production (Bulati et al., 2014; Harris et al., 2000; Sanz et al., 2008), rendering these cells key mediators in the control of the immune response and/or in the generation or maintenance of inflammation (Klinker and Lundy, 2012; Lund, 2008; Lund and Randall, 2010; Rosser and Mauri, 2015; Vazquez et al., 2015).

As previously stated, the reduced number of circulating B lymphocytes is a typical feature of ageing (Strindhall et al., 2007), and changes in the relative frequencies of the different B cell subsets have been reported (Bulati et al., 2011; Colonna-Romano et al., 2009; Frasca et al., 2011; Lin et al., 2016; Naradikian et al., 2016). The univocal evaluation of specific B cell subsets is complicated, both by variations between individuals (Lin et al., 2016), and by the use of different phenotype markers to characterize them, which are correlated with their functional/developmental stage, activation status, recirculation properties or anatomic location. So, different functional B cell subsets have been identified in humans using many cellular markers, and, among these IgD, CD27, CD24 and CD38, as well as surface Igs (IgM, IgG, IgA).

However, “core” subsets may be identified by evaluating only IgD and CD27 expression on CD19B cells (Kaminski et al., 2012). As above discussed, on the basis of the expression of these two markers, circulating B cells can be divided into different functional subsets: (1) IgD<sup>+</sup>CD27<sup>-</sup> naïve B cells, (2) IgD<sup>+</sup>CD27<sup>+</sup> memory unswitched B cells, (3) IgD<sup>-</sup>CD27<sup>+</sup> memory switched (IgG<sup>+</sup> or IgA<sup>+</sup>) B cells and (4) IgD<sup>-</sup>CD27<sup>-</sup> double negative memory switched

(IgG<sup>+</sup> or IgA<sup>+</sup>) (DN) B cells, that as reviewed (Bulati et al., 2011), show characteristics of senescent/exhausted memory B cells. The unswitched cells also express IgM and have been designated as IgM memory B cells (IgD<sup>+</sup>IgM<sup>+</sup>CD27<sup>+</sup>), while some of the IgD<sup>-</sup>CD27<sup>+</sup> (memory switched) are IgM<sup>+</sup> and have been designated as “IgM-only” memory B cells (Buffa et al., 2013; Bulati et al., 2011, 2014; Colonna-Romano et al., 2009; Frasca et al., 2011; Kaminski et al., 2012; Shi et al., 2005). On the basis of this kind of classification, it has been shown a significant age-related reduction of IgD<sup>+</sup>CD27<sup>-</sup> naïve B cells (Colonna-Romano et al., 2009; Gupta et al., 2005; Lin et al., 2016), no age-related changes in IgD<sup>+</sup>CD27<sup>+</sup> memory unswitched and IgD<sup>-</sup>CD27<sup>+</sup> memory switched B cells (Bulati et al., 2011; Colonna-Romano et al., 2009), while IgD<sup>-</sup>CD27<sup>-</sup> DN memory B cells are significantly increased in the elderly and in centenarians (Bulati et al., 2011; Colonna-Romano et al., 2009; Frasca et al., 2011). Other authors, instead, have described the increase of naïve B cells in the elderly (Frasca et al., 2008), but they considered these cells as CD19<sup>+</sup>CD27<sup>-</sup>IgG<sup>-</sup>IgA<sup>-</sup>, so by lack of switched receptor rather than by the presence of IgD. Moreover the same authors have described an age-related decrease of memory switched B cells, identifying them as IgG<sup>+</sup>/IgA<sup>+</sup>CD27<sup>+</sup> (Frasca et al., 2012b) (Table 1). For these reasons, a further characterization of naïve/memory B cells, identified by IgD and CD27 markers, was carried out, and it revealed, in each of the four subpopulations, a particular surface Igs (IgM, IgG or IgA) expression. Briefly, it has been shown that both naïve (IgD<sup>+</sup>CD27<sup>-</sup>) and memory unswitched (IgD<sup>+</sup>CD27<sup>+</sup>) B cells, express only IgM, in both young and elderly subjects. Concerning DN (IgD<sup>-</sup>CD27<sup>-</sup>) memory cells, most of them were IgG<sup>+</sup> or IgA<sup>+</sup> (Table 3), and less than 10% were IgM<sup>+</sup>. Also in this case it was not found any age-related differences in the relative expression of IgM, IgG or IgA isotypes. In contrast, the proportion of IgG, IgA and IgM memory B cells in the switched memory compartment (IgD<sup>-</sup>CD27<sup>+</sup>) resulted to be different between young and old, with a significant decrease of IgM<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup> memory B cells (Table 1) in the elderly (Buffa et al., 2011). This result is in agreement with previous data demonstrating a reduction of “IgM-only” memory B cells in the elderly (Shi et al., 2005), who are hence more susceptible to infection with encapsulated bacteria. It has been also shown a not significant increase in the expression of IgG and IgA on switched memory B cells from old donors (Buffa et al., 2011), although the relative proportions of the different isotypes of memory cells mirror the relative proportion of serum immunoglobulin of the different isotypes (Listì et al., 2006). Moreover, together with the switched immunoglobulins expression, evaluating the number of somatic mutations in the IgG VH regions of IgD<sup>-</sup>CD27<sup>+</sup> and IgD<sup>-</sup>CD27<sup>-</sup> B cells in young and elderly donors, it has been estimated the effect of ageing on B cell receptor hypermutation. The results have shown that, in both young and elderly subjects, IgG<sup>+</sup>IgD<sup>-</sup>CD27<sup>-</sup> DN memory B cells have significant fewer mutations (Table 3) than IgG<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup> switched memory B lymphocytes, although the rate of mutations observed in the DN B cell subset of old people is significantly reduced when compared to the rate of mutations observed in DN cells from young subjects. Moreover, there were no differences between the two age groups in the IgG<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup> switched memory B cell compartment (Buffa et al., 2011).

The increase of the DN memory B cells in the elderly (Bulati et al., 2011; Colonna-Romano et al., 2009), together with the reduced rate of mutation, also described by Fecteau et al. (2006), might be due to the disconnected generation of these cells from germinal centres, as it has been demonstrated that ageing negatively affects the germinal centre formation in secondary lymphoid tissues (William et al., 2002). The hypothesis is that, IgD<sup>-</sup>CD27<sup>-</sup> DN B cells could derive from IgD<sup>-</sup>CD27<sup>+</sup> memory switched B cells, which have lost CD27 expression, or, alternatively, due to their low number of SHM, DN B cells could directly originate from IgD<sup>+</sup>CD27<sup>-</sup> naïve B cells that are

**Table 3**  
Phenotypic and molecular characteristics of IgD-CD27- DN/LM B cells in the elderly.

Characteristics	Meaning	References
Switched slg (IgG, IgA) Short Telomeres Lower Expression of Co-stimulatory Molecules Reduced SHM	Memory cells	Colonna-Romano et al. (2009)
Not Impaired GrB Production CCR6 <sup>+</sup> CCR7 <sup>+</sup> Increased miR-155, miR-16 and miR-93 Increased TNF- $\alpha$ /IL-6 mRNA Increased p16 <sup>INK4</sup> mRNA spontaneous phosphorylation of AMPK and p38MAPK	Do not act as APC DN B cells could derive from memory switched B cells, which have lost CD27 expression, or, alternatively, DN B cells could directly originate from naïve B cells that are triggered by polyclonal stimuli Effector Function Pro-inflammatory Trafficking Phenotype Senescent-Associated Secretory Phenotype (SASP)	Buffa et al. (2011) Bulati et al. (2014) Frasca et al. (2017)

triggered by polyclonal stimuli, as microbial products (Bulati et al., 2011; Colonna-Romano et al., 2009; Palma et al., 2014).

Using another cellular approach, that takes into account the developmental B cell markers, CD24 and CD38, it is possible to identify four different B cell population, as (1) CD19<sup>+</sup>CD38<sup>high</sup>CD24<sup>high</sup>, transitional B cells, that include immature and IgD<sup>+</sup>CD27<sup>-</sup> naïve B cells, (2) CD19<sup>+</sup>CD38<sup>int</sup>CD24<sup>int</sup>, defined as mature naïve B cells, that are mainly IgD<sup>+</sup>CD27<sup>-</sup>, (3) CD19<sup>+</sup>CD38<sup>-</sup>CD24<sup>high</sup> primarily memory B cells, which show a variety of different memory phenotypes, including IgD<sup>-</sup>CD27<sup>-</sup> DN B cells, and (4) a not thus far described CD19<sup>+</sup>CD38<sup>-</sup>CD24<sup>-</sup> population of B cells, which include both IgD<sup>-</sup>CD27<sup>+</sup>switched memory and IgD<sup>-</sup>CD27<sup>-</sup> DN B (Blair et al., 2010; Buffa et al., 2013; Carsetti et al., 2004). Interestingly, it has been demonstrated an age-related increase of these CD19<sup>+</sup>CD38<sup>-</sup>CD24<sup>-</sup> B cells (Table 1), which also seem to be involved in inflammation, as they are able to produce TNF- $\alpha$  (Buffa et al., 2013; Palma et al., 2014).

Moreover, recently, an age-related accumulation of a subset of B cells, with unique phenotypic and functional features, termed age-associated B cells (ABC), that resemble IgD<sup>-</sup>CD27<sup>-</sup>DN B cells, has been identified in mice and humans (Table 1) (Naradikian et al., 2016; Rubtsova et al., 2015). These cells are characterized by a T-BET driven transcriptional program, robust responsiveness to TLR7 and TLR9 ligands, and a propensity for IgG2a production. Despite ABC (CD19<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>CD21<sup>-</sup>IgD<sup>-</sup>) and DN (CD19<sup>+</sup>IgG<sup>+</sup>IgD<sup>-</sup>CD27<sup>-</sup>) B cells cannot be compared, due to their different phenotypic characterization, they share some characteristics. Indeed, it has been proposed that ABC are a memory subset generated by nucleic acid containing antigens in the context of a pro-inflammatory cytokine milieu, are consider exhausted B cells and they also arise during humoral autoimmune diseases (Naradikian et al., 2016; Rubtsova et al., 2015). Furthermore, an in vivo study has suggested that these cells are also an abundant source of TNF- $\alpha$  and inhibit survival of B-cell precursors (Ratliff et al., 2013).

The conflicting data in the literature, regarding the distribution of naïve/memory B cells in the elderly, and the presence of exhausted/senescent B cells with different phenotypes (DN, CD24<sup>-</sup>CD38<sup>-</sup> or ABC), strengthen the idea of using shared markers to uniquely characterize the memory/naïve B cell subpopulations (Kaminski et al., 2012).

#### 4.2. DN B lymphocytes are exhausted/senescent cells involved in inflamm-ageing, but, in some circumstances, might be 'active'

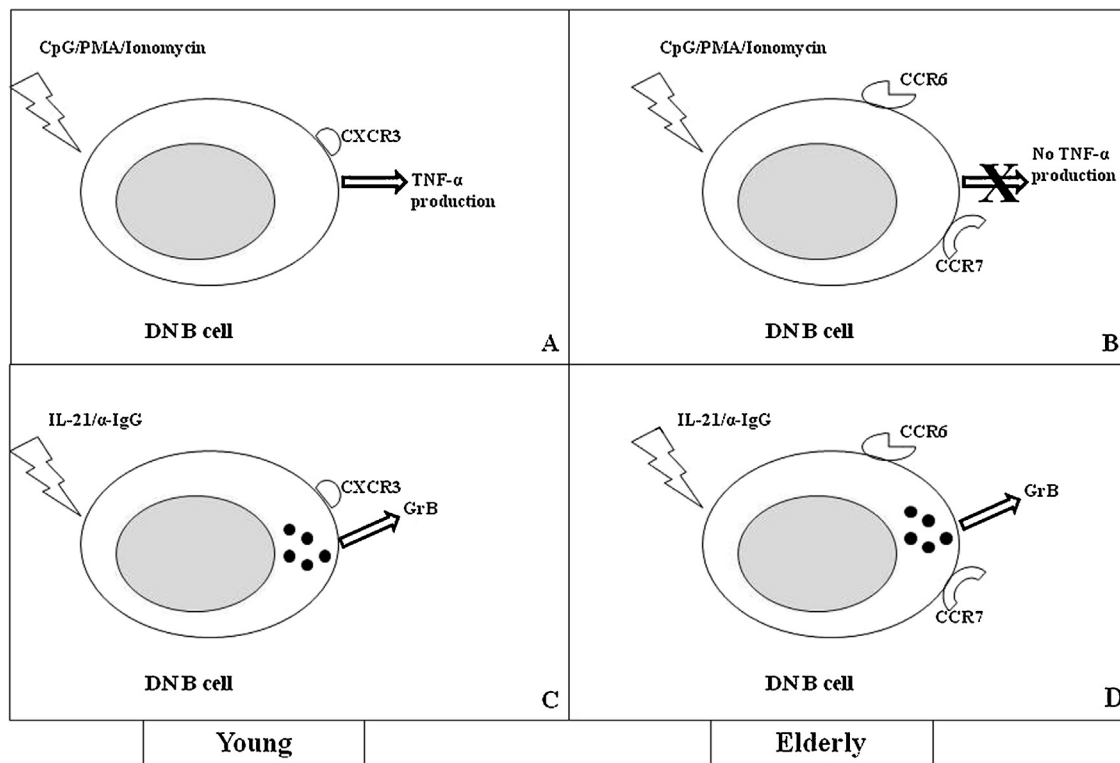
IgD<sup>-</sup>CD27<sup>-</sup>DN B cells have been also reported to be expanded, in percentage, in other models of "chronic stimulation", as in patients affected by Systemic Lupus Erythematosus (SLE) or Rheumatoid Arthritis (RA), in HIV-infected people, in subjects challenged with Respiratory Syncytial Virus and in Alzheimer's disease (AD)

patients (Bulati et al., 2015; Fecteau et al., 2006; Mahmood et al., 2015; Palma et al., 2014; Sanz et al., 2008; Wei et al., 2007). So, probably, the increase in percentage of the DN B cell population observed in the elderly, might be related to the typical inflammatory microenvironment, characterized by a general increase in plasma levels of pro-inflammatory cytokines and other inflammatory mediators (inflamm-ageing). Notably, neither in the elderly nor in the abovementioned models of chronic inflammation, it has been reported the increase of the absolute number of DN B cells, as these conditions are characterized by lymphopenia. It can be stated that, as demonstrated for T cells (Pawelec, 2014a,b), DN B cells could be senescent/exhausted memory cells, and their expansion might be the manifestation of a physiologic modification time-related (ageing) or a pathologic deregulation (SLE or RA patients, HIV infected subjects, AD patients) of the immune system.

Although the different phenotype approach used to identify naïve and memory B cells, there is a consensus according to the age-related increase in percentage of late/exhausted memory B cells or antigen experienced pro-inflammatory B cell subsets (Buffa et al., 2013; Bulati et al., 2011; Colonna-Romano et al., 2009; Fecteau et al., 2006; Naradikian et al., 2016), which could be the by-product and/or the cause of inflamm-ageing. The term "exhausted" could be reported to terminally differentiated-senescent cells, that express the cell cycle regulator p16<sup>INK4</sup>, which induces cell cycle arrest, even these cells remain metabolically active, and undergo distinct phenotypic alterations, as well as telomere shortening and decondensation of peri-centromeric satellite DNA (Farr et al., 2016; Pinti et al., 2016). These cells accumulate in multiple tissues with ageing, and produce dysfunctional molecules (Farr et al., 2016; Pinti et al., 2016).

As demonstrated, IgD<sup>-</sup>CD27<sup>-</sup> peripheral B cells, namely senescent/exhausted B cells (Bulati et al., 2011; Colonna-Romano et al., 2009; Frasca et al., 2017; Kaminski et al., 2012), have shorter telomeres (see below), show lower expression of co-stimulatory molecules (Table 3) (Colonna-Romano et al., 2009), seem to be pre-activated, as they produce pro-inflammatory cytokines before stimulation (Frasca et al., 2012a), and are "refractory" to undergo in vitro class switch when stimulated by antigens and mitogens (Fig. 1) (Buffa et al., 2011, 2013; Colonna-Romano et al., 2009; Fecteau et al., 2006; Pinti et al., 2016). So, the hallmark of senescent cells is the acquisition of a senescence-associated phenotype, which involves the chronic secretion of numerous pro-inflammatory cytokines, chemokines, growth factors and proteases (Pinti et al., 2016).

Concerning the difference in telomere length between naïve and memory B cells in humans, the studies are controversial. Indeed, Colonna-Romano et al. (2009), reported that memory B cells have shorter telomeres than naïve B cells, while Son et al. (2003) reported that naïve and memory B cells have similar telomere length, whereas the first study shown that memory B cells have



**Fig. 1.** Figure depicts trafficking phenotype and ability to respond to two different stimulations of DN B cells from both young and elderly donors. As shown in panels A and C, DN B cells from young donors express CXCR3 chemokine receptor, that enables these cells to reach tissues during a common inflammatory reaction. In panels B and D are shown DN B cells from elderly that express the two chemokine receptors CCR6 and CCR7, which drive these cells to chronic inflamed tissues. In panel A and panel B are depicted the different behaviour of DN B cells, from young and elderly people, respectively, when stimulated with a strong activation signal (CpG/PMA/Ionomycin). As shown, DN B cells from young donors (panel A) could be activated and produce TNF- $\alpha$ , while DN B cells from elderly (panel B) do not respond to this stimulus. In Panel C and D are shown as, both in young and elderly, DN B cells are able to produce GrB, when stimulated with human recombinant IL-21 and anti-human IgG.

longer telomeres than naïve B cells (Martens et al., 2002). Probably, these controversial data may be due to the different approach used to differentiate naïve from memory B cells in the cited studies.

There is no doubt that a link between “inflamm-aging” and acquired immune responses may be identified, both in the production of chemokines, and in the expression of their receptors. Although there is much promiscuity within the chemokine network, it is well known that certain combinations of chemokines and their receptors enable immune cells to reach specific tissues (Kunkel and Butcher, 2003; Perez-Andres et al., 2010; Stein and Nombela-Arrieta, 2005). It has been reported that, with ageing, B cells undergo a remodelling on the expression of their chemokine receptors profile, and in particular in the memory compartment, including IgD<sup>+</sup>CD27<sup>-</sup> senescent/exhausted DN memory B cells, which seem to be able to reach chronic inflamed tissues (Bulati et al., 2014). Indeed, it has been shown that IgD<sup>+</sup>CD27<sup>-</sup> DN B cells from young donors, express CXCR3 (Fig. 1), a chemokine receptor that consents cells to reach the inflammatory sites, where its ligand, monokine-induced by interferon- $\gamma$  (CXCL9) and IP-10 (CXCL10), are highly expressed during a common inflammatory reaction (Farber, 1997; Kunkel and Butcher, 2003). Surprisingly, with ageing, DN B cells lose the expression of CXCR3 molecule, but express higher levels of CCR6 and CCR7 (Fig. 1, Table 3), which, as demonstrated (Comerford et al., 2010; McNamee et al., 2013; Müller and Lipp, 2003; Schutyser et al., 2003; Welsh-Bacic et al., 2010), are involved in the migration to the inflammatory sites, in this case, in chronic inflamed tissues. These are interesting data that could be discussed in terms of “inflamm-aging”. The hypothesis is that the typical inflammatory milieu of aged people influences the trafficking ability of B cells rendering them more sensitive to both cytokines and others pro-inflammatory molecules which are over-

produced in the elderly (Salvioli et al., 2013; Singh and Newman, 2011). This hypothesis is also supported by results obtained in a cohort of highly inflamed AD patients. In this study (Bulati et al., 2015) it has been demonstrated that in AD patients there is a reduction of both total and naïve B cells and the increase of DN memory B cells. Moreover, DN B cells express higher levels of both CCR6 and CCR7 chemokine receptors when compared to healthy elderly. Interestingly, these data are shown only in severe AD patients and not in mild AD subjects (Bulati et al., 2015).

It is well known that B cells, with an appropriate stimulation, are able to produce cytokines, chemokines or other effector molecules (Hagn and Jahrsdörfer, 2012; Klinker and Lundy, 2012; Lund, 2008; Lund and Randall, 2010; Rosser and Mauri, 2015). In previously papers it has been shown that IgD<sup>+</sup>CD27<sup>-</sup> DN B cells seem to be refractory to undergo in vitro activation. Indeed, stimulating DN B cells, with CpG, that binds TLR9, or with a polyclonal mitogen of B cells that binds the B cell receptors (anti-BCR), of both young and elderly subjects, these cells are not able to be activated and to produce cytokines (Colonna-Romano et al., 2009). Moreover, using a strong activation signal, as CpG/PMA/Ionomycin, DN B cells from young donors could be activated and produce TNF- $\alpha$ , while DN B cells from elderly do not respond to this stimulus (Fig. 1) (Buffa et al., 2011). Interestingly, the in vitro stimulation of the CD24<sup>+</sup>CD38<sup>-</sup> B cells, that include both switched memory (IgD<sup>+</sup>CD27<sup>+</sup>) and DN (IgD<sup>+</sup>CD27<sup>-</sup>) B cells, using the strong activation signal (CpG/PMA/Ionomycin), make these cells able to produce higher amount of the pro-inflammatory cytokine TNF- $\alpha$ , but without significant differences between young and old people (Buffa et al., 2013). As CD38<sup>-</sup>CD24<sup>+</sup> B cell are largely expanded in elderly, it can be speculated that also CD24<sup>+</sup>CD38<sup>-</sup> could contribute to “inflamm-aging”.



So, the ability of B cells to produce effector molecules depends both on the triggering of its receptors (BCR, CD40 or TLRs), and the microenvironment (Hagn and Jahrsdörfer, 2012; Klinker and Lundy, 2012; Lund, 2008; Lund and Randall, 2010; Rosser and Mauri, 2015). In the last years, it has been demonstrated that the combination of the pro-inflammatory IL-21 and BCR stimulation, in the absence of CD40 triggering, enables B cells to produce and secrete the active form of the cytotoxic serine protease granzyme B (GrB), that plays a critical role in early anti-viral immune responses, in the regulation of autoimmune mechanisms and in cancer immune-surveillance (Hagn and Jahrsdörfer, 2012). Moreover, both in elderly (Agrawal et al., 2012) and in SLE patients (Dolff et al., 2011), which shown increased percentage of IgD<sup>-</sup>CD27<sup>-</sup>DN B cells (Colonna-Romano et al., 2009; Sanz et al., 2008; Wei et al., 2007), increased levels of IL-21 have been reported. In a recent paper it has been demonstrated that, when stimulated with human recombinant IL-21 and anti-human IgG (BCR triggering), DN B cells from both young and elderly people, produce GrB (Bulati et al., 2014), without any difference between the two age groups (Fig. 1). So, as previously discussed, in elderly, IgD<sup>-</sup>CD27<sup>-</sup>DN B cells are increased and show a trafficking phenotype that consent them to reach chronic inflamed tissues or tertiary lymph node. As their capacity to produce GrB is not age-impaired and, almost certainly, in other condition they could produce other kinds of pro-inflammatory molecules, they certainly exert a biological function, probably having an active role in inflamm-ageing and immunosenescence (Table 3).

It is important to stress that, not only the phenotypic modifications and the ability to respond to different stimuli, but also the functional quality of the B cell compartment certainly influences the individual's response. On this purpose, it has been above discussed that unstimulated total B cells from elderly produce significantly higher levels of TNF- $\alpha$  than those produced by young subjects, and these negatively correlates with B cells function (Frasca et al., 2014). In a recent paper (Frasca et al., 2017), it has been investigated the capacity of naïve/memory B cell subsets to express multiple senescent-associated secretory phenotype markers (SASP), such as cell senescence (p16<sup>INK4</sup>), miRNAs (miR-155, miR-16, miR93) and pro-inflammatory cytokines (TNF- $\alpha$ , IL-6). The authors have demonstrated that unstimulated memory, but not naïve B cells, from both young and elderly people, express the mRNA for TNF- $\alpha$ , IL-6 and p16<sup>INK4</sup>, and the pro-inflammatory miR-155, miR-16 and miR-93. Levels of these molecules were higher in B cells from elderly compared to young and, among memory B cell subsets, IgD<sup>-</sup>CD27<sup>-</sup>DN B cells, also known as late memory (LM), showed the highest levels of SASP markers (Table 3), which are associated with the activation of NF- $\kappa$ B transcription activity, in the absence of cell activation (Frasca et al., 2017). Moreover, DN/LM B cells also shown spontaneous phosphorylation of AMPK and p38MAPK (Table 3) (Frasca et al., 2017), which are associated with cell exhaustion and senescent phenotype, suggesting that a reprogramming of cellular metabolism occurs in senescent cells, and this metabolic adaption induces global changes in cellular metabolic programs through the regulation of gene transcription (Blagih et al., 2012).

## 5. Conclusion

It is known that inflammation is important for survival in the earlier stage of life to fight infections and for the tissues repair, but it can have adverse and detrimental effects on aged individuals ("antagonistic pleiotropic theory", Goto, 2008). Indeed, as discussed, elderly are characterized by a subclinical chronic inflammatory condition termed "inflamm-ageing", which contributes to the development of a variety of age-associated diseases such

as metabolic, cardiovascular, autoimmune and neurodegenerative diseases and cancer (Bucci et al., 2014; Frasca and Blomberg, 2016; Fulop et al., 2016; Grasse et al., 2016; Grubeck-Loebenstein et al., 2009; Johnson and Cambier, 2004; Rubtsova et al., 2015; Salvioli et al., 2013). The condition of inflamm-ageing provides a continuous mild antigenic challenge that leads to a progressive stimulation, or depletion, of the immune system cells and to filling of the immunological space by activated/exhausted lymphocytes with altered functions (Bulati et al., 2011, 2014; Fulop et al., 2016; Larbi and Fulop, 2014; Naradikian et al., 2016; Pawelec, 2014a,b; Pinti et al., 2016; Rubtsova et al., 2015).

It is clear that ageing is a complex process that is influenced by multiple factors at multiple levels. Nonetheless, it is undeniable that inflammation plays an important role in this phenomenon. It was widely discussed above as inflamm-ageing seems to have a pivotal role on the age-related changes at systemic, molecular and cellular levels that leads to B cell compartment impairment. Indeed, inflamm-ageing contributes in all stages of development and maturation of B cells, from B lymphopoiesis to plasma cells differentiation, but the precise interplay between inflamm-ageing and the age-related changes of B cell compartment, remains to be elucidated. A pivotal role in the impairment of B cell branch of the immune system is certainly played by senescent/exhausted DN B cells. Indeed, as discussed, these cells are increased in the elderly, and in other models of chronic inflammation, and they show a pro-inflammatory trafficking and a SASP phenotype. It is also known that the age-related accumulation of senescent cells represents a favourable environment for the development of pro-inflammatory age-related diseases (Campisi, 2003, 2011). These results make the study of B cells in the aged an important tool for monitoring, not only the immunosenescence phenomenon *per se*, but, as these modifications reflect the inflammatory events, also the chronic inflammatory diseases, such as cancers, HIV, AD and autoimmune phenomena, and the effectiveness of vaccines.

As discussed, healthy elderly and individual with chronic diseases, as HIV infected patients, shared similar features of B cells impairment, as loss of protective immunity and poor response to vaccinations. As in elderly, impaired AID expression and poor effectiveness of influenza vaccination has been also shown in HIV-positive individuals (Cagigi et al., 2013a), where, also the increased frequency of IgD<sup>-</sup>CD27<sup>-</sup>DN B cells is negatively related to the strength of humoral response to the vaccine (Cagigi et al., 2013b). Moreover, another study correlates the impaired antibody response to influenza vaccination, in both HIV infected or uninfected ageing women, to inflammation, measured by high levels of plasma IL-21 and TNF- $\alpha$  (Parmigiani et al., 2013). All these data suggest that, the evaluation of the AID expression, the frequency of DN B cells and the levels of pro-inflammatory cytokines, could be useful tools for vaccination design, in terms of number of vaccine doses, in order to achieve immune protection in both elderly and chronic inflamed individuals.

Further evidence demonstrating the pro-inflammatory nature of DN B cells, and their potential role as useful marker for the monitoring of pro-inflammatory diseases, is coming from a recent clinical study on RA patients, with a significantly expanded population of DN B cells (Mahmood et al., 2015). The authors have studied the modulation of IgD<sup>-</sup>CD27<sup>-</sup>DN B cells after treating their patients with the anti-IL6R drug Tocilizumab. They obtained a significant reduction of this subpopulation. This result confirms the role of inflammation as the cause/effect of DN B cells increase and, in the meantime, suggests DN B cell as an important candidate marker for the monitoring of the pharmacological therapy in RA and, hopefully, in other autoimmune or inflammatory disorders.

Moreover, the molecular modifications of B cells could be used to monitor both cancer and autoimmune diseases in the elderly. As it has been demonstrated an age-related increase of miR-155

and miR16, directly related to the inflammatory status of elderly (Frasca et al., 2015a, 2017), monitoring these two biomarkers, in both total and DN B cells, could have a prognostic significance for B cell cancer (Due et al., 2016), and autoimmune disorders (Churov et al., 2015).

Finally, in the last years, altered regulation of the signalling pathways has been suggested to be one of the most important cause of immunosenescence, and the modulation of these pathways represent a major challenge to “restore” the immune response to functional levels. Indeed, alterations in the stimulatory and/or inhibitory signalling have been demonstrated to occur in the immune responses of both innate and acquired immunity during ageing, in particular in neutrophils and T lymphocytes, and these events lead to a less efficient function of the immune system (Fulop et al., 2014, 2016). The authors suggest that combined approaches that consist of maintaining adequate early feed-forward activation while down-regulating feedback inhibition would be beneficial in restoring several T cell functions in ageing (Fulop et al., 2014, 2016). It can be speculate that also for B cells it could be possible to restore their impaired function. For example, by acting on the level of phosphorylation of AMPK or p38MAPK, it could be evaluated *in vitro* if class switch and antibody production will be improved.

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