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"B CELL CHANGES IN AGING"

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Coordinatore	Tutor
Prof. Calogero Caruso	Prof.ssa Giuseppina Colonna-Romano

Dott. Adriana Martorana

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CHAPTER 1

Introduction

1.1 The feature of aging

In modern society, the increase of life expectancy of global population, as a consequence of improving life standards, health care progresses and vaccinations, will contribute to the increase of the number of older persons (aged 60 years or over) by 2050. Indeed it is estimated that approximately the most part of 2 billion people on Earth will be made up by elderly (Gui J, 2012). For these reasons, tailored strategies will be adopted by national health systems to improve the quality of life for older adults by alleviating or mitigating the most frequent adverse effects of aging and age-related diseases (Dewan SK et al., 2012).

Aging is a complex process that involves anatomic structures, physiological and social processes and cellular, molecular, tissues changes (Goldberg AL et al., 2002; Singh T and Newman AB, 2011). Although variable for each individual, it is a gradual and progressive phenomenon, under genetic and environmental control. Both a diminished ability to maintain homeostasis of the organism and the reduced capacity to respond to environmental stimuli are correlated with the increased predisposition of elderly people to illness and death (Troen BR, 2003; Rattan SI, 2008). Clinical observations indicate that some infections are more prevalent and have poorer outcomes in the elderly than in younger adults and are the fourth most common cause of death in older people (Heron MP and Smith BL, 2007). Indeed, the elderly population is more susceptible to influenza, pneumococci, (Nicholson KG et al., 1997) respiratory syncytial virus (RSV) and group B streptococcus (GBS) but also from opportunistic infections and re-emergent chronic infections

such as herpes zoster (Ginaldi L et al., 2001; Bulati M et al., 2011; Dewan et al., 2012; Oviedo-Orta E et al., 2013). The major reason for the increased susceptibility to infections in the elderly is the impairment of the immune system, called immunosenescence (Franceschi C et al., 1995; Pawelec G et al., 2005), which consists in progressive and cumulative modifications that affect both innate and instructive immune reactions (Franceschi C et al., 1995; Pawelec G et al., 2005). Thus, clonotypic and innate immunity have a key role in the control of the survival of the elderly, since the susceptibility to these diseases depends, in part, on a good and functional immune system (Licastro F et al., 2005; Candore G et al., 2006a). In particular, Franceschi (Franceschi C et al., 1995) according to the "remodeling theory of aging", proposed that immunosenescence is not a decline of immune functions but is its reorganization. Indeed, adaptive immunity deteriorates while innate immunity is largely conserved or even up-regulated with the age (Cossarizza et al., 1991). Moreover, it was demonstrated that even though elderly people are able to do anamnestic responses, they are not capable to establish a good primary type response against new antigens (Fagnoni FF et al., 2000), hence, for them, it is difficult overcome infections (Globerson A and Effros RB, 2000). In addition, the vaccine efficacy is decreased in old population because of the diminished of antibody responses to primary immunization with protein antigens such as hepatitis B vaccine, tetanus toxoid, rabies vaccines and for others factors, such as alimentation, pulmonary disease, diabetes mellitus, cancer, autoimmune and heart disease (Roman BE et al., 2013; Krawinkel MB et al., 2012; Moore SE et al, 2012; Oviedo-Orta E et al., 2013).

In addition to the remodeling of immune system, aging is also characterized by chronic low-grade inflammation state, named "inflamm-aging" (Franceschi C et al., 2000a). Indeed, aged people show the increase in the production of inflammatory mediators, such as cytokines and acute phase proteins, which act as predictors of mortality independent on pre-existing morbidity. Many factors, including augmented amount of fat tissue, decreased production of sex steroid, smoking and chronic disorders as atherosclerosis, seem to contribute to this status (Krabbe K et al., 2004), although the most important cause of inflamm-aging may be the chronic antigenic load encountered during life and which affects the immune system. It is believed that pro-inflammatory status with the genetic background are linked to the majority of age-associated diseases as atherosclerosis, Alzheimer's disease, cancer, type 2 diabetes and sarcopenia because prolonged activation causes chronic inflammation that damages organs (Franceschi C et al., 2000a,b; De Martinis et al., 2005; Licastro F et al., 2005; Vasto S et al., 2007).

One of the most aspect of inflamm-aging is the presence of elevate circulating levels of pro-inflammatory factors (IL-1β, IL-6, TNFα, and prostaglandin E2) and anti-inflammatory mediators, (IL-1 receptor antagonist, soluble TNF receptor, IL-10, transforming growth factor beta (TGF-β), acute phase proteins, C-reactive protein, and serum amyloid A) and contributes to the decreased ability of the elderly to mount an appropriate immune response following an infectious challenge (Gomez CR et al., 2010; Bruunsgaard H et al., 2010; Ginaldi L et al., 2001; Trzonkowski P et al., 2004).

It was demonstrated that TNF-α induces the production of Amyloid beta peptide in Alzheimer disease patients but it is also involved in atherosclerosis development because of the increase of the proliferation in smooth muscle cells (Wick G et al., 2000; Saurwin-Teissl M et al., 2000).

Another change that occurs with aging is the decrease of the serum levels of EGFR and EGF that regulates cell growth, proliferation and differentiation (Shurin GV et al., 2007).

Therefore, inflamm-aging, in a global perspective, could be seen as the common biological factor responsible for the decline and the onset of disease in old people, but at the present, this phenomenon has become more complicated than what was surmised in the past. Indeed, inflamm-ageing acts at different levels of complexity, from molecule to cell, from organ to organ system and also to organism. Thus it is difficult to predict exactly the changes related to age in different organs and cell types in body. Moreover, to this regard, it has been proposed that the inflamm-aging is the result of overlapping between the level of pro-inflammatory molecules in the bloodstream and their production in different cells and tissues (Salvioli S et al., 2013; Cevenini E et al., 2013). As a consequence, the balance between inflammatory and anti-inflammatory stimuli modifies cells microenvironment that affects organs and tissues, leading to the ‘mosaic of ageing’ (Cevenini E et al., 2008) that causes a remodeling in epigenetic and gene expression with aging (Cevenini E et al., 2013). Concerning this, it is known that IL-6, a cytokine involved in the growth of cancer cells, such as breast cancer (Sansone P et al., 2007; Studebaker AW et al.,2008), also has detrimental effects when produced

in excess in skeletal muscle (e.g. sarcopenia) whereas is normally released during exercise (Beyer I et al., 2012) and has beneficial in muscle metabolism (Pedersen BK et., 2008).

In addition, senescent cells, over the time, could participate to this process with opposing effects: adopting a state of permanent cell cycle arrest, as response to damaging agents (e.g. oxygen free radicals), with beneficial effects (tumor suppression and tissue repair) or promoting cancer progression and ageing with deleterious effects on health of the organism (Rodier F et al., 2011; Cevenini E et al., 203). Recent findings suggest that the number of cells that express senescent markers increases with age and that their clearance is performed by immune system but it is not known about whether this process changes with age or in age-related disease, or if these cells escape to this mechanism (Campisi J et al., 2007). In conclusion, it is fundamental to clarify the complex process of inflamm-aging to improve targeted therapeutic interventions.

A number of experimental evidences suggested that another cause of immunosenescence is the persistent viral infection as it has been demonstrated for Citomegalovirus (CMV) (Vescovini R et al., 2007; Vescovini R et al., 2010; Derhovanessian E et al., 2011) that appears accelerate immune aging (Pawelec G et al., 2005; Akbar AN and Fletcher JM, 2005). The relation between CMV infection and poor health status was assessed in OCTO/NONA longitudinal studies (Wikby A et al., 1994 Wikby A et al., 2002) in Swedish population. It has been reported that CMV infection is part of a group of features called Immune Risk Profile (IRP) that are useful to predict mortality (Pawelec G et al., 2005). Other parameters that contribute to IRP are high levels of late stage

differentiated $CD8^+$ T cells, low levels of $CD4^+$cell count, poor T cells proliferative response to mitogens, inversion of CD4:CD8 ratio, low IL-2 production and decreased B cells count. In addition, it has been reported the CMV seropositivity and high levels of IL-6 are predictor of frailty and mortality (Schmaltz HN et al., 2005; Wang GC et al., 2010). Moreover, it has also been demonstrated a correlation between CMV infection and the progression of AIDS (Griffiths PD et al., 2006). Besides, it has been suggested that in elderly people the inflamm-aging could be independent from CMV serostatus as demonstrated by a recent longitudinal study (Bartlett DB et al., 2012).

1.2 The effects of aging on the immune system

1.2.1 T lymphocytes

- *Structural and environmental changes in thymus*

The thymus is the lymphoid organ responsible for the development of self-restricted, self-tolerant, immunocompetent T cells that mature through a series of proliferation and differentiation stages dependent upon receiving instructions from the specialized thymic microenvironment (Anderson G, et al., 2001; Ma D et al., 2013).

With advancing age, there is an involution of the thymus (Taub DD and Longo DL, 2005; George AJ et al., 1996; Lynch HE et al., 2009) and both intrinsic and extrinsic factors are thought to contribute toward this process. Indeed, structural, phenotypical, and architectural changes of

thymic microenvironment have been observed (Chinn IK, et al., 2012). These processes include down regulation of various thymic epithelial cell (TEC) markers such keratin, MHC class II together with alterations of cortical and medullary markers (Li L et al., 2003; Bertho JM, et al., 1997; Palmer DB et al., 2013). Furthermore, the structural integrity of the thymic niche is disrupted with age, including disorganization of the cortical and medullary junction; together with an increase of fibrosis, adipose tissue, and the accumulation of senescent cells in the aged thymus (Gui J, et al., 2007; Dixit VD, 2010). The thymic involution occurs in two phases: the first is associated with the physiological growth and the second is linked to the age-related changes (Aw D et al., 2012). In particular, the kinetic of this process is not uniform throughout life. Indeed, it is characterized by a rapid early decline, after which it seems to proceed at a steady rate (Palmer DB et al., 2013; George AJ et al., 1996) and then perhaps may end in later life (Nasi M et al., 2006; Mitchell WA et al., 2010).

- *Age –related modifications in T cell subsets*

These age–related modifications result in a progressive decrease of the percentage and absolute number of circulating $CD3^+$ T lymphocytes and $CD4^+$ and $CD8^+$ T cells subsets (Pawelec G et al., 2002; Cossarizza A et al.,1996). Moreover, it is also observed the decline in output of newly developed T cells. As a consequence, there is a reduction of number of circulating naive T cells that do not permit to replenish the naïve T cells lost in the periphery and also to maintain the size of T cell repertoire (Kohler S et al., 2005; Naylor K et al., 2005; Fulop T et al., 2013). In this way, aged subjects become less responsive to immune stimulation and

vaccination, and likely prone to develop cancer, autoimmune disorders and chronic inflammatory diseases (Fulop T et al., 2013).

In addition, aging of acquired immunity is associated with accumulation of memory and effector T cells as result of lifelong exposure to new and persistent infections (Fulop T et al., 2013; Saule P et al., 2006). Particularly, several studies have suggested the increase of the number of highly differentiated $CD28^-$ T cells, especially within the $CD8^+$ T-cell subset in the elderly. This subset, that lacks of CD28 expression, is considered a biomarker for immunosenescence (Effros RB et al., 1994**;** Pawelec G et al., 2008). In addition, $CD8^+$ $CD28^-$ T cells exhibit reduced antigen receptor diversity, defective antigen-induced proliferation and a shorter replicative lifespan while showing enhanced cytotoxicity regulatory functions (Oviedo-Orta E et al., 2013; Weng NP, et al., 2009) and resistence to apoptosis (Pawelec G et al., 2008).

There are several surface markers which can be used to classify T cells. The most utilized are CD28, CD27, CD45, CCR7, CD62L, CD95, CD95L (Gupta S et al., 2005). For long time, memory and naïve human T cells were discriminated on the basis of CD45RA antigen, expressed primarily on naïve T lymphocytes and CD45RO expressed on memory T cells. But it is noted that a population of late-differentiated memory T cells re-express CD45RA, so this marker is not entirely useful to discriminate naïve and memory T cells (Hamman D et al., 1997). Actually, the most widely accepted phenotyping model for $CD8^+$ T and $CD4^+$ T includes Naïve T lymphocytes (N: $CCR7^+$, $CD27^{+++}$, $CD28^{+++}$, $CD45RA^+$), that are also characterized by the absence of CD95 and the expression of CD62L ; Central Memory cells (CM: $CCR7^+$, $CD27^{++}$,

$CD28^{++}$, $CD45RA^{-}$), principally located in the lymph nodes, they represent the lesser differentiated memory population (Nociari MM, et al., 1999); Effector Memory cells (EM: $CCR7^{-}$, $CD27^{\pm}$, $CD28^{\pm}$, $CD45RA^{-}$), the highly mature T cell population found in extranodal tissues and mucous membranes. Furthermore, this last subset of lymphocytes seems to be the responsible for the tissue damages in autoimmune disorders because EM T cells are found in inflamed non-lymphoid tissues where secrete high amounts of effector cytokines (INF-γ and TNF-α), chemokines (RANTES) and acquire cytotoxic activity via granzyme/perforin granule exocytosis pathway (Kaech SM et al., 2002; Wherry EJ et al., 2003). Another interesting memory T cell population is named TEMRA: these are terminally differentiated memory lymphocytes re-expressing CD45RA (TEMRA: $CCR7^{-}$, $CD27^{-}$, $CD28^{-}$, $CD45RA^{+}$). It has been demonstrated a strong correlation between chronological age and the frequency and absolute number of this population in most humans. It has been suggested that many of these TEMRA cells are not able to produce cytokines, to mediate cytotoxic activity, besides they show senescence-related proliferative defects (Effross RB, 2011; Fulop T et al.,2013). However, it has been recently demonstrated that, in some circumstances, TEMRA might secrete cytokines and express high levels of granzyme B and perforin (Libri VR et al., 2011). So, they may be important for protection against certain infections *in vivo* (Bruns H et al., 2009; Di Mitri D et al., 2011).

Another classification of naïve/memory T cells is based on the expression of CD57 and KLRG1, two inhibitory receptors present on late-stage differentiated cells and considered as markers of senescence.

Indeed, KLRG1 is expressed by $CD4^+$and $CD8^+$ T lymphocytes and NK cells; differently CD57 is mainly expressed on $CD8^+$and NK cells, and at lower levels, is express by $CD4^+$ (Ouyang Q et al., 2003; Larbi A et al., 2009; Pellicanò M et al., 2011).

Many evidences have shown that CMV, which establishes persistent, usually asymptomatic, life-long infection, has an enormous impact on the distribution of T cell subsets in most old people, which show a clonal expansion of CMV-specific CD4 and CD8 T lymphocytes (Looney RJ et al., 1999; Chidrawar S et al., 2009; Almanzar G et al., 2005, Pawelec G et al., 2005). CMV-specific T cells typically display an effector memory phenotype of late-stage differentiation. CMV also modulates innate immunity and induces the production of cytokines and chemokines which affect T cell immunity. Therefore, the typical consequence of aging is the progressive filling of the "immunological space" by activated lymphocytes in response to chronic/continuous stress either from pathological or physiological antigenic stimuli (Goto M, 2008).

In the aged, it is also observed a high frequency of regulatory T (Treg) cells and an imbalance in Treg response and in production of IL-17. Treg cells from elderly play an immunosuppressive role *in vitro* and express high levels of IL-10 and TGF-β (Simone R et al., 2008; Oviedo-Orta E et al., 2013). On the contrary, Th17 cells that act against bacteria, activating and recruiting neutrophils, are also reduced in the elderly (Lee JS et al., 2011; Oviedo-Orta E et al., 2013).

*1.2.2 **B lymphocytes***

- *Age-related modifications in B cell compartment*

The most of the literature on immunosenescence has focused on T cell impairment, but it has been also demonstrated the impairment of the B cell branch in aging. Indeed, B cell compartment is affected in elderly people, thus, humoral immune response is modified in aged both in the quality and quantity of the antibodies produced and in the size of developing B cells subsets (Cancro et al., 2009; Frasca D and Blomberg 2011; Buffa S et al., 2011). Indeed, it has been abundantly demonstrated that the percentage and absolute number of total $CD19^+$ B Cells decrease in aged (Colonna-Romano et al., 2003; Frasca D et al., 2008; Veneri D et al., 2009), although the B lymphopoiesis persists during adult life (Rossi MI et al., 2003). It seems that defects in hematopoietic stem cells (HSC) in bone marrow, is partly responsible for the B cell impairment (Kogut I et al., 2012; Bulati M et al., 2011). Indeed, the lowered output of naïve B cells from aged might be caused by the shift toward the production of myeloid progenitors at expense of lymphoid progenitors. This phenomenon seems to be connected to the up-regulation of genes involved in myeloid differentiation (Rossi DJ et al., 2005; Chambers SM et al., 2007) and in the alteration of cytokine milieu in the bone marrow (e.g TGF-β), other than to the impaired of V-DJ rearrangement. (Challen GA et al., 2010; Gibson KL et al., 2009). Moreover, it has been suggested that the shrinkage of the B cell repertoire (Guerretaz LM et al., 2008) and the shortening of telomeres might be implicated (Warren LA et al., 2009; Weng NP et al., 2008). In humans, several studies have reported changes in B cells or in the antibody repertoire (Dunn-Walters DK et al., 2010),

particularly in the heavy chain of BCR. Indeed, upon encountering of antigen, mature naïve B cells migrate to secondary lymphoid organs where they organize germinal centres and undergo immunoglobulin affinity maturation (Carsetti R et al., 2004). During this stage, class switch recombination (CSR) takes place, this leads to production of different isotypes (IgG, IgA, IgE) with diverse effector functions, and somatic hypermutation (SHM), in which the V domains of immunoglobulin may increase their affinity by accumulation of mutations. These processes, are both dependent on the expression of the enzyme activation-induced cytidine deaminase (AID) (Pan-Hammarstrom Q, et al., 2007; Cagigi A et al., 2009) and are fundamental for the quality of the immune response and for the development of an efficient serologic memory to prevent re-infections. Indeed, Frasca D et al., (2008) have shown that the expression of E2A-encoded transcription factor E47 (E47) and AID in B cells progressively decrease with age. As a consequence, elderly people show a significant collapse both in repertoire diversity and clonal expansion. Indeed, an increased oligoclonality and a reduced frequency of somatic hypermutation in the elderly response to pneumococcal vaccination has been reported (Kolibab et al., 2005). This is linked with a poor health status, frailty and a reduced protective humoral immune responses (Cancro MP et al., 2009; Dunn-Walters DK et al., 2010; Gibson KL 2009; Scholtz JL et al., 2013). These data indicate a relative deficit in the ability of elderly people to mount primary responses to newly encountered antigenic epitopes, and also suggest that, a decline in T cell help or innate immune functions could also contribute to this change (Thompson WW et al., 2003; Kogut I et al.,

2012). Beside, with aging, the levels of total and specific serum Ig isotypes are modified. Indeed, although the number of total B lymphocytes is reduced, the amount of IgG, IgA and, to a lesser extent IgE, is augmented. In a different way, the levels of IgM decrease or not change, while those of IgD decline (Listì F et al., 2006; Frasca D et al., 2010).

The study of B cell subsets is complicated by ample differences between individuals, as well as the variety of phenotyphing approaches employed (Ademokun A, et al 2010). In particular, using $CD19^+$, IgD and CD27 (core markers) the following major circulating B cell subsets can be distinguished (Kaminski et al.,2013; Wei C et al., 2011): naïve B cells (IgD^+CD27^-); unswitched memory (IgD^+CD27^+) B cells also referred to as natural effector B cells that express IgM too; classical switched memory B cells (IgD^-CD27^+) that express IgG^+ or IgA^+ and Double Negative (DN) memory B lymphocytes (IgD^-CD27^-) (Colonna-Romano G et al., 2009; Shi Y et al., 2003) Indeed, naïve B cells are IgD^+, while classical memory B lymphocytes express switched immunoglobulins (IgG, IgA, IgE) (Klein U et al, 1998) and CD27, a typical marker of memory B cells correlated with the presence of somatic hypermutations in Ig genes (Agematzu K et al., 2000).

It has been demonstrated that naïve B cells are significantly reduced in elderly subjects (Gupta s et al., 2005; Colonna-Romano G et al., 2008), although other authors have observed the increase of percentage but not in the absolute number with age of these cells (Shi Y et al., 2005; Frasca et al., 2008). Concerning memory B cells, that are the responsible for driving the rapid secondary antibody response, Frasca et al., (2012) have

shown a decrease of switched memory B lymphocytes both in percentage and in absolute number suggesting a defect to undergo class switch. Unlike the above-mentioned data, Colonna-Romano G et al., (2003, 2009) have reported no significant changes of memory B cells identified as $CD19^{+}CD27^{+}$ in the elderly, moreover Macallan DC et al., (2005) have shown an increased proliferative ability of switched memory B cells. Moreover, DN B cells are memory B cells which have down-regulated the CD27 marker (Anolik JH et al., 2004; Fecteau JF et al., 2006; Wei C et a., 2007; Colonna-Romano G et al., 2009; Cagigi A et al., 2009). In fact, for the most part of them, DN B cells have switched the heavy chain of immunoglobulin molecule in IgG^{+}, others (more than 20%) in IgA^{+}, whereas less than 10% are IgM^{+}. Beside, these cells have low levels of ABCB1 transporter (Colonna-Romano G et al., 2009), a protein responsible for the transport of molecules across cell membranes (Efferth T et al., 2003) and very low levels of Bcl2 that should preserve cells from apoptosis. It has been also verified that DN not act as antigen presenting cells (APC) because express reduced levels of HLA-DR, CD80 and CD40 that are useful for antigen presentation and T-B cooperation. It has also been described that shown that DN B cells of the elderly donors have very short telomeres compared to the same subpopulation of young donors. Moreover, these cells are not responder to CpG stimulation although can be activated with $F(ab')_2$ (anti-BCR) (Colonna-Romano G et al., 2009). In addition, keeping in mind that DN B cells from elderly subjects show an intrinsic *in vitro* activation, there is not a link between their capacity to proliferate and the ability to produce cytokines as TNF-α and IL-10 also when stimulated with strong stimuli (CpG/PMA/Ionomycin) (Buffa S et

al., 2011). However, others reported that DN B cells can be stimulated *in vitro* to secrete immunoglobulins against tetanus toxoid and influenza virus (Wirths and Lanzavecchia et al., 2005).

Furthermore, it has been demonstrated that DN B lymphocytes are enlarged significantly in percentage but not in absolute number in healthy elderly (Colonna-Romano et al., 2009). The increased of these cells has also been demonstrated in healthy subjects challenged with respiratory syncityal virus (RSV) (Sanz I et al., 2008); in active Lupus patients (Wei C et al., 2007) and in HIV patients (Cagigi A et al., 2009) and it might be the result of persistent stimulation of immune system. In elderly people, DN B cells show a reduced rate of the mutations evaluated in the V region of IgG genes when compared with young (Buffa S et al., 2011). Thus, the increase of the double negative memory B cells in the elderly together with the reduced rate of mutation might be due to the disconnected generation of these cells from germinal centers, as it has been demonstrated that ageing negatively affects the germinal center formation in secondary lymphoid tissues (William J et al., 2002; Frasca D et al., 2005) or might represent the first wave of memory B cells (Blink EJ et al., 2005; Inamine A et al., 2005) or terminally differentiated memory B cells. Alternatively, it has been hypothesized that the increase of these memory B cells subset might be also the manifestation of an age-related physiologic modification (elderly) or a pathologic deregulation (SLE patients) of the immune system (Bulati et al., 2011).

Among IgM memory B cells, it is also possible to distinguish $IgM^{+}IgD^{+}CD27^{+}$ and $IgM^{+}IgD^{-}CD27^{+}$ called “IgM only”. It is reported that IgM memory B cell subsets, that is the predominant B subset, is

reduced with age and might be involved in defective immune response against infections by encapsulated bacteria (Shi Y et al., 2005; Buffa S et al., 2011) so, increasing the predisposition to pneumococcal infection (Frasca D and Blomberg ., 2011; Buffa et al., 2011).

Using additional markers, as CD24 and CD38 it is possible to identify a recently produced population (Transitional B cells) (Fecteau JF et al., 2006; Palanichamy A et al., 2009; Blair PA et al., 2010; Berkowska MA et al., 2011). They express high levels of both CD24 and CD38 ($CD38^{hi}CD24^{hi}$ Transitional B cells).

It has been demonstrated that the human immature transitional $CD19^{+}$ CD24hiCD38hi B cells have regulatory function. Indeed, these lymphocytes, also called Breg, are the main interleukin-10-producing subset, that suppress the differentiation of T helper 1 cells (Blair et al., 2010). It has been observed that they expand in patients suffering of lymphoma and autoimmune diseases, such as rheumatoid arthritis (RA) and SLE (Palanichamy A et al., 2009; Blair PA et al., 2010). In particular, Blair et al (2010) has also reported that in SLE patients, these cells loss their suppressive capacity, producing less IL-10. In association with previous evidence showing defects in Treg cell activity in SLE, the authors have suggested the impairment of regulatory cell functions in this and other autoimmune diseases.

B lymphocytes and cytokine/chemokine production

Interestingly, B cells have effector and regulatory functions other than antibody production such as T cell and dendritic cell regulation and cytokine and chemokine production (Sanz I et al., 2007; Martin F and Chan AC, 2006; Harris DP et al., 2011). Indeed, in literature, naïve and

memory B cells have been distinguished also by producing different pro- and anti-inflammatory cytokines. Particularly, naïve B cells produce anti-inflammatory cytokines, whereas memory B cells are the main responsible for pro-inflammatory cytokines production (Duddy ME et al., 2004, 2007; Sanz I et al., 2007, 2008; Lund FE, 2008). Buffa et al., (2011) has demonstrated that $CD27^+$ and CD27- B cells from young and elderly subjects produce different kind of cytokines. Indeed, in both age-groups, the physiological (α-CD40/IL-4) stimulation induces a good IL-10 and TNF-α production by unswitched memory B cells. Fascinatingly, in older people, naïve B cells are also highly activated to produce cytokine under these conditions. On the other hand, it has been also demonstrated that a strong stimulation (CpG/PMA/Ionomycin) (Bouaziz JD et al., 2010) activates the production of IL-10 by both IgD^+CD27^- (classical naïve) and IgD^+CD27^+ (memory unswitched) B cells in young and elderly subjects. Thus, it has been suggested that naïve B cells from young donors need a sufficiently strong stimulus to be activated *in vitro*, while naïve B cells from elderly subjects are able to produce high basal levels IL-10 and TNF-α as they might be basically activated by bacteria or viruses (such CMV). As a consequence, Buffa S et al.,(2011) have supposed that cytokines production in young subject might control the size of immune response while in the elderly, the higher production of cytokine by naïve B cells may be related to overstimulation of the immune system. In this regard, Rieger A and Bar-Or A (2008) have proposed that the IL-10 production by naïve B cells might be a control mechanism to prevent the exacerbation of inflammation in an autoimmune context, whereas IL-10 production by memory B cells might be participate in the resolution of the disease.

Due to the above reported data, the different distribution of B cell subpopulations in the elderly and their ability to produce pro- or anti-inflammatory cytokines might play a central role in the generation of the inflammatory environment typical of age (Agrawall N and Gupta SK, 2010; Licastro F et al., 2005).

Moreover, another link between inflamm-aging and adaptive immune responses might be identified in the expression of chemokines' receptors. Indeed, certain combination of chemokines and their receptors guide all the immune cells and also B lymphocytes to specific tissues (Kunkel EJ and Butcher EC, 2003). Accordingly, CXCR4, CXCR5, CCR6 and CCR7 have been identified as receptor that drive B cells to lymph node attract by a combination of CXCL12, CXCL13, CCL20 and CCL19 respectively while CXCR3 leads B cells to sites of inflammation (Comeford I et al., 2010; Welsh-Bacic et al., 2011).

Recently, several lines of evidences have suggested that the combination of interleukin-21 (IL-21) and BCR stimulation, in absence of CD40 ligation, enables B cells to produce and secrete active form of cytotoxic serine protease granzyme B (GrB) that induces apoptosis of target cells independently from perforin, but using mannose-6-phosphate or fluid phase endocytosis (Hagn M et al., 2009; Kurschus FC et al., 2005; Gross C et al., 2003). It has been proposed that GrB-secreting B cells might exert cytotoxic functions and partecipate in early cell-mediated immune response during inflammatory and neoplastic process. Indeed, it has been demonstrated that GrB secretion by B cell might play a significant role in early antiviral immune responses, in the regulation of

autoimmune diseases and in cancer immunosorvelliance (Hagn M et al., 2009).

1.3 Centenarian offspring: a model of successful aging

As previously discussed, healthy aging and lifelong has been also related with genetics (Roush W, 1996; Troen BR, 2003; Franceschi C et al., 2005; Candore G et al., 2006a; Salvioli S et al., 2006; De Benedictis and Franceschi C, 2007).

Centenarians represent the best example of extreme longevity in humans that have escaped neonatal mortality, illnesses in the pre-antibiotic era (Candore G et al., 2010), and the major age-related diseases, such as cancer (Akushevich I et al., 2012; Pavlidis N et al., 2012), cardiovascular diseases, diabetes and dementia (Franceschi C and Bonafe M`, 2003; Terry DF et al., 2003, 2004), so reaching the extreme limit of human life in healthy and good clinic conditions. For this reasons, they represent the best prototypes of successful aging (Franceschi C et al., 1995) and are considered a good model for the study healthy aging. Studies on centenarians might allow the identification of key factors associated with exceptional longevity in humans. Moreover, centenarians are equipped with well-preserved and efficient immunological defense mechanisms, and optimal combinations of an appropriate lifestyle and genetic background (Franceschi C et al., 1995). Moreover, it has been demonstrated that they have a genetic predisposition to produce high

amounts of anti-inflammatory cytokine or lower level of pro-inflammatory ones (Salvioli S et al., 2006; Franceschi C et al., 2007: Salvioli S et al., 2013). However, it has been reported that they also produce high levels of IL-6 and other inflammatory markers (Gerly R et al., 2000; Gangemi S et al., 2005; Salvioli S et al., 2013). In order to explain this paradox, it has been suggested that, in these individuals, the balance between pro- and anti-inflammatory cytokine could be have positive role toward the development of those age-related diseases having a strong inflammatory pathogenetic component and might compensate the concomitant development of strong and effective anti-inflammatory responses (Candore G et al. 2006; Franceschi C et al. 2007; Vasto S et al. 2008, 2009; Baggio G et al., 1998). Furthermore, centenarians are equipped with well-preserved and efficient immunological defense mechanism, and optimal combination of lifestyle and genetic background. Looking the immune system, some parameters, such as NK cell number and activity, are conserved in centenarians and similar to those found in young subjects. Nevertheless, centenarians show a decrease of B cells and naive T lymphocytes, accumulation of expanded clone of memory T cells, a progressive increase of CD28-cytotoxicT cells, the accumulation of expanded clones of memory T cells and a shrinkage of T cell repertoire as aged people. In addition, it is observed a complex reshaping of the cytokine network and an age-relate increase in adhesion molecules on the lymphocytes surface (Cossarizza A et al., 1996).

According to epidemiological data in different populations, there is a familial component of longevity. Indeed, these studies demonstrate that parents, siblings and offspring of long-lived subjects have a significant

survival advantage, a high probability to have been or to became long-living persons and to have a lower risk to undergo the most important age-related diseases compared with the age-matched controls population (Terry DF et al., 2004a, 2004b). Especially, centenarian offspring (CO), have genetic and functional advantages that predispose them to healthy ageing and longer survival (Terry DF et al., 2004a, 2004b) and are considered the best example of successful aging (Franceschi C et al 1995; Franceschi C et al., 2008). These findings support the hypothesis that CO are a suitable target of ageing studies because they have an appropriate control group, i.e. age-matched healthy elderly subjects, who haven't a familial history of longevity (Derhovanessian E et al., 2010). It is also observed that CO as their controls share the same incidents of cancer, dementia, osteoporosis, Parkinson's disease but lower risk of cardiovascular disease, myocardial infarction and stroke (Terry DF et al., 2004a, 2004b).

Data in literature on B cell branch demonstrate that CO do not show the typical naïve/memory shift observed in elderly (Colonna-Romano G et al., 2010). Indeed, although a decreased B cell count was observed both in CO and in their age-matched controls, in the offspring of centenarians, naïve B cells ($IgD^{+}CD27$-) were more abundant whereas DN B cells (IgD-CD27-) were significantly decreased, looking similar to the young (Colonna-Romano et al., 2010). It is well known that the quality and the size of the humoral immune response decline with age (Frasca D et al., 2005; Miller JP and Cancro MP, 2007; Gibson KL et al., 2009; Dunn-Walters DK et al., 2010; Bulati M et al., 2011) and that these changes are correlated by lower antibody responses and decreased production of high-

affinity antibodies. The evaluation of IgM secreted in the serum by CO shows that the values are within the range of the levels observed in young subjects (Colonna-Romano et al., 2010).

Taken together these data support the idea that centenarian offspring have an advantage both to fight the main age-related diseases and to properly respond against new infections, prolonging their life. Alternatively, immunogenetic profile may give this result.

1.3 Alzheimer's Disease (AD): a model of unsuccessful aging

Alzheimer's disease (AD) is the most common cause of dementia in elderly people. It is a progressive and irreversible neurodegenerative disease characterized by functional impairment, amnesia and other cognitive dysfunctions (Martin Prince RB, 2011). It can be considered as one of the most example of unsuccessful aging. Currently, AD is diagnosed by the presence of memory and cognitive impairment, early brain atrophy in several brain regions detected by structural MRI, a characteristic pattern of decreased glucose metabolism in parietal-temporal association cortices shown by FDG-PET analysis (Fox NC et al., 2001; Sultana R et al., 2013) but, a firmness diagnosis can be made only post-mortem analysis upon brain autopsy. With this analysis it has revealed that the two main neuropathologycal hallmarks of AD include extracellular deposit of senile plaques and neurofibrillary tangles (NFTs).

The former is characterized by the association of amyloid β-peptide, dystrophic neuritis, activated microglia and reactive astrocytes; while, the latter consist of iperphosphorylated tau-proteins that cause the collapse of microtubules (Nussbaum RL and Ellis CE, 2003). The production of Aβ plaques is the result of the cleavage of amyloid precursor protein (APP), a transmembrane glycoprotein expressed in an ubiquitous way, that is synthesized in the endoplasmatic reticulum and it is involved in the neuronal and dendritic growth and synapse formation (Priller C et al., 2006). APP is processed by two different pathways: the physiological pathway and the pathogenic or amyloid pathway. The first involves the α-secretase enzyme which cuts within the APP domain, generating two fragments, P3 and AICD, two γ-secretase substrates while the second pathway is due to the consecutive action of β and γ secretases, catalyzing the release of Aβ40 and Aβ42 fragments that will settle, joining into plaques (Selkoe, 2001; Bird TD, 2005. Alzheimer's disease occurs predominantly after the age of sixty, although there are rare cases of onset between thirty-fifty years. Most cases of Alzheimer's disease are sporadic even though there is a small subset of cases that have an earlier age of onset and have a strong genetic basis. It is accepted that there are many factors that increase the incidence of this disease. Indeed, a number of studies have found that mutations on APP, Presenilin-1 (PS1) and Presenilin-2 (PS2), enzymes/cofactors are involved in the APP digestion, lead to a preferential production of Aβ42, the toxic fragment, compared to the not toxic fragment Aβ40 (Bird TD, 2005). It is also known that Apoliproprotein E (ApoE) can bind amyloid beta peptide and localize in senile plaques, supporting the theory that this protein plays a key role in

the clereance of Aβ42 (Nussbaum and Ellis, 2003). Moreover, some researchers have shown that cholesterol may influence APP degradation pathway inhibiting α-secretase activity but enhancing β- and γ-secretase functions (Crestini A et al., 2006; Grimm MO et al., 2011). The most important risk factor seems to be the age (Leuner K, et al., 2012). Indeed, the aging brain is exposed to a lifetime of changes and insults such as oxidative stress, trauma, damaged molecules, inflammation which may support the beginning of neurodegeneration. Furthermore, it has been observed that in very old women (over eighty years), the risk to developing this disease is greater than in males so it seems that gender may be considered another AD risk factor (Nussbaum RL and Ellis CE, 2003).

Brain inflammation is a typical hallmark of Alzheimer's disease. Microglia, astrocytes and neurons are responsible for inflammatory reactions. Indeed, inflammatory mediators could directly promote AD by interference in the APP-metabolism (Fassbender K et al., 2000).

It has been shown that Aβ peptide is able to stimulate the synthesis and secretion of IL-1, IL-6 and IL-8 by microglial cells, activating the inflammatory response. In the nervous tissue, these cytokines induce APP and, as consequence, Aβ synthesis that, in turn, will increase cytokines production by glial cells and neurons. If the activation of cells persists and becomes chronic, these cytokines contribute to neurodegeneration (Akiyama H et al., 2000). Moreover, the combined action of TNF-α and IFN-γ not only stimulates Aβ42 synthesis, but leads to reduced secretion of soluble APP protein, generally considered as a protein with neuroprotective attitudes. Inflammation induced by the accumulation of

Aβ peptide is not a local phenomenon that concerns only the brain of AD patients, but a systemic process that affects the entire organism (Britschgi M et al., 2007). Therefore, a reciprocal relationship between Aβ42 and inflammatory mediators might exist (Griffin WS et al., 2002). Different studies have shown an increase in chemokines production, like MIP-1α, RANTES and MCP-1 by PBMC of AD patients and the expression of CCR5 on brain endothelial cells (Li M et al., 2009; Reale M et al., 2008). The expression of CCR2 and CCR5 on T cells and CCR5 on B cells on AD patients are increased after *in vitro* stimulation with r-Aβ peptide (Pellicanò M et al., 2010).

It has been recently suggested that blood derived cells seem to accumulate in the AD brain and that immunological changes characterizes this pathology. Indeed it has been reported a different distribution and reactivity of immune cells and the presence of antibodies direct to CNS-specific amyloid beta peptides (Rogers J et al., 1998; Fiala M et al., 2005; Monsonego A et al., 2003). According to this evidences, several studies have described a communication between central and systemic immune response. It has been suggested that neuroinflammation might induce the afflux of central nervous system proteins, as Aβ peptide, or inflammatory mediators across the blood-brain-barrel (BBB). This process might cause immune response and recruitment of myeloid or lymphocytic cell into the brain (Britchgi M and Wyss-Coray, 2007). Moreover, it has been demonstrated that peripheral blood cells (PBMCs) from AD patients are able to produce high levels of cytokines, such as IL-1β,and IL-6 compared to PBMCs of controls subjects (Speciale L et al., 2007). Other studies have shown Aβ-peptide stimulates MIP-1α expression on peripheral T

cells and expression of its receptors CCR5 on brain endothelial cells. This modification promotes T cells crossing of BBB and migration towards brain (Man SM et al., 2006).

Sign of a systemic involvement have been described in peripheral blood lymphocytes. Indeed, it has been reported a significant decrease in B and T cells numbers while the number of NK cells does not change (Ritchartz-Saltzburger E et al., 2007). Concerning T compartment, the most change is seen in $CD4^+$ lymphocytes because, AD patients show a significant decline of naïve ($CD45RA^+CCR7+$) and a simultaneous increase of effector memory ($CD45RA^-CCR7^-$) and TEMRA: (terminal effector memory RA) ($CD45RA^+CCR7^-$) T cells, when compared to age matched controls (Larbi A et al., 2009). Furthermore, recently it has been described an higher frequency of activated T cells ($CD4^+CD25^+FoxP3^-$) in AD patients compared to old controls (Pellicanò M et al., 2011).

CHAPTER 2

Outline of the thesis

2. OUTLINE OF THE THESIS

The progressive increase of lifespan and the consequent growth of the elderly population have focused the attention of scientific community on aging. Particularly, researchers have concentrate their efforts trying to understand the mechanisms that could lead to longevity.

Aging is a natural process that occurs in cells, tissues and organs (Cevenini E et al., 2008; 2013). One of the most important characteristics of aging is the progressive deregulation of immune responses, resulting in an increased susceptibility to infectious diseases and pathological conditions relating to the inflammation and the onset of autoimmune diseases (Troen BR, 2003). The age related modifications in immune competence are called “immunosenescence” (Franceschi C et al., 1995; Pawelec G et al., 2005). This phenomenon is a complex process that involves both the innate and adaptive immune compartment (Franceschi C et al., 1995; Licastro F et al., 2005; Pawelec G et al., 2005). Lifelong and chronic antigenic load are the major driving force of immunosenescence. The consequences of this occurrence are the progressive filling of the immunological space by activated lymphocytes in response to chronic/continuous stressor agents, the constant decline in the number of naive T cells, the reduction of new B cell precursors, the extended survival of memory B and T cells, the increase in homeostatic proliferation and clonal expansion. All those factors contribute to the limited repertoire and the collapse of cell diversity that are frequently correlated with poor health status (Cancro MP et al., 2009; Gibson KL, 2009; Dunn-Walters DK et al., 2010; Scholtz JL et al., 2013)..

Nevertheless, some people can reach the extreme limit of life-span in good clinical conditions, escaping major age-related diseases, as centenarians do. Indeed, they may represent the prototypes of successful aging Franceschi C et al., 2005; Terry DF et al., 2003; 2004). This event seems to be the result of balance between environmental and genetic factors (Franceschi C et al., 1995). Studies of centenarian pose the challenge of whom to use as control. Differently from their parents, Centenarian offspring (CO) have an appropriate control group, i.e. healthy elderly, who haven't a familiar history of longevity (Derhovanessian E et al., 2010). Moreover, offspring of centenarians have a genetic background that could predispose them to healthy aging and longer survival rather their age-matched controls whose parents died at an average life expectancy (Terry DF et al., 2004).

The aim of this thesis is to study the immunological profile of elderly people and a group of people "genetically advantaged" for longevity (CO), to evaluate if exist a correlation between familial longevity and the immune system, focusing our attention on the B cell branch and particularly on double negative (DN) B cells.

As a model of unsuccessful aging, we focused our attention on immune system patients suffering by Alzheimer's disease (AD).

In **chapter 3** ("*A novel B cell population revealed by a CD38/CD24 gating strategy: CD38(-)CD24(-) B cells in centenarian offspring and elderly people", 2013*) we show a different distribution of naïve/memory B cell subsets in the elderly and in centenarian offspring. We also characterize a novel population of late memory B cells and perform

functional analysis to evaluate cytokines productions induced by *in vitro* activation with CpG/PMA/Ionomycin.

In **chapter 4 (“***Evidence for Less Marked Potential Signs of T-Cell Immunosenescence in Centenarian Offspring Than in the General Age-Matched Population*”, 2013) we show a phenotypic analysis of the T-cell arm of adaptive immunity in a group of centenarian offspring comparing them with equally elderly people without a familial history of longevity. In particular, we have analyzed the expression of CD27, CD28, CD45RA, CD45RO and CD57 on $CD4^+$ and $CD8^+$ to evaluate the different stage of differentiation of $CD4^+$ and $CD8^+$ subsets between young, elderly, centenarian offspring and age-matched controls. The aim of this study is to evaluate some possible differences that can be related with the increased lifespan expectancy of centenarian offspring.

In **chapter 5 (“***Trafficking phenotype and production of Granzyme B by Double Negative B cells (IgG^+IgD^-CD27-) in the elderly”*, 2014) we investigate the expression of CCR7, CCR6,CXCR4, CXCR5 and CD62L on naïve/memory B subpopulations in young and elderly subjects to evaluate if the pro-inflammatory status, typical of aged people, could influence the trafficking phenotype of these cells. Furthermore, in order to evaluate whether DN memory B cells are able to act as Granzyme B (GrB) producing cells, we study 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.

In **chapter 6** *(“Double Negative ($CD19^+IgD^-CD27^-$) B lymphocytes: a new insight from telomerase activity in healthy elderly, in centenarian offspring and in Alzheimer’s disease patients”, manuscript in*

preparation) we analyze cell surface expression of two BCR-inhibitory receptors, CD307d and CD22 to evaluate whether the low proliferative ability of Double Negative (DN) B cells might be related to over-expression of these receptors. Furthermore, in order to evaluate whether DN B lymphocytes might play any role in the immune response we assessed the proliferative response of these cells after stimulation *in vitro* with different kind of stimuli. Because of DN B cell are capably to vigorously proliferate after CpG/α-IgG/CD40 *in vitro*, we analyzed whether this stimulus might modifies telomerase activity in young and elderly subjects. In addition, to verify if relative activity of telomerase (RTA) might be a useful test to evaluate the efficiency of immune system we also performed the test using B cells obtained from genetically advantage people, centenarian offspring, and unsuccessfully aged people, as patient affected by severe Alzheimer's disease.

In **chapter 7** (*"Immunophenotype and trafficking profile in Alzheimer's disease patients", manuscript in preparation*) we evaluate the different distribution of the circulating B cells subpopulation in the two different groups of AD patients object of the study (Severe and Mild) comparing them one each other and with their age-matched healthy controls. Moreover, we analyze the expression of CCR7, CCR6, CXCR3, CXCR4 and CXCR5 chemokines receptors on healthy elderly donors, severe AD and mild AD patients, in order to evaluate whether in AD patients, $IgD^{-}CD27^{-}$ DN B cells show the pro-inflammatory trafficking profile.

In **chapter 8 (***"Immunosenescence, inflammation and Alzheimer's disease", 2012)* we present a review about the modifications of the

immune system with aging (immunosenescence), showing immunological changes on cellular and humoral branches. Moreover, we focus on changes of some immunoinflammatory parameters observed in patients affected by AD.

In **chapter 9 (“***Genetics of longevity. data from the studies on Sicilian centenarians”, 2012*) we report current literature data on centenarians. We discuss about genetic background and immune system of these subjects that reach the extreme limits of human life. In particular, we also report our data gathered for 10 years in Sicilian centenarians, concerning the relationship between gender and longevity, the role of some immune and inflammatory genes or epigenetic age-related modifications and ageing and longevity.

In **chapter 10** (“*Centenarian Offspring: a model for Understanding Longevity”,2013*) we summarize several aspects that permit to consider centenarian offspring a suitable model to understand successful aging. We discuss their genetic background, cardiovascular and immunological profile and cognitive status.

Finally, in **chapter 11**, a summary and general discussion of the results are presented.

CHAPTER 3

"A novel B cell population revealed by a CD38/CD24 gating strategy: $CD38^{-}CD24^{-}$ B cells in centenarian offspring and elderly people"

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A novel B cell population revealed by a CD38/CD24 gating strategy: $CD38^{-}CD24^{-}$ B cells in centenarian offspring and elderly people

Silvio Buffa · Mariavaleria Pellicanò · Matteo Bulati · Adriana Martorana · David Goldeck · Calogero Caruso · Graham Pawelec · Giuseppina Colonna-Romano

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Abstract The B cell arm of adaptive immunity undergoes significant modifications with age. Elderly people are characterized by impaired B cell responses reflected in a reduced ability to effectively respond against viruses and bacteria. Alterations of immunity with advancing age (immunosenescence) have been widely studied in centenarians who are considered a good example of successful aging. In recent years, attention has shifted to centenarian offspring (CO) as a model of people genetically advantaged for healthy aging and longevity. Here, we describe the preliminary characterization of a proposed new population of memory B cells, defined as $CD19^{+}CD38^{-}CD24^{-}$, which we find at higher frequencies in the elderly but less so in CO than healthy age-matched random controls. In addition, we found a decreased expression of RP105 (CD180), a toll-like receptor-associated molecule, on these cells. CD180 downregulation may potentially be a marker of immunosenescence. Moreover, we show that these $CD19^{+}CD38^{-}CD24^{-}$ B cells produce TNF and hypothesize that their observed expansion in the elderly might contribute to the increased inflammatory status sometimes designated "inflamm-aging."

Keywords B cell · CD38 · CD24 · CD180 · Immunosenescence · Centenarian offspring

Introduction

B cells are key mediators of immunity. The humoral immune response includes production of antibodies against pathogens and cytokines interacting with other components of the immune system. Early stages of B cell development occur in the bone marrow from hematopoietic stem cells. The early progenitors of B lymphocytes develop into pro-, pre-, and immature B cells (LeBien and Tedder 2008) that, after they are controlled for autoreactivity (Carsetti et al. 1995; Palanichamy et al. 2009), leave the bone marrow and enter the blood as transitional B cells (Allman et al. 1993; Chung et al. 2003; Mauri and Ehrestein 2008). In humans, peripheral blood naïve and memory B cells have been described on the basis of the differential

Silvio Buffa and Mariavaleria Pellicanò contributed equally to this work. Graham Pawelec and Giuseppina Colonna-Romano contributed equally to this work.

S. Buffa · M. Bulati · A. Martorana · C. Caruso · G. Colonna-Romano (✉)
Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies (DIBIMEF), University of Palermo,
Corso Tukory 211,
Palermo 90134, Italy
e-mail: giuseppina.colonnaromano@unipa.it

M. Pellicanò · D. Goldeck · G. Pawelec
Department of Internal Medicine II, Center for Medical Research, Tübingen Aging and Tumor Immunology Group, University of Tübingen,
Tübingen, Germany

expression of IgD and CD27 (Anolik et al. 2004; Shi et al. 2005; Wei et al. 2007; Frasca et al. 2008), as follows: IgD^+CD27^- cells are naïve; IgD^-CD27^+ cells are memory cells including the unswitched memory cells also known as marginal zone-like B cells (Weller et al. 2004) and the IgM^- memory B cell population identified as $IgM^+IgD^+CD27^-$; IgD^-CD27^+ cells are classical switched memory B cells also including the "IgM-only" memory B cells identified as $IgM^+IgD^-CD27^+$, and finally, IgD CD27 , double-negative memory B cells (Fecteau et al. 2006; Colonna-Romano et al. 2009).

More recently, a different flow cytometric approach has been used to distinguish naïve from memory B cells (Allman et al. 2001, 2004; Carsetti et al. 2004; Palanichamy et al. 2009; Blair et al. 2010). The use of two developmentally regulated markers, CD24 and CD38, in association with the B-lineage marker CD19, allowed the identification of three different B cell populations: CD19 $CD38^{high}CD24^{high}$, the previously mentioned transitional B cells that also include immature B cells; $CD19^+CD38^{int}CD24^{int}$ defined as mature B cells; and the final step of maturation in the periphery, $CD19^-CD38^-CD24^{high}$ so-called "primarily memory B cells." In a recent paper (Chaplin et al. 2011), differentiation from transitional to mature B cells was induced by stimulation with CD180 (RP105), a toll-like receptor (TLR)-4 homologue expressed by monocytes, macrophages, dendritic cells, and B lymphocytes which regulates TLR-4 signaling and induces B cell proliferation.

Many papers have focused on modifications of the immune system in the elderly (immunosenescence) likely to contribute to their increased morbidity and mortality. It has been widely reported that elderly people show changes in B cell number, low levels of antibody production, and poor responses to recall antigens, and a collapse in B cell receptor repertoire diversity correlated with poor health status and the impairment of antibody response (Miller and Cancro 2007; Kumar and Burns 2008; Cancro et al. 2009; Gibson et al. 2009; Dunn-Walters and Ademokun 2010; Frasca et al. 2010; Frasca and Blomberg 2011; McElhaney et al. 2012). Lower numbers and percentages of B cells also form part of the cluster of immune parameters collectively known as the "Immune Risk Profile" associated with 2-, 4-, and 6-year mortality of the very elderly in the Swedish OCTO/NONA longitudinal studies (Pawelec et al. 2005). We and others have previously demonstrated that in elderly people, IgD^+CD27^- naïve B cells are significantly reduced (Gupta et al. 2005; Colonna-Romano et al. 2008). In contrast, Chong et al. (2005) demonstrated that the percentage of circulating naïve B cells, identified as $CD27^-$, were significantly higher in the aged subjects than young subjects. This topic is still a somewhat controversial finding, as previously reviewed by us (Bulati et al. 2011). Moreover, double-negative (DN) B cells (IgD^-CD27^-) are significantly increased in the elderly (Colonna-Romano et al. 2009; Bulati et al. 2011), as well as under certain pathological conditions, such as systemic lupus erythematosus (SLE) (Anolik et al. 2004; Wei et al. 2007), chronic HIV infection (Cagigi et al. 2009), and in healthy subjects challenged with respiratory syncytial virus (Sanz et al. 2008).

We have recently focused on some characteristics of the naïve/memory B cell compartment of centenarian offspring (CO). This is a special population of elderly people that, like their centenarian parent(s), could have genetic and functional advantages that predispose them to healthy aging and longer survival (Terry et al. 2003, 2004). We have shown that CO fail to show the age-associated increase of DN (IgD^-CD27^-) B cells seen in the general elderly population. Consistent with this, the level of serum IgM in CO is within the range of the levels observed in young subjects (Colonna-Romano et al. 2010).

As outlined above, several distinct memory B cell populations have been identified in humans, but associations between their specific phenotype and their functions remain to be clarified. Here, we report the characterization of a proposed new population of memory B cells identified as $CD19^+CD38^-CD24^-$. Moreover, we show that these cells are expanded in the general elderly but not in CO who show similar levels to those observed in young donors, suggesting a more "youthful" B cell constellation. A more detailed examination of CO B cells was then performed to evaluate the expression of CD27, IgM, and IgD. It is known that IgM memory B cells are reduced in the elderly, thus predisposing them to pneumococcal infection (Shi et al. 2005; Buffa et al. 2011). Here again, we show that CO have a "younger" B cell profile. Indeed, the percentage of these cells are not as reduced in CO as in their age-matched controls and is more similar to the percentage observed in young people. Moreover, we have examined the expression of CD180 on total B cells and found that elderly people have a significant increase of the $CD19^+CD180^+$ B cell subset. Finally, we have evaluated the expression of CD180 on B cell populations, identified by the

differential expression of IgD/CD27 and CD38/CD24, in order to determine the age-related modulation of this marker on these cells.

Material and methods

Subjects

A total of 35 healthy Sicilians were included in the study. Twelve subjects aged 70.1–8.3 years, who were the offspring of at least one centenarian parent, were compared with seven age-matched controls without a centenarian parent (aged 69.1+9 years), eight young (28.5±1.9 years), and eight old (86.4–3.8 years). All subjects were in good health according to their clinical history, and none of them had neoplastic, infectious, or autoimmune diseases or received any medications influencing immune function at the time of the study. The study received approval from the local ethics committee, and all participants gave their informed consent.

Whole blood was collected by venepuncture in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Lympholyte (Cedarlane Laboratories Limited, Ontario, Canada) and stored frozen.

Flow cytometric immunophenotyping of B cell subsets from human peripheral blood of young, elderly, centenarian offspring, and age-matched controls

To characterize the phenotype of B lymphocyte subsets, extracellular labeling was performed with anti-CD19-PE, CD24-Pe-Cy7, CD5-V450, IgD-FITC, IgM-PerCP-Cy5.5 (BD Biosciences), CD27-Qdot605 (Invitrogen), and CD38-eFluor650 (eBioscience). Cell viability was determined with RedVid (Invitrogen). All staining steps were performed in PBS, 2 % FCS, 2 mM EDTA, and 0.01 % Na azide (PFEA) buffer. Blocking of nonspecific binding sites was accomplished using human immunoglobulin Gamunex (Bayer, Leverkusen, Germany) or mouse serum (Caltag/Invitrogen, Karlsruhe, Germany). For each experiment, cells or mouse/rat κ-chain Comp Beads (Becton Dickinson) were stained with the corresponding fluorochrome-labeled antibodies and incubated for 20 min at 4 °C in the dark. Human-unstained cells were used as negative controls. After washing with PFEA, the cells or beads were resuspended and measured on a LSR-II flow cytometer using the acquisition software FACSDiva (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star, Portland, OR). For data analysis, dead cells (RedVid-positive) were excluded. $CD19^+$ living cells were gated within the side/forward scatter (SSC/FSC) lymphocyte gate.

Stimulation of PBMC with cytosine guanine/phorbol myristate acetate/ionomycin for B cell IL-10, IL-6, and TNF production

PBMC (10^6 cells/ml) were suspended in X-vivo 15 (Lonza, Walkersville, MD, USA) with or without cytosine guanine (CpG)-B 2006 (3 μg/ml, Tib Molbiol), phorbol myristate acetate (PMA) (50 ng/ml), ionomycin (1 mg/ml), and monensin sodium salt (2 mM, Sigma Aldrich) in 24-well flat-bottom plates for 5 h, at 37 °C (CO_2 5 %). Cells were harvested, washed, and directly stained with anti-CD19-PerCP-Cy5.5, CD24-Pe-Cy7, IgD-FITC, CD180-PE (BD Biosciences), CD27-Qdot605 (Invitrogen), and CD38-eFluor650 (eBioscience). For indirect immunofluorescence, antihuman CD5 (clone UCHT2) culture supernatant hybridoma cell line (Zentrum für Medizinische Forschung, Tübingen, Germany) was used as primary antibody. The secondary antibody was rat anti-mouse PO (Invitrogen). Cells were permeabilized with Citofix/Citoperm (BD Biosciences). Finally, cells were stained with anti-IL-10-APC (Miltenyi Biotec), TNF-AlexaFluor700, and IL-6-V450 (BD Biosciences), washed, and analyzed.

Statistical analyses

All statistical analyses were performed with Graph Pad Prism 4.0 using the Mann Whitney nonparametric *U* test to compare two independent groups. Statistical significance was expressed as $P<0.05$, $P<0.01$, and $P<0.001$ as shown in the figures. All values are expressed as mean+standard error of the mean (SEM).

Results

Characterization of $CD38^+/CD24^+$ B cell subsets

To better characterize the memory/naïve phenotype of $CD38^+/CD24^+$ B cell subsets, we have gated $CD19^+CD38^{hi}CD24^{hi}$ "classical transitional," $CD19^+CD38^{int}CD24^{int}$ "mature naïve," and

CD19 CD38 CD24^{+} "primarily memory" B cells and further evaluated them on the basis of the expression of IgD and CD27, widely used to identify naïve and memory B cells (Fig. 1). Moreover, as a fourth population that lacks both CD24 and CD38 is clearly present, we also extended the IgD/CD27 characterization to this population. In the figure, CD19^{+}CD38^{++}CD24^{-} plasma cells are also shown. Most of the CD19^{+}CD38^{hi}CD24^{hi} cells (plot I) are IgD^{-}CD27^{-} (85.4 %), thus showing that classical transitional B cells have a naïve phenotype, although about 12 % of them are IgD^{+}. Again, CD19^{+}CD38^{int}CD24^{int} cells (plot II) are principally IgD^{+}CD27^{-}, although about 15 % of them express CD27. Moreover, the CD19^{+}CD38^{-}CD24^{-} B lymphocytes (plot III), here referred to as primarily memory B cells, show a variety of different phenotypes. Indeed, some of them (26.6 %) are IgD^{+}CD27^{+} (unswitched memory); others (44 %) are IgD^{-}CD27^{+} (switched memory); 25 % of them are naïve as they express IgD but are negative for CD27 expression, and those that have a very low percentage (4.3 %) are IgD^{-}CD27^{-} (DN). Our analysis of the fourth population (CD19^{-}CD38^{-}CD24^{-}) (plot IV) indicated that these cells were predominantly IgD^{-}, including the IgD^{-}CD27^{+} (switched memory) and the IgD^{-} CD27^{-} (double negative) B cell subsets, although 8.3 % of them are naïve (IgD^{+}CD27^{-}). Therefore, during maturation, it seems that B cells progressively modulate both CD38/CD24 and IgD/CD27 expressions, although not synchronously. In order to characterize these populations in more detail, we have evaluated the expression of additional immunological markers (Fig. 2);

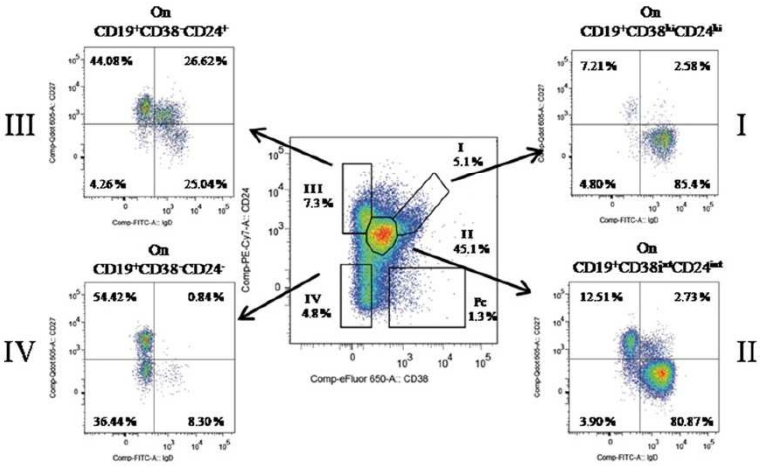

Fig. 1 Phenotypic characterization of CD38/CD24 B cell subsets. CD19^{+}CD38^{hi}CD24^{hi} (*I*), CD19^{+}CD38^{int}CD24^{int} (*II*), CD19^{+}CD38^{-}CD24^{+} (*III*), and CD19^{+}CD38^{-}CD24^{-} (*IV*) were evaluated on the basis of the expression of IgD and CD27. Moreover, it is also depicted the gate of CD19^{+}CD38^{+} CD24^{+} plasma cells. In figure, the phenotypic characterization of an 82-year-old sample is shown. For data analysis, dead cells (RedVid positive) were excluded. CD19^{+} living cells were gated within the SSC/FSC lymphocyte gate. The evaluation of CD38 and CD24 within CD19^{+} B cells leads to the identification of four naïve/memory B cell subsets. This kind of characterization confirmed that the majority of CD19^{+}CD38^{hi}CD24^{hi} have a naïve (IgD^{+}CD27^{-}) phenotype, although about 12 % of them are IgD^{-}. The CD19^{+}CD38^{int}CD24^{int} B cells are principally naïve, whereas about 15 % of them express CD27. The analysis of CD19^{+}CD38^{-}CD24^{+} B cells reveals a variety of different phenotypes: some of them are IgD^{+}CD27^{+} (unswitched memory); others are IgD^{-}CD27^{+} (memory switched); about 25 % of them are naïve as they express IgD, but lack CD27 expression, and those that have a very low percentage (4 %) are IgD^{-}CD27^{-} (DN). Moreover, CD19^{+}CD38^{-}CD24^{-} B cells, principally IgD^{-}, are IgD CD27^{+}/IgD CD27^{-}

as no differences have been observed among the groups of age studied, the figure shows representative histograms of IgM, CD5, and CD180 expression. As shown, the largest proportion of $CD19^+CD38^{hi}CD24^{hi}$ cells (≥80 % IgD^+CD27^-) was IgM^+CD5^+, whereas of $CD19^+CD38^{int}CD24^{int}$ cells (≥80 % IgD^-CD27^-), IgM^-CD5^+, and of $CD19^+CD38^-CD24^-$ cells (IgD^+CD27^+), IgM^+CD5^-, as previously demonstrated (Carsetti et al. 2004; Palanichamy et al. 2009; Blair et al. 2010). Finally, the CD19 CD38 CD24 population ($IgD^-CD27^{\pm}$) not thus far described was found to be IgM $CD5^-$. Moreover, the analysis of CD180 expression revealed that almost all CD180-negative B cells are gated within the $CD19^+CD38^-CD24^-$ population, whereas classical transitional, mature naïve, and primarily memory cells were positive for this marker. Results depicted in Fig. 2 are representative of all subjects (young, old, centenarian offspring, and age matched) analyzed.

CpG + PMA/ionomycin stimulates intracellular cytokine production by human blood B cells in vitro

A typical feature of aging is a chronic, low-grade inflammation characterized by a general increase in the production of pro-inflammatory cytokines and inflammatory markers (Cevenini et al. 2010). Memory and naïve B cells can produce different cytokines and chemokines; in particular, memory B cells produce high levels of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α, suggesting that B cells might take part in the generation or in the maintenance of the inflammatory environment of the elderly (Agrawal and Gupta 2011). To study the functional properties of peripheral blood B lymphocytes, we tested their ability to produce cytokines "in vitro" on stimulation with a combination of CpG, PMA, and ionomycin. We evaluated the production of IL-10, an anti-inflammatory cytokine, and IL-6 and TNF-α, two pro-inflammatory cytokines, by CD38/CD24 B cell subsets. These cytokines have been extensively studied in aging, as they are involved in the control (IL-10) or maintenance (IL-6 and TNF-α) of inflammation, one of main characteristics of aged people (inflamm-aging) (Franceschi et al. 2000a; Lio et al. 2003; Sanjabi et al. 2009). Our analysis revealed that in all the subjects studied, 5 h after stimulation, the main IL-10-producing cells were of the $CD19^+CD38^{hi}CD24^{hi}$ phenotype (Table 1). In contrast, $CD19^+CD38^-CD24^+$ and CD19 CD38 CD24 cells are the more responsive B cell populations involved in TNF-α production (Table 2). As no differences were detected (not shown) regarding the age classes, the tables show the mean fluorescent intensity (MFI) values (mean+SEM) of all the subjects studied. On the other hand, this kind of stimulation was not able to induce IL-6 production. Indeed, we did not detect

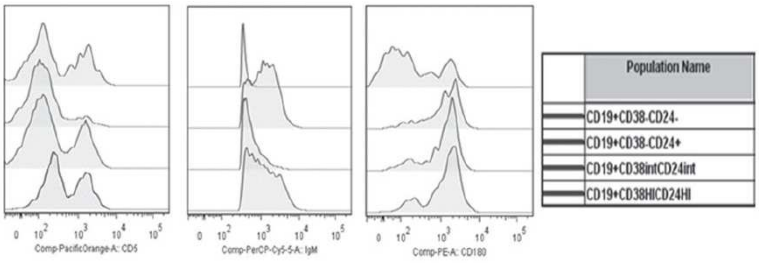

Fig. 2 Evaluation of IgM, $CD5_1$ and CD180 on CD38/CD24 B cells. A typical experiment showing the expression of the immunological markers on $CD19^+CD38^{hi}CD24^{hi}$, CD19 $CD38^{int}CD24^{int}$, CD19 CD38 CD24 , and $CD19^+$CD38 CD24 B cell subsets. Histograms are a representative of all subjects (young, old, CO, and age matched) analyzed. As shown, CD5 is well expressed on $CD19^-CD38^{hi}CD24^{hi}$ and $CD19^+CD38^-CD24^-$ (MFI 789 and 704, respectively); a lower level is expressed on $CD19^+CD38^{int}CD24^{int}$ (MFI 570), whereas $CD19^-CD38^-CD24^-$ cells show the lowest level of expression of CD5 (MFI 278). Concerning IgM_1 $CD19^-CD38^{hi}CD24^{hi}$ and $CD19^-CD38^-CD24^-$ cells are IgM^+ (MFI 1,601 and 1,489, respectively); $CD19^+CD38^{int}CD24^{int}$ cells are IgM^+ (MFI 682), whereas CD19 CD38 CD24 cells show the lowest level of expression of IgM (MFI 391). The evaluation of CD180 on CD38/CD24 B cell subsets has revealed that $CD19^+CD38^{hi}CD24^{hi}$ (MFI 1,578), $CD19^-CD38^{int}CD24^{int}$ (MFI 1,413), and $CD19^+CD38^-CD24^-$ (MFI 1,507) cells are $CD180^-$, whereas $CD19^+CD38^-CD24^-$ cells show the lowest level of expression of CD180 (MFI 484), resulting to CD180

Table 1 IL-10 production by CD38/CD24 B cell subsets with or without CpG/PMA/ionomycin stimulation

IL-10										
RPMI					CpG/PMA/ionomycin					
CD38/CD24 B cell subsets MFI values (mean ± SEM)	$CD38^{hi}CD24^{hi}$ (1,126.4 ± 208.3)	$CD38^{int}CD24^{int}$ (611.7 ± 127.6)	$CD38^{int}CD24^{int}$ (481.4 ± 45.1)	CD38 CD24 (341.6 ± 57.7)	CD38/CD24 B cell subsets MFI values (mean ± SEM)	$CD38^{hi}CD24^{hi}$ (2,150.2 ± 316.7)	$CD38^{int}CD24^{int}$ (1,039.4 ± 276)	$CD38^{-}CD24^{+}$ (784 ± 75.9)	CD38 $CD24^{-}$ (576 ± 123.7)	
$CD38^{hi}CD24^{hi}$ (1,126.4 ± 208.3)	–	p1 = **0.03**	p2 = **0.02**	p3 = **0.005**	$CD38^{hi}CD24^{hi}$ (2,150.2 ± 316.7)	–	p1 = **0.002**	p2 = **0.001**	p3 = **0.0001**	
$CD38^{int}CD24^{int}$ (611.7 ± 127.6)	p1 = **0.03**	–	p4 = 0.6	p5 = **0.03**	$CD38^{int}CD24^{int}$ (1,039.4 ± 276)	p1 = **0.002**	–	p4 = 0.1	p5 = **0.04**	
$CD38^{-}CD24^{+}$ (481.4 ± 45.1)	p2 = **0.02**	p4 = 0.6	–	p6 = **0.01**	$CD38^{-}CD24^{+}$ (784 ± 75.9)	p2 = **0.001**	p4 = 0.1	–	p6 = **0.02**	
$CD38^{-}CD24^{-}$ (341.6 ± 57.7)	p3 = **0.005**	p5 = **0.03**	p6 = **0.01**	–	$CD38^{-}CD24^{-}$ (576 ± 123.7)	p3 = **0.0001**	p5 = **0.04**	p6 = **0.02**	–	

Statistically significant differences are marked in bold

A total of 35 healthy Sicilians were analyzed. $CD19^{+}$ $CD38^{hi}CD24^{hi}$ is the main B cell subset involved in IL-10 production. Data are expressed as MFI values (mean±SEM). p1 = $CD19^{+}$ $CD38^{hi}CD24^{hi}$ vs $CD19^{+}$ $CD38^{int}CD24^{int}$; p2 = $CD19^{+}$ $CD38^{hi}CD24^{hi}$ vs $CD19^{+}$ $CD38^{-}CD24^{+}$; p3 = $CD19^{+}$ $CD38^{hi}CD24^{hi}$ vs $CD19^{+}$ $CD38^{-}CD24^{-}$; p4 = $CD19^{+}$ $CD38^{int}CD24^{int}$ vs $CD19^{+}$ $CD38^{-}CD24^{+}$; p5 = $CD19^{+}$ $CD38^{int}CD24^{int}$ vs $CD19^{+}$ $CD38^{-}CD24^{-}$; p6 = $CD19^{+}$ $CD38^{-}CD24^{+}$ vs $CD19^{+}$ $CD38^{-}CD24^{-}$

Table 2 TNF-α production by CD38/CD24 B cell subsets with or without CpG/PMA/ionomycin stimulation

TNF-α										
RPMI						CpG/PMA/ionomycin				
CD38/CD24 B cell subsets MFI values (mean ± SEM)	$CD38^{hi}CD24^{hi}$ (202.1 ± 19.2)	$CD38^{int}CD24^{hi}$ (94 ± 14.2)	CD38 $CD24^{+}$ (97.3 ± 15.8)	CD38 CD24 (76.7 ± 12)	CD38/CD24 B cell subsets MFI values (mean ± SEM)	$CD38^{hi}CD24^{hi}$ (297.4 ± 26.2)	$CD38^{int}CD24^{int}$ (341.9 ± 51.1)	CD38 CD24 (574.5 ± 80.1)	CD38 CD24 (653 ± 152.7)	
$CD38^{hi}CD24^{hi}$ (202.1 ± 19.2)	–	p1 = **0.003**	p2 = **0.0004**	p3 = **0.0001**	$CD38^{hi}CD24^{hi}$ (297.4 ± 26.2)	–	p1 = 0.8	**p2 = 0.05**	**p3 = 0.006**	
$CD38^{int}CD24^{int}$ (94 ± 14.2)	**p1 = 0.003**	–	p4 = 0.7	p5 = 0.3	$CD38^{int}CD24^{int}$ (341.9 ± 51.1)	**p1** = 0.8	–	**p4 = 0.04**	**p5 = 0.1**	
CD38 $CD24^{+}$ (97.3 ± 15.8)	**p2 = 0.0004**	**p4** = 0.7	–	p6 = 0.4	CD38 $CD24^{+}$ (574.5 ± 80.1)	**p2 = 0.05**	**p4 = 0.04**	–	p6 = 0.7	
CD38 CD24 (76.7 ± 12)	**p3 = 0.0001**	p5 = 0.3	p6 = 0.4	–	CD38 CD24 (653 ± 152.7)	**p3 = 0.006**	p5 = 0.1	p6 = 0.7	–	

Statistically significant differences are marked in bold

A total of 35 healthy Sicilians were analyzed. The major contribution to the production of TNF is seen from $CD38^{-}$ $CD24^{+}$ and $CD38^{-}$ $CD24^{-}$ cells. Data are expressed as MFI values (mean ± SEM). p1 = CD19^{+} $CD38^{hi}$ $CD24^{hi}$ vs CD19^{+} $CD38^{int}$ $CD24^{int}$; p2 = CD19^{+} $CD38^{hi}$ $CD24^{hi}$ vs CD19^{+} $CD38^{-}$ $CD24^{+}$; p3 = CD19^{+} $CD38^{hi}$ $CD24^{hi}$ vs CD19^{+} $CD38^{-}$ $CD24^{-}$; p4 = CD19^{+} $CD38^{int}$ $CD24^{int}$ vs CD19^{+} $CD38^{-}$ $CD24^{+}$; p5 = CD19^{+} $CD38^{int}$ $CD24^{int}$ vs CD19^{+} $CD38^{-}$ $CD24^{-}$; p6 = CD19^{+} $CD38^{-}$ $CD24^{+}$ vs CD19^{+} $CD38^{-}$ $CD24^{-}$

a significant increase in production of IL-6 compared to unstimulated PBMC (not shown).

Age-related changes result in altered distribution of naïve/memory subsets

We have determined whether the distribution of B cell phenotypes including the newly defined subset is different in the elderly compared to the young, and whether this is different in centenarian offspring compared to age-matched and elderly controls (Table 3). We did not observe any significant differences in the $CD19^{+}CD38^{hi}CD24^{hi}$ (Fig. 3a) or $CD19^{+}CD38^{int}CD24^{int}$ (Fig. 3b) populations between any groups of subjects tested. However, we observed a trend towards higher proportions of $CD19^{+}CD38^{-}CD24^{-}$ cells in the elderly. Interestingly, the percentage of these cells in centenarian offspring are more similar to what is observed in young donors than the age-matched (AM) controls (Fig. 3c). Most strikingly, the $CD19^{+}CD38^{-}CD24^{-}$ population was highly significantly enriched in the older subjects. Again, their distribution in CO fell between the young and the elderly in the general population (Fig. 3d).

CD180-negative B cells and CD180 expression on CD38/CD24 and IgD/CD27 B cell subsets in different age groups

It is known that immune system of elderly people shows some characteristics typical of chronically stimulated subjects (CMV-positive elderly subjects and HIV and SLE patients). Moreover, circulating B cells have been reported to lack the TLR-4 homologue CD180 (RP105) in chronically stimulated SLE patients (Koarada and Tada 2012). We therefore analyzed the percentage of $CD19^{+}CD180^{-}$ B lymphocytes in PBMC of young, elderly, CO, and their AM controls. Figure 4a shows a significant increase of this population in the elderly relative to the young. However, no differences were seen between CO and AM controls, although the percentage of $CD180^{-}$ B cells in CO and AM controls are higher than in the young but lower than in the old donors. Next, we have evaluated the level of expression of CD180 in the four subsets identified on the basis of the markers CD38 and CD24 in the four age groups (Fig. 4b). The phenotypic characterization of CD38/CD24 B cells has shown that most CD19 CD38 CD24 B cells are CD180 negative (data shown in Fig. 2). Our comparative analysis between young, CO, AM, and old (O) subjects did not reveal age-associated changes in the level of CD180 expression. Moreover, as we have observed that $CD19^{+}$CD38 CD24 B cells mainly include both switched memory ($IgD^{-}CD27^{+}$) and DN ($IgD^{-}CD27^{-}$) B cells, we have also evaluated the expression of CD180 on B cell subsets identified on the basis of the expression of IgD and CD27 (Fig. 4c). Interestingly, in all the subjects, this analysis revealed a progressive decrease of CD180 expression through the different stages of maturation, from naïve (IgD^{+}CD27) to memory-switched ($IgD^{-}CD27^{+}$) and DN ($IgD^{-}CD27^{-}$) B cells and from $CD19^{+}CD38^{hi}CD24^{hi}$ to $CD19^{+}$CD38 CD24 B cells, although the comparative study did not show any changes between age classes.

Age-related differences in the distribution of IgM CD27 (naïve mature), $IgD^{+}IgM^{+}CD27^{+}$ (natural effector), and $IgD^{-}IgM^{+}CD27^{+}$ (IgM only) B cell phenotypes

The complexity of the memory B cell pool is further documented by the evidence of different IgM^{+} B cell

Table 3 Distribution of CD38/CD24 B cell phenotypes in young, centenarian offspring, age-matched controls, and old subjects

CD38/CD24 B cell subsets	Age groups				*P* value					
	Y	CO	AM	O	p1	p2	p3	p4	p5	p6
$CD19^{+}CD38^{high}CD24^{high}$	4.1±1.3	2.8±0.7	3.9±1.3	3.8±1.5	0.9	0.5	0.9	0.7	0.8	0.7
$CD19^{+}CD38^{int}CD24^{int}$	66.8±3.1	54.3±11.5	54.8±4.3	54.5±9.6	0.4	1	0.1	1	1	1
$CD19^{+}$CD38 $CD24^{+}$	4.1±0.8	4.2±0.9	5.7±1.1	6.6±0.6	0.06	0.2	0.4	1	0.2	0.7
$CD19^{+}CD38^{-}CD24^{-}$	0.9±0.3	3.1±1.4	15.4±3.1	9.4±4.7	**0.03**	**0.02**	**0.01**	0.06	0.3	0.5

Statistically significant differences are marked in bold

Percentage values are expressed as mean±SEM. Differences between age groups were analyzed with the Mann–Whitney test. p1=Y vs O; p2=CO vs AM; p3=Y vs AM; p4=Y vs CO; p5=CO vs O; p6=AM vs O

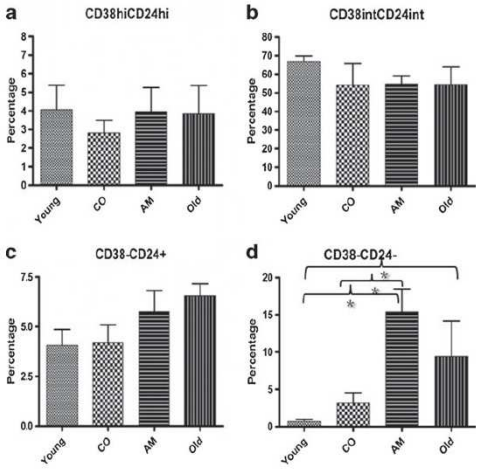

Fig. 3 Distribution of B cell phenotypes in young, old, centenarian offspring, and age-matched controls. Percentage of $CD19^{+}CD38^{hi}CD24^{hi}$ (**a**) and $CD19^{+}CD38^{int}CD24^{int}$ (**b**) B cell populations do not change when analyzed in different age groups. **c** Trend towards an increase in the age-related $CD19^{+}CD38^{-}CD24^{+}$ B cell subset. **d** Significant increase of the $CD19^{+}CD38^{-}CD24^{-}$ B cell subset in elderly people (old and AM) when compared to young. CO, like young, show a lower percentage of this population when compared to their age-matched controls. Differences between age groups were analyzed with the Mann Whitney test. $^{*}P<0.05$

phenotypes presumably representing different stages of B cell differentiation. We and others have demonstrated that IgM B lymphocytes are reduced in the elderly (Shi et al. 2005; Buffa et al. 2011). As the previous phenotypic characterization has not revealed differences in the IgM expression, we chose to analyze IgM^{+} B cell phenotypes on our samples according to the Berkowska gating strategy (Berkowska et al. 2011), based on the major subset of $CD38^{dim}CD24^{dim}$, excluding transitional B cells (Fig. 5). So, $CD38^{dim}CD24^{dim}$ B cells can be separated into three IgM populations: $IgM^{+}CD27^{-}$ (naïve mature), $IgD^{+}IgM^{-}CD27^{+}$ (natural effector), and $IgD^{-}IgM^{+}CD27^{+}$ (IgM only) (Fig. 5a). A comparison between our subjects confirmed (Shi et al. 2005; Buffa et al. 2011) a different distribution of B cells identified on the basis of these markers between young and old donors and revealed that CO possess a more "youthfully" distributed immune profile compared with age-matched controls. Indeed, the percentage of $IgM^{+}CD27^{-}$ (naïve mature) cells (Fig. 5b) are significantly greater in CO, a group of people presumably advantaged for longevity. The analysis of the other two $IgM^{+}CD27^{+}$ memory B cell phenotypes revealed significantly fewer of these memory cells in the CO (Fig. 5c, d).

Discussion

During aging, the immune system progressively loses its ability to fight off infections and to respond as quickly or as efficiently to different stimuli. Moreover, the inability of old individuals to effectively respond to vaccines results in less effective immunizations against viruses and bacteria, thus increasing the risk of infections and diseases (Miller and Cancro 2007; Cancro et al. 2009; Gibson et al. 2009; Dunn-Walters and Ademokun 2010; Frasca and Blomberg 2011). Indeed, it is well documented that the immune response to vaccination declines with age, although the reasons for this are poorly understood (Kumar and Burns 2008; Frasca et al. 2010; McElhaney et al. 2012). It is also known that in the elderly, there is an impairment of both innate and adaptive immune responses. As we age, the numbers of critical cells in the immune system change, and many may become less functional. However, innate immunity seems to be better preserved, while more severe age-dependent changes occur in the adaptive immune system (Franceschi et al. 2000b). There are clear changes in B cell generation and repertoire, and as a consequence of this and changes in the T cells, elderly people show defective humoral immunity (Cancro et al. 2009; Bulati

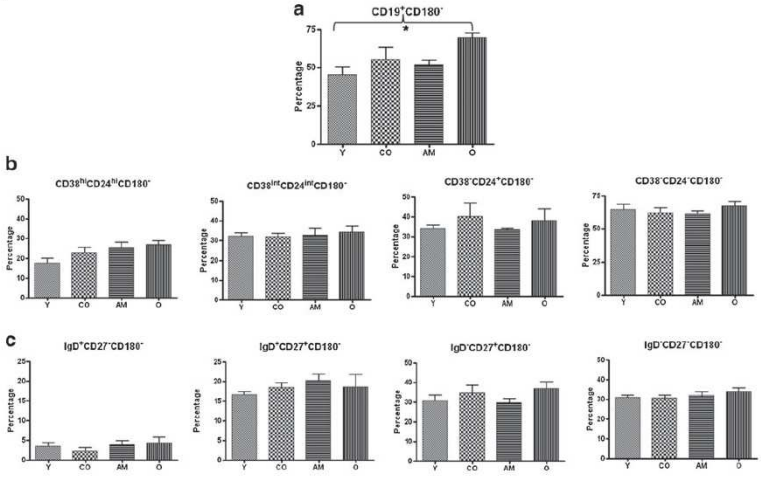

Fig. 4 CD180-negative B cells and CD180 expression on CD38/CD24 and IgD/CD27 B cell subsets. **a** Evaluation of $CD19^{+}CD180$ (RP105) B cell population among the different age groups analyzed. Old people show a significant increase in the percentage of the B cell subset lacking CD180 expression, when compared with young. **b** Histograms show CD180-negative B cells among $CD38^{hi}CD24^{hi}$, $CD38^{int}CD24^{int}$, $CD38^{-}CD24^{+}$, and $CD38^{-}CD24^{-}$ B cell compartments in young (*Y*), centenarian offspring (*CO*), age-matched (*AM*), and old (*O*) subjects. A progressive increase in percentage of CD180-negative cells from $CD38^{hi}CD24^{hi}$ to $CD38^{-}CD24^{-}$ cells has been demonstrated without age-related changes between groups studied. **c** Expression of CD180 on naïve memory B cells identified on the basis of the expression of IgD and CD27. A progressive down-modulation of CD180 from naïve ($IgD^{+}CD27^{-}$) to DN ($IgD^{-}CD27^{-}$) B cells has shown but, again, no change between age classes. Differences between age groups were analyzed with the Mann–Whitney test. $^{*}P<0.05$. *Bar graphs* indicate mean±SEM

et al. 2011; Frasca and Blomberg 2011). There is a decrease both in percentage and absolute number of total B lymphocytes (Pawelec et al. 2005; Shi et al. 2005; Frasca et al. 2008; Veneri et al. 2009; Bulati et al. 2011), and in previous papers, we (Colonna-Romano et al. 2009) and others (Gupta et al. 2005) have demonstrated that the percentage of naïve B cells are significantly reduced in aged individuals. However, this is still a somewhat controversial finding as some authors reported an increase, a decrease, or no changes in the naïve/memory B cell compartments, as we have reviewed (Bulati et al. 2011). The $CD27^{+}$ memory B cells and, in particular, the IgM memory $CD19^{+}IgM^{+}IgD^{+}CD27^{+}$ cells, which are considered to be the recirculating equivalent of murine marginal zone cells, have been shown to decline in both proportion and numbers with age (Shi et al. 2005; Buffa et al. 2011). In a previous study, we documented the expansion of CD19 IgD CD27 cells, a DN memory B cell population, in the elderly (Colonna-Romano et al. 2009). This population is also expanded in subjects chronically stimulated (Anolik et al. 2004; Wei et al. 2007; Sanz et al. 2008; Cagigi et al. 2009) and might be considered the result of long-enduring stimulation. However, not all subjects or population groups are equally susceptible to the effects of long-term chronic stimulation of the immune system. Indeed, recent evidence has shown a well-preserved immune profile of a group of healthy elderly centenarian offspring who seem to have genetic and functional advantages associated with the reduced risk of disability with age (Terry et al. 2003, 2004). We have demonstrated that the naïve B cells subset is well preserved in CO compared with the age-

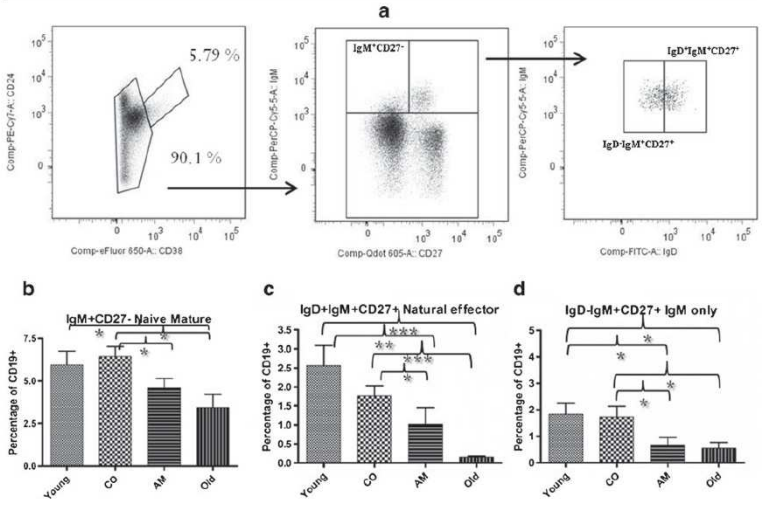

Fig. 5 Diversity in the human IgM$^-$ B cell compartment and distribution in young and old, centenarian offspring (*CO*), and their age-matched (*AM*) controls. **a** Within the CD19$^+$ B cell compartment, B lymphocytes are characterized on the basis of CD38 and CD24 expression. According to the Berkowska et al. (2011) gating strategy, CD38^{dim}CD24^{dim} B lymphocytes were subdivided on the basis of IgM and CD27 expressions, defining IgM$^-$CD27$^-$ naïve mature. Subsequently, on the basis of IgD expression, IgM$^+$CD27$^+$ B cells were separated into IgD$^+$CD27$^+$ unswitched memory and IgD$^-$ IgM$^+$CD27$^+$ switched memory cells. IgD$^-$ IgM$^-$CD27$^+$ IgM only. **b** Young donors have a significant higher percentage of naïve mature cells compared to the elderly. A direct comparison between CO and aged people (AM and old subjects) emphasizes that the former shows a significantly higher percentage of this IgM population. **c** A substantial and significant age-related decrease was observed in the natural effector subset. **d** Age-related decrease of the IgM-only B cell subset observed in old and age-matched controls compared with young and centenarian offspring subjects. Differences between age groups were analyzed with the Mann–Whitney test. */P<0.05; **/P<0.01; ***/P<0.001

matched controls. The mirror image of this is a more restricted memory pool, which includes, in addition, a less expanded DN compartment (Colonna-Romano et al. 2010). Collectively, our data suggest age-related changes, but they are of course cross-sectional in the present study and therefore can only formally demonstrate differences between groups. Other investigations, such as a longitudinal follow-up studies, will be necessary to affirm a real age-related change.

Recently, several new B cell populations have been described, although their phenotype has not been always associated with established biological function. In the present work, we gained insight into the naïve/memory B cell compartment focusing, in particular, on the different age groups analyzed. According to the gating strategy proposed by Carsetti et al. (2004), our study has focused on four distinct B cell populations: CD19$^+$CD38^{hi}CD24^{hi} (classical transitional), CD19$^+$CD38^{int}CD24^{int} (mature naïve), CD19$^+$CD38$^-$CD24$^+$ (primarily memory), and in addition, a not previously described CD19$^+$CD38$^-$CD24$^-$ population. A previous study reported that the frequency of transitional B cells is rapidly reduced during the first years of life, stabilizing after 5 years of age (Morbach et al. 2010). Here, we found that there are no differences in the percentage of transitional and mature naïve B cells, comparing young and the three different groups of old donors CO, AM, and elderly, but we did observe a higher proportion of CD19$^+$CD38 CD24$^+$ primarily memory cells in young and CO compared to AM, although the difference did not achieve statistical significance. However, the

CD19$^+$CD38$^-$CD24$^-$ putative new population was significantly increased in the elderly, and the percentage of this population in CO is more similar to that observed in young than in elderly or age-matched donors. This may therefore contribute to their immunological "youthfulness." Apart from this, the other B cell populations identified on the basis of the expression of CD38/CD24 show the same distribution in the different groups studied. The evaluation of both IgD and CD27 on CD38/CD24 B cells also supports the developmental dynamic from transitional B cells that are mainly IgD$^+$CD27$^-$ (naïve), through mature naïve B cells, in which we observe the progressive appearance of CD27, which is, finally, most strongly expressed on the primarily memory CD19$^+$CD38$^-$CD24$^+$, together with the progressive loss of IgD. CD19$^+$CD38$^-$CD24$^+$ B lymphocytes, also known as primarily memory B cells, show a variety of different phenotypes. Although more than 70 % are CD27$^+$, the simultaneous evaluation of IgD and CD27 has revealed that some of them (27 %) are IgD$^+$CD27$^+$ (unswitched memory); others (44 %) are IgD$^-$CD27$^+$ (switched memory); 25 % of them are naïve, as they express IgD, and are negative for CD27 expression, and those that have a very low percentage (4.3 %) are IgD$^-$CD27$^-$ (DN). Some authors have shown that this population should include only memory B cells, although their analyses have revealed a percentage of CD27-negative B cells gated inside the aforementioned subset (Carsetti et al. 2004; Blair et al. 2010). Unfortunately, those authors did not report the percentage of CD27$^-$, so we cannot numerically compare our data with the data reported in their papers. Further analyses are necessary to elucidate this point. Focusing on CD19$^+$CD38$^-$CD24$^-$ B lymphocytes, we did not observe any changes in distribution of IgD/CD27 phenotypes in the different age groups. Moreover, here, we show that about half of these cells are IgD CD27$^+$-switched memory, not different in the elderly (Colonna-Romano et al. 2009; Buffa et al. 2011), whereas almost 30 % of them are IgD CD27$^-$ DN B cells that are increased in the elderly but not in CO (Colonna-Romano et al. 2009, 2010). It is of note that a fraction of the IgD$^-$CD27$^+$ subset also includes IgD IgM$^+$CD27$^+$ memory B cells that we and others have described as being decreased in the elderly (Shi et al. 2005; Buffa et al. 2011). Probably, their low percentage does not influence the percentage of CD19$^+$CD38$^-$CD24$^-$ B cells in the different age groups. The evaluation of IgM has revealed that this marker is strongly expressed on CD19$^+$CD38^{hi}CD24^{hi} and on IgD$^+$CD27$^-$ cells (Colonna-Romano et al. 2009) and tends to disappear from both CD19$^+$CD38$^-$CD24$^-$ and IgD$^-$CD27$^-$ cells. We have also evaluated CD5 expression on CD38/CD24 B cell phenotypes. Our analysis revealed that all different subpopulations had different levels of CD5. Our characterization of CD38/CD24 B cells has confirmed that CD19$^+$CD38^{hi}CD24^{hi} cells were positive for CD5. Interestingly, CD19$^-$CD38$^-$CD24$^-$ cells were also positive for the same marker. This molecule is present in adulthood on all B cell subsets, but at a low and variable frequency. CD5 expression is associated with the presence of the B cell receptor (BCR), and it appears in the bone marrow; it has been shown to be a negative regulator of BCR signaling, and its expression may be a marker of antigen exposure (Gary-Gouy et al. 2000, 2002; Carsetti et al. 2004). Additional characterization of these CD19$^+$CD38$^-$CD24$^-$ B lymphocytes has demonstrated here that almost all are CD180 negative. This marker, also known as RP105, is a toll-like receptor-associated molecule, originally identified as a B cell surface molecule mediating activation and proliferation in mice. Data from the literature indicate that RP105-deficient mice are hypo-responsive to TLR-4 and TLR-2 stimulation (Ogata et al. 2000; Nagai et al. 2005). Recently, increasing interest in this molecule has led several investigators to evaluate CD180 in humans and, in particular, in subjects suffering from autoimmune diseases. It has been demonstrated that the percentage of CD180-negative B cells are significantly increased in systemic lupus erythematosus and, to a lesser extent, in rheumatoid arthritis patients, compared to healthy controls. Its expression declines during the inactive stages of disease, suggesting that the expansion of this population is correlated with disease activity (Koarada and Tada 2012). The phenotypic characterization of the above-described subset has defined a profile of activated B cells producing immunoglobulins and anti-dsDNA antibodies. Moreover, approximately 50 % of these CD180-negative B cells had intracellular IgG (Koarada et al. 1999), suggesting that they could be induced to differentiate into IgG-secreting B cells by certain stimuli. As autoimmune phenomena are a common feature in the elderly, we have evaluated the expression of this marker on total B lymphocytes in different age groups, observing a reduced expression with age. Indeed, CD180-negative B cells are significantly increased in the very elderly donors (age range 86.4+3.8) when compared to the young (age range 28.5±1.9), but not in CO and AM

(age range 70.1±8.3 and 69.1±9, respectively). Furthermore, we assessed the level of expression of CD180 in the four subsets identified on the basis of the markers CD38 and CD24 in the four age groups. In any event, our comparative analysis between young, CO, AM, and O subjects did not reveal age-associated differences in the level of CD180 expression. Interestingly, the evaluation of CD38 CD24 CD180 B cells gated inside $CD19^+$ B cell population has shown a trend (not significant) towards higher proportions in elderly donors and age-matched controls when compared with young donors and centenarian offspring (data not shown). Moreover, as we have observed that $CD19^+CD38^-CD24^-$ B cells, which are increased in elderly people when compared to young donors and in age-matched controls more than in centenarian offspring, mainly include both switched memory (IgD^-CD27^+) and DN (IgD^-CD27^-) B cells, we have also evaluated the expression of CD180 on IgD/CD27 B cell subpopulations. As expected, we saw a lower expression of this marker from naïve to memory-switched and DN B cells, although the comparative analysis did not show any changes between age classes. Data obtained from CD180 evaluation on CD38/CD24 and IgD/CD27 phenotypes cannot be considered similar, although, in both gating systems, CD180-negative B cells are increased from naïve to memory B cells. Thus, reduced expression of the TLR-associated molecule CD180 on total B cells is observed in the elderly, and an age-related increase of DN and of $CD19^+CD38^-CD24^-$ B cells, at least defective for CD180, could be related to the increased susceptibility to Gram-negative bacterial diseases of elderly people. Indeed, it is known that elderly people are more susceptible than the young to pneumococcal infection. The decline of the IgM memory B cell pool in the elderly could also be involved in defective immune responses against infections by encapsulated bacteria.

On the basis of IgM, CD27, and IgD expression, IgM B lymphocytes can be subdivided into IgM^+CD27^- "naïve mature," $IgD^-IgM^+CD27^+$ IgM-only, and $IgD^+IgM^+CD27^+$ "natural effector" B cells. As the previous phenotypic characterization of CD38/CD24 B cells has not revealed differences in the IgM expression, we chose to analyze IgM^+ B cell phenotypes on our samples according to the Berkowska gating strategy (Berkowska et al. 2011), based on the major subset of $CD38^{dim}CD24^{dim}$. We analyzed IgM B cell subsets, comparing data between subjects included in this study. We have confirmed data from the literature demonstrating impairment decrease of IgM B lymphocytes in the elderly (Shi et al. 2005; Buffa et al. 2011). On the contrary, a further evaluation of CO B cell populations revealed that the percentage of these cells are not reduced to the same extent as their age-matched controls and remain more similar to that observed in young people. These data reinforce our previous hypothesis according to which CO have a younger B cell profile. This hypothesis is corroborated by the reduced number of $CD19^+CD38^-CD24^-$ B cells and a higher level of IgM B cell subsets in CO, compared to AM donors. This immunological advantage, likely due to a good bone marrow reservoir, could help CO both to fight the main age-related diseases and to maintain healthy aging.

Here, we have also performed a preliminary functional study on the CD38/CD24 B cells to better understand their biological role and function. We tested their ability to produce pro- and anti-inflammatory cytokines on strong stimulation with CpG/PMA/ionomycin in vitro. We know that cytokines are considered key players in maintaining lymphocyte homeostasis. Their function is to induce and modulate the nature of the response after an immune insult, or in contrast, they may cause non-responsiveness and active immune suppression (Sanjabi et al. 2009). Moreover, it is known that elderly people show a pro-inflammatory microenvironment that has been related to the increased risk of morbidity and mortality (Licastro et al. 2005; Vasto et al. 2007). Concerning the anti-inflammatory cytokine IL-10, also our stimulation system provides data consistent with a biological role of $CD19^+CD38^{hi}CD24^{hi}$ cells as regulatory cells, i.e., a B cell population involved in IL-10 production. Our comparative analysis did not reveal any differences between the four age groups analyzed. We have previously demonstrated that under the same conditions, IgD CD27 (naïve) B cells produce high levels of IL-10 in the elderly (Buffa et al. 2011). Although we have confirmed that $CD19^+CD38^{hi}CD24^{hi}$ cells are mainly IgD^+CD27^+, our analysis of CD38/CD24 populations revealed a certain percentage of naive IgD/CD27 in all four populations analyzed, extending the potential role of IL-10-producing cells. Finally, regarding the pro-inflammatory cytokine TNF-α, the $CD19^+CD38^-CD24^+$ and $CD19^+CD38^-CD24^-$ B cell subsets, which seem to be sensitive to biological and not chronological aging, are the main B cells involved in the in vitro production of this pro-inflammatory cytokine, with no differences between the groups studied. At the

present time, we cannot state which population is more responsible for the impaired cytokine production. Nonetheless, as TNF-α is a pro-inflammatory cytokine recently reported to be increased in a mouse model made of aged B cells (Frasca et al. 2012), we hypothesize that switched memory (IgD^-CD27^+) and/or DN (IgD^-CD27^-) B cells could be responsible for this cytokine production. We suggest that the expansion of these B cell subsets in the elderly might contribute to the increased inflammatory status designated "inflammaging."

Taken together, our data document the phenotypic and biological characteristics of a $CD19^+CD38^-CD24^-$ B cell population and suggest that these cells might act as memory B cells involved in inflammation. Analysis with additional markers, such as IgG, IgA, HLA, CD138, CD43, and CD70, may be useful for a deeper characterization of this subset, as well as investigating them under pathological conditions. We also need to evaluate if they might act as antibody (Ab)-producing cells or develop into Ab-secreting cells, and in particular, whether these cells could be responsible for autoantibody production. Moreover, our data emphasize that modifications to B cell immunity could play a pivotal role during processes associated with aging. A better understanding of B cell immunosenescence is crucial for the improvement of the quality of life of the growing elderly population worldwide. Hence, B cell immune profiling must be included in any attempts to develop useful biomarkers of human immunosenescence. The study of B cell phenotypes in the peripheral blood of centenarian offspring revealed that many parameters are well preserved and more closely resemble that of the young than of either their age-matched controls or old subjects. In any event, other immune parameters, such as CD180 expression and cytokine production, seem not suggest a complete preservation of the B cell branch in the offspring of long-lived subjects. Further analyses are necessary to correlate the environmental and genetic background with a well-preserved immune system of centenarian offspring that could support the hypothesis of a "familial youth" of the immune system.

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References

Agrawal S, Gupta S (2011) TLR1/2, TLR7, and TLR9 signals directly activate peripheral blood naive and memory B cell subsets to produce cytokines, chemokines, and hematopoietic growth factors. J Clin Immunol 31(1):89–98

Allman DM, Ferguson SE, Lentz VM, Cancro MP (1993) Peripheral B cell maturation. II. Heat-stable antigen(hi) splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. J Immunol 151(9):4431–4444

Allman D, Lindsley RC, DeMuth W, Rudd K, Shinton SA, Hardy RR (2001) Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. J Immunol 167(12):6834–6840

Allman D, Srivastava B, Lindsley RC (2004) Alternative routes to maturity: branch points and pathways for generating follicular and marginal zone B cells. Immunol Rev 197:147–160

Anolik JH, Barnard J, Cappione A, Pugh-Bernard AE, Felgar RE, Looney RJ, Sanz I (2004) Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus. Arthritis Rheum 50(11):3580–3590

Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, He B, Biermann K, Lange JF, van der Burg M, van Dongen JJ, van Zelm MC (2011) Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood 118(8):2150–2158

Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, 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

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

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

Cancro MP, Hao Y, Scholz JL, Riley RL, Frasca D, Dunn-Walters DK, Blomberg BB (2009) B cells and ageing: molecules and mechanisms. Trends Immunol 30(7):313–318

Carsetti R, Köhler G, Lamers MC (1995) Transitional B cells are the target of negative selection in the B cell compartment. J Exp Med 181(6):2129–2140
Carsetti R, Rosado MM, Wardmann H (2004) Peripheral development of B cells in mouse and man. Immunol Rev 197:179–191
Cevenini E, Caruso C, Candore G, Capri M, Nuzzo D, Duro G, Rizzo C, Colonna-Romano G, Lio D, Di Carlo D, Palmas MG, Scurti M, Pini E, Franceschi C, Vasto S (2010) Age-related inflammation: the contribution of different organs, tissues and systems. How to face it for therapeutic approaches. Curr Pharm Des 16:609–618
Chaplin JW, Kasahara S, Clark EA, Ledbetter JA (2011) Anti-CD180 (RP105) Activates B cells to rapidly produce polyclonal Ig via a T cell and MyD88-independent pathway. J Immunol 187(8):4199–4209
Chong Y, Ikematsu H, Yamaji K, Nishimura M, Nabeshima S, Kashiwagi S, Hayashi J (2005) CD27(−) (memory) B cell decrease and apoptosis-resistant CD27(−) (naive) B cell increase in aged humans: implications for age-related peripheral B cell developmental disturbances. Int Immunol 17(4):383–390
Chung JB, Silverman M, Monroe JG (2003) Transitional B cells: step by step towards immune competence. Trends Immunol 24(6):343–349
Colonna-Romano G, Bulati M, Aquino A, Vitello S, Lio D, Candore G, Caruso C (2008) B cell immunosenescence in the elderly and in centenarians. Rejuvenation Res 11 (2):433–439
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 Develop 130 (10):681–690
Colonna-Romano G, Buffa S, Bulati M, Candore G, Lio D, Pellicanò M, Vasto S, Caruso C (2010) B cells compartment in centenarian offspring and old people. Curr Pharm Des 16(6):604–608
Dunn-Walters DK, Ademokun AA (2010) B cell repertoire and ageing. Curr Opin Immunol 22(4):514–520
Fecteau JF, Côté 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(6):3728–3736
Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, De Benedictis G (2000a) Inflamm-aging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci 908:244–254
Franceschi C, Bonafe M, Valensin S (2000b) Human immunosenescence: the prevailing of innate immunity, the failing of clonotypic immunity, and the filling of immunological space. Vaccine 18(16):1717–1720
Frasca D, Blomberg BB (2011) Aging affects human B cell responses. J Clin Immunol 31(3):430–435
Frasca D, Landin AM, Lechner SC, Ryan JG, Schwartz R, Riley RL, Blomberg BB (2008) Ageing down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. J Immunol 180(8):5283–5290
Frasca D, Diaz A, Romero M, Landin AM, Phillips M, Lechner SC, Ryan JG, Blomberg BB (2010) Intrinsic defects in B cell response to seasonal influenza vaccination in elderly humans. Vaccine 28(51):8077–8084
Frasca D, Romero M, Diaz A, Alter-Wolf S, Ratliff M, Landin AM, Riley RL, Blomberg BB (2012) A molecular mechanism for TNF-α-mediated downregulation of B cell responses. J Immunol 188(1):279–286
Gary-Gouy H, Bruhns P, Schmitt C, Dalloul A, Daëron M, Bismuth G (2000) The pseudo-immunoreceptor tyrosine-based activation motif of CD5 mediates its inhibitory action on B-cell receptor signaling. J Biol Chem 275(1):548–556
Gary-Gouy H, Harriague J, Bismuth G, Platzer C, Schmitt C, Dalloul AH (2002) Human CD5 promotes B-cell survival through stimulation of autocrine IL-10 production. Blood 100(13):4537–4543
Gibson KL, Wu YC, Barnett Y, Duggan O, Vaughan R, Kondeatis E, Nilsson BO, Wikby A, Kipling D, Dunn-Walters DK (2009) B-cell diversity decreases in old age and is correlated with poor health status. Ageing cell 8 (1):18–25
Gupta S, Su H, Bi R, Agrawal S, Gollapudi S (2005) Life and death of lymphocytes: a role of immunosenescence. Immun Ageing 23;2:12
Koarada S, Tada Y (2012) RP105-negative B cells in systemic lupus erythematosus. Clin Dev Immunol 2012:259186
Koarada S, Tada Y, Ushiyama O, Morito F, Suzuki N, Ohta A, Miyake K, Kimoto M, Nagasawa K (1999) B cells lacking RP105, a novel B cell antigen, in systemic lupus erythematosus. Arthritis Rheum 42(12):2593–2600
Kumar R, Burns EA (2008) Age-related decline in immunity: implications for vaccine responsiveness. Expert Rev Vaccines 7(4):467–479
LeBien TW, Tedder TF (2008) B lymphocytes: how they develop and function. Blood 112(5):1570–1580
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
Lio D, Scola L, Crivello A, Colonna-Romano G, Candore G, Bonafè M, Cavallone L, Marchegiani F, Olivieri F, Franceschi C, Caruso C (2003) Inflammation, genetics, and longevity: further studies on the protective effects in men of IL-10-1082 promoter SNP and its interaction with TNF-alpha-308 promoter SNP. J Med Genet 40:296–299
Mauri C, Ehrenstein MR (2008) The 'short' history of regulatory B cells. Trends Immunol 29(1):34–40
McElhaney JE, Zhou X, Talbot HK, Soethout E, Bleackley RC, Granville DJ, Pawelec G (2012) The unmet need in the elderly: how immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines. Vaccine 30(12):2060–2067
Miller JP, Cancro MP (2007) B cell and ageing: balancing the homeostatic equation. Exp Gerontol 42(5):396–399
Morbach H, Eichhorn EM, Liese JG, Girschick HJ (2010) Reference values for B cell subpopulations from infancy to adulthood. Clin Exp Immunol 162(2):271–279
Nagai Y, Kobayashi T, Motoi Y, Ishiguro K, Akashi S, Saitoh S, Kusumoto Y, Kaisho T, Akira S, Matsumoto M, Takatsu K, Miyake K (2005) The radioprotective 105/MD-1 complex links TLR2 and TLR4/MD-2 in antibody response to microbial membranes. J Immunol 174(11):7043–7049

Ogata H, Su I, Miyake K, Nagai Y, Akashi S, Mecklenbräuker I, Rajewsky K, Kimoto M, Tarakhovsky A (2000) The toll-like receptor protein RP105 regulates lipopolysaccharide signaling in B cells. J Exp Med 192(1):23–29

Palanichamy A, Barnard J, Zheng B, Owen T, Quach T, Wei C, Looney RJ, Sanz I, Anolik JH (2009) Novel human transitional B cell populations revealed by B cell depletion therapy. J Immunol 182(10):5982–5993

Pawelec G, Akbar A, Caruso C, Solana R, Grubeck-Loebenstein B, Wikby A (2005) Human immunosenescence: is it infectious? Immunol Rev 205:257–268

Sanjabi S, Zenewicz LA, Kamanaka M, Flavell RA (2009) Anti-inflammatory and proinflammatory roles of TGF-beta, IL-10, and IL-22 in immunity and autoimmunity. Curr Opin Pharmacol 9(4):447–453

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

Shi Y, Yamazaki T, Okubo Y, Uchara Y, Sugane K, Agematsu K (2005) Regulation of aged humoral immune defense against pneumococcal bacteria by IgM memory B cell. J Immunol 175(5):3262–3267

Terry DF, Wilcox M, McCormick MA, Lawler E, Perls TT (2003) Cardiovascular advantages among the offspring of centenarians. J Gerontol A Biol Sci Med Sci 58(5):M425–M431

Terry DF, Wilcox MA, McCormick MA, Perls TT (2004) Cardiovascular disease delay in centenarian offspring. J Gerontol A Biol Sci Med Sci 59(4):385–389

Vasto S, Candore G, Balistreri CR, Caruso M, Colonna-Romano G, Grimaldi MP, 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

Veneri D, Ortolani R, Franchini M, Tridente G, Pizzolo G, Vella A (2009) Expression of CD27 and CD23 on peripheral blood B lymphocytes in humans of different ages. Blood Transfus 7(1):29–34

Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, Lee EH, Milner EC, 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(10):6624–6633

Weller S, Braun MC, Tan BK, Rosenwald A, Cordier C, Conley ME, Plebani A, Kumararatne DS, Bonnet D, Tournilhac O, Tchernia G, Steiniger B, Staudt LM, Casanova JL, Reynaud CA, Weill JC (2004) Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harbouring a pre-diversified immunoglobulin repertoire. Blood 104(12):3647–3654

CHAPTER 4

"Evidence for less T cell immunosenescence in centenarian offspring than the general elderly population"

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Evidence for Less Marked Potential Signs of T-Cell Immunosenescence in Centenarian Offspring Than in the General Age-Matched Population

Mariavaleria Pellicanò,[1,*] Silvio Buffa,[2,*] David Goldeck,[1] Matteo Bulati,[2] Adriana Martorana,[2] Calogero Caruso,[2] Giuseppina Colonna-Romano,[2,*] and Graham Pawelec[1,*]

[1]Department of Internal Medicine II, Center for Medical Research, University of Tübingen, Tübingen Aging and Tumor Immunology Group, Tübingen, Germany.
[2]Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Palermo, Italy.

*These authors contributed equally to this work.

Address correspondence to Mariavaleria Pellicanò, PhD, Department of Internal Medicine II, Center for Medical Research, University of Tübingen, Tübingen Aging and Tumor Immunology Group, Waldhörnlestr. 22, 72072 Tübingen, Germany. Email: mariavaleria.pellicano@uni-tuebingen.de

People may reach the upper limits of the human life span at least partly because they have maintained more appropriate immune function, avoiding changes to immunity termed "immunosenescence." Exceptionally long-lived people may be enriched for genes that contribute to their longevity, some of which may bear on immune function. Centenarian offspring would be expected to inherit some of these, which might be reflected in their resistance to immunosenescence, and contribute to their potential longevity. We have tested this hypothesis by comparing centenarian offspring with age-matched controls. We report differences in the numbers and proportions of both $CD4^+$ and $CD8^+$ early- and late-differentiated T cells, as well as potentially senescent $CD8^+$ T cells, suggesting that the adaptive T-cell arm of the immune system is more "youthful" in centenarian offspring than controls. This might reflect a superior ability to mount effective responses against newly encountered antigens and thus contribute to better protection against infection and to greater longevity.

Key Words: T cells—Immunosenescence—Aging—Longevity—Centenarian offspring.

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INCREASING age is accompanied by a decreased ability of the immune system to protect against new antigenic challenges or to control chronic infections, and increased immune dysregulation also reflected in chronically elevated signs of systemic inflammation. These age-associated changes over the life span, loosely termed *immunosenescence* and *inflamm-aging*, are associated with a decline in the normal functioning of the different components of the immune system. Hence, there is a reduction in responsiveness as well as a functional deregulation of immune effector cells and an alteration of the cytokine and chemokine environment in the elderly population (1). T cells play a major role in defense against infection and in the secretion of pro- and anti-inflammatory factors, but in the elderly people, the balance of T-cell phenotypes in the peripheral blood is markedly different from that seen in most young people, in a manner most commonly interpreted as indicating impaired functionality in the former. This is thought to contribute to poorer vaccine responses, increasing incidence of infection, and reactivation of latent viruses in old people (2). Cross-sectional studies indicate that the most noticeable differences in young and old people lie in the frequencies and absolute numbers of naïve and different differentiation stages of memory T cells. These data are generally interpreted as a dynamic process of decreasing frequency of naïve T cells and increasing frequency of memory T cells with age, due to conversion of naïve cells to memory cells after antigen contact, although in the absence of longitudinal studies, this assumption cannot be formally proven. However, limited longitudinal studies in the very elderly people have shown that an accumulation of late-stage differentiated $CD8^+$ T cells resulting in an inverted CD4:CD8 ratio and poor T-cell proliferative responses to mitogens formed the central plank of the "immune risk profile" (IRP), which did indeed predict mortality at 2-, 4-, and 6-year follow-up (3). It is of note that the number of naïve $CD8^+$ T cells was not part of the IRP. Intriguingly, cytomegalovirus (CMV) infection, however, was part of the IRP and has been postulated to be the main driver of the accumulation of late-stage differentiated $CD8^+$ T cells in the at-risk group. It is now clear that CMV itself influences T-cell phenotype distribution, independently of age, but becoming more marked in elderly populations with a higher percentage of CMV-infected individuals (4–6). In some studies, CMV infection is closely related to both a reduction of $CD8^+$ naïve T cells and increase of $CD8^+$ late-differentiated

effector memory (6), whereas in other studies, the loss of naïve cells was reported to be age dependent but not CMV dependent (7).

Because essentially no older individuals can escape from all age-related diseases, such as atherosclerosis, osteoporosis, sarcopenia, or insulin resistance, defining the elderly individuals as healthy is challenging. For this reason, the term "delayed aging" may better describe a group of people, such as centenarians, who have presumed genetic and functional advantages resulting in a lesser risk of developing major age-related diseases and, as a consequence, survive longer in a good condition (8). Studies of families with exceptionally long-lived participants have suggested that genetic background may contribute a very significant advantage in terms of longevity compared with the general population (9). Older individuals in such families also exhibit more "youthful" immune profiles in that their immune signatures are more similar to those of younger individuals than to age-matched (AM) controls. When infected with CMV, they appear resistant to the alterations in T-cell phenotype distribution caused by this virus in the general population (10). Centenarian offspring (CO) may be a special population of older individuals that, like their centenarian parent(s), could also have genetic and functional advantages that predispose them to healthy aging and longer survival (11–13). Data on B cells have shown that CO appear more similar to young donors than AM sporadic controls (14) and do not show the typical naïve–memory shift observed in the elderly people (15). Recently, we also demonstrated that the IgM memory B-cell pool present in CO is not reduced to the same extent as in their AM controls and remains more similar to that observed in young people (16). Here, we asked whether the T-cell compartment of CO is also resistant to the imputed age-associated changes observed in AM controls having no history of familial longevity. We report higher numbers and percentages of $CD28^+CD27^-CD45RA^+CD45RO^-$ naïve cells within the $CD4^+$ as well as $CD8^+$ subsets in CO compared with the general elderly population, whereas there were fewer CD28 CD27 CD45RA CD45RO late-differentiated $CD8^+$ T cells in CO. Moreover, CO had lower numbers and percentages of $CD8^+CD57^+$ putatively senescent T cells compared with the general population of elderly people. These data on CO T-cell distribution imply that cellular immune capacity is likely to be better preserved in CO than AM random controls and could provide a survival advantage under certain conditions of environmental exposures to pathogens.

Methods

Participants

A total of 21 Sicilian CO have been identified and investigated (age range: 70.1 ± 8.3; 10 men and 11 women), with at least one centenarian parent (>99 years). Fifteen AM controls (age range: 69.1 ± 9.7; 7 men and 8 women), 10 old (O) participants (age range: 86.4 ± 3.8; 5 men and 5 women), and 12 young participants (age range: 28.5 ± 1.9; 7 men and 5 women) from Sicily were also included in the study. All participants were in good health at the moment of the recruitment, as revealed by blood tests (complete blood cell count, erythrocytes, C-reactive protein, liver function tests, iron, proteins). The controls were collected from the same population as the patient cohort. Characteristics of these individuals and their clinical history are summarized in Table 1.

The University Hospital Ethics Committee approved the study, and written informed consent was obtained from all participants according to Italian law.

All participants were tested for CMV serostatus by ELISA using CMV-IgG-ELISA PKS assays (Genesis Diagnostics, United Kingdom). All the elderly participants were positive for CMV antibody (CO, AM, and O), whereas none of the young participants were infected.

Whole blood was collected by venepuncture in vacutainer tubes containing ethylenediaminetetraacetic acid, at the same time of day for all participants. Peripheral blood

Table 1. Characteristics and Clinical History of Individuals Studied

	Young People	Centenarian Offspring	Age Matched	Old People
Participants	12	21	15	10
Gender	7 men, 5 women	10 men, 11 women	7 men, 8 women	5 men, 5 women
Age, mean (*SD*)	28.5 (1.9)	70.1 (8.3)	69.1 (9.7)	86.4 (3.8)
Prevalence of disease, *n* (%)				
Myocardial infarction	0 (0.0)	0 (0.0)	2(13.3)	2 (20)
Stroke	0 (0.0)	1 (4.8)	2 (13.3)	1 (10)
Hypertension	0 (0.0)	3 (14.3)	5 (33.3)	2 (20)
Cancer	0 (0.0)	1 (4.8)	2 (13.3)	2 (20)
Rheumatoid arthritis	0 (0.0)	0 (0.0)	1 (6.7)	1 (10)
Level of education, *n* (%)				
Only primary education			2 (13.3)	10 (100)
Secondary education		19 (90.5)	11 (73.3)	
Higher education	12 (100)	2 (9.5)	2 (13.3)	

mononuclear cells were separated using a Ficoll/Hypaque gradient (Cedarlane Laboratories Limited, Ontario, Canada) and viably cryopreserved according to standard protocols.

Flow Cytometry

To analyze T-lymphocyte subsets, peripheral blood mononuclear cells were adjusted to 1×10^6/mL and stained with antibodies. Direct immunofluorescence was performed with anti-CD3-AlexaFluor700, CD8-PercP, CD28-PercP-Cy5.5 (Becton Dickinson, Heidelberg, Germany), CD4-Qdot705 (Invitrogen, Karlsruhe, Germany), CD27-Qdot605 (Invitrogen), CD45RO-eFluor650 (eBioscience, San Diego, CA). CD45RA-PacificBlue (BioLegend, Biozol, Eching, Germany), and CD57-FITC (Immunotools, Friesoythe, Germany).

Cell viability was determined with RedVid (Invitrogen). All staining steps were performed in PFEA buffer (phosphate-buffered saline, 2% fetal calf serum, 2 mM ethylenediaminetetraacetic acid, and 0.01% Na azide). Blocking of nonspecific binding sites was accomplished using human immunoglobulin GAMUNEX (Bayer, Leverkusen, Germany) or mouse serum (Caltag/Invitrogen, Karlsruhe, Germany). For each experiment, cells or mouse/rat κ-chain Comp Beads (Becton Dickinson) were stained with the corresponding fluorochrome-labeled antibodies and incubated for 20 minutes at 4°C in the dark. Human unstained cells were used as negative controls. After washing with PFEA, the cells or beads were resuspended and measured using an LSR-II flow cytometer and the acquisition software FACSDiva (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). For data analysis, dead cells (RedVid-positive) were excluded. $CD3^-$ living cells were gated within the side scatter/forward scatter (SSC/FSC) lymphocyte gate. Further analysis was performed using $CD3^+CD4^+$ and $CD3^+CD8^+$ gated populations.

Statistics

All statistical analyses were performed with Graph-Pad Prism 4.0 using the Mann–Whitney nonparametric *U* test to compare two independent groups. Differences were considered significant with a p value ≤ .05. Significant differences are indicated by $*p \le .05$, $**p \le .01$, $***p \le .001$. Significant differences between young (Y) versus old (O) participants are not indicated because the purpose of this study is to compare CO with sporadic AM controls, not with the young participants, where differences are clearly significant.

Results

T-Cell Phenotype Distribution in CO, AM Controls, and Old and Young Participants

To determine the T-cell immune profile of CO compared with the general population, we first investigated the distribution of $CD3^+$, $CD4^+$, and $CD8^+$ T cells. Characteristics of individuals studied and their clinical history are summarized in Table 1. Young people tended to have a higher percentage of $CD4^+$ and fewer $CD8^+$ T lymphocytes than the elderly people and, as a consequence, their average CD4:CD8 ratio was greater (Table 2). There was a slight but nonsignificant increase in the mean percentage of $CD4^+$ T cells within the $CD3^+$ T-cell population in CO as a group compared with the AM controls. A similar slight trend is also visible for $CD8^+$ T cells, but in this case, CO had a lower percentage compared with AM controls. These slight shifts also affected the CD4:CD8 ratio. As a consequence, CO show a higher ratio compared with their AM controls (Table 2). The CD4:CD8 ratio cutoff for inclusion in the IRP is less than or equal to 1; the data presented here indicate that, as expected, no young controls fell into this group, whereas 3/10 old people (30%) had a CD4:CD8 ratio of less than or equal to 1 (Figure 1). In this respect, CO and AM controls differed little ($p > .05$), with 5/21 CO (23.8%) and 4/15 AM (26.6%) falling into the IRP group by this criterion (Figure 1).

Costimulatory Molecules on $CD4^+$ and $CD8^+$ T Cells

Next, the expression of the costimulatory molecules CD27 and CD28 on $CD4^+$ and $CD8^+$ cells was analyzed. These two markers are useful to identify the stage of differentiation of T cells because they are expressed in the early stage and not in the latest stage of differentiation with CD28 downregulated before CD27 on $CD8^+$ T cells and vice versa in $CD4^+$ cells (17,18). We therefore compared T-cell differentiation status between the four groups analyzed. The percentage of early differentiated $CD4^+CD27^+$ cells (Figure 2A) in young people is significantly higher than in any of the elderly groups (CO, AM, O). Nonetheless, within the elderly population, CO have on average a significantly greater percentage of $CD4^+CD27^+$ cells than their AM controls (or the old group). The same result was observed

Table 2. Lymphocyte Subpopulations in Young People, Centenarian Offspring (CO), Age-Matched (AM) Controls, and Old People

Lymphocyte Subpopulations	Young People (age 28.5 ± 1.9), mean ± *SD*	CO (age 70.1 ± 8.3), mean ± *SD*	AM (age 69.1 ± 9.7), mean ± *SD*	Old People (age 86.4 ± 3.8), mean ± *SD*
$CD3^+$	73.4 ± 16.7	71.5 ± 8.7	72.2 ± 7.2	73.9 ± 8.1
$CD4^+$	58.2 ± 11.2	55.5 ± 18.9	47.1 ± 14.3	48.8 ± 15.6
$CD8^+$	23.3 ± 6.6	24.2 ± 9.9	30.3 ± 11.6	31.6 ± 8.4
CD4:CD8 ratio	2.6 ± 0.9	2.4 ± 1.9	1.9 ± 1.6	1.8 ± 1

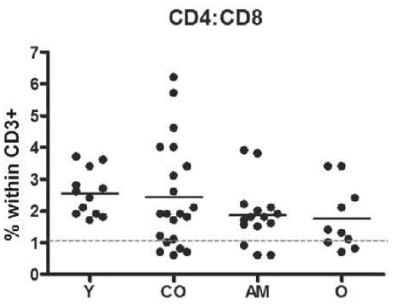

Figure 1. CD4:CD8 ratio in young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people. Peripheral blood mononuclear cells were stained with CD3, CD4, and CD8 antibodies. The CD4:CD8 ratio was calculated within $CD3^+$ cells. Bars represent medians. Significant differences valuated by Mann–Whitney nonparametric *U* testing are indicated by $^*p \le .05$, $^{**}p \le .01$, $^{***}p \le .001$. The horizontal dotted line indicates a CD4:CD8 ratio of ≤1.

analyzing the absolute cell number (data not shown). For $CD4^-CD28^+$ cells, we observed a similar pattern, with a higher expression of CD28 in young people compared with the older group; CO again had more $CD4^+CD28^-$ cells than their AM controls, although in this case, the difference did not reach significance ($p = .06$, data not shown).

Within the CD8 subset, again, significantly higher percentages of $CD27^-$ and $CD28^+$ cells were observed in young people compared with any of the elderly groups (CO, AM, O). Also here, there was a significant difference between CO and AM controls for the expression of CD27 as percentages (Figure 2B) and absolute numbers (data not shown). A trend toward a higher percentage of $CD28^+$ cells was observed ($p = .1$, data not shown).

Naïve and Memory T Cells Within the $CD4^+$ and the $CD8^+$ Populations

Naïve and memory T cells can be identified by analyzing the expression of two isoforms of the protein phosphatase CD45 (CD45RA and CD45RO), with naïve cells more likely to express CD45RA and memory cells CD45RO. Although assessing the expression of CD45RA and CD45RO provides some information on T-cell differentiation status (Figures 3B and 4B), they are not by themselves sufficient to distinguish between naïve and memory cells as CD45RA is also reexpressed by late-differentiated memory cells (19). For this reason, we have analyzed the expression of CD45RA and CD45RO together with CD27 and CD28 to identify T-cell phenotypes in detail. As previously reported, we considered the phenotype $CD27^+CD28^+CD45RA^-CD45RO^-$ as the naïve T cells and $CD27^-CD28^-CD45RA^+CD45RO^+$ as the late-differentiated cells (20).

We confirmed here that naïve $CD4^+$ ($CD27^+CD28^+CD45RA^+CD45RO^-$) cells are more frequent in young people than in any of the groups of elderly people (Figure 3A). The percentage of naïve cells in AM controls (and old participants) is again significantly lower than in CO ($p = .005$, Figure 3A and B). The same result was observed analyzing the absolute cell counts of naïve $CD4^+$ ($p = .002$, Figure 3C). Differences were more noticeable when we analyzed naïve and late memory $CD8^+$ T cells in detail. There were fewer naïve $CD8^+$ T cells in all elderly groups (Figure 4A and B), but CO again had a significantly higher proportion of naïve $CD8^+CD27^+CD28^+CD45RA^+CD45RO^-$ cells than their AM controls ($p = .005$). Also in this case, the absolute cell number of naïve $CD8^+$ cells is significant higher in CO compared with AM ($p = .002$, Figure 4C). Reciprocally, a higher percentage of late-differentiated $CD4^+$ cells ($CD27^-CD28^-CD45RA^-CD45RO^+$) in the older groups is less evident in CO although this difference did not reach significance, neither as percentage ($p = .1$, Figure 5A) or absolute number of cells ($p = .1$, Figure 5B).

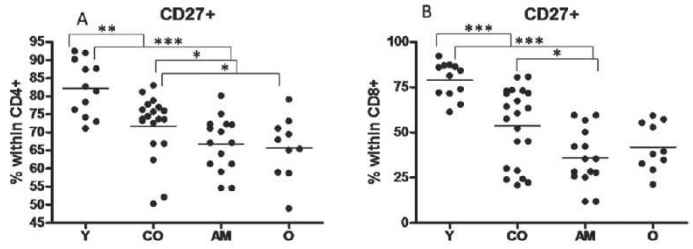

Figure 2. Costimulatory molecule CD27 expression in young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people. Peripheral blood mononuclear cells of 12 young controls (age range: 28.5 ± 1.9; 7 men and 5 women), 21 CO (age range: 70.1 ± 8.3; 10 men and 11 women), 15 AM controls (age range: 69.1 ± 9; 7 men and 8 women), and 10 old (O) participants (age range: 86.4 ± 3.8; 5 men and 5 women) were stained for CD3, CD4, CD8, and CD27. The percentages of $CD27^-$ (early differentiated) lymphocytes are shown for $CD4^+$ (A) and $CD8^+$ T cells (B). Bars represent medians. Significant differences evaluated by Mann–Whitney nonparametric *U* testing are indicated by $^*p \le .05$, $^{**}p \le .01$, $^{***}p \le .001$.

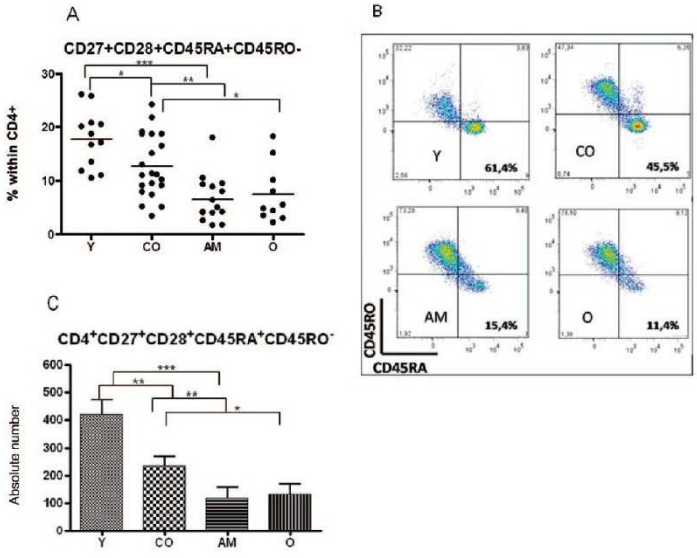

Figure 3. CD4+ naïve cells. Peripheral blood mononuclear cells from young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people as in Figure 2 were stained with CD3, CD4, CD28, CD27, CD45RA, and CD45RO to identify naïve cells. Percentages (A) and absolute cell counts (C) of $CD28^+CD27^+CD45RA^+CD45RO^-$ naïve cells of 12 young controls, 21 CO, 15 AM controls, and 10 old participants. Bars represent medians. (B) Representative dot plots of naïve CD4+ T-cell distribution in a young control (upper left), in a CO (upper right), in an AM (lower left), and in an old participant (lower right). Significant differences are indicated by $*p \le .05$, $**p \le .01$, $***p \le .001$.

Along the same lines, the percentage of late-differentiated ($CD8^+CD27^-CD28^-CD45RA^-CD45RO^-$) cells was significantly lower in CO compared with both AM controls and old people both as percentage ($p = .003$, Figure 5C) and absolute number of cells ($p = .002$, Figure 5D).

Potentially Senescent $CD4^+$ and $CD8^+$ T Cells

Previous studies have suggested that lifelong antigenic exposure may lead to increased frequencies of end stage–differentiated $CD8^+$ T cells that have often been designated "senescent" in the literature. These cells are characterized *inter alia* by the expression of the inhibitory receptor CD57 (21). Therefore, we analyzed the percentage of $CD57^+$ cells, finding that the older groups had a significantly higher percentage of $CD57^+$ cells than young people. Although for $CD4^+$ cells, there were no differences between CO and AM controls or older people ($p > .05$, data not shown), percentages of $CD8^+CD57^+$ cells were significantly lower in CO than in AM controls and older people ($p = .0005$, Figure 6), suggesting that fewer "senescent" T cells are accumulating in CO. The same trend was present when we analyzed absolute cell numbers.

Discussion

Compromised and dysregulated immunity is commonly assumed to be a cause of the increased susceptibility and sensitivity to infectious disease and poor response to vaccination in old people. The immune system, which is constantly challenged by external and internal agents, undergoes dramatic changes with age. These cumulative changes include those resulting from decreased thymic output following puberty, alterations in lymphocyte population dynamics in late life, and reduced intracellular signaling within those cells (22,23). Alterations in T-cell immunity imputed to occur dynamically with aging are reflected in altered numbers and proportions of T-cell phenotypes. One of the consequences of the developmentally programed involution of the thymus in humans is the rapidly declining output of naïve T lymphocytes after puberty. In aged humans, after the age of 70, naïve $CD4^+$ T cells have declined to such an extent that

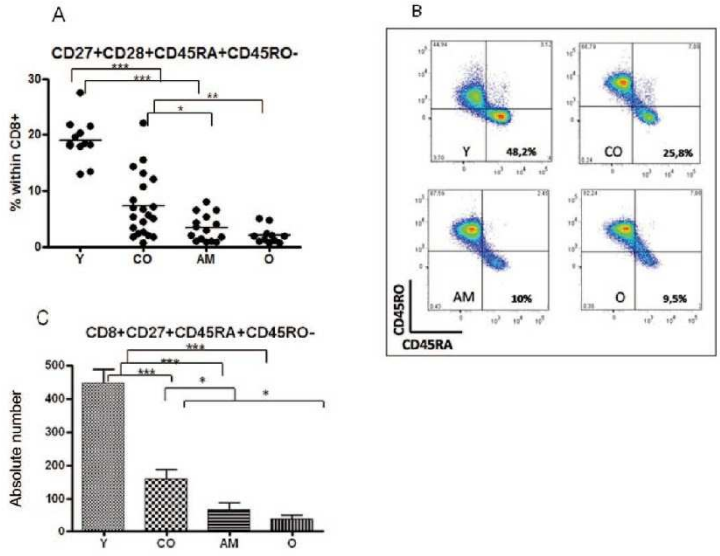

Figure 4. CD8 naïve cells. Peripheral blood mononuclear cells from young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people as in Figure 2 were stained with CD3, CD8, CD28, CD27, CD45RA, and CD45RO to identify naïve cells. Percentages (A) and absolute cell count (C) of $CD28^+CD27^+CD45RA^+CD45RO^-$ naïve cells of 12 young controls, 21 CO, 15 AM controls, and 10 old participants. Bars represent medians. (B) Representative dot plots of naïve $CD8^+$ T-cell distribution in a young control (upper left), in a CO (upper right), in an AM (lower left), and in an old participant (lower right). Significant differences are indicated by $^*p \le .05$, $^{**}p \le .01$, $^{***}p \le .001$.

their capacity to help B cells to provide efficient humoral responses may be compromized (24–26). As a result of thymic involution, the peripheral T-cell repertoire is not replenished, correlating with the impaired immune response that characterizes old individuals (27). Thus, the elderly people have low percentages of naïve T cells that display numerous functional defects and are associated with mortality, primarily from infectious diseases. The mirror image of this situation is an increased representation of late-differentiated memory T cells that occurs as a consequence of a lifetime's exposure to microbial and other agents.

Similar changes in the $CD8^-$ subpopulation take place even earlier in life than in $CD4^+$ T cells (28,29). In particular, in the CD8 compartment, late-differentiated $CD45RA^+CD28^-$ T cells accumulate with age and their increased frequencies inversely correlate with vaccine responses (30–32). Thus, it is clear that T cells become significantly less able to engage in effective immune responses and to become functional memory cells as we age, as also seen in mice (33). Many of these $CD8^-$ T cells with a late-differentiated phenotype are likely to be CMV specific and their accumulation is much less marked in older CMV-seronegative individuals (6,8–10). Indeed, it was demonstrated that repeated exposure to antigens directly affects the T-cell arm and pathogens and thus directly contribute to immunosenescence (29,34). Chronic CMV infection has an enormous impact on all the changes observed in elderly people. It seems that not only chronological age alone but also persistent CMV infection "accelerates" what is commonly interpreted as immune aging, materially influencing the distribution of T-cell phenotypes in the peripheral blood of old individuals (1,10,35–37). In general, $CD4^-$ as well as $CD8^+$ T cells are affected by chronic CMV response, although the magnitude of the effect is greater for $CD8^+$ T cells (38,39), and other herpes viruses probably contribute little (40).

Recently, we have demonstrated that CMV-seropositive individuals from families enriched for longevity have higher percentages of naïve T cells and lower percentages of CD45RA-reexpressing and late-differentiated effector memory T cells than the general AM population. These people also have lower levels of the proinflammatory marker C-reactive protein compared with AM CMV-seropositive

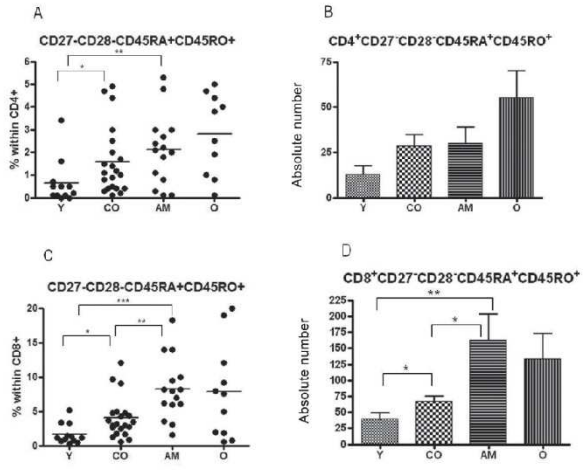

Figure 5. $CD4^+$ and $CD8^+$ late-differentiated cells. Peripheral blood mononuclear cells from young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people as in Figure 2. The percentages and absolute cell count of late differentiated $CD28^-CD27^-CD45RA^-CD45RO^+$ within $CD4^+$ cells (A and B) and within $CD8^+$ cells (C and D) are shown. Significant differences between the four groups analyzed evaluated by Mann–Whitney nonparametric U testing are indicated by $^*p \le .05$, $^{**}p \le .01$, $^{***}p \le .001$.

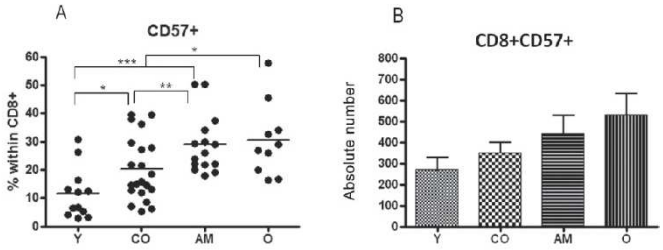

Figure 6. $CD8^+CD57^+$ "senescent" cells. Peripheral blood mononuclear cells from young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people as in Figure 2 were stained with CD3, CD8, and CD57. Percentages (A) and absolute number (B) of $CD8^+CD57^+$ cells in 12 young controls, 21 CO, 15 AM controls, and 10 old people are shown. Bars represent medians. Differences between the four groups analyzed evaluated by Mann–Whitney nonparametric U testing are indicated by $^*p \le .05$, $^{**}p \le .01$, $^{***}p \le .001$.

controls, suggesting a lower proinflammatory status despite CMV infection (less "inflamm-aging"). The analysis of immune signatures of offspring genetically enriched for longevity has revealed marked differences compared with the general elderly population that might better result in protection not only from infectious disease but also from cardiovascular morbidity and other inflammatory diseases such as diabetes (10). Such family studies facilitate the identification of genes that are also expected to affect on immune function in old age.

In the present study, we performed a phenotypic analysis of the T-cell arm of adaptive immunity in a group of Sicilian CO, a special population of elderly people presumed to be genetically advantaged for longevity, and who may share

some of the same characteristics as members of long-lived families. Here, we have analyzed very rare people with at least one centenarian parent (100–107 years of age) and compared these results with those of a previous article on familial longevity (10) in which the individuals studied ranged from 40 to 70 years of age with at least one parent having at least one sibling that lived to be more than 90. Thus, the present study specifically sought phenotypic similarities in cohorts derived in a very different manner (offspring of sporadic vs familial longevity). To emphasize the likely genetic contribution, rather than the environmental, we selected the present cohort from a southern European population, which will have experienced very different exposures (food, weather conditions, culture, pathogens, etc.). Also given the fact that these populations are likely to be genetically different, we sought to identify prevalent-shared immune signatures despite all these possible confounding differences in the population. The results indicate some striking similarities between the two different cohorts, emphasizing the potential biological relevance to longevity of these findings under quite different conditions.

We have recently demonstrated that offspring of a centenarian parent also have a "younger" B-cell as well as T-cell profile that could help them resist infection (14,16). Here, we have analyzed the T-cell immune profile of CO (age range: 70.1±8.3) compared with equally elderly people without a familial history of longevity (age range: 69.1±9.7), and also with unrelated very elderly individuals (age range: 86.4±3.8). All were evaluated for CMV status and found to be uniformly positive. Thus, any differences seen between CO and other elderly people cannot be solely due to CMV infection, whereas differences between any of the elderly groups and the young groups, who were all CMV-negative, are mostly likely caused by CMV.

The distribution of the $CD4^+$ and $CD8^-$ subsets within $CD3^+$ T cells was analyzed in terms of the CD4:CD8 ratio because this is one of the immunological parameters included in the IRP in the Swedish OCTO/NONA longitudinal studies (41–43). As there are few studies of the IRP in different populations, it remains of interest to explore its presence and relevance in non-Swedish cohorts. We found that CO had a higher CD4:CD8 ratio compared with their AM controls, again appearing more similar to the CMV-negative young people in this respect, despite being CMV-positive. As previously demonstrated for the B-cell arm of the immune system (14,15), the T-cell arm is probably better preserved in CO than in sporadic AM controls. This could be a consequence of an increased thymic output, due to slower involution or, alternatively, to less primarily CMV-driven peripheral post thymic expansion that causes a significant contraction of the peripheral T-cell receptor repertoire in most elderly people (44). This remains to be investigated, for example, by assessing T cell receptor (TREC) levels.

Analysis of CD27 and CD28 expression, which defines an early stage of T-cell differentiation, confirmed higher percentages in young people compared with the elderly people. Comparison of the four groups revealed that the percentages of $CD4^+CD27^-$ cell subsets were significantly higher in the presumed genetically advantaged CO group compared with their AM controls, with CO again looking more similar to the young groups. For CD28 expression on $CD4^+$ and $CD8^+$ cells, the differences between CO and controls remained trends but did not reach statistical significance. Next, we evaluated naïve T cells within the $CD4^+$ and $CD8^+$ populations. Our recent detailed classification defined them as $CD27^+CD28^+CD45RA^+CD45RO^-$ (20). As expected, $CD8^+$ naïve T cells were more frequent in young donors than in the general elderly population, but here we also saw fewer $CD4^+$ naïve cells, not always seen in every published study or in our own previous studies on other populations. This may be due to population effects and the relatively small numbers of donors tested here, or to true population differences in Sicilians. Using these phenotyping panels, we had previously observed fewer $CD4^-$ naïve cells in Western populations only under pathological conditions (notably, Alzheimer's disease) (20,45). However, we have observed a more pronounced effect on $CD4^+$ naïve cells in a non-European population where people are considered old at a much earlier chronological age than in the West and might well be expected to have a higher pathogen load (46). The patterns observed in the Sicilian elderly population seem to fall midway between those of the commonly studied northern European and U.S. populations, and the rural Pakistani population studied by Alam and colleagues.

The presence of fewer naïve cells is commonly mirrored by accumulations of memory cells, which have a more restricted repertoire for recognizing pathogens to which the individual was previously exposed. Consistent with the higher level of naïve T cells in CO, percentages of late-differentiated $CD8^+$ memory T cells ($CD27^-CD28^-CD45RA^+CD45RO^+$) were significantly lower in CO than in the general elderly population and were in fact similar to those in the young group. Although the differences between young (CMV−) and elderly (CMV+) groups can mostly be attributed to the expansion of CMV-specific clones, the lowest percentage of memory T cells observed in CO (who were also all CMV+) may be due to more efficient maintenance of immune surveillance against this potentially dangerous pathogen. Consistent with this, the analysis of putatively senescent CD57-expressing cells within $CD8^+$ T lymphocytes, that previous studies have reported as increased in elderly people when compared with young people (21), also indicated that CO showed a phenotype more similar to the CMV-negative young people than the CMV-positive AM controls and very old people. In this respect, CO do appear very similar to the offspring of long-lived parents in familial longevity studies (10). Seeking shared genetic parameters in these two disparate populations might therefore assist in

identifying the nature of the genes possibly contributing to extended human longevity.

Taken together, data reported here suggest that CO do not show the typical naïve/memory trend observed in the random elderly people, despite all being CMV-positive. The adaptive immune system (both B- and T-cell arms) appears "better conserved" in the offspring of centenarians (ie, more like that seen in younger CMV-negative people), and less susceptible to the major effects that CMV infection has on the general population. This may contribute to their ability to resist typical age-related diseases and imbue them with an increased probability to reach the extreme limits of human life as their centenarian parent did. The similarity between these results and those from the Leiden Longevity Study of familial longevity suggests the presence of longevity-promoting genes shared between quite different populations conveying resistance to the effects of CMV infection on immune signatures commonly believed to be mark deleterious immune function.

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Conflict of Interest

The authors declare no competing financial interests.

References

1. Larbi A, Franceschi C, Mazzatti D, Solana R, Wikby A, Pawelec G. Aging of the immune system as a prognostic factor for human longevity. *Physiology*. 2008;23:64–74.
2. McElhaney JE, Zhou X, Talbot HK, et al. The unmet need in the elderly: how immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines. *Vaccine*. 2012;30:2060–2067.
3. Pawelec G, Ferguson FG, Wikby A. The SENIEUR protocol after 16 years. *Mech Ageing Dev*. 2001;122:132–134.
4. Pawelec G, Akbar A, Caruso C, Solana R, Grubeck-Loebenstein B, Wikby A. Human immunosenescence: is it infectious? *Immunol Rev*. 2005;205:257–268.
5. Chidrawar S, Khan N, Wei W, et al. Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals. *Clin Exp Immunol*. 2009;155:423–432.
6. Derhovanessian E, Larbi A, Pawelec G. Biomarkers of human immunosenescence: impact of Cytomegalovirus infection. *Curr Opin Immunol*. 2009;21:440–445.
7. Lazuardi L, Jenewein B, Wolf AM, Pfister G, Tzankov A, Grubeck-Loebenstein B. Age-related loss of naïve T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes. *Immunology*. 2005;114:37–43.
8. Franceschi C, Bonafè M, Valensin S. Human immunosenescence: the prevailing of innate immunity, the failing of clonotypic immunity, and the filling of immunological space. *Vaccine*. 2000;18:1717–1720.
9. Schoenmaker M, de Craen AJ, de Meijer PH, et al. Evidence of genetic enrichment for exceptional survival using a family approach: Leiden Longevity Study. *Eur J Hum Genet*. 2006;14:79–84.
10. Derhovanessian E, Maier AB, Beck R, et al. Hallmark features of immunosenescence are absent in familial longevity. *J Immunol*. 2010;185:4618–4624.
11. Barzilai N, Gabriely I, Gabriely M, Iankowitz N, Sorkin JD. Offspring of centenarians have a favorable lipid profile. *J Am Geriatr Soc*. 2001;49:76–79.
12. Terry DF, Wilcox MA, McCormick MA, et al. Lower all-cause, cardiovascular, and cancer mortality in centenarians' offspring. *J Am Geriatr Soc*. 2004;52:2074–2076.
13. Rose G, Passarino G, Scornaienchi V, et al. The mitochondrial DNA control region shows genetically correlated levels of heteroplasmy in leukocytes of centenarians and their offspring. *BMC Genomics*. 2007;8:293.
14. Colonna-Romano G, Buffa S, Bulati M, et al. B cells compartment in centenarian offspring and old people. *Curr Pharm Des*. 2010;16:604–608.
15. Colonna-Romano G, Bulati M, Aquino A, et al. A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people. *Mech Ageing Dev*. 2009;130:681–690.
16. Bulla S, Pellicanò M, Bulati M, et al. A novel B cell population revealed by a CD38/CD24 gating strategy: CD38-CD24- B cells in centenarian offspring and elderly people. *Age*. 2012; forthcoming.
17. Romero P, Zippelius A, Kurth I, et al. Four functionally distinct populations of human effector-memory CD8+ T lymphocytes. *J Immunol*. 2007;178:4112–4119.
18. Koch S, Larbi A, Derhovanessian E, Özcelik D, Naumova E, Pawelec G. Multiparameter flow cytometric analysis of CD4 and CD8 T cell subsets in young and old people. *Immun Ageing*. 2008;5:6.
19. Hamann D, Baars PA, Rep MH, et al. Phenotypic and functional separation of memory and effector human CD8+ T cells. *J Exp Med*. 1997;186:1407–1418.
20. Pellicanò M, Larbi A, Goldeck D, et al. Immune profiling of Alzheimer patients. *J Neuroimmunol*. 2012;242:52–59.
21. Ibegbu CC, Xu YX, Harris W, Maggio D, Miller JD, Kourtis AP. Expression of killer cell lectin-like receptor G1 on antigen-specific human CD8+ T lymphocytes during active, latent, and resolved infection and its relation with CD57. *J Immunol*. 2005;174:6088–6094.
22. Nikolich-Žugich J, Li G, Uhrlaub JL, Renkema KR, Smithey MJ. Age-related changes in CD8 T cell homeostasis and immunity to infection. *Semin Immunol*. 2012;24:356–364.
23. Larbi A, Pawelec G, Wong SC, Goldeck D, Tai JJ, Fulop T. Impact of age on T cell signaling: a general defect or specific alterations? *Ageing Res Rev*. 2011;10:370–378.
24. Eaton SM, Burns EM, Kusser K, Randall TD, Haynes L. Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses. *J Exp Med*. 2004;200:1613–1622.
25. Maue AC, Eaton SM, Lanthier PA, Sweet KB, Blumerman SL, Haynes L. Proinflammatory adjuvants enhance the cognate helper activity of aged CD4 T cells. *J Immunol*. 2009;182:6129–6135.
26. Goronzy JJ, Weyand CM. T cell development and receptor diversity during aging. *Curr Opin Immunol*. 2005;17:468.
27. Berent-Maoz B, Montecino-Rodriguez E, Dorshkind K. Genetic regulation of thymocyte progenitor aging. *Semin Immunol*. 2012;24:303–308.
28. Effros RB, Cai Z, Linton PJ. CD8 T cells and aging. *Crit Rev Immunol*. 2003;23:45.
29. Weiskopf D, Weinberger B, Grubeck-Loebenstein B. The aging of the immune system. *Transplant International*. 2009;22:1041–1050.
30. Goronzy JJ, Fulbright JW, Crowson CS, Poland GA, O'Fallon WM, Weyand CM. Value of immunological markers in predicting responsiveness to influenza vaccination in elderly individuals. *J Virol*. 2001;75:12182–12187.
31. Saurwein-Teissl M, Lung TL, Marx F, et al. Lack of antibody production following immunization in old age: association with CD8(+)

CD28(-) T cell clonal expansions and an imbalance in the production of Th1 and Th2 cytokines. *J. Immunol.* 2002;168:5893–5899.
32. Trzonkowski P, Myśliwska J, Pawelec G, Myśliwski A. From bench to bedside and back: the SENIEUR Protocol and the efficacy of influenza vaccination in the elderly. *Biogerontology.* 2009;10:83–94.
33. Haynes L, Swain SL. Aged-related shifts in T cell homeostasis lead to intrinsic T cell defects. *Semin Immunol.* 2012;24:350–355.
34. Kern F, Bunde T, Faulhaber N, et al. Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. *J Infect Dis.* 2002;185:1709.
35. Almanzar G, Schwaiger S, Jenewein B, et al. Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. *J Virol.* 2005;79:3675–3683.
36. Pawelec G, Derhovanessian E, Larbi A, Strindhall J, Wikby A. Cytomegalovirus and human immunosenescence. *Rev Med Virol.* 2009;19:47–56.
37. Pawelec G, McElhaney JE, Aiello AE, Derhovanessian E. The impact of CMV infection on survival in older humans. *Curr Opin Immunol.* 2012;24:507–511.
38. Akbar AN, Fletcher JM. Memory T cell homeostasis and senescence during aging. *Curr Opin Immunol.* 2005;17:480–485.
39. Sylwester AW, Mitchell BL, Edgar JB, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J Exp Med.* 2005;202:673–685.
40. Derhovanessian E, Maier AB, Hähnel K, et al. Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated CD4+ and CD8+ T-cells in humans. *J Gen Virol.* 2011;92(pt 12):2746–2756.
41. Wikby A, Johansson B, Olsson J, Löfgren S, Nilsson BO, Ferguson F. Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune. *Exp Gerontol.* 2002;37:445–453.
42. Nilsson BO, Ernerudh J, Johansson B, et al. Morbidity does not influence the T-cell immune risk phenotype in the elderly: findings in the Swedish NONA Immune Study using sample selection protocols. *Mech Ageing Dev.* 2003;124:469–476.
43. Wikby A, Ferguson F, Forsey R, et al. An immune risk phenotype, cognitive impairment, and survival in very late life: impact of allostatic load in Swedish octogenarian and nonagenarian humans. *J Gerontol A Biol Sci Med Sci.* 2005;60:556–565.
44. Kohler S, Wagner U, Pierer M, et al. Post-thymic in vivo proliferation of naive CD4+ T cells constrains the TCR repertoire in healthy human adults. *Eur J Immunol.* 2005;35:1987–1994.
45. Larbi A, Pawelec G, Witkowski JM, et al. Dramatic shifts in circulating CD4 but not CD8 T cell subsets in mild Alzheimer's disease. *J Alzheimers Dis.* 2009;17:91–103.
46. Alam I, Goldeck D, Larbi A, Pawelec G. Aging affects the proportions of T and B cells in a group of elderly men in a developing country-a pilot study from Pakistan. *Age (Dordr).* 2012; forthcoming.

CHAPTER 5

"Trafficking phenotype and production of Granzyme B by Double Negative B cells ($IgG^{+}IgD^{-}CD27^{-}$) in the elderly"

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

Matteo Bulati, Silvio Buffa, Adriana Martorana, Giuseppina Candore, Domenico Lio, Calogero Caruso, Giuseppina Colonna-Romano *

Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Corso Tukory 211, 90134 Palermo, Italy

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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

* Corresponding author at: Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Corso Tukory 211, Palermo 90134, Italy. Tel.: +39 0916555908; fax: +39 0916555933.
E-mail address: giuseppina.colonnaromano@unipa.it (G. Colonna-Romano).

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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, Auburn, 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

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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 (Cornerford 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^+)	$CXCR5^{[low]}CD62L^+$	$CCR6^+CXCR5^{[low]}CD62L^-$
Double negative (IgD^-CD27^-)	$CXCR3^+$	$CCR7^+CCR6^+$

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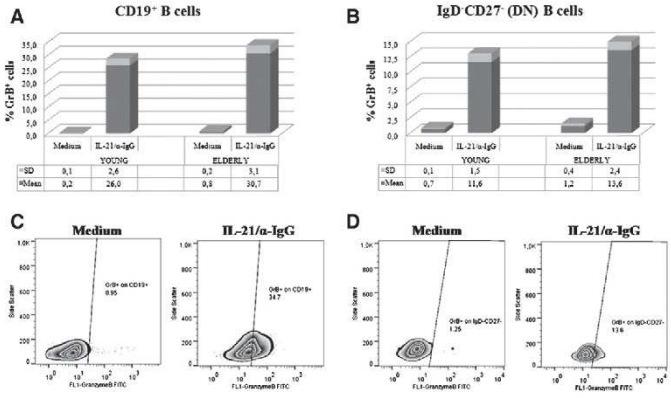

Fig. 1. IL-21 and BCR engagement induce GrB in total human CD19$^+$ B cells and in IgD$^-$ CD27$^-$ (DN) B lymphocytes. Magnetically sorted B cells or IgD$^-$ CD27$^-$ (DN) B lymphocytes were cultured in AIM-V medium for 16 h and incubated in the presence/absence of both recombinant human IL-21 (50 ng/ml) 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 chemo-attraction 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

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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.

Acknowledgments

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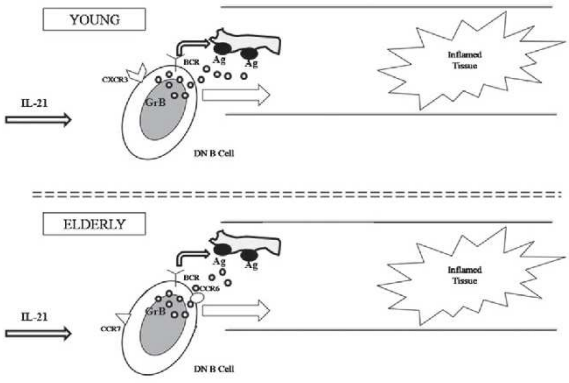

Fig. 2. The "intriguing scenario" of the DN B cells in the inflamed tissue. Properly stimulated, DN B lymphocytes of young donors are able not only to migrate into inflammatory sites (CXCR3 expression), but herein they exert their function producing GrB (upper side of the figure). In the elderly, the 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).

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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

Aiello, J.H., Strasny, 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., Olivieri, 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

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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.

Please cite this article as: Bulati, M., et al., Trafficking phenotype and production of granzyme B by double negative B cells ($IgG^{+}IgD^{-}CD27^{-}$) in the elderly, Exp. Gerontol. (2014), http://dx.doi.org/10.1016/j.exger.2013.12.011

CHAPTER 6

"Double Negative ($CD19^+ IgD^- CD27^-$) B lymphocytes: a new insight from telomerase activity in healthy elderly, in centenarian offspring and in Alzheimer's disease patients"

(Manuscript in preparation)

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Double Negative ($CD19^+IgG^+IgD^-CD27^-$) B lymphocytes: a new insight from telomerase in healthy elderly, in centenarian offspring and in Alzheimer's disease patients.

Adriana Martorana[1], Matteo Bulati[1], Silvio Buffa[1], Carmela Rita Balistreri[1], Delia Azzarello[2], Cecilia Camarda[2], Giuseppina Colonna-Romano[1]

[1]Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies (DIBIMEF), University of Palermo, Italy

[2]Department of Experimental Biomedicine and Clinical Neurosciences, University of Palermo, Italy

Abstract

Immunosenescence is characterized by the impairment of both cellular and humoral immunity, so elderly people show a reduced ability to respond against new infections and vaccines. Moreover, ageing is associated with decrease of B lymphocyte percentage and absolute number. We and others have previously demonstrated a significant increase of Double Negative (DN) ($CD19^+IgG^+IgD^-CD27^-$) B cells in elderly people and in chronic stimulated subjects (HIV, SLE, RSV).In previous works, we have demonstrated that DN B cells of elderly donors have very short telomeres compared to the same subpopulation of young donors, show a low frequency of somatic mutation and are not responder to CpG stimulation, although they can be weakly activated with $F(ab')_2$ (anti-BCR). In order to understand whether the low attitude to proliferate after the *in vitro* stimulation of DN B cells depends on the expression of inhibitory receptors, we have assessed the expression of CD307d and CD22 on naïve/memory B cell subsets focusing our attention on DN B cells. Then, we have evaluated the proliferative response of DN B cells after different kinds of stimuli in young and elderly donors. We have demonstrated that the refractoriness to proliferate of DN B cells does not depend on the expression of inhibitory receptors, but it is limited to certain stimulators. Indeed, when DN B cells are stimulated

engaging contemporarily BCR and TLR9, they become able to proliferate and reactivate the relative telomerase activities (RTA). In the present study we also compared the telomerase reactivation activity in a group of people genetically advantaged for longevity as Centenarian offspring are and in a group of severe Alzheimer's disease patients who represent an example of unsuccessful ageing model. In conclusion, we suggest that the monitoring of DN B cells might be a useful parameter to evaluate the quality of aging process.

Introduction

Advancing age yields numerous immune system changes, as evidenced by blunted primary and recall response, feeble vaccine efficacy and increased prevalence of inflammatory pathologies (Frasca et al. 2004; Schenkein et al. 2008; Cancro et al. 2009; Shaw et al., 2010; Frasca et al. 2011). Accordingly, the knowledge of how age impacts the production and the behaviour of B cells, as well as the modulation of the humoral immune response, are fundamental to understanding the age-related phenomenon known as immunosenescence. B cells are key mediators of immunity having effector and regulatory functions, other than antibody production (Harris et al., 2000; Martin and Chan, 2006; Sanz et al., 2007), through the secretion of different cytokines. These in turn play an important role in the regulation of normal immune responses, but also contributing to human autoimmune diseases (Youinou et al., 2005; Harris et al., 2005a,b).

Nowadays B cell branch has been extensively studied and different B cells subsets, such as transitional, naïve, memory and plasmablasts have been identified by using of different cellular markers, for instance IgD, CD27, CD24, CD38 although do not exist a general consensus to classify B cell subpopulation in humans (Kaminski et al., 2012). However, it has been suggested a kind of classification to identify "core" subsets on peripheral $CD19^+$ B cells which could represents an useful biomarker in some autoimmune disease (Kaminski et al., 2012). To this perspective,naïve and different memory B cells subsets have been described (Shi et al., 2003; Tangye and Hodgkin 2004; Fecteau et al., 2006, Wei et al., 2007; Bulati et al. 2011). In particular, in humans, peripheral blood naïve and memory B cells can be divided, on the basis of differential expression of IgD and CD27, into different functional cell subsets: naïve IgD^+CD27^- cells; memory unswitched IgD^+CD27^+ cells, (some of which are the so called "IgM memory"

$IgM^+IgD^+CD27^+$), classical switched memory B cells IgD^-CD27^+ that also include the $IgM^+IgD^-CD27^+$ (IgM only) and finally - Double Negative (DN) memory B lymphocytes IgD^-D27^- (Shi et al., 2003; Colonna-Romano et al., 2009).

Advanced age is *per se* a condition characterized by lack of B clonotypic immune response to new extracellular pathogens. It is to note that aging affects the humoral branch of the immune system because the B cell number is reduced in the elderly (Faria et al., 2008; Frasca et al., 2008; Veneri et al., 2009). Indeed, it is known that the proportion of different subsets is also altered (as reviewed by Bulati et al., 2011).We and others (Colonna-Romano et al., 2003; Gupta et al., 2005) have shown that in the elderly there is a significant decrease in naïve (IgD^+CD27^-) B cells and no significant reciprocal increase of $CD27^+$ memory B lymphocytes (Klein et al., 1998; Agematsu et al., 2000). Moreover, our and other groups have also demonstrated the increase in percentage but not in absolute number of $CD19^+IgG^+IgD^-CD27^-$ (DN) B cells in different cohort of subjects. Indeed, this B cell population is expanded both in the elderly (Colonna-Romano et al., 2009; Bulati et al., 2011) and in patients suffering of chronic immune inflammation, such as chronic HIV infections (Cagigi et al., 2009), systemic lupus erythematosus (SLE) (Anolik et al., 2004; Wei et al., 2007) and in healthy subjects challenged with respiratory syncytial virus (RSV) (Sanz et al., 2008). (Fecteau et al., 2006; Wei et al., 2007; Sanz et al., 2008; Cagigi et al., 2009; Colonna-Romano et al., 2009; Bulati et al., 2011).

However, not all subjects or population groups are equally susceptible to the effects of long-term chronic stimulation of the immune system. Recently, we have reported that in a genetically advantaged cohort of centenarian offspring (CO), distribution of naïve/memory B cells subsets is more similar to that observed in young subjects instead of their age-matched (AM) controls (70-80 years old) that have not a background of longevity. Indeed, CO do not show the typical naïve/memory shift observed in the elderly and DN B cells are not increased, while naïve B cell subset is well preserved. These data on centenarian offspring support the hypothesis of a "familiar youth" of the immune system, due to their favorable genetic background, that can be a big advantage both to fight the main age-related diseases and to properly respond to vaccinations, also suggesting a good bone marrow cell reservoir. Recently, we have evaluated the distribution of naïve/memory B cell subset in a Sicilian cohort of Alzheimer's disease (AD) patients. Our preliminary results corroborate data from literature that

indicates a reduction both in percentage than absolute number of total B cells (Speciale L et al., 2007; Xue et al., 2009; Pellicanò et al., 2010) but interestingly, DN B cells are significantly increased in severe AD patients, when compared to age-matched healthy elderly donors, suggesting the effect of chronic stimulation on the humoral B cell branch (*manuscript in preparation*).

In our previously paper, we have estimate the ability of DN B cells to go toward cycle of replication evaluating the telomere length. As "classical memory" $IgD^{-}CD27^{+}$ B cells, also DN B cells show features of memory lymphocytes with short telomeres. We have also demonstrated that DN B cells of elderly donors have very short telomeres compared to the same subpopulation of young donors (Colonna-Romano et al., 2009). Moreover, these cells are not responder to CpG stimulation, although they can be weakly activated with $F(ab')_2$ (anti-BCR)(Colonna-Romano et al. 2009). In addition, keeping in mind that DN B cells from elderly subjects show an intrinsic "in vitro" activation, there is not a link between their capacity to proliferate and the ability to produce cytokines as TNF-α and IL-10 also when stimulated with strong stimuli (CpG/PMA/Ionomycin) (Buffa S et al., 2011).

Recently, a new memory B lymphocyte population was discovered by Moir et al. (2008) in HIV-viremic individuals. This subset ($CD20^{+}CD27^{-}CD21^{-}$), called tissue-like memory B cells, lacks the expression of CD27 and shows a typical profile of pro-inflammatory trafficking receptors that is in agreement with migration to chronically inflamed tissues and away from lymphoid tissues that favour the cooperation between B and T cells. Similarly to DN B lymphocytes, these cells express significantly high levels of some chemokine receptors that drive cells to the inflamed tissues (Bulati et al., 2014). Furthermore, tissue-like memory cells are characterized by high levels expression of inhibitory receptors, compared with memory and naïve B cells, such as CD307d, CD22 and CD72 that, probably, do not allow high levels of proliferation ability of these B cells in response to B cell stimuli (Moir et al., 2008).

In the present study, we have evaluated in young and elderly donors, the proliferative response of DN B cells after different kinds of stimuli. In order to understand whether the low attitude to proliferate after the *in vitro* stimulation of DN B cells depends on the expression of inhibitory receptors, such as exhausted tissue-like memory B lymphocytes in HIV-viraemic individuals (Day et al., 2006; Trautmann et al., 2006), we have decided to investigate cell surface expression of CD307d and CD22 in naïve and

memory B cells in young and elderly donors. Our results demonstrate that, although DN cells of elderly donors are per se poorly responders, we show that the refractivity to proliferate of DN B cells does not depend on the expression of inhibitory receptors, but it is limited to certain stimulators. Indeed, in the present paper we observed that engaging contemporary both the innate (to which they are not sensitive) and the adaptive receptors, DN B cells are able to proliferate. As consequence, we compared the potential relationship between proliferation and relative telomerase activities (RTA) in DN B cells in young and elderly subjects in order to understand the biology of these cells and the age-related modulation of response.

In conclusion, an adequate stimulation might render DN B lymphocytes active players in the immune response. Noteworthy, here we also report data obtained from CO and severe AD patients that suggest us that the evaluation of the reactivity of DN cells might be a useful parameter to evaluate the quality of ageing process.

Materials and methods

Subjects

A total of 63 Sicilian subjects were included in the study: 20 young (age range 25-40 years) and 20 elderly (age range 78-90 years), 8 Centenarian Offspring (CO) (age range 60-70), 7 age-matched controls (AM) (age range 63-74) and 8 Alzheimer's disease patients (AD) (age range 69-76). Among this group we have performed phenotype analysis, to evaluate the BCR-inhibitory receptors (CD307d and CD22) expression in B cell subpopulations (20 young and 20 elderly) and we have also performed functional analysis (B cell proliferation and relative telomerase activity, RTA, after *in vitro* stimulation on total B cells and DN subset) (10 young, 10 elderly, 8 Centenarian Offspring (CO), 7 age-matched controls (AM) and 8 Alzheimer's disease patients (AD).

AD subjects included in the study were assessed with a multidimensional protocol including: demographic characteristics, medical history, pharmacological treatments, clinical, neuropsychological and neurological examination, standard laboratory blood tests and neuro-imaging study with CT and/or MRI scan. The exclusion criteria were: a diagnosis of severe systemic disorder, the presence of psychosis, a history of significant head

injury or substance abuse. Diagnosis of probable AD was according to standard clinical procedures and followed the DSM-IV criteria [American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders: DSM-IV (Text Revision). Washington, American Psychiatric Association, 2000] and the diagnosis of AD was based on the criteria of the National Institute for Neurological and Communicative Disorders and Stroke-Alzheimer Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann et al.,1984). Cognitive perfor mance and alterations were measured according to the Mini Mental State Examination (MMSE) and the Global Deterioration Scale.

Healthy controls (HC), randomly selected from the same population as the patient cohort, had complete neurological examinations and were judged to be in good health based on their clinical history and blood tests (complete blood cell count, erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, liver function tests, iron, proteins, cholesterol, triglycerides). The University Hospital Ethics Committee approved the study, and informed consent was obtained from all guardians of patients and controls according to Italian law. Whole blood was collected by venopuncture in vacutainer tubes containing ethylenediamine tetraacetic acid. The samples were kept at room temperature and used within 2 h for the various experiments.

B lymphocytes immunomagnetic separation

Peripheral blood mononuclear cells (PBMCs) were isolated by use of density-gradient Ficoll-Lympholyte (Cedarlane Laboratories Limited, Ontario, Canada) centrifugation. PBMCs were adjusted to $1*10^6$/ml in RPMI 1640 medium (Euroclone, Devon, UK) supplemented with 10% heat-inactivated Fetal Bovin Serum (Euroclone), 1% penicillin/streptomicin, 10 mM HEPES, and 1 mM L-Glutamin. B lymphocytes were separated from PBMCs by immunomagnetic sorting, as described by Miltenyi et al. (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.

Antibodies and Flow Cytometry for phenotypic analysis

Purified B cells were stained with different combinations of the following monoclonal antibodies: anti-IgD_{FITC}, anti-$CD27_{PE}$ or anti-$CD27_{APC}$, anti-$CD22_{PE\text{-}Cy5}$(BD, Pharmingen) and anti-$CD307d_{PE}$ (FcRL4) (BioLegend).

Cells were washed twice and analyzed. All measurements were made with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with the same instrument setting. At least 10^4 cells were analyzed using CellQuestPro (Becton Dickinson, San Jose, CA, USA) software.

Stimulation of B cells in vitro

Purified B cells ($1x10^5$/200 µl) were cultured in 96-well round-bottom plates, in complete RPMI medium with 10% Fetal Bovin Serum in absence or presence of 2 µg/ml of anti-BCR [$F(ab')_2$] (Jackson ImmunoResearch Laboratories, Inc, Philadelphia) 3µg/ml of CpG-B 2006 oligodeoxynucleotide (TIB Molbiol, Genova, Italy), and 500ng/ml of anti-human CD40 purified (BD, Pharmingen) for 72h, at 37°C in 5% CO2.

Proliferation assay

Cell proliferation assay was performed by Ki67 evaluation. Cultured cells, stimulated or not, were washed and stained with anti-IgD_{PE} or anti anti-IgG_{PE}, anti-$CD19_{PECy5}$ and anti-$CD27_{APC}$ for 30 minutes at 4°C. Then cells were washed twice and re-suspended with 250 µl of BD Fixation/Permeabilization solution for 20 minutes at 4°C. After two wash in BD Perm/Wash solution buffer, cells were stained in the dark for 30 minutes on ice with 20µl of anti-$Ki67_{FITC}$ (Becton Dickinson). Subsequently, cells were washed twice and the Ki67 positive cells were analyzed. All measurements were made with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with the same instrument setting. At least 10^4 Cells were analyzed using CellQuestPro (Becton Dickinson, San Jose, CA, USA) software.

DN B ($CD19^+IgG^+CD27^-$) lymphocytes sorting with FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA)

After 72hrs of culture, B cells, stimulated and not stimulated as above mentioned, were stained with 20µl of anti-IgG_{FITC}, anti-$CD27_{PE}$ and anti-$CD19_{APC}$ (Pharmingen, BD Bioscience, Mountain View, CA, USA) for 30 min at 4°C. Next, cells were washed and 1 ml of PBS/BSA (4%) was added. After defining the sorting region gate of $CD19^+IgG^+CD27^-$ (Double Negative, DN B cells) population, we optimized the sample concentration, verifying the event rate and the sort rate to maximize the efficiency of cell separation. Finally, DN B lymphocytes were collected in cytometry tubes and used for telomerase activity measurements.

Detection of telomerase activity by TRAP assay

For quantitative analysis of telomerase activity, a Telomeric Repeat Amplification Protocol (TRAP) (Kim et al., 1997) and a photometric enzyme immunoassay were performed using Telo*TAGGG* Telomearse PCR Elisa^{Plus} kit (Roche Diagnostics, Indianapolis, USA), according to the manufacture's protocol. This precisely involved elongation and amplification of telomerase reaction products to allow highly sensitive detection of telomerase activity by a photometric enzyme immunoassay. In addition, cellular extracts from human $CD19^+$ or DN B lymphocyte cultures under baseline conditions or activated, incubated for 72hrs and sorted (as above described) were utilized.

Briefly, we firstly obtained pellets of sorted $CD19^+IgG^+CD27^-$ cells at 3,000g for 5 min at 2-8°C. They were lysed directly in sterile reaction tubes using the lysis buffer provided in the kit. Protein lysate was kept on ice for 30 min and centrifuged at 16,000g for 20 min at 2-8°C. Protein concentration was measured by standard methods. Subsequently, the supernatants obtained were utilized in quantity of 0.5-10 μg total protein for the TRAP reaction, having the assurance to prepare for each sample a negative control by heat inactivation of its aliquot at 85°C for 20 min. In performing the TRAP reaction, high control template (concentration 0.1 amol/μl; quantity used for each reaction 1μl), a reaction mixture (25 μl for each sample), an Internal standard (IS;5 μl for each sample) provided in the kit were also utilized. Thus, sterile tubes (each containing a total of 30μl of the master mix-25 μl of reaction mixture and 5 μl of IS- and a suitable volume of each negative or positive sample or 1 μl of control template) were transferred to thermal cycler (MyCycler, Biorad). A combined primer elongation/amplification reaction was performed by the following protocol: primer elongation 10-30 min at 25 °C 1 cycle; telomerase inactivation 5 min at 94°C 1 cycle; amplification (denaturation 30s at 94°C; annealing 30s at 50 °C; polymerization 90s 72°C) for 30 cycles; 10 min at 72 °C for 1 cycle; hold 4°C. During the reaction, telomerase add telomeric repeats (TTAGGG) to the 3'end of the biotin-labeled primer. The elongation products, as well as the IS included in the same reaction tube, are then amplified. The PCR products were split into two aliquots, denatured, bound to a streptavidin-coated 96-well plate and hybridized to a digoxigenin (DIG)-labeled telomeric repeat-specific probes, specific for the telomeric repeats and IS. The resulting products were immobilized via the biotin label to streptavidin-coated 96-well microplate. Immobilized amplicons were then detected with an antibody against digoxigen that is conjugated to horseradish peroxidase (anti-DIG-HRP) and the sensitive peroxidase substrate TMB. . Sample absorbance was measured

at 450 nm (reference wavelength 690 nm) using an ELISA plate reader within 30 min after the addition of the stop reagent. Absorbance values were then utilized to calculate the relative telomerase activity of each sample using an appropriate formula provided in the kit ‘s protocol.

Statistical analysis

All statistical analyses were performed with Graph Pad Prism 4.0 using the Mann-Whitney nonparametric U test to compare two independent groups. Statistical significance was expressed as $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***) as shown in the figures. All values are expressed as mean± standard error of the mean (SEM).

RESULTS

Expression of BCR- inhibitory receptors (CD307d and CD22) in B cell subpopulations

To evaluate if any analogy exists between tissue-like memory B cells described in the blood of HIV-viraemic individuals and DN B cells observed in our models of aging, we have evaluated the expression of two BCR-inhibitory receptors,CD307d (FcRL4) and CD22. Indeed, it has been suggested that the low proliferative capacity of tissue-like memory B cells and their reduced replication history in vivo might be related to the over-expression of inhibitory receptors (Moir et al., 2008).As we have previously demonstrated, DN B cells, that are increased in the elderly, proliferate less than naïve B cells when stimulated in vitro with different stimulators. The evaluation of these inhibitor receptors on naïve/memory B cells identified on the basis of IgD/CD27 expression revealed a different situation from data described in literature. As shown in table 1, both in young and elderly donors, CD307d is mainly expressed on memory unswitched B cells and it is significantly reduced on naïve B cells and lost by memory switched and double negative B cells. Intriguingly, memory unswitched B cells from old donors show significantly higher levels of this molecule compared to memory unswitched B cells from young donors.

Concerning CD22 analysis, its expression is not related to the different ability of the four B cell subpopulations to proliferate. Indeed, our analysis revealed a higher degree of expression in all B cell subpopulations independently from the age of donors and so, no significant difference was observed between young and elderly subjects (Table 1).

Taken together these data suggest that there is not a relationship between the expression of the two inhibitory receptors and the ability to proliferate of DN B cells.

Total $CD19^+$B cells and DN B cells are strongly activated by the simultaneous triggering of adaptive (BCR) and innate (TLR9) immune receptors

The attitude of total $CD19^+$ B cells to proliferate, after stimulation with a combination of different stimuli, was evaluated in a group of young and elderly donors to assess whether any difference could be related to the age of the subjects or, differently due to the kind of stimulation. As known, during aging the B cell branch of the immune system is impaired resulting in a loss of function and a less protective effect against a variety of pathogens. So, the correlation stimulus-activation might be helpful to understand the biology of these cells and the effector mechanisms. We tested total $CD19^+$ B cells to the proliferative effects of TLR9 ligand CpG alone, or with α-IgG/α-CD40 that engages the adaptive receptor BCR. Moreover, we have also engaged both the innate and the adaptive receptors culturing the cells with both the stimulators. Our data confirm the reluctance of $CD19^+$ B cells of elderly subjects to properly respond against innate and adaptive stimulation. On the contrary, in young donors the same stimuli induce a feeble but growing response. Interestingly, when contemporary engaged, the innate and adaptive receptors of total B cells cause a strong proliferation both in young than elderly subjects (Figure 1). As we are interested on DN B cells and to assess whether these cells of the elderly might play any role in the immune response, we have evaluated their ability to be activated by different kinds of stimuli. As total B lymphocytes, also DN B cells of elderly donors are able to proliferate by the contemporary engagement of the innate and adaptive receptors (Figure 1).

Relative Telomerase Activity (RTA) in $CD19^+$B lymphocytes upon "different" in vitro stimulation

As known, chronic stimulation of immune cells and ageing are characterized by telomere erosion: this phenomenon causes the reduced proliferative ability of immune cells together with the reduction of clonal expansion after antigen stimulation. To verify whether the ability to respond to the stimulation with CpG, α-IgG and α-CD40 modifies telomerase expression in total B cells and

in naive/memory B cells subsets, we cultured B cells of our different groups of study with different stimuli. Our preliminary results demonstrate that RTA evaluation seem to be a very sensitive method to evaluate the size of activation of B cells: indeed as shown in figure 2, activation of total B cells with 2 different concentration of CpG alone, or with α-IgG and α-CD40 or, finally with CpG, α-IgG and α-CD40, causes a telomerase activation that is dependent on the quality and on the quantity of the stimulation (Figure 2).

Relative Telomerase Activity (RTA) in DN B cell subset upon "triple" in vitro stimulation

To verify if RTA might be a useful test to evaluate the efficiency of immune system in the elderly, we also performed the test using B cells obtained from genetically advantaged people, as Centenarian offspring (CO) are (Colonna-Romano et al., 2010) and unsuccessfully aged people as patient affected by severe Alzheimer's disease (AD).

As depicted in Figure 3, the evaluation of RTA in DN obtained from young, old, CO and severe AD patients shows that the activation of telomerase in DN cells by the combined stimulation of innate and adaptive stimuli mirrors the "immunological" age of the donors. In fact old donors show a reduce RTA when compared with young donors, CO, as described in other models are in the middle between Y and O and finally severe AD patients show very low levels of RTA.

INHIBITORY RECEPTOR Median (P25-P75)	Naive (IgD^+CD27^-)	Memory Unswitched (IgD^+CD27^+)	Memory Switched (IgD^-CD27^+)	Double Negative (DN) (IgD^-CD27^-)	p1	p2	p3	p4	p5	p6	
YOUNG	68.6 (66.2-71)	205.5 (168.6-242.5)	19.3 (16.9-21.8)	15.7 (14.6-16.7)	**0.04**	**0.05**	**0.004**	**0.002**	**0.001**	NS	**CD307d**
ELDERLY	85.8 (64.8-118)	577.4 (526.6-877)	30.7 (29.9-35.4)	21.2 (20-27.8)	**0.005**	**0.05**	**0.04**	**0.002**	**0.001**	NS	
p Young vs Elderly	NS	**0.03**	NS	NS							
YOUNG	421.3 (383.5-443.2)	436.9 (366.9-459.7)	289.6 (246.6-307.1)	353.3 (299.7-437.5)	NS	NS	NS	**0.01**	NS	NS	**CD22**
ELDERLY	449.5 (437.6-570)	562.2 (411-581.8)	248.3 (231.9-287.9)	359.7 (232.9-378)	NS	**0.001**	**0.008**	**0.009**	**0.02**	NS	
p Young vs Elderly	NS	NS	NS	NS							

Table 1.Evaluation of BCR-inhibitory receptors (CD307d and CD22) expression on B cell subpopulations obtained from 20 Young (age range 25-40) and 20 Elderly donors (age range 78-90). Purified B lymphocytes were stained with anti-IgD and anti-CD27to identify the four IgD/CD27 B cell subsets. Subsequently, naïve/memory B cells were evaluated for CD22 and CD307 (FcRL4) expression. Both in young and elderly donors, CD307d (FcRL4) is mainly expressed on memory unswitched B cells and it is significantly reduced on naïve B cells and lost by memory switched and double negative B cells. Memory unswitched B cells from elderly subjects show significantly higher levels of this molecule compared to memory unswitched B cells from young donors. CD22 is well expressed at in all B lymphocytes subpopulations both in young and elderly donors. Values are expressed as median and range of mean fluorescence intensities (MFI) ± SEM. p1= Naïve Vs Memory Unswitched; p2= Naïve Vs Memory Switched; p3= Naïve Vs DN; p4= Memory Unswitched Vs Memory Switched; p5= Memory Unswitched Vs DN; p6= Memory Switched Vs DN. NS=Not Significant. Statistical analysis were evaluated by Mann-Whitney nonparametric U testing are indicated by p (GraphPad Prism 4).

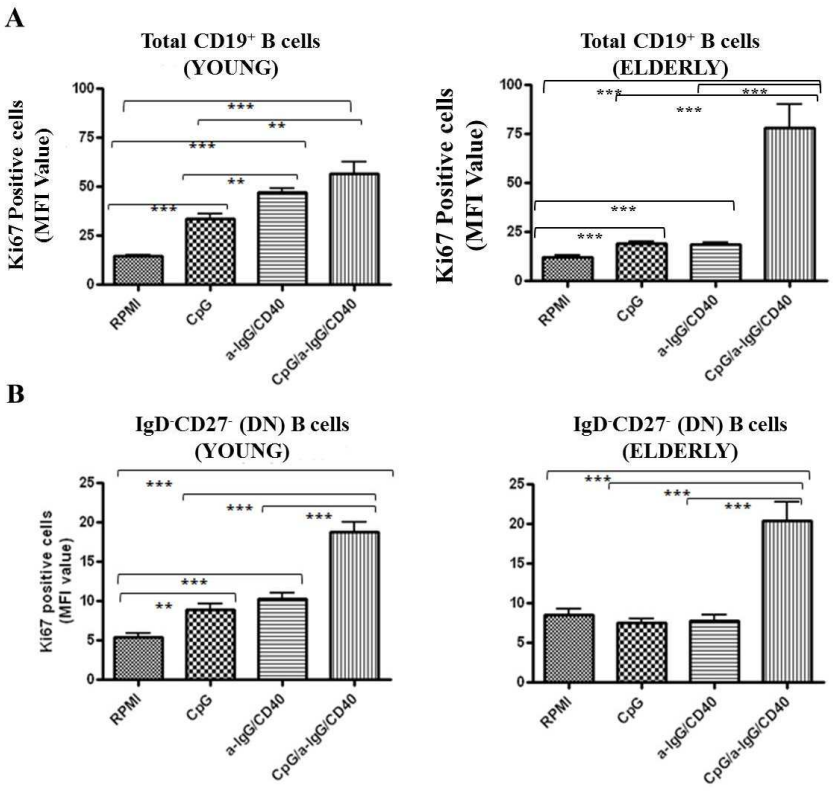

Figure 1. Intracitoplasmatic expression of Ki67 onCD19$^+$B cells and Double Negative (DN) B cells cultured in RPMI, CpG,α-IgG/CD40 and CpG/α-IgG/CD40 for 72hr in 10 young (age-range 25-40) and 10 elderly (age-range 78-90) subjects. Purified B lymphocytes were cultured with different stimuli and then were stained with Ki-67, anti-IgD, anti-CD19 and anti-CD27 to assess proliferative ability after in vitro stimulation. (A) Total CD19$^+$ B cells from young donors (left side of the figure) slightly proliferate to different stimuli but at higher levels with combination of adaptive and innate stimulation;total CD19$^+$ B cells from elderly people (right side of the figure) are less responder to the stimulation with CpG and α-IgG/CD40 but are well activate by the "triple" stimuli CpG/α-IgG/CD40. (B) DN cell from young subjects (left side of the figure) maintain the same trend of proliferation of the total CD19$^+$ B cells, indeed these cells show a significant level of Ki67 expression when stimulated with CpG/α-IgG/CD40; DN B lymphocytes from elderly donors (right side of the figure) proliferate significantly with CpG/α-IgG/CD40, even though these cells have higher in vitro basal level of activation. Values are expressed as median and range of mean fluorescence intensities (MFI) ± SEM. Significant differences are evaluated by Mann-Whitney nonparametric U testing and are indicated by *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$. Moreover, we reported significant differences between the proliferative ability of DN B cells in young vs elderly subjects in not stimulated culture ($p<0.0015$) and after stimulation with α-IgG/CD40 ($p<0.03$), whereas we did not observed significant differences between the proliferative

ability of DN B cells in young vs elderly subjects after stimulation with CpG and CpG/α-IgG/CD40.

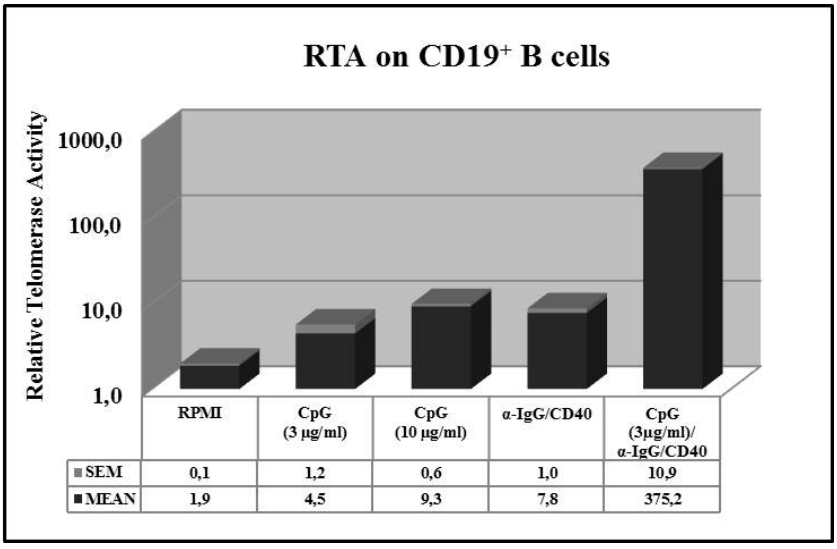

Figure 2. Relative Telomerase activity (RTA) (mean and SEM) evaluatedin $CD19^{+}$lymphocytes from (5) young subjects (age-range 25-40) purified by immunomagnetic sorting and activate withdifferent stimuli: CpG (10µl/ml), CpG (3µl/ml), a-IgG/CD40 and CpG/α-IgG/CD40). Telomerase activity was measured by TRAP assay as described in *Materials and Method.* Values are shown in $\log_{10}$ scale. Significant differences are evaluated by Mann-Whitney nonparametric U testing (GraphPad Prism 4).

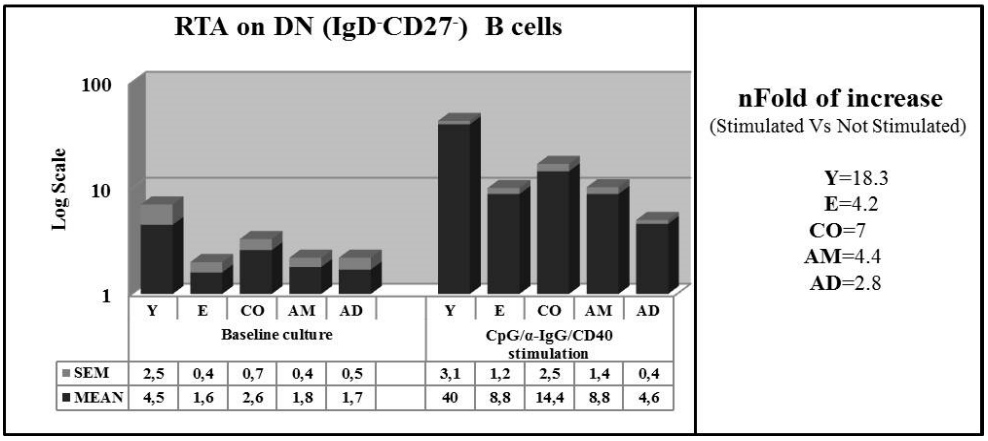

Figure 3. The Relative Telomerase Activity (RTA) in Double Negative (DN) B lymphocytes ($IgG^+IgD^-CD27^-$) was measured in not stimulated and stimulated culture with CpG/α-IgG/CD40 for 72h in 7 young controls(Y) (age-range 25-40), 8 elderly subjects (E) (age-range 78-90), 8 Centenarian Offspring (CO) (age range 60-70),7 age-matched controls (AM)(age range 63-74) and 8 Alzheimer's disease patients (AD) (age range 63-74). DN B cells were separated from other subsets by sorting with FACSCalibur flow cytometer.Values are expressed as mean of MFI±SEM.Significant differencesvaluated by Mann–Whitney nonparametric U testing (GraphPad Prism 4). In the right side of the figure are represented the fold of increase between stimulated and unstimulated culture. The p values are: p<0,0003 (young), P<0,0002 (elderly), P<0,0003 (Centenarianoffspring), <0,0006 (Age-Matched) and <0,0006 (Alzheimer's disease).

Discussion

It is well established that both innate and adaptive immunity in the elderly are impaired and related to the increased susceptibility to infectious disease, autoimmunity and cancer. T and B cell branch are involved in immunosenescence (Ouyang et al. 2003; Pawelec et al., 2005; Pawelec and Larbi., 2008; Frasca et al., 2004, 2010, 2011; Schenkein et al., 2008; Cancro et al., 2009; Bulati et al., 2011; Frasca and Blomberg 2011). In the elderly we have demonstrated the increase in percentage, but not in absolute number, of a "Double negative" ($IgG^+IgD^-CD27^-$) memory B cells population (Colonna-Romano et al., 2009) and the contemporary reduction of naïve (IgD^+CD27^-) B lymphocytes (Colonna-Romano et al., 2009; Gupta et al., 2005), that is crucial for the response to new antigens. However, there are controversial findings about the increase, decrease or no change in the naïve/memory B cells, as we have reviewed (Bulati et al., 2011). The increase of DN B lymphocytes in the old subjects might be associated with their typical

inflammatory micro-environment (Licastro et al., 2005; Vasto et al., 2007; Singh and Newman, 2011), characterized by a general increase in plasma levels of pro-inflammatory cytokines and other inflammatory mediators. Moreover, this B cell subset has also been described in patients affected by HIV, SLE and challenged with RSV (Fecteau et al., 2006; Wei et al., 2007; Sanz et al., 2008; Cagigi et al., 2009). Accordingly with this evidence, their increase could be the consequence of pathologic-long-enduring stimulation or, alternatively, it might be related to the exhaustion of memory B cells chronically stimulated in the elderly (Colonna-Romano et al., 2009).

A link between "Inflamm-aging" and adaptive immune responses may be identified in the expression of chemokine receptors. Recently, we have shown that naïve and memory B cells from young and elderly donors express different chemokine receptors. Focusing on DN B cells, we have described the higher expression of CCR6 and CCR7 in elderly donors, while the same subset obtained by young subjects have revealed the sole expression of CXCR3 (Bulati et al., 2014). The meaning of the changing in the chemokine receptors profile might be related to the inflammatory environment, typical of ageing, rendering these cells more sensitive to pro-inflammatory molecules and cytokines (Singh and Newman, 2011; Salvioli et al., 2013) and in this way DN B cells become able to migrate in inflamed sites (Bulati et al., 2014).

Moreover, we have hypothesized that DN might be an exhausted pool of memory B cells (Colonna-Romano et al., 2009) because of their low replicative ability after *in vitro* stimulation with physiological (α-CD40/IL-4; CpG) or not-physiological strong stimulus (CpG/PMA/Ionomycin). Recently, we have shown as these cells are not able to produce cytokines such as TNF-α and IL-10 (Buffa et al., 2011), even if others reported that DN B cells can be stimulated *in vitro* to secrete immunoglobulins against tetanus toxoid and influenza virus (Wirths and Lanzavecchia et al., 2005).

Two different groups (Moir et al., 2008; Hao et al., 2011) have described other two exhausted B cell populations. The first group have observed, in HIV-viraemic patients, the increase of a population of memory, tissue-like memory B cells ($CD20^{+}CD27^{-}CD21^{-}$), that express significantly high levels of CXCR3 and CXCR6, and low levels of CCR7, CD62L and CXCR5 than classical memory or naïve B cells. Furthermore, tissue-like cells are characterized by the expression at high levels of inhibitory receptors, compared with memory and naïve B cells, such as CD307d, CD22 and CD72 that, probably, prevent the proliferation capacity of these B cells in response to B cell stimuli (Moir et al., 2008). Hao's group (2011) has described a mature B cell subset that accumulates with age in old mice. They lack the

expression of CD21/35, CD23, CD43 and AA4.1, and are also IgD negative. Moreover, these cells express low levels of CD62L and CXCR5 and high levels of CXCR4. This population is not activated, because of low levels of MHC-II and CD86 expression and they seem to be refractory to activation by adaptive immune receptors. Indeed this cells poorly respond to BCR and CD40 triggering, but they yet respond to TLR9 or TLR7 ligation and proliferate most actively to combined TLR and BCR stimuli (Hao et al., 2011). In the present study, to better understand whether the low proliferative capacity of DN B cells after *in vitro* stimulation may be related to the expression of inhibitory receptors, such as exhausted tissue-like memory B lymphocytes in HIV-viraemic individuals (Day et al., 2006; Trautmann et al., 2006), we have decided to analyze cell surface expression of CD307d and CD22 in naïve and memory B cells in young and elderly donors. In particular, CD307d (FcRL4), is a protein that has the capacity to inhibit BCR signaling by binding to SHP-1 and SHP-2 (Ehrhardt et al., 2005), that is exclusively presents on B cells, although it is not found in the earliest stages of bone marrow B cell development. Low level of CD307d has been found on mature and germinal centre B cells, but higher expression of this molecule has been observed in a subpopulation of memory B cells (Matesanz-Isabel et al., 2011). It has been suggested that $CD307d^+$ B cells could represent a specialized tissue subpopulation of memory B cells (Ehrhardt et al., 2005). Thus, it would be interesting to determine whether CD307d is a good marker for identifying different memory B-cell subsets. In this regard, Moir et al. (2008) report that a population of memory B cells ($CD20^+CD27^-CD21^-$) positive for FcRL4 ($CD307d^+$) have an increased frequencies in the peripheral blood of human HIV-viraemic patients and have an "exhausted" phenotype. But, in healthy individuals $FcRL4^+$ B cells are largely restricted to mucosal tissues and mesenteric lymph nodes, and are very rare in peripheral blood, lymph nodes and the spleen (Ehrhardt et al., 2005). These cells do not express the typical memory B cells marker, CD27, almost all of them have undergone class switching, mainly to IgG, whereas a smaller proportion express IgA and they lack the expression of markers that identify germinal centre B cells and plasma cells (Ehrhardt et al., 2005; Falini et al., 2003). The $CD307d^+$ B cells detected by Moir et al. (2008) share many phenotypic features with the tissue-based $FcRL4^+$ memory B cells, indicating that, in the disease situation of viraemic HIV-positive patients, $FcRL4^+$ B cells might exit from mucosal tissues and circulate through the blood. Another intriguing feature is that reported by Cagigi et al. (2009). Indeed they also found a significant increase in percentage of a not classical IgG^+CD27^- or IgA^+CD27^- memory B cell

population in HIV-infected patients, that could be the same population, identified with different phenotypic markers by Moir et al. (2008).

Concerning CD22, is a type I membrane protein that is expressed at low levels on pre- and immature B cells and maximally on mature B cells (Nitschke et al., 1997). Furthermore, it is an inhibitory coreceptor of the BCR (Sieger et al., 2013) and, like other co-receptors, is required to modulate the antigen receptor signal in response to the stimuli coming from the local microenvironment (Cyster and Goodnow, 1997). This molecule was originally identified as a B-cell-associated adhesion protein that appeared to function in the regulation of B-cell activation, but the ability of CD22 to inhibit antigen-induced signaling depends upon its proximity to the BCR (Walker and Smith, 2008). Indeed, while CD22 appears to inhibit signals that derive from the BCR, it might also initiate positive signals when react to itself, or perhaps with other surface receptors (Walker and Smith, 2008).

The evaluation of the expression of BCR inhibitory receptors CD307d and CD22 in all B subpopulations in young and elderly donors revealed that there are not differences between the two age groups studied, with the exception of for CD307d on memory unswitched from elderly subjects. In this way, the reduced ability of B lymphocytes from old donors to respond to immune challenge cannot be fully attributed to a mechanism lying on the expression of these molecules. It is interesting to note that, differently to tissue-like B cells identified by Moir et al.,(2008), DN B cells do not express CD307d. This result might suggest that tissue-like B cells and DN B cells are differently controlled in their ability to proliferate after BCR engagement.

As DN B lymphocytes do not express inhibitors receptors, to understand whether these cells might be a by-product of B cell activation or whether they might play any role in the immune response, we have decided to stimulated B cells *in vitro* by CpG alone, then α-IgG/CD40 and also the contemporary engagement of adaptive and innate immune receptors (BCR and TLR9). Our results show that in young subjects total $CD19^+$ cells and all B subsets respond after stimulation in vitro albeit at different levels. Indeed, they proliferate at higher levels with triggering of BCR/TLR9, respectively less with adaptive or innate stimuli. In old donors, the situation is different because $CD19^+$ lymphocytes and naïve, memory unswitched and double negative B cells do not proliferate after physiological stimuli (CpG alone, then α-IgGCD40) but proliferate with the double stimulation although al lower levels observed in young donors. This could reflect that simultaneous

BCR and TLR stimulation might activate some pathway involved in immune function.

Telomerase is a ribonucleoprotein enzyme that plays an essential role in cell proliferation by compensating for the loss of telomere (Greider et al, 1998) and protecting chromosomes (Epel et al., 2010). Its activity is highly regulated, indeed it is constitutively expressed in germ line cells (Atzmon G et al., 2010; Martens et al., 2002; Weng et al., 1997) and in tumor cells while is absent in most of human somatic cells (Atzmon G et al., 2010; Kim et al., 1994), exceptlymphocytes that are the unique cellular population characterized by high telomerase expression even during advanced phases of differentiation.Moreover, during resting phases B and T subpopulations express h-TERT at low levels, but upon activation a significant increase is observed (Lobetti-Bodoni et al., 2010; Son et al., 2003;Weng et al., 1997; Donaldson et al., 1999). Additionally, it is known that the chronic life stress (Damjanovic et al., 2007; Epel et al., 2004) is cause of shortening of telomeres and both raised (Damjanovic et al., 2007) and decrease of telomerase activity (Epel et al., 2004). Nowadays, the meaning of this paradox is unclear. Generally, telomerase activity is under dynamic control. For example, the acute stress, e.g. cortisol (Choi et al., 2008) may compromise its activity while mitogenic stimulation, e.g. antigen stimulation for B lymphocytes (Igarashi and Sakaguki, 1997), may induce itself (Epel et al., 2010). In fact, telomerase activation occurs in conjunction with cell activation after the encounter with a cognate antigen (Dolcetti and De Rossi, 2012).

It is also known that B lymphocytes have longer telomeres and higher telomerase activity than T cells (Dolcetti and De Rossi, 2012; Martens et al., 2002; Weng et al., 1997). In particular, with regard to B subtype, germinal centre B cells, a subpopulation with extensive clonal expansion and antigen-driven selection, show longer telomeres than those of naïve and memory lymphocytes and also that $CD27^+$ memory B cells have longer telomeres than $CD27^-$ population (Dolcetti and De Rossi, 2012; Martens et al., 2002; Weng et al., 1997).

In agreement with these evidence, our group have demonstrated that DN B cells and memory B cells have shorter telomeres than naïve B cells. Especially in elderly people, classical memory (IgD^-CD27^+) and double-negative B cells (IgD^-CD27^-), that have a very similar telomere length, have shorter telomere compared to young subjects (Colonna-Romano et al., 2009). An open debate about the role of shortened telomeres and decrease telomerase activity in the

aging of immune system is the main aim of investigations in immunosenescence (Lobetti-Bodoni et al., 2010; Son et al., 2000). For this reason, and because of it is believed that the up-regulation of telomerase might play a role in maintaining lymphocytes replicative potential and function (Son et al., 2003) we have examined whether relative telomerase activity was induced after stimulation with CpG/α-IgG/CD40 in CD19+ cells in young donors and in DN B lymphocytes in young and elderly subjects. We have seen that in stimulated B cells from young people the relative telomerase activity approximately increased about 200 fold and also that in double negative B cells the levels of induced telomerase activity was higher in young than elderly. Further, we have expanded this study to centenarian offspring and subject affected by a severe form of Alzheimer's disease. It is known that the CO are a good model to study successful aging inasmuch have favourable background, escaping the main age-related disease. Indeed, they have a good reservoir of naïve B cells that allow them to keep fighting off new infections. As we have previously demonstrated, CO also have less double negative B lymphocytes than their age-related counterpart that are not genetically advantaged for longevity (Colonna-Romano et al., 2010). On the other hand, Alzheimer's disease is the most common cause of dementia in the elderly subjects and it is estimated that the number of affected individuals is predicted to triplicate by 2050 (Wimo et al., 2003; Rubio-Perez et al., 2012; Hebert et al., 2000; Martorana et al., 2012). Moreover, it has been demonstrated that AD patients show many modifications of immune and inflammatory systems (Pellicanò et al., 2012; Larbi A et al., 2009; Richartz-Salzburger E et al., 2007). Particularly, our group and others (Martorana A et al., 2012; Pellicanò et al., 2010; Speciale L et al., 2007; Xue et al., 2009) have reported a decrease both in percentage and absolute number of total B cells from AD patients when compared to their age-matched healthy controls. Recently, we have also observed the increase of DN B cells in severe AD subjects (manuscript in preparation). Furthermore, it is known that telomerase seems to be protect neurons from amyloid Aβ-induced apoptosis (Zhu et al., 2000; Panossian et al., 2003) and that there are a significant telomeres shortening in PBMC from AD than age-matched controls (Panossian et al., 2003). Here, we have observed that, after stimulation, telomerase activity of double negative B cells in centenarian offspring was higher than healthy elderly and lower than young subjects so we confirm that they behave halfway between young and elderly people. Differently, we have seen that stimulation induced telomerase reactivation of double negative B cells of AD patients but at lower level than other groups studied.

Taken together, our data suggest that if properly stimulated, DN B cells acquire the ability to begin new cellular cycles as demonstrated by RTA values. Age is not *per se* a limiting factor for the telomerase function, although it has influence on the level of expression of this enzyme. The monitoring level of the enzymatic function such as other functional analysis (cytokine and chemokine expression, production of pro- and anti-inflammatory mediators, cytotoxic activity, etc) might represent a rapid and immediate additional tool for monitoring the physiologic and pathological ageing process.

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Authorship

Contribution: A.M. and G.C.-R designed the experiments; A.M. and CR.B.performed experiments; A.M., S.B. and M.B. analyzed the data; G.C.-R. provided material necessary for performing experiments; A.M., S.B. G.C.-R wrote the manuscript; all authors have seen and approved the final draft of the manuscript.

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Correspondence: Giuseppina Colonna-Romano, Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Corso Tukory 211 Palermo, 90134 Italy; e-mail: giuseppina.colonnaromano@unipa.it.

References

- Agematsu, K., Hokibara,S., Komiyama, A., 2000. CD27: a memory B-cell marker. Immunol. Today, 204-206.
- Anolik JH, Barnard J, Cappione A, Pugh-Bernard AE, Felgar RE, Looney RJ, Sanz I. Rituximab improves peripheral B cell

abnormalities in human systemic lupus erythematosus. Arthritis Rheumat. 40:3538-3590 (2004)

- Atzmon G, Cho M, Cawthon RM, Budagov T, Katz M, Yang X, Siegel G, Bergman A, Huffman DM, Schechter CB, Wright WE, Shay JW, Barzilai N, Govindaraju DR, and Suh Y. Genetic variation in human telomerase is associated with telomere length in Ashkenazi centenarians. PNAS 2010;107:1710-1717.
- Buffa S, Bulati M, Pellicanò M, Dunn-Walters DK, Wu YC, Candore G, Vitello S, Caruso C, Colonna-Romano G. B cell immunosenescence: different features of naive and memory B cells in elderly. Biogerontology. 12(5):473-483 (2011)
- Bulati M, Buffa S, Martorana A, Candore G, Lio D, Caruso C, Colonna-Romano G. Trafficking phenotype and production of Granzyme B by Double Negative B cells (IgG(+)IgD(-)CD27(-)) in the elderly. Exp Gerontol. 2014 Jan 2. pii: S0531-5565(13)00367-7. doi: 10.1016/j.exger.2013.12.011. [Epub ahead of print].
- Bulati M, Buffa S, Candore G, Caruso C, Dunn-Walters DK, Pellicanò M, Wu YC, Colonna Romano G. B cells and immunosenescence: a focus on IgG+IgD-CD27- (DN) B cells in aged humans. Ageing Res Rev. 2011 Apr;10(2):274-84.
- 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 hyper-mutated antibodies during chronic HIV-1 infection. PLoS One 4(5), e5427.
- Cancro MP, Hao Y, Scholz JL, Riley RL, Frasca D, Dunn-Walters DK, Blomberg BB (2009) B cells and ageing: molecules and mechanisms. Trends Immunol 30: 313–318.
- Choi J, Fauce SR, Effros RB. Reduced telomerase activity in human T lymphocytes exposed to cortisol. Brain Behav Immun. 2008 May;22(4):600-5.
- 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 Develop. 130, 681-690.
- Colonna-Romano G, Buffa S, Bulati M, Candore G, Lio D, Pellicanò M, Vasto S, Caruso C. B cells compartment in centenarian offspring and old people. Curr Pharm Des. 2010;16(6):604-8.

- Colonna Romano G, Bulati M, Aquino A, Scialabba G, Candore G, Lio D, Motta M, Malaguarnera M, Caruso C. B cells in the aged: CD27, CD5 and CD40 expression. Mech Ageing Dev. 124:389-393 (2003).
- Cyster JG, Goodnow CC. Tuning antigen receptor signaling by CD22: integrating cues from antigens and the microenvironment. Immunity. 1997 6(5):509-17.
- Damjanovic AK, Yang Y, Glaser R, Kiecolt-Glaser JK, Nguyen H, Laskowski B, Zou Y, Beversdorf DQ, Weng NP. Accelerated telomere erosion is associated with a declining immune function of caregivers of Alzheimer's disease patients. J Immunol. 2007, 179(6):4249-54.
- Day, C.L., D.E. Kaufmann, P. Kiepiela, J.A. Brown, E.S. Moodley, S. Reddy, E.W. Mackey, J.D. Miller, A.J. Leslie, C. DePierres, et al. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature. 443:350–354.
- Dolcetti R, De Rossi A. Telomere/telomerase interplay in virus-driven and virus-independent lymphomagenesis: pathogenic and clinical implications.Med Res Rev. 2012 Mar;32(2):233-53. doi: 10.1002/med.20211. Epub 2010 Jun 14.
- Donaldson L, Fordyce C, Gilliland F, Smith A, Feddersen R, Joste N, Moyzis R, Griffith J. Association between outcome and telomere DNA content in prostate cancer.J Urol. 1999 Nov;162(5):1788-92.
- Ehrhardt, G.R., Hsu, J.T., Gartland, L., Leu, C.M., Zhang, S., Davis, R.S., Cooper, M.D., 2005. Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells. J. Exp. Med. 202, 783–791.
- Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, Cawthon RM. Accelerated telomere shortening in response to life stress. Proc Natl Acad Sci USA. 2004, 101(49):17312-5.
- Epel ES, Lin J, Dhabhar FS, Wolkowitz OM, Puterman E, Karan L, Blackburn EH. Dynamics of telomerase activity in response to acute psychological stress.Brain Behav Immun. 2010 May;24(4):531-9. doi: 10.1016/j.bbi.2009.11.018. Epub 2009 Dec 16.
- 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 JF, Côté G, Néron S. 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 (2006).

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

- Frasca, D., and Blomberg, B.B., 2011. Aging Affects Human B Cell Responses. J. Clin. C., 2005. Innate immunity and inflammation in ageing: a key for understanding age-related diseases. Immun. Ageing 18, 2-8.
- Frasca D, Diaz A, Romero M, Landin AM, Phillips M, Lechner SC, Ryan JG, Blomberg BB (2010) Intrinsic defects in B cell response to seasonal influenza vaccination in elderly humans.Vaccine 28(51):8077-8084

- 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., Riley, R.L., and Blomberg, B.B., 2004. Effect of age on the immunoglobulin class switch. Crit. Rev. Immunol. 24, 297-320.
- Greider CW. Telomeres and senescence: the history, the experiment, the future. Curr Biol. 1998 Feb 26;8(5):R178-81. Review.

- Gupta S, Su H, Bi R, Agrawal S, Gollapudi S. Life and death of lymphocytes: a role of immunosenescence. Immun Ageing. 23:2-12 (2005)
- 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.

- Harris, D. P., S. Goodrich, A. J. Gerth, S. L. Peng, and F. E. Lund. 2005a. Regulation of IFN-γ production by B effector 1 cells: essential roles for T-bet and the IFN-γ receptor. J. Immunol. 174: 6781-6790.

- Harris D. P., S. Goodrich, K. Mohrs, M. Mohrs, and F. E. Lund. 2005b. Cutting edge: the development of IL-4-producing B cells (B effector 2 cells) is controlled by IL-4, IL-4 receptor α, and Th2 cells. J. Immunol. 175: 7103–7107.
- Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, Johnson LL, Swain SL, Lund FE. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol. 2000 Dec;1(6):475-82. PubMed PMID: 11101868.
- Hebert LE, Scherr PA, Bienias JL, Bennett DA, Evans DA: Alzheimer disease in the US population: prevalence estimates using the 2000 census. Arch Neurol 2003, 60:1119–1122.
- Igarashi H, Sakaguchi N. Telomerase activity is induced in human peripheral B lymphocytes by the stimulation to antigen receptor. Blood. 1997, 89(4):1299-307.
- 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.
- Kim NW, Wu F. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). Nucleic Acids Res 1997;25:2595–2597.
- Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW.Specific association of human telomerase activity with immortal cells and cancer. Science. 1994 Dec 23;266(5193):2011-5.
- Klein U., Rajewsky K , Kuppers R., 1998. Human immunoglobulin (Ig)M+ IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as general marker for somatically mutated (memory)B cells. J.Exp. Med.188,1679-1689.
- Larbi A, Pawelec G, Witkowski JM, Schipper HM, Derhovanessian E, Goldeck D, Fulop T: Dramatic shifts in circulating CD4 but not CD8 T cell subsets in mild Alzheimer's disease. J Alzheimers Dis 2009, 17:91–103.
- Licastro F, Candore G, Lio D, Porcellini E, Colonna-Romano G, Franceschi C, Caruso C.Innate immunity and inflammation in ageing:

a key for understanding age-related diseases. Immun Ageing. 2005 May 18;2:8.

- Lobetti-Bodoni C, Bernocco E, Genuardi E, Boccadoro M, Ladetto M. Telomeres and telomerase in normal and malignant B-cells. Hematol Oncol. 2010 Dec;28(4):157-67. doi: 10.1002/hon.937. Review.
- Martens UM, Brass V, Sedlacek L, Pantic M, Exner C, Guo Y, Engelhardt M, Lansdorp PM, Waller CF, Lange W. Telomere maintenance in human B lymphocytes. Br J Haematol. 2002 Dec;119(3):810-8.
- Martin F, Chan AC. B cell immunobiology in disease: evolving concepts from the clinic.Annu Rev Immunol. 2006;24:467-96.
- Martorana A., Bulati M., Buffa S., Pellicanò MV, Caruso C., Candore G, Colonna-Romano G. Immunosenescence, inflammation and Alzheimer's disease.Longevity & Healthspan 2012, 1:8 doi:10.1186/2046-2395-1-8 Wimo A, Jonsson L, Winblad B: An estimate of the worldwide prevalence and direct costs of dementia in 2003. Dement Geriatr Cogn Disord 2006, 21:175–181.
- Matesanz-Isabel J, Sintes J, Llinàs L, de Salort J, Lázaro A, Engel P. New B-cell CD molecules. Immunol Lett. 2011, 134(2):104-12.
- McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinicaldiagnosis of Alzheimer’sdisease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer’sDisease. Neurology 1984;34:939-944.
- 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 AC, O'Shea MA, Roby G, Kottilil S, Arthos J, Proschan MA, Chun TW, Fauci AS. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. J Exp Med. 2008 Aug 4;205(8):1797-805.
- Nitschke L, Carsetti R, Ocker B, Köhler G, Lamers MC. CD22 is a negative regulator of B-cell receptor signalling.Curr Biol. 1997 Feb 1;7(2):133-43.
- 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.

- Panossian L.A., Porter V.R., Valenzuela H.F., Zhua X., Erin Reback , Masterman D., Cummingsb J.L., Effros R.B.. Telomere shortening in T cells correlates with Alzheimer's disease status. Neurobiology of Aging 24 (2003) 77–84.
- Pawelec, G., and 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.
- Pellicanò M, Larbi A, Goldeck D, Colonna-Romano G, Buffa S, Bulati M, Rubino G, Iemolo F, Candore G, Caruso C, Derhovanessian E, Pawelec G: Immune profiling of Alzheimer patients. J Neuroimmunol 2012, 242:52–59.
- Pellicanò M, Bulati M, Buffa S, Barbagallo M, Di Prima A, Misiano G, Picone P, Di Carlo M, Nuzzo D, Candore G, Vasto S, Lio D, Caruso C, Colonna-Romano G: Systemic immune responses in Alzheimer's disease: in vitro mononuclear cell activation and cytokine production. J Alzheimers Dis 2010, 21:181–192.
- Richartz-Salzburger E, Batra A, Stransky E, Laske C, Köhler N, Bartels M, Buchkremer G, Schott K: Altered lymphocyte distribution in Alzheimer's disease. J Psychiatr Res 2007, 41:174–178.
- Rubio-Perez JM, Morillas-Ruiz JM: A review: inflammatory process in Alzheimer's disease, role of cytokines. ScientificWorldJournal 2012, 2012:756357.
- 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.

- Sanz I, Anolik JH, Looney RJ. B cell depletion therapy in autoimmune diseases. Front Biosci. 12:2546-2567 (2007)
- Schenkein JG, Park S, Nahm MH (2008) Pneumococcal vaccination in older adults induces antibodies with low opsonic capacity and reduced antibody potency. Vaccine 26:5521–5526
- Shaw AC, Joshi S, Greenwood H, Panda A, Lord JM. Aging of the innate immune system. Curr Opin Immunol. 2010; 22: 507-13.
- ShiY, Agematsu K, Ochs HD, Sugane K. Functional analysis of human memory B-cell subpopulations: IgD+CD27+ B cells are crucial in secondary immune response by producing high affinity IgM. Clin Immunol 2003;108: 128-37.
- Sieger N, Fleischer SJ, Mei HE, Reiter K, Shock A, Burmester GR, Daridon C, Dörner T. D22 ligation inhibits downstream B cell receptor signaling and Ca(2+) flux upon activation. Arthritis Rheum. 2013 Mar;65(3):770-9. doi: 10.1002/art.37818.
- Singh, T., Newman, A.B., 2011. Inflammatory markers in population studies of aging. Ageing Res. Rev. 10(3), 319-329.
- Son NH., Murray S., Yanovski j., Hodes RJ., Weng N., 2000.Lineage-specific telomere shortening and unaltered capacity for telomerase expression in human t and B lymphocytes with age. J Immunol., 1191-1196.
- Son NH, Joyce B, Hieatt A, Chrest FJ, Yanovski J, Weng NP.Stable telomere length and telomerase expression from naïve to memory B-lymphocyte differentiation.Mech Ageing Dev. 2003 Apr;124(4):427-32.
- Speciale L, Calabrese E, Saresella M, Tinelli C, Mariani C, Sanvito L, Longhi R, Ferrante P: Lymphocyte subset patterns and cytokine production in Alzheimer's disease patients. Neurobiol Aging 2007, 28:1163–1169.
- Tangye SG, Hodgkin PD. Divide and conquer: the importance of cell division in regulating B-cell responses. Immunology. 2004 Aug;112(4):509-20.
- Trautmann, L., L. Janbazian, N. Chomont, E.A. Said, S. Gimmig, B. Bessette, M.R. Boulassel, E. Delwart, H. Sepulveda, R.S. Balderas, et al. 2006. Upregulation of PD-1 expression on HIV-specific CD8+ T

cells leads to reversible immune dysfunction. Nat. Med. 12:1198–1202.

- 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.
- Veneri, D., Ortolani, R., Franchini, M., Tridente, G., Pizzolo, G., Vella, A., 2009. Expression of CD27 and CD23 on peripheral blood B lymphocytes in humans of different ages. Blood Transfus. 7, 29–34.
- Walker JA, Smith KG.CD22: an inhibitory enigma.Immunology. 2008, 123(3):314-25.
- 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.
- Weng NP, Granger L, Hodes RJ. Telomere lengthening and telomerase activation during human B cell differentiation. Proc Natl Acad Sci USA 1997; 94: 10827–10832.
- Wimo A, Jonsson L, Winblad B. An estimate of the worldwide prevalence and direct costs of dementia in 2003. Dement Geriatr Cogn Disord. 2006;21(3):175-81.
- 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.
- Xue SR, Xu DH, Yang XX, Dong WL: Alterations in lymphocyte subset patterns and co-stimulatory molecules in patients with Alzheimer disease. Chin Med J (Engl) 2009, 122:1469–1472.
- Youinou P, Jamin C, Pers JO, Berthou C, Saraux A, Renaudineau Y. B lymphocytes are required for development and treatment of autoimmune diseases. Ann N Y Acad Sci. 2005 Jun;1050:19-33.
- Zhu H, Fu W, Mattson MP. The catalytic subunit of telomerase protects neurons against amyloid beta-peptide-induced apoptosis. J Neurochem 2000;75:117–24.

CHAPTER 7

"Immunophenotype and trafficking profile in Alzheimer's disease patients"

(Manuscript in preparation)

Manuscript in preparation

Immunophenotype and trafficking profile in Alzheimer's disease patients

Bulati M[1*], Buffa S[1*], Martorana A[1], Gervasi F[2], Camarda C[3], Azzarello DM[3], Colonna-Romano G[1]

[1]Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies (DIBIMEF), University of Palermo

[2]Laboratory of experimental Oncohematology and cell cultures for clinical use, ARNAS CIVICO, Palermo

[3]Department of Experimental Biomedicine and Clinical Neuroscience, University of Palermo

*These authors contributed equally to this work.

Abstract

Alzheimer's Disease (AD) is the most common cause of dementia. It is known that, other than age, the systemic inflammation represent one of the risk factor for this pathology. Recent evidences suggest that it is not only the central nervous system (CNS) that can be blamed for inflammatory response in AD, but also cells from periphery. The chronic stimulus given by the accumulation of Amyloid β, that characterize the Alzheimer's disease, may trigger the immune reactions inducing a chronic inflammation. In the brain tissue, there is an activation of adaptive immunity, that try to eliminate the initially insult both from brain and periphery. This inflammatory scenario could lead towards a recruitment of myeloid and lymphocytic cells into the CNS. As the immune status in the periphery is strictly linked with the brain environment, the study of the different peripheral lymphocyte subpopulations in AD, results to be important. In our previously study, it has been demonstrated a decrease of total $CD19^+$ B cells in AD patients when compared with their healthy age-matched controls, suggesting the involvement of B cells in AD. Moreover, we have demonstrated a different distribution of B lymphocyte subpopulations in elderly subjects, in which the pro-inflammatory milieu seems to influence the profile of trafficking receptor in the increased Double Negative (DN, IgD^-CD27^-) B cell population. Thus, in this study

we have assessed the distribution of specific naïve/memory B lymphocyte subpopulations in severe and mild AD patients comparing them with their age-matched controls. Furthermore, we have evaluated in DN B cells, the expression of some chemokine receptors involved in cell migration toward inflamed tissue in AD patients and in healthy elderly age-matched controls. Here, we show that there is a significant decrease of naïve ($IgD^{+}CD27^{-}$) B cells and a significant increase of DN B cells in severe AD subject compared with healthy age-matched control, while mild AD subjects behave as their age-matched healthy controls. Besides, we also show that DN B cells in AD patients have an enhanced pro-inflammatory trafficking profile. Indeed, the increased expression of the chemokine receptor CCR6 seems to depend on the severity of the pathology, while CCR7, increase only in severe AD patients, supporting the hypothesis that the increase of DN B cells is correlated with chronic inflammation. Thus, we conclude that AD status might affects the distribution of specific naïve and DN memory B cells and that might also influence the pro-inflammatory profile of trafficking receptor in DN B cells, driving these lymphocytes to inflamed brain tissue.

Introduction

Alzheimer's disease is the most common cause of dementia involving about 13 million people worldwide. This pathology, that leads a loss, usually progressive and severe, of brain function, does not represent only a medical but also a social problem. The economic impact of dementia is enormous. Globally, costs for people with dementia is over than 1% of gross domestic product (GDP) (Wimo et al., 2010; de Vugt and Verhey, 2013).

The single greatest risk factor for Alzheimer's disease is age. While the disease can occur in younger people, even in their 30s and 40s, the risk grows considerably after age 65, and it is estimated that 50% of those who pass their eightieth birthday will be stricken. Indeed, during aging, brain is massively exposed to a great variety of stressors, such as trauma, oxidative stress, tissues damages, inflammation, which may be correlated with the beginning of the neuro-degeneration (Fulop et al., 2013a, 2013b). Pathological changes in the AD brain include neuronal and synapse loss, senile plaques and neurofibrillary tangles, that are normally present in normal brain aging, but in AD they are more severe and, at the beginning, the degeneration involves

specific regions of the cerebral cortex, as hippocampus, entorhinal and temporoparietal cortex (Sardi et al., 2011). These areas are important not only for memory, but also for other cognitive functions. The crucial step in AD pathogenesis is the production of amyloid beta (Aβ), that is the result of cleavage of a larger peptide, named amyloid precursor protein (APP), which is overexpressed in AD (Griffin, 2006). The highly insoluble and proteolysis-resistant fibrillar 42-aminoacid form of β-amyloid ($A\beta_{42}$ peptide), leads to a formation of senile plaques that accumulate and deposit in the parenchyma brain of the AD patients. Even if the elderly are the most involved population, aging alone cannot be considered as the only cause of this disease. There are other risk factors besides age: family history of Alzheimer's, stress, serious illness or injury, inadequate physical and social activity and poor diet (Fulop et al., 2013a, 2013b). A number of additional pathogenic mechanisms that overlap with Aβ plaques and neurofibrillary tangles include inflammation (Strous and Shoenfeld, 2006), oxidative damage (Evseev et al., 2001), iron deregulation (Combarros et al., 2005), mitochondrial dysfunction (Arshavsky, 2006). Inflammation clearly occurs in pathologically vulnerable regions of the AD brain, indeed senile plaques result from the accumulation of several other proteins and a chronic inflammatory reaction around deposits of amyloid. Microglia, astrocytes, and neurons are responsible for the inflammatory reaction. Activated cells strongly produce inflammatory mediators such as pro-inflammatory cytokines, chemokines, macrophage inflammatory proteins, monocyte chemo-attractant proteins, prostaglandins, leukotrienes, thromboxanes, coagulation factors, reactive oxygen species, nitric oxide, complement factors, proteases, protease inhibitors, pentraxins, and C-reactive protein (Veneri et al., 2009; Appay et al., 2011; Bulati et al., 2011; Frasca et al., 2012; Pawelec et al., 2012). The microglia activation can be due to local or systemic inflammation. In fact, a strong local inflammatory stimulus, such as a previous head trauma, is a risk factor for AD and several epidemiological studies clearly show that blood elevations of acute phase proteins, markers of systemic inflammatory stimuli, may be risk factors for cognitive decline and dementia. For a long time lymphocytes were not considered as major players in brain immunity and consequently in neurodegenerative diseases. Recent evidences suggest that it is not only the central nervous system (CNS) that can be blamed for inflammatory response in AD, but also cells from periphery (Bonotis et al., 2008; Ciccocioppo et al., 2008; Miscia et al., 2009; Liu et al., 2010; Pellicanò et al., 2010, 2012; Martorana et al., 2012). It is also known that in AD, the integrity of blood brain barrier (BBB), which normally act protecting and isolating the brain

from organism's immune reactions, is compromised by multiple microtrauma, microvascular pathologies and inflammation. This results in an increased permeability of BBB that leads to the abolition of the immunological privilege of CNS. It is now well accepted that adaptive immunity plays a role in normal brain physiology as well as in neurodegenerative diseases (Kipnis et al., 2004, 2008; Schwartz and Schechter, 2010). The chronic stimulus given by the accumulation of Aβ, or other insults that characterize the Alzheimer's disease, may trigger the immune reactions inducing a chronic inflammation, not only in the brain tissue but also in periphery. Herein, the immune system seeks to eliminate the overproduced Aβ by mounting an acute inflammatory response (Feng and Sun, 2011). This results in an activation of adaptive immunity, production of specific antibodies and T cells activation to eliminate the initially insult both from brain and periphery. This inflammatory scenario could lead towards a recruitment of myeloid and lymphocytic cells into the CNS (Britschgi and Wyss-Coray, 2007). So, in this context it has been demonstrated the presence of both $CD4^+$ and $CD8^+$ T lymphocytes in brain parenchyma of AD patients (Town et al., 2005; Li et al., 2009; Fulop et al., 2013a, 2013b). The overall immune response and the persistent microglial activation could result in a chronic inflammation process contributing to AD development and progression (Fulop 2013a, 2013b).

The "peripheral lymphocytes" topic, as a different tool for early diagnosis of AD, has been examined by different groups, even if with conflicting results (Britschgi and Wyss-Coray, 2007; Speciale et al., 2007; Larbi et al., 2009; Pellicanò et al., 2010). A reduction of total $CD3^+$ T cells were demonstrated (Xue et al., 2009). Specific lymphocyte subpopulations have also been investigated, although there are many discordant results (Britschgi and Wyss-Coray, 2007; Speciale et al., 2007; Larbi et al., 2009; Xue et al., 2009; Pellicanò et al., 2010). The involvement of B cells in the complex cellular interactions active in AD patients is suggested by the modification of their B cell compartment when compared with healthy age-matched controls. We have previously demonstrate a decrease, both in percentage and in absolute number, of total $CD19^+$ B cells in AD patients when compared with their healthy age-matched controls (Pellicanò et al, 2010). In this paper we focused on the naïve/memory B cell subsets identified with the combination of the "core" IgD and CD27 surface markers (Kaminski et al., 2012), trying to assess whether the involvement of the B cell branch in AD patients also affects the distribution of specific lymphocyte subpopulations. This is also

based on our previous paper (Colonna-Romano et al., 2009; Buffa et al., 2011; Bulati et al., 2011) in which we showed a different distribution of B lymphocyte subpopulations in elderly subjects, in particular a significant decrease of naïve (IgD^+CD27^-) B cells and a significant increase of Double Negative (DN, IgD^-CD27^-) memory B cells. Moreover, as we have demonstrate in healthy elderly subjects a pro-inflammatory profile of trafficking receptor in DN B cells (Bulati et al., 2014), we evaluated the expression of some chemokine receptors involved in cell migration in AD patients and in healthy elderly age-matched controls. It is known that the combinations of chemokines and their receptors guide the immune cells, and also B cells, to specific tissues. In particular CXCR4, CXCR5, CCR6 and CCR7 have been identified as receptors that drive B cells to lymph node and allow B cells to recirculate, while CXCR3 leads B cells to sites of inflammation (Kunkel and Butcher, 2003). Moreover, many authors report the involvement of CCR6 in the recruitment of immune system cells in sites of inflammation (Schutyser et al., 2003; Comerford et al., 2010; Welsh-Bacic et al., 2011). Concerning the role of CCR7 as pro-inflammatory receptor, its involvement has been shown in autoimmune and infectious diseases, such as rheumatoid arthritis, Helicobacter pylori-induced gastritis, and Sjögren's syndrome (Müller and Lipp, 2003). Besides, the CCR7/CCL19/CCL21 chemokine axis involvement 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).

Surprisingly, both in mild and in severe AD patients, we found a trafficking phenotype of DN B cells consistent with the migration of these cells into the inflamed tissues.

Materials and methods

1. Subjects studied

Fifty Sicilian subjects were studied, 20 severe (age range 65-85 years) and 15 mild (age range 65-91 years) Alzheimer's Disease (AD) patients and 15 healthy age-matched controls (age range 65-81 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, or their care-givers, gave informed consent according to the Italian law.

Healthy controls (HC), randomly selected from the same population as the patient cohort, had complete neurological examinations and were judged to be in good health based on their clinical history and blood tests (complete blood cell count, erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, liver function tests, iron, proteins, cholesterol, triglycerides). The University Hospital Ethics Committee approved the study, and informed consent was obtained from all guardians of patients and controls according to Italian law. Whole blood was collected by venopuncture in vacutainer tubes containing ethylenediamine tetraacetic acid. The samples were kept at room temperature and used within 2 h for the various experiments.

AD subjects were assessed with a multidimensional protocol including: demographic characteristics, medical history, pharmacological treatments, clinical, neuropsychological and neurological examination, standard laboratory blood tests and neuro-imaging study with CT and/or MRI scan. The exclusion criteria were: a diagnosis of severe systemic disorder, the presence of psychosis, a history of significant head injury or substance abuse. Dementia was diagnosed according to DSM-IV criteria [American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders: DSM-IV (Text Revision). Washington, American Psychiatric Association, 2000] and the diagnosis of AD was based on the criteria of the National Institute for Neurological and Communicative Disorders and Stroke-Alzheimer Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann G et al.,1984). According to Mini-Mental State Examination [MMSE] (Folstein et al., 1975), 20 patients were affected by severe dementia (score < 17), whereas 15 were affected by mild-to-moderate grade of dementia (score >17< 24). Patients with vascular dementia were not included in the study. Since treatment with acetyl-cholinesterase inhibitors may modulate cytokine expression (Reale et al., 2004), patients were included before starting that therapy. The clinical protocol included the following: cognitive assessment, functional and behavioural assessment, evaluation of vascular risk factors (VRF) and somatic co-morbidity.

2. Cell preparation

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 $1x10^6$/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.

3. Antibodies and Flow Cytometry panels

PBMCs were stained with different combinations of the following monoclonal antibodies: anti-IgD_{FITC}, anti-$CD196_{PE}$ (CCR6), anti-$CD197_{PE}$ (CCR7), anti-$CD183_{APC}$ (CXCR3), anti-$CD184_{PE}$ (CXCR4, Fusin), anti-$CD185_{PE\text{-}Cy7}$ (CXCR5) (BD, Pharmingen), anti-$CD19_{PB}$, anti-$CD27_{AlexaFluor750}$ or anti-$CD27_{PE\text{-}Cy7}$ (Beckman Coulter, Miami, FL, USA). Cells were washed twice and analyzed. All measurements were made with a CyAN ADP flow cytometer (Beckman Coulter, Miami, FL, USA) with the same instrument setting. At least 10^4 cells were analyzed using FlowJo (Tree Star) software.

4. 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.

Results

Naïve(IgD^+CD27^-) B cells are decreased and Double Negative (IgD^-CD27^-) B cells are increased in Severe Alzheimer's Disease Patients.

We have evaluated the different distribution of the circulating B cells subpopulation in the two different groups of AD patients studied (Severe and

Mild) comparing them one each other and with their age-matched healthy controls. First, as shown in Figure 1, we confirm (Pellicanò et al., 2010) a significant decrease, both in percentage and in absolute number, in total B cell population in severe AD subjects compared to healthy elderly, but no significant difference between these last ones and mild AD patients.

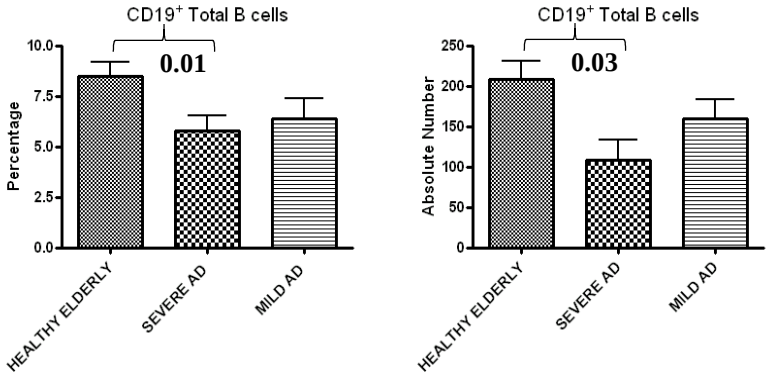
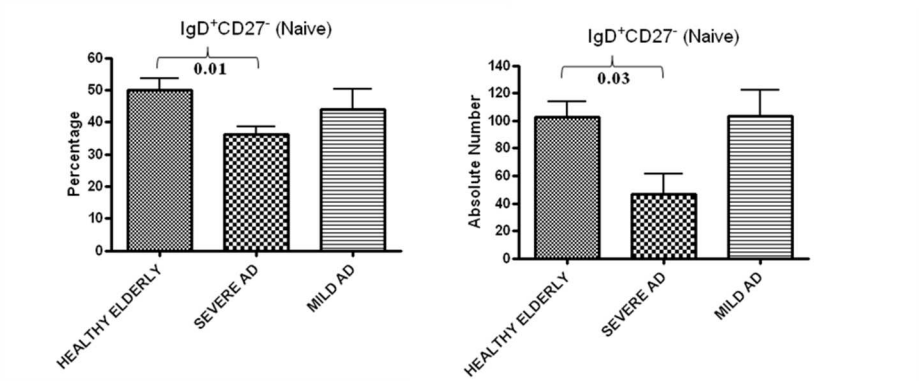


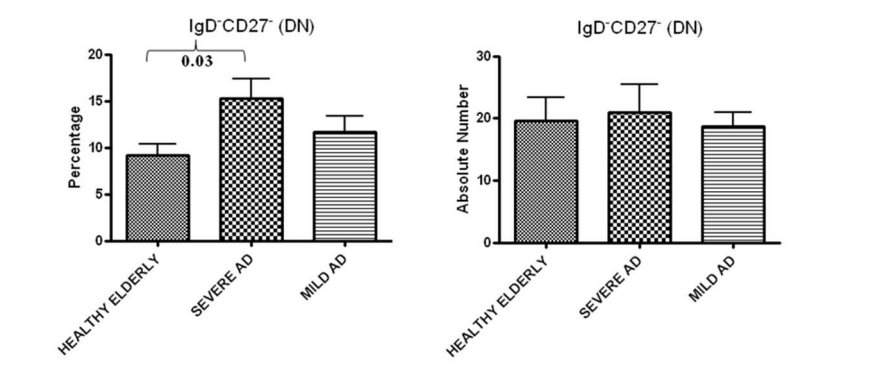
Figure 1. Evaluation of percentage and absolute number of $CD19^+$ B lymphocytes in peripheral blood of healthy elderly, severe AD and mild AD patients. Data are expressed as mean ± SD. Statistically significant differences was evaluated with Mann-Whitney test and is indicated on the top of the histogram, only when it occurs.

Concerning the four B cells subpopulations, identified with the "core" markers IgD and CD27, we have found, in severe AD subjects, a further significant decrease in (IgD^+CD27^-) naïve B cells (Figure 2, panel A), both in percentage and absolute number, and a connected marked increase in Double Negative (IgD^-CD27^-) memory B cells (Figure 2, panel B), in percentage but not in absolute number, compared to age-matched healthy donors,. We did not find any significant differences on Unswitched (Figure 2, panel C) and Switched (Figure 2, panel D) memory B lymphocytes in severe AD patients, while Mild AD patients behave as healthy elderly subjects.

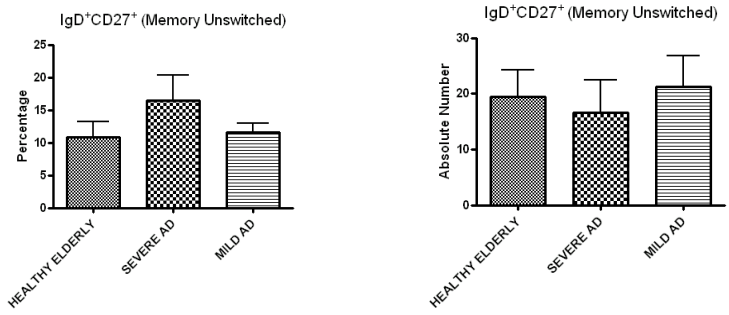
(A)


IgD+CD27- (Naive)
0.01
Percentage
60
50
40
30
20
10
0
HEALTHY ELDERLY
SEVERE AD
MILD AD
IgD+CD27- (Naive)
0.03
Absolute Number
140
120
100
80
60
40
20
0
HEALTHY ELDERLY
SEVERE AD
MILD AD

(B)


IgD-CD27- (DN)
0.03
Percentage
20
15
10
5
0
HEALTHY ELDERLY
SEVERE AD
MILD AD
IgD-CD27- (DN)
Absolute Number
30
20
10
0
HEALTHY ELDERLY
SEVERE AD
MILD AD

(C)


IgD+CD27+ (Memory Unswitched)
Percentage
25
20
15
10
5
0
HEALTHY ELDERLY
SEVERE AD
MILD AD
IgD+CD27+ (Memory Unswitched)
Absolute Number
30
20
10
0
HEALTHY ELDERLY
SEVERE AD
MILD AD

(D)

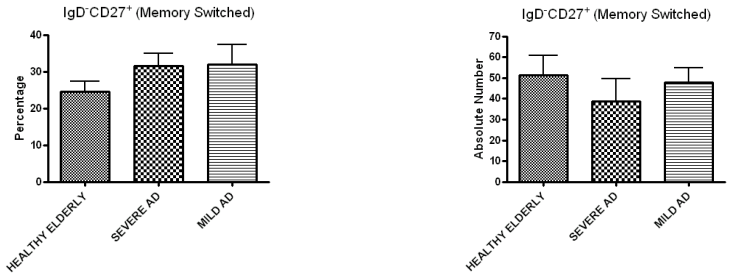


Figure 2. Percentage and absolute number of the four B cell subpopulation, identified by using the "core" markers IgD and CD27, in healthy elderly donors, severe AD patients and mild AD patients. Data are expressed as mean ± SD. Statistically significant differences was evaluated with Mann-Whitney test and is indicated on the top of the histogram, only when it occurs. (A) $IgD^{+}CD27^{-}$ Naïve B cells. (B) $IgD^{-}CD27^{-}$ Double Negative (DN) B cells. (C) $IgD^{+}CD27^{+}$ Unswitched Memory B cells. (D) $IgD^{-}CD27^{+}$ Switched Memory B cells.

Profile of trafficking receptors in DN B cell subpopulation.

In order to evaluate whether also in AD patients, $IgD^{-}CD27^{-}$ DN B cells show the typical pro-inflammatory trafficking profile (Bulati et al., 2014), we have assessed, on these population of lymphocytes, the expression of CCR7, CCR6, CXCR3, CXCR4 and CXCR5 chemokines receptors on healthy elderly donors, severe and mild AD patients. Concerning CXCR4 and CXCR5, we have found, in both groups of AD patients (severe and mild), exactly similar results to those of their age-matched controls, indeed these two chemokines receptors are not present in DN B cells (data not shown). The pro-inflammatory CXCR3 chemokine receptor of all groups studied shows no significant differences between them (data not shown). Surprisingly, we found intriguing results for the other two pro-inflammatory chemokines receptors CCR6 and CCR7. Concerning

CCR6 we show (Figure 3) a significantly increased expression (p= 0.008 healthy elderly vs mild AD; p= 0.006, healthy elderly vs severe AD) of this chemokine receptor related to the severity of Alzheimer's disease. CCR7 expression is significantly increased (Figure 4) in severe AD patients, but not in mild AD subjects (p= 0.02, severe AD vs healthy elderly; p= 0.009, severe AD vs mild AD). A similar behaviour is mirrored in total B cells (Figure 5), suggesting that the expression of these chemokines receptors could be influenced by the pro-inflammatory milieu of AD patients, and that, CCR6 and, probably, CCR7 could drive B cells to inflamed brain tissue.

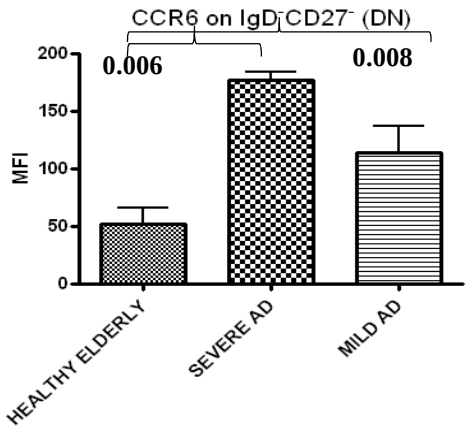


Figure 3. Evaluation of CCR6 expression on $IgD^{-}CD27^{-}$ DN B cells of healthy elderly, severe AD patients and mild AD patients. Data are expressed as mean ± SD of Mean Fluorescence Intensity (MFI). Statistically significant differences was evaluated with Mann-Whitney test and is indicated on the top of the histogram, only when it occurs.

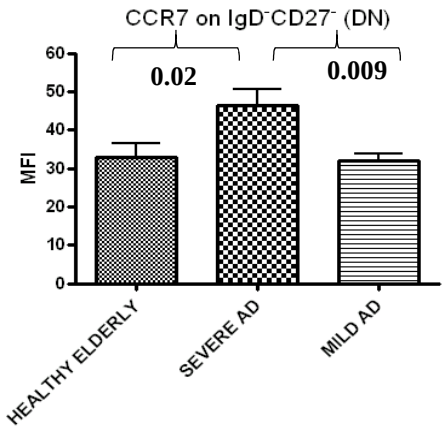


Figure 4. Evaluation of CCR7 expression on IgD$^-$CD27$^-$ DN B cells of healthy elderly, severe AD patients and mild AD patients. Data are expressed as mean ± SD of Mean Fluorescence Intensity (MFI). Statistically significant differences was evaluated with Mann-Whitney test and is indicated on the top of the histogram, only when it occurs.

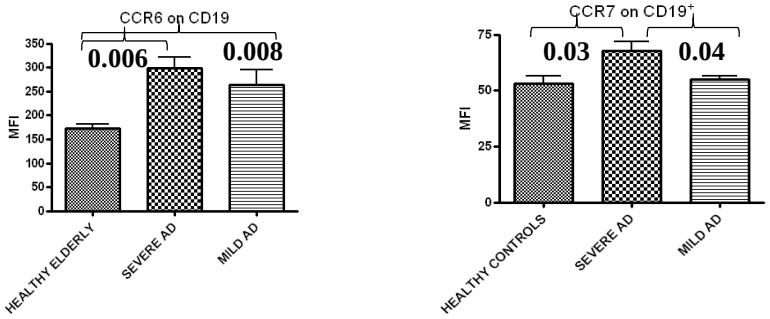


Figure 5. Evaluation of CCR6 and CCR7 expression on CD19$^+$ B cells of healthy elderly, severe AD patients and mild AD patients. Data are expressed as mean ± SD of Mean Fluorescence Intensity (MFI). Statistically significant differences was evaluated with Mann-Whitney test and is indicated on the top of the histogram, only when it occurs.

Discussion

Although Alzheimer's disease is a neurodegenerative pathology, it has been widely demonstrated the involvement of a dysregulation of the immune response in AD pathogenesis. Even if it is still unclear whether the inflammatory processes are a primary or a subsequent event, it has been suggested that flogistic process has an important role in disease development (Sardi et al., 2011). It is also known that in the elderly there is a low-grade chronic and systemic inflammation (inflamm-aging), which is always controlled and asymptomatic, and it can constitute a major determinant of frailty and age-associated diseases (Gupta and Pansari, 2003; Sardi et al., 2011; Salvioli et al., 2013). A growing body of evidences demonstrates that Aβ-plaques induce an inflammatory reaction in the brain (Vom Berg et al., 2012; Heneka et al., 2013; Monsonego et al., 2013), whereas the oligomeric forms of Aβ42 exert synaptotoxicity (Haass and Selkoe, 2007; Walsh and Selkoe, 2007). It has also been shown the pathological effects of Aβ on brain vasculature, that lead to the "cerebral amyloid angiopathy", a phenomenon that causes vascular inflammation, brain haemorrhages, compromised perivascular drainage and altered blood flow (Thal et al., 2008). Moreover, the loss of integrity of BBB, may lead to a passage of proteins in cerebro spinal fluid (CSF), causing a compromised role of BBB in preserving the brain as an "immune sanctuary" (Sardi et al., 2011). Inflammatory processes such as microgliosis, astrocytosis, dystrophic microglia, complement activation, cytokine elevation and acute-phase protein changes are thought to represent a response to the accumulation of Aβ in the vasculature and parenchyma of the brain (Monsonego et al., 2013). The compromised immune system associated with aging may substantially impact on these processes and lead to compromised brain function and neuronal repair processes, which enhance the progression of AD (Monsonego et al., 2013). In AD patients there is an evident systemic inflammation, indeed they present higher than normal levels of pro-inflammatory cytokines and chemokines both in the periphery as well as in the brain (Reale et al., 2008; Lee et al., 2009). So immune system cells can be stimulated by these pro-inflammatory mediators, including also Aβ and various other agents, and the result may be an enhanced immune cell differentiation (Pellicanò et al., 2010, 2012; Goldeck et al., 2013). So, it is clear that there is a link between immune status in the periphery and the brain. Indeed, in AD patients, immune cells migrate

along a chemokine gradient from the periphery through the BBB into the brain (Fiala et al., 1998), or they are recruited in the CNS by other pro-inflammatory mediators, such as Aβ, produced in the inflamed brain of AD (Britschgi and Wyss-Coray, 2007). For these reasons, an accurate study of the phenotype of the different peripheral lymphocytes subpopulation in AD, results to be important for monitoring the stage and the severity of the pathology. To this day, the only way to confirm a diagnosis of Alzheimer's is by autopsy of brain tissue. However, physical, psychological, and neurological examination can lead to a relatively accurate diagnosis, partly by eliminating other possible causes of dementia and partly by identifying the key signs or manifestations of Alzheimer's. Indeed severe and mild-to-moderate AD subjects were included in the study by using cognitive, functional and behavioural assessment and the evaluation of vascular risk factors and somatic co-morbidity. In our previously studies on immunosenescence of the B cell branch, we have demonstrated a significantly decrease in total $CD19^+$ B lymphocytes, both in percentage and in absolute number, and a remodeling of the B cell subpopulation with ageing (Colonna Romano et al., 2009; Bulati et al., 2011). Indeed, we have reported a significantly decrease, in percentage, of the IgD^+CD27^- naïve B cells and a simultaneous increase, in percentage, of the IgD^-CD27^- double negative (DN) memory B lymphocytes. We characterized these DN B cells as a population of switched (IgG^+/IgA^+) memory B cells with short telomeres and they are poorly responder to conventional stimuli, indicating them as senescent cells (Colonna Romano et al., 2009; Buffa et al., 2011). It has been also demonstrated that the increase of these cells is correlated to the chronic stimulation of the immune system, as in Systemic Lupus Erythematosus or HIV patients and healthy subjects challenged with Respiratory Syncytial Virus (Wei et al., 2007; Sanz et al., 2008; Cagigi et al., 2009). DN B cells have also a pro-inflammatory phenotype of trafficking receptors, as they express elevated levels of CCR6 and CCR7, indicating that the typical age-associated chronic-inflamed milieu influence the leukocytes migration (Bulati et al., 2014). Moreover, if adequately stimulated, these cells are able to proliferate and reactivate telomerase (Martorana et al., *manuscript in preparation*), and, in the presence of IL-21, they produce Granzyme B (Bulati et al., 2014). In the present paper we investigate on the B cell arm of the adaptive immune system of a cohort of AD patients in two different stages of the pathology, severe and mild AD patients, compared them each other and with their age-matched healthy controls. We show a decrease of $CD19^+$ B cells, both in percentage and in absolute number, in severe AD patients when

compared with their age-matched healthy controls. Concerning the B cell subpopulation identified by using the "core" markers IgD and CD27, we show a significantly decrease, both in percentage and in absolute number, of naïve (IgD^+CD27^-) B cells in severe AD subject compared with healthy age-matched controls. Besides, there is a significant increase, only in percentage, of DN (IgD^-CD27^-) B cells in severe AD subject compared with healthy age-matched controls. Mild AD subjects behave as their age-matched healthy controls. This data is very interesting because, due to the inflammatory nature of AD, it supports the hypothesis that the increase of DN B cells is correlated with chronic inflammation. Another intriguing data is that obtained from the trafficking phenotype. Indeed, concerning the pro-inflammatory chemokine receptor CCR6, its increase depends on the severity of the pathology. Recently it has been observed an increase of this chemokine receptor also in T cells obtained from AD patients (Goldeck et al., 2013). Other authors report that, together with adhesion molecules, like selectins and integrins, CCR6 influences T cell migration through the choroid plexus into the CSF (Sallusto et al., 2012). Moreover, in a mouse model of AD-like disease, it has been shown a higher CCR6 expression both in brain and in periphery of these mice, which the authors suggested was due to the systemic inflammation in AD (Subramanian et al., 2010). Besides, CCR7 expression is significantly increased only in severe AD patients, but not in mild AD subjects. These data suggest that the expression of these two pro-inflammatory chemokines receptors could be influenced by the pro-inflammatory milieu of AD patients, and that, CCR6 and, probably, CCR7 could drive B cells to inflamed brain tissue.

Conclusions

Alzheimer's Disease is a progressive, irreversible and debilitating disease and, currently, there is no effective preventive or disease modifying therapy or treatments available. Immunotherapy represents a potentially disease modifying strategy aimed at reducing the pathological lesions of AD and facilitating cognitive improvement. Many clinical trials are currently underway (Madeo and Frieri, 2013). The main goal of these therapies is to reduce the production of and/or enhance the clearance of cerebral Aβ plaques. Although mouse models of AD have shown promising results for both passive

and active immunotherapy, more investigations are needed before this approach can be applied in practice in humans (Fu et al., 2010). Clinical trials have not yet shown a significant effect on cognitive decline for A-beta immunotherapy despite a reduction is plaque burden (von Bernhardi, 2010; Aisen and Vellas, 2013). Another issue is how to monitor therapeutic progress. For this reasons, the use of different biomarkers could be important to detect pre-clinical disease, select individuals with mild cognitive impairment (MCI) and predict which patients may benefit most from therapy. So, biomarkers could be useful for the determination of disease risk but are also invaluable in establishing a diagnosis. For example, complement proteins show promise as possible biomarkers and seem to be linked to AD pathology (Thambisetty et al., 2011). Nowadays, there are several types of peripheral biomarkers under investigation, but more work is required before they can be considered clinically useful (Mayeux and Schupf 2011). Thus, also the phenotypic studies of the peripheral lymphocyte subpopulations might be useful as biomarkers of AD for monitoring the effectiveness of therapeutic interventions. In this view, the results discussed in this paper could be used as additional empirical support aimed at developing standard operating procedures for AD biomarkers in the diagnostic routine and clinical trial.

References

Aisen PS and Vellas B. Editorial: passive immunotherapy for Alzheimer's disease: what have we learned, and where are we headed? 2013. J Nutr Health Aging, 17: 49-50

Appay V, Fastenackels S, Katlama C, Ait-Mohand H, Schneider L, Guihot A, Keller M, Grubeck-Loebenstein B, Simon A, Lambotte O, Hunt PW, Deeks SG, Costagliola D, Autran B, Sauce D: Old age and anti-cytomegalovirus immunity are associated with altered T-cell reconstitution in HIV-1-infected patients. AIDS 2011, 25:1813-1822.

Arshavsky YI. Alzheimer's disease, brain immune privilege and memory: a hypothesis. J Neural Transm 2006;113:1697–707.

Bonotis K, Krikki E, Holeva V, Aggouridaki C, Costa V, et al. (2008) Systemic immune aberrations in Alzheimer's disease patients. J Neuroimmunol 193: 183–187.

Britschgi M and Wyss-Coray T: Systemic and acquired immune responses in Alzheimer's disease. Int Rev Neurobiol 2007, 82:205–233.

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. Biogerontol. 12(5), 473-83.

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

Bulati M, Buffa S, Martorana A, Candore G, Lio D, Caruso C,Colonna-Romano. Trafficking phenotype and production of Granzyme B by Double Negative B cells (IgG(+)IgD(-)CD27(-)) in the elderly.Exp Gerontol. 2014 pii: S0531-5565(13)00367-7. doi: 10.1016/j.exger.2013.12.011.

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 hyper-mutated antibodies during chronic HIV-1 infection. PLoS One 4(5), e5427.

Carlesimo GA, Caltagirone C, Gainotti G (1996) The mental deterioration battery: normative data, diagnostic reliability and quantitative analyses of cognitive impairment. Eur Neurol 36:378–384.

Ciccocioppo F, Lanuti P, Marchisio M, Gambi F, Santavenere E, Pierdomenico L, Bascelli A, Velluto L, Gambi D, Miscia S. 2008. Expression and phosphorylation of protein kinase C isoforms in Abeta(1–42) activated T lymphocytes from Alzheimers disease. Int. J. Immunopathol. Pharmacol. 21, 23–33.

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 Develop. 130, 681-690.

Combarros O, Infante J, Rodriguez E, Llorca J, Pena N, Fernandez-Viadero C, et al. CD14 receptor polymorphism and Alzheimer's disease risk. Neurosci Lett 2005;380:193-196.

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.

Cummings JL, Mega M, Gray K, Rosenberg-Thompson S, Carusi DA, GornbeinJ. The Neuropsychiatric Inventory: comprehensive assessment of psychopathology in dementia. Neurology 1994;44:2308–2314.

de Vugt ME and Verhey FR. The impact of early dementia diagnosis and intervention on informal caregivers. Prog Neurobiol. 2013, 110:54-62

Evseev VA, Davydova TV, Mikovskaya OI. Dysregulation in neuroimmunopathology and perspectives of immunotherapy. Bull Exp Biol Med 2001;131:305-308.

Feng Y, Li L, Sun XH. Monocytes and Alzheimer's disease.Neurosci Bull. 2011, (2):115-22

Fiala M, Zhang L, Gan X, Sherry B, Taub D, et al. (1998) Amyloid-beta induces chemokine secretion and monocyte migration across a human blood–brain barrier model. Mol Med 4: 480–489.

Folstein MF, Folstein SE, McHugh PR, "Mini-mental state": a practical method for grading the cognitive state of patients for the clinician, *J Psychiatr Res* **48** (1975), 189-198.

Frasca D, Diaz A, Romero M, Phillips M, Mendez NV, Landin AM, Blomberg BB: Unique biomarkers for B-cell function predict the serum response to pandemic H1N1 influenza vaccine. Int Immunol 2012, 24:175–182.

Fu HJ, Liu B, Frost JL and Lemere CA (2010). Amyloid-beta immunotherapy for Alzheimer's disease. CNS Neurol Disord Drug Targets, 9: 197-206

Fulop T, Lacombe G, Cunnane S, Le Page A, Dupuis G, Frost EH, Bourgade-Navarro K, Goldeck D, Larbi A, Pawelec G. Elusive Alzheimer's disease: can immune signatures help our understanding of this challenging disease? Part 1: clinical and historical background. Discov Med. 2013a, 15(80):23-32.

Fulop T, Lacombe G, Cunnane S, Le Page A, Dupuis G, Frost EH, Bourgade-Navarro K, Goldeck D, Larbi A, Pawelec G. Elusive Alzheimer's disease: can immune signatures help our understanding of this challenging disease? Part 2: new immune paradigm. Discov Med. 2013b, 15(80): 33-42.

Goldeck D, Larbi A, Pellicanó M, Alam I, Zerr I, Schmidt C, Fulop T, Pawelec G. Enhanced Chemokine Receptor Expression on Leukocytes of Patients with Alzheimer's Disease. 2013. PLoS One, 8 (6).

Griffin WS (2006) Inflammation and neurodegenerative diseases. Am J Clin Nutr (suppl):470S-474S.

Gupta A, Pansari K. Inflammation and Alzheimer's disease. Int J Clin Pract 2003;57:36–9.

Haass C and Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid b-peptide. Nat Rev Mol Cell Biol 2007; 8:101–12.

Heneka MT, Kummer MP, Stutz A et al. NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. Nature 2013; 493:674–8.

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.

Katz S, Ford AB, Moskowitz RW, Jackson BA, Jaffe MW. Studies of illness in the aged. The index of ADL: a standardized measure of biological and psychosocial function. JAMA 1963; 185:914-919.

Kipnis J, Avidan H, Caspi RR, Schwartz M. Dual effect of CD4+CD25+ regulatory T cells in neurodegeneration: a dialogue with microglia. Proc Natl Acad Sci USA. 2004. 101 Suppl 2:14663-14669.

Kipnis J, Derecki NC, Yang C, Scrable H.Immunity and cognition: what do age-related dementia, HIV-dementia and 'chemo-brain' have in common? Trends Immunol. 2008. 29(10):455-463.

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

Larbi A, Pawelec G, Witkowski JM, Schipper HM, Derhovanessian E, Goldeck D, Fulop T (2009) Dramatic shifts in circulating CD4 but not CD8 T cell subsets in mild Alzheimer's disease. *J Alzheimers Dis* **17**, 91-103.

Lawton MP and Brody EM (1969) Assessment of older people: self maintaining and instrumental activities of daily living. Gerontologist 9:179–186

Lee KS, Chung JH, Choi TK, Suh SY, Oh BH, et al. (2009) Peripheral cytokines and chemokines in Alzheimer's disease. Dement Geriatr Cogn Disord 28: 281–287.

Li M, Shang DS, Zhao WD, Tian L, Li B, Fang WG, Zhu L, Man SM, Chen YH. Amyloid beta interaction with receptor for advanced glycation end products up-regulates brain endothelial CCR5 expression and promotes T cells crossing the blood-brain barrier. J Immunol. 2009. 182(9):5778-88.

Liu YJ, Guo DW, Tian L, Shang DS, Zhao WD, et al. (2010) Peripheral T cells derived from Alzheimer's disease patients overexpress CXCR2 contributing to its transendothelial migration, which is microglial TNF-alpha-dependent. Neurobiol Aging 31: 175–188.

Luzzatti C, Willmes K, De Bleser R et al (1994) New normative data for the Italian version of the Aachen Aphasia Test (AAT). Arch Psicol Neurol Psichiatria 55:1086–1131.

Madeo J and Frieri M. Alzheimer's Disease and Immunotherapy. Aging and Disease 2013, 4: 210-220.

Martorana A, Bulati M, Buffa S, Pellicanò M, Caruso C, Candore G, Colonna-Romano G. Immunosenescence, inflammation and Alzheimer's Disease. Longevity & Healtspan 2012. 1:8.

Mayeux R and Schupf N. Blood-based biomarkers for Alzheimer's disease: plasma Abeta40 and Abeta42, and genetic variants. 2011. Neurobiol Aging, 32 Suppl 1: S10-19

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinicaldiagnosis of Alzheimer'sdisease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer'sDisease. Neurology 1984;34:939-944.

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.

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

Miscia S, Ciccocioppo F, Lanuti P, Velluto L, Bascelli A, et al. (2009) Abeta(1-42) stimulated T cells express P-PKC-delta and P-PKC-zeta in Alzheimer disease. Neurobiol Aging 30: 394–406.

Monsonego A, Nemirovsky A, Harpaz I. CD4 T cells in immunity and immunotherapy of Alzheimer's disease. 2013, Immunology, 139, 438–446.

Pawelec G, McElhaney JE, Aiello AE, Derhovanessian E: The impact of CMV infection on survival in older humans. Curr Opin Immunol 2012, 24:1–5.

Pellicano M, Bulati M, Buffa S, Barbagallo M, Di Prima A, et al. (2010) Systemic immune responses in Alzheimer's disease: in vitro mononuclear cell activation and cytokine production. J Alzheimers Dis 21: 181–192.

Pellicanò M, Larbi A, Goldeck D, Colonna-Romano G, Buffa S, Bulati M, Rubino G, Iemolo F, Candore G, Caruso C, Derhovanessian E, Pawelec G: Immune profiling of Alzheimer patients. J Neuroimmunol 2012, 242:52–59.

Reale M, Iarlori C, Gambi F, Feliciani C, Salone A, Toma L, DeLuca G, Salvatore M, Conti P, Gambi D (2004) Treatment with an acetylcholinesterase inhibitor in Alzheimer patients modulates the expression and production of the proinflammatory and anti-inflammatory cytokines. *J Neuroimmunol* **148**, 162-171.

Reale M, Iarlori C, Feliciani C, Gambi D (2008) Peripheral chemokine receptors, their ligands, cytokines and Alzheimer's disease. J Alzheimers Dis 14: 147–159.

Sallusto F, Impellizzieri D, Basso C, Laroni A, Uccelli A, et al. (2012) T-cell trafficking in the central nervous system. Immunol Rev 248: 216–227.

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.

Sardi F, Fassina L, Venturini L, Inguscio M, Guerriero F, Rolfo E, Ricevuti G. Alzheimer's disease, autoimmunity and inflammation. The good, the bad and the ugly. Autoimmun Rev. 2011 Dec;11(2):149-153.

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

Schwartz M and Shechter R.Systemic inflammatory cells fight off neurodegenerative disease. Nat Rev Neurol. 2010 Jul;6(7):405-10.

Speciale L, Calabrese E, Saresella M, Tinelli C, Mariani C, Sanvito L, Longhi R, Ferrante P 2007 Lymphocyte subset patterns and cytokine production in Alzheimer's disease patients. *Neurobiol Aging* **28**, 1163-1169.

Spinnler H, Tognoni G et al. (1987) Italian group on the neuropsychological study of ageing: Italian standardization and classification of neuropsychological tests. Ital J Neurol Sci 6(suppl 8):1–120

Strous RD and Shoenfeld Y. To smell the immune system: olfaction, autoimmunity and brain involvement. Autoimmun Rev 2006;6:54-60.

Subramanian S, Ayala P, Wadsworth TL, Harris CJ, Vandenbark AA, et al. (2010) CCR6: a biomarker for Alzheimer's-like disease in a triple transgenic mouse model. J Alzheim Dis 22: 619–629.

Thal DR, Griffin WS, de Vos RA, Ghebremedhin E. Cerebral amyloid angiopathy and its relationship to Alzheimer's disease. Acta Neuropathol 2008; 115:599–609.

Town T. Inflammation, immunity, and Alzheimer's disease. CNS Neurol Disord Drug Targets 2010;9:129–31.

Thambisetty M, Simmons A, Hye A, Campbell J, Westman E, Zhang Y, Wahlund LO, Kinsey A, Causevic M, Killick R, Kloszewska I, Mecocci P, Soininen H, Tsolaki M, Vellas B, Spenger C and Lovestone S. Plasma biomarkers of brain atrophy in Alzheimer's disease. 2011, PLoS One, 6: e28527

Town T, Tan J, Flavell RA, Mullan M. T-cells in Alzheimer's disease. Neuromolecular Med. 2005;7(3):255-64.

Veneri D, Ortolani R, Franchini M, Tridente G, Pizzolo G, Vella A: Expression of CD27 and CD23 on peripheral blood B lymphocytes in humans of different ages. Blood Transfus 2009, 7:29–34.

Vom Berg J, Prokop S, Miller KR et al. Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease-like pathology and cognitive decline. Nat Med 2012; 18:1812–9.

von Bernhardi R. Immunotherapy in Alzheimer's disease: where do we stand? Where should we go? 2010. J Alzheimers Dis, 19: 405-421

Walsh DM and Selkoe DJ. Ab oligomers-a decade of discovery. J Neurochem 2007; 101:1172-1184.

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.

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.

Wimo A, Jönsson L, Bond J, Prince M, Winblad B. Alzheimer Disease International. The worldwide economic impact of dementia 2010. Alzheimers Dement. 2013, 9(1):1-11

Xue SR, Xu DH, Yang XX, Dong WL: Alterations in lymphocyte subset patterns and co-stimulatory molecules in patients with Alzheimer disease. Chin Med J (Engl) 2009, 122:1469-1472.

CHAPTER 8

"Immunosenescence, inflammation And Alzheimer's disease"

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REVIEW Open Access

Immunosenescence, inflammation and Alzheimer's disease

Adriana Martorana[1], Matteo Bulati[1], Silvio Buffa[1], Mariavaleria Pellicanò[1,2], Calogero Caruso[1], Giuseppina Candore[1] and Giuseppina Colonna-Romano[1*]

Abstract

Ageing impacts negatively on the development of the immune system and its ability to fight pathogens. Progressive changes in the T-cell and B-cell systems over the lifespan of individuals have a major impact on the capacity to respond to immune challenges. The cumulative age-associated changes in immune competence are termed immunosenescence that is characterized by changes where adaptive immunity deteriorates, while innate immunity is largely conserved or even upregulated with age. On the other hand, ageing is also characterized by "inflamm-ageing", a term coined to explain the inflammation commonly present in many age-associated diseases. It is believed that immune inflammatory processes are relevant in Alzheimer's disease, the most common cause of dementia in older people. In the present paper we review data focusing on changes of some immunoinflammatory parameters observed in patients affected by Alzheimer's disease.

Keywords: Immunosenescence, Alzheimer's disease, Inflammation, Cytokine, Chemokine, Lymphocyte, Ageing

Review

Ageing and the immune system

During the past century, humans have gained more years of average life expectancy than in the last 10,000 years. Currently, people are living much longer than they used to; and the longer they live, the longer their bodies are exposed to environmental factors that increase the risk of age-associated diseases. The reduction of the response to environmental stimuli is associated with an increased inclination towards illness and death. In western countries, the mortality rate increases in people over 65 years old, if compared with younger individuals, by 100-fold for stroke or chronic lung disease, by 92-fold for heart disease, by 89-fold for influenza and correlated pneumonia infections, and by 43-fold for cancer [1]. Ageing is the consequence of the collapse of self-organizing systems and reduced ability to adapt to the environment, and it has been suggested that normal human ageing is associated with a loss of complexity in a variety of anatomic structures and physiological processes [2]. These losses lead to physical inability, impaired mental functional capacity and organ and apparatus deregulation [3], with the consequence of increased susceptibility to diseases and death. On the contrary, healthy ageing seems directly correlated with a good functioning of the immune system, suggesting that it is related to both environmental factors and genetic background. Indeed, many studies have focused on genetic determinants of longevity in genes regulating the immune-inflammatory response [4-7].

Ageing impacts negatively on the development of the immune system and its ability to function. Progressive changes in the T-cell and B-cell systems over the lifespan of individuals have a major impact on the capacity to respond to immune challenges. These cumulative age-associated changes in immune competence are termed immunosenescence. According to the remodeling theory of ageing proposed several years ago [8], the current data on human immunosenescence describe a complex scenario where adaptive immunity deteriorates, while innate immunity is largely conserved or even up-regulated with age. Under an evolutionary perspective, antigens are the cause of a persistent lifelong antigenic stress, responsible for the accumulation of effector $CD8^+/CD28^-$ T cells, the decrease of naïve T lymphocytes ($CD45RA^+CD62L^+$)

* Correspondence: giuseppina.colonnaromano@unipa.it
Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, corso Tukory 211, 90134 Palermo, Italy
Full list of author information is available at the end of the article

and the marked shrinkage of the T-cell repertoire with age [9-14]. The humoral compartment is also affected in the aged [15-20]; indeed, B-cell numbers are decreased and the B-cell repertoire is influenced by ageing through the quality of antibody response [21-25], and this decreased B-cell diversity is associated with poor health status [26-28]. Immunosenescence is thus not a random deteriorative phenomenon, as was hypothesized in 1989 in "the network theory of aging", but could be envisaged as the result of the continuous challenge of the unavoidable exposure to a variety of potential antigens such as viruses and bacteria, but also food and self-molecules among others [12,13,29-31].

Immunosenescence therefore materially contributes to the decreased ability of the older person to control infectious diseases, which is also reflected in the observed poor response to vaccination [25,32-34]. In recent years, the idea of the immunological risk phenotype (IRP) that includes some immunological parameter changes that predict survival has been suggested [35-37]. A good immune system in the older person is tightly correlated to health status, and, as aforementioned, some immunological parameters are often markedly reduced in these subjects (Table 1). On the contrary, infectious diseases, cancer, autoimmune diseases and inflammatory chronic diseases such as atherosclerosis, heart diseases and Alzheimer's disease (AD) are frequent in this phase of life [38]. Indeed, much experimental and clinical evidence has suggested that the immune system is implicated, with a variable degree of importance, in almost all age-related or associated diseases.

Ageing is accompanied by a chronic low-grade inflammatory state demonstrated by the increased serum levels of inflammatory mediators such as cytokines and acute phase proteins in the aged [39,40]. The most important role in this basal pro-inflammatory status in the older person seems to be played by chronic antigenic stress, which, interacting with the genetic background, potentially triggers the onset of age-related inflammatory diseases [6,7,41]. The inflammatory process is a physiological phenomenon that is necessary for the elimination of pathogenic viruses or bacteria, but the prolonged period to which aged people are exposed may lead to chronic inflammation that inevitably damages several organs. Chronic inflammation appears to be involved in the pathogenesis of all age-related diseases such as AD, atherosclerosis, diabetes, sarcopenia and cancer [4,42-47].

Inflammation, Alzheimer's disease and immune response

AD is the most common cause of dementia in older people and it is estimated that 27 million people are affected worldwide [48,49]. As the life expectancy of the population increases, the number of affected individuals is predicted to triple by 2050 [49,50]. Age is therefore the main risk factor in AD, although early-onset disease can occur before age 60. AD may not be an inevitable occurrence of the aging process, but it is a disease with significant genetic roots. Indeed, genetics is important not only in predicting susceptibility but also the age of disease onset in the older person [51]. Other important risk factors are environmental events in early life as well as childhood IQ [52] and gender. In most studies, women were found to be at greater risk for AD. However, it is not clear whether this effect is due to genetic or hormonal differences between males and females or whether it is a surrogate marker of other still unmeasured socioeconomic factors [53].

AD is a progressive brain disorder affecting regions of the brain that control memory and cognitive functions. The two major neuropathologic hallmarks of AD are extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tangles. The production of Aβ, a decisive event in AD, is the result of the cleavage of amyloid precursor protein (APP), whose levels are high in AD.

APP has important developmental functions in cell differentiation and in the organization of synapses [54]. According to the Aβ hypothesis, AD begins with the abnormal processing of APP. Proteolysis of extracellular domains by sequential β-secretases and γ-secretases results in a family of peptides that form the β-amyloids (Aβ). Among these Aβ peptides, the more insoluble ($A\beta_{42}$) has a propensity for self-aggregation into fibrils that form the senile plaques characteristic of AD pathology. Neurofibrillary tangles are composed of the tau-protein and in healthy neurons are integral components of microtubules, while in AD tau-protein becomes hyperphosphorylated and this phenomenon leads to the tangles binding to each other and forming tangled threads [55].

Brain inflammation is a pathological hallmark of AD, and we know that inflammation is a response to eliminate both the initial cause of cell injury as well as the necrotic cells and tissues resulting from the original insult. If tissue health is not restored, inflammation becomes a chronic condition that continuously erodes the surrounding tissues [55]. Inflammation clearly occurs in pathologically susceptible regions in brain AD, with increased expression of acute-phase proteins and pro-inflammatory cytokines [6,7,49,56-58]. The cells responsible for the inflammatory reaction are microglia, astrocytes, and neurons. These activated cells produce high levels of inflammatory mediators such as pro-inflammatory cytokines and chemokines, prostaglandins, leukotrienes, thromboxanes, coagulation factors, free radicals as reactive oxygen species and nitric oxide, complement factors, proteases and protease inhibitors, and C-reactive protein [49,58]. The hypothesis is that Aβ plaques and tangles stimulate a chronic inflammatory

Table 1 Modifications of T-cell and B-cell systems in older humans

T cells and B cells or B-cell products	Lymphocyte subpopulations	Change	Reference
$CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$	Total T cells, T helper cells,	Decrease	[9]
(percentage and absolute number)	cytotoxic T lymphocytes		[14]
$CD3^+CD45RA^+CD62L^+$	Naive T cells	Decrease	[10]
(percentage)			[11]
			[12]
			[13]
$CD8^+CD28^-$	Effector T cells	Increase	[10]
(percentage)			[11]
			[12]
			[13]
$CD19^+$	Total B cells	Decrease	[24]
(percentage and absolute number)			[25]
			[16]
			[17]
			[18]
$CD19^+CD5^+$	B1 cells	Decrease	[15]
(percentage and absolute number)			
$CD19^+IgD^+CD27^-$	Naive B cells	Decrease	[19]
(percentage)			
$CD19^+IgD^-CD27^-$	Double Negative B cells	Increase	[19]
(percentage)			[24]
			[20]
IgG, IgA		Increase	[21]
		No change	[22]
IgD, IgM		Decrease	[21]
IgE		No change	[21]
(after specific immunization)			[22]
		Decrease	[23]
Autoantibodies		Increase	[27]
			[26]

reaction [59]. Inflammatory mediators, in turn, enhance APP production and the amyloidogenic processing of APP to induce $A\beta_{42}$ peptide production. These circumstances also inhibit the generation of a soluble APP fraction that has a neuroprotective effect [60,61]. On the contrary, Aβ induces the expression of pro-inflammatory cytokines in glial cells in a vicious cycle [62,63].

To date, the timing with which neuroinflammation is believed to influence AD is unknown. In particular, clinical and experimental evidence from different transgenic models has suggested that a pro-inflammatory process might precede plaque deposition [64]. A recent paper correlates the increased levels of C-reactive protein with the formation of senile plaques [65]. C-reactive protein has been shown to exist in two forms: the monomeric form, which has pro-inflammatory properties [66,67]; and the circulating pentamer form [68]. Authors have recently shown that the aggregated forms of Aβ plaques lead to the formation of the pro-inflammatory monomeric form of C-reactive protein, which exacerbates local inflammation [65].

There is currently much evidence suggesting the involvement of a systemic immune response in AD. Indeed, numerous investigations suggest that in addition to the central nervous system (CNS) cells, blood-derived cells can also be blamed for the inflammatory response and seem to accumulate in the AD brain [69-71]. Other studies have shown changes in the distribution and reactivity of immune cells in the blood [63,72-75]. Britschgi and Wyss-Coray have shown that there is communication between CNS and cells and factors involved in the systemic immune response [74]. In particular,

neuroinflammation induces the efflux of proteins, such as Aβ, or inflammatory mediators from CNS across the blood–brain-barrier (BBB); this may cause systemic immune reaction and recruitment of myeloid or lymphocytic cells into the CNS.

Indeed, it is known that BBB has a "monitoring role" between the immune system and AD to protect the brain from the entry of macromolecules, like immunoglobulins, and cells, including immunocompetent cells. A recent assumption supposes that microvascular diseases, often associated with AD, microtraumas and inflammation could cause the abnormal permeability of the BBB. The consequence of this impairment is the anomalous presence of serum proteins in the cerebrospinal fluid and in the brain, including Aβ. In the brain Aβ can bind astrocytes, starting a degenerative and inflammatory process. Finally, autoantibodies bound to neurons can induce $A\beta_{42}$ internalization and deposition, increasing brain damage [74,76].

Under physiological conditions T lymphocytes are few in the brain, although they are able to cross the BBB. The T-lymphocyte number increases in AD patients, especially in the hippocampus and temporal cortex. Herein, activated microglia increase the expression of MHC I and II, which allows the migration of T cells [76].

Communication between the CNS and the immune system in AD could thus influence both the lymphocyte distribution in the blood and the production of immune mediators [74]. Therefore, despite T cells being able to enter the brain tissue, it is also possible that T cells exert their effects without entering the CNS. Indeed, peripheral blood mononuclear cells (PBMCs) from AD patients produce higher levels of pro-inflammatory cytokines, such as IL-1β and IL-6, compared with PBMCs from control subjects [6,7,77]. Other studies have shown that Aβ stimulates macrophage inflammatory protein (MIP)-1α overexpression by peripheral T cells and its receptor CCR5 expression on brain endothelial cells necessary for T cells crossing the BBB [78]. Moreover, other altered immune parameters were documented, such as decreased percentages of naive T cells and an increase of memory T cells, an increased number of $CD4^+$ T lymphocytes that lack the co-stimulatory molecule CD28, and a reduction of $CD4^+CD25^{high}$ regulatory T cells [79].

Figure 1 shows the hypothesis that supports the involvement of the immune system in the pathogenesis of AD.

Systemic immune profile in Alzheimer's disease

At present a correct diagnosis of AD, characterized by pathological changes in the AD brain (that include neurological loss, extracellular amyloid plaques and intracellular neurofibrillar tangles), can be only evaluated by post-mortem autopsy, although a recent study [61] emphasized the role of soluble Aβ oligomers as a key factor responsible for the early pre-plaque formation. Activation of microglia occurs in the early stages of the disease, even before plaque formation, and is correlated with early cognitive deficits. As a consequence of the microglial activation and the deregulation of nerve grow factor metabolism, these authors have indicated matrix metalloproteinase-9 as a possible biomarker for signaling the early stages of ongoing CNS inflammation [61]. Another study has put in evidence the use of imaging techniques for early detection of glial activation prior to plaque deposition [80].

The evaluation of some modified parameters obtainable from the blood of patients could therefore be a goal for the research on AD.

The knowledge of the aforementioned systemic inflammation in AD patients has suggested a new research area that focuses on leukocyte modifications, as it would be desirable to have methods available that allow the use of peripheral blood from patients to identify "prognostic" or disease markers.

In this scenario, many authors have identified changes in lymphocyte distribution and in cytokine levels in the plasma of AD patients [75,79,81] that support the involvement of the immune system in AD. Many studies have reported alterations of both the innate and acquired immune system [74], although there are many discordant results (Table 2). Indeed, our group and others [63,82,83] have reported a decrease both in the percentage and the absolute number of total B cells from AD patients when compared with age-matched healthy controls. We did not observe any changes for the other main lymphocyte subpopulations [63]. On the contrary, Xue and colleagues have shown a significant reduction of $CD3^+$ T cells, but no changes in $CD4^+$ and $CD8^+$ T-cell subsets [83]. Richartz-Salzburger and colleagues confirm the decrease of $CD3^+$ and $CD8^+$ T cells, but showed a slight increase of $CD4^+$ cells [81]. Larbi and colleagues emphasized the dramatic changes within the $CD4^+$ T-cell compartment, with a reduction of naïve $CD4^+CD45RA^+CCR7^+$ and a simultaneous increase of effector memory $CD4^+CD45RA^-CCR7^-$ T cells and of terminal effector memory RA $CD4^+CD45RA^+CCR7^-$ T cells [79]. Again, the authors have demonstrated a reduction of $CD4^+CD25^{high}$ cells, potentially considered regulatory T cells [79].

More recently, the use of larger numbers of surface markers confirmed the significant reduction of naïve $CD4^+$ T cells, identified as $CD4^+CD28^+CD27^+CD45RA^+CD45RO^-$ in AD patients, compared with age-matched controls and a contemporary increase of $CD4^+CD28^-CD27^-CD45RA^+CD45RO^+$ late differentiated memory T cells [75]. The further evaluation of CD57 and KLRG-1, commonly considered senescence markers on these cells,

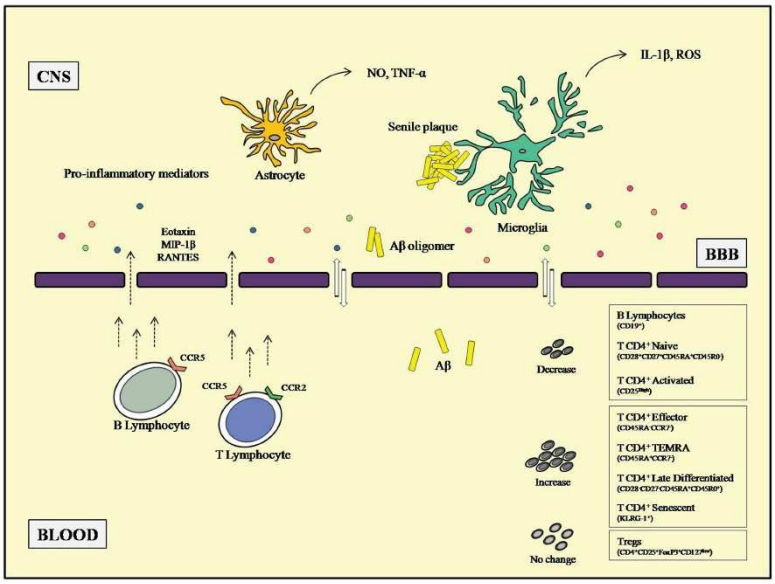

Figure 1 Communication between the central nervous system and systemic immune responses in Alzheimer's disease patients. Inflammation clearly occurs in pathologically susceptible regions of the Alzheimer's disease (AD) brain. Neurodegeneration and neuroinflammation can result in changes of central nervous system (CNS) proteins (for example, amyloid-beta (Aβ) peptide) or inflammatory mediators (acute-phase proteins and pro-inflammatory cytokines and chemokines) across the blood–brain-barrier (BBB). These CNS-derived proteins and mediators may induce systemic immune reactions and/or recruit lymphocytic cells into the CNS. The cells responsible for the inflammatory reaction in CNS are activated microglia and astrocytes. The hypothesis is that Aβ plaques and tangles stimulate a chronic inflammatory reaction. Other than CNS resident cells, blood-derived cells can also be blamed for inflammatory response and seem to accumulate in the AD brain due to the expression of chemokine receptors. The changes in lymphocyte distribution in the AD patient's blood are also depicted.

has demonstrated a significant increase of late differentiated KLRG-1^{+}CD4^{+} T cells in AD patients compared with age-matched healthy controls. No differences have been reported concerning CD57 expression on CD4^{+} T cells when comparing AD patients and their controls [75]. Moreover, the deep characterization of regulatory T cells as CD4^{+}CD25^{+}FoxP3^{+}CD127^{low} has demonstrated no differences between the two groups studied, thereby revealing that the previously reported data [79] are referred to activated T cells (CD4^{+}CD25^{+}) instead of regulatory cells. Table 2 describes the reported data.

Regarding CD8^{+} T cells, no modifications are reported in AD patients when compared with their age-matched controls. Indeed, this might be due to the well-known role of CD8^{+} T cells in age-related changes strictly correlated with chronic cytomegalovirus infection, which is a feature common to almost all older people (as well as AD patients) [35-37].

$A\beta_{42}$ and *in vitro* peripheral blood mononuclear cell activation

A recent hypothesis suggests that persistent stimulation of the immune system by Aβ peptides leads to B-cell and T-cell responses, as well as to the release of inflammatory mediators.

Although the Aβ aggregates are mainly found in the brain amyloid plaques, the soluble forms, monomers

Table 2 Main modifications of lymphocytes subpopulations between Alzheimer's disease patients and age-matched controls

Phenotype	Lymphocyte subpopulation	Changesin Alzheimer disease	Reference
$CD19^+$	Total B cells	Decrease	[82]
(percentage)			[83]
			[63]
$CD19^+$	Total B cells	Decrease	[82]
(absolute number)			[63]
CD3	Total $^-$ cells	No change	[63]
(percentage)		Decrease	[81]
			[83]
CD3 $CD8^+$(percentage)	Cytotoxic $^-$ lymphocytes	No change	[63] [83]
		Decrease	[81]
CD3 $CD4^+$	T helper cells	No change	[63]
(percentage)			[83]
		Increase	[81]
CD3 $CD4^+$CD45RA $CCR7^+$	Naïve $CD4^+$ T	Decrease	[79]
(percentage)	cells		
CD3 $CD4^+$CD28 CD27 CD45RA CD45RO	Naïve $CD4^+$ T	Decrease	[75]
(percentage)	cells		
CD3 $CD4^+$CD45RA CCR7	Effector memory	Increase	[79]
(percentage)	$CD4^+$ T cells		
CD3 $CD4^+$CD45RA CCR7 (percentage)	Terminal effector memory RA cells	Increase	[79]
CD3 $CD4^+$CD28 CD27 CD45RA CD45RO	Late differentiated	Increase	[75]
(percentage)	$CD4^+$ T cells		
CD3 $CD4^+CD25^{high}$	Activated $CD4^+$ T	Decrease	[79]
(percentage)	cells		
CD3 $CD4^+$CD25 $FoxP3^+CD127^{low}$(percentage)	Regulatory T cells	No change	[75]
$CD3^-CD4^+KLRG-1^+$(percentage)	Senescent $CD4^+$ T cells	Increase	[75]

and oligomers, predominate in the plasma where they may interact with the cells of the immune system [84].

Activation markers and chemokine receptors are overexpressed in unstimulated AD cells when compared with controls. This is evidence for the pro-inflammatory status of AD [6,7,85,86]. In this scenario, we have reported an *in vitro* response of T cells to recombinant $A\beta_{42}$ ($rA\beta_{42}$). Indeed the CD69 activation marker is overexpressed in $rA\beta_{42}$-stimulated AD cells when compared with their controls [63]. Moreover, we have also reported an increased expression of the chemokine receptors CCR2 and CCR5 only on T cells of AD patients after *in vitro* stimulation by $rA\beta_{42}$, whereas B cells overexpress CCR5 after the same *in vitro* treatment. The modulated expression of these receptors might enhance the migration of lymphocytes across the brain microvascular endothelial cells [87,88]. Strictly related to the expression of chemokine receptors is the observation that peripheral T lymphocytes of AD patients produce higher MIP-1α levels than age-matched controls [78]. This observation, together with the expression of the MIP-1α receptor CCR5 on the human brain microvascular endothelial cells, might explain the migration of T cells and B cells across the BBB. Microglial cells also produce MIP-1α. It has been demonstrated that MCP-1 via CCR2, expressed on brain endothelial cells, contributes to increased brain endothelial permeability [74,78]. In contrast to these data, we did not observe any significant overproduction of MIP-1α in PBMCs *in vitro* stimulated by $rA\beta_{42}$. This discrepancy might be due to the different experimental systems used since the production/binding of MIP-1α *in vivo* or *in vitro* was assessed

using human brain microvascular endothelial cells [78]. Moreover, in AD patients we and others [63,89] have demonstrated an increased production of RANTES, which is one of CCR5/s ligands (Table 3).

The role of $A\beta_{42}$ in the generation of an "inflammatory milieu" is also suggested by the observation that *in vitro* stimulation of PBMCs by $rA\beta_{42}$ induces the production of different chemokines and cytokines, rendering these cells active players in the inflammatory response in AD patients [63]. In fact, after an *in vitro* stimulation of PBMCs, AD patients have shown a significantly high production of the inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ. We have also reported an increase of the anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist, so we hypothesized that this situation might balance the overproduction of the above-described pro-inflammatory cytokines. As previously stated, however, there is an efflux of amyloid from CNS that can prime lymphocytes. Some authors have demonstrated a reduction of both pro-inflammatory and anti-inflammatory cytokines, hence assuming a general impairment of immune functions in AD patients, whereas others have demonstrated a decrease of IL-10, an increase of MIP1-α and an increase of IFN-γ, respectively [74,78,82,88]. Methodological differences (mitogen or Aβ stimulation) among the different studies, including inclusion criteria for both AD patients and healthy controls, might explain the great variability of data (Table 3).

Table 3 Cytokines, growth factors, chemokines and chemokine receptors on Alzheimer's disease patients after *in vitro* stimulation

	Stimulated vs. unstimulated AD patients	Reference
Cytokines		
IL-1β,IL-6,TNF-α,IL-1ra	Increase	[63]
IFN-γ	Increase	[63]
		[82]
IL-10	Decrease	[77]
	Increase	[63]
Growth factors		
GM CSF,G CSF	Increase	[63]
Chemokines		
Eotaxin,MIP 1β	Increase	[63]
RANTES	Increase	[89]
MIP 1α	No change	[63]
Chemokine receptors		
CCR2 and CCR5 on T cells	Increase	[63]
CR5on B cells	Increase	[63]

AD, Alzheimer's disease; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-1ra, IL-1 receptor antagonist; MIP, macrophage inflammatory protein.

Since monocytes are the main source of IL-6 and TNF-α and they possibly efficiently bind $A\beta_{42}$ via CD36, the pattern of cytokine production observed by us is the one to be expected. Besides, we have previously demonstrated an increased expression of the scavenger receptor CD36 on monocytes from AD subjects in unstimulated and stimulated cultures that could be related to their efficient role to bind plasmatic Aβ which in turn causes the production of cytokines, chemokines, and reactive oxygen species, hence activating the signaling cascade necessary for cellular migration, adhesion, and phagocytosis [63].

In addition, the engagement of monocytes might render these cells more efficient in T-cell activation [90]. Some studies have suggested receptors for advanced glycosylation end products as possible candidates for the role of soluble Aβ receptors. These receptors have been found on $CD4^+$ T-cell surfaces and are known to bind various molecules including Aβ; ligation of receptors for advanced glycosylation end products results in cell activation and inflammatory response [91]. Another possible receptor might be Toll-like receptor-4 [92,93], expressed on $CD4^+$ T cells, for which the potentially modulatory effect upon ligation by Aβ may even be direct.

Conclusions

Many modifications of immune and inflammatory systems have been reported in patients affected by AD. These changes might be the consequence of the overproduction of Aβ that can activate the blood cells, rendering them active producers of inflammatory mediators. On the contrary, the role of the genetic background namely the polymorphisms of genes involved in the immune-inflammation must be considered to fully elucidate the complex mechanisms that play a role in the generation of AD. Moreover, as a high proportion of women are affected by AD, especially at a very advanced age, it is important to consider the role played both by hormones and levels of education regarding the different propensity of males and females to develop disease. Fascinatingly, other important risk factors that could be related to the typical pro-inflammatory status of older people are environmental events in early life as well as childhood IQ.

Abbreviations

Aβ: amyloid beta; AD: Alzheimer's disease; APP: amyloid precursor protein; BBB: blood–brain barrier; CCR: chemokine receptor type; CNS: central nervous system; IFN: interferon; IL: interleukin; IQ: intelligence quotient; IRP: Immunoregikal risk phenotype; KLRG 1: killer cell lectin-like receptor subfamily G member 1; MHC: major histocompatibility complex; MIP: macrophage inflammatory protein; PBMC: peripheral blood mononuclear cell; $rA\beta_{42}$: recombinant amyloid beta 42; RANTES: regulated upon activation, normal T cell expressed, and secreted; TNF: tumor necrosis factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AM, MB, SB and GC-R wrote the first draft. Subsequent drafts were written by AM, who had the overall supervision of the review processing. All authors edited the paper and approved its final version.

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Author details

[1]Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, corso Tukory 211, 90134 Palermo, Italy. [2]Department of Internal Medicine II, Center for Medical Research, Tübingen Aging and Tumor Immunology Group, University of Tübingen, Waldhörnlestraße 22, 72072 Tübingen, Germany.

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References

1. Troen BR: **The biology of ageing.** *Mt Sinai J Med* 2003, **70:**3–22.
2. Goldberger AL, Peng CK, Lipsitz LA: **What is physiologic complexity and how does it change with aging and disease?** *Neurobiol Aging* 2002, **23:**23–26.
3. Wick G, Berger P, Jansen-Dürr P, Grubeck-Loebenstein BA: **Darwinian-evolutionary concept of age-related diseases.** *Exp Gerontol* 2003, **38:**13–25.
4. Candore G, Colonna Romano G, Balistreri CR, Di Carlo D, Grimaldi MP, Listì F, Nuzzo D, Vasto S, Lio D, Caruso C: **Biology of longevity: role of the innate immune system.** *Rejuvenation Res* 2006, **9:**143–148.
5. Franceschi C, Motta L, Motta M, Malaguarnera M, Capri M, Vasto S, Candore G, Caruso C: **The extreme longevity: the state of the art in Italy.** *Exp Gerontol* 2008, **43:**45–52.
6. Di Bona D, Plaia A, Vasto S, Cavallone L, Lescai F, Franceschi C, Licastro F, Colonna Romano G, Lio D, Candore G, Caruso C: **Association between the interleukin-1β polymorphisms and Alzheimer's disease: a systematic review and meta-analysis.** *Brain Res Rev* 2008, **59:**155–163.
7. Di Bona D, Vasto S, Capurso C, Christiansen L, Deiana L, Franceschi C, Hurme M, Mocchegiani E, Rea M, Lio D, Candore G, Caruso C: **Effect of interleukin-6 polymorphisms on human longevity: a systematic review and meta-analysis.** *Ageing Res Rev* 2009, **8:**36–42.
8. Franceschi C, Cossarizza A: **Introduction: the reshaping of the immune system with age.** *Int Rev Immunol* 1995, **12:**1–4s.
9. Cossarizza A, Ortolani C, Paganelli R, Barbieri D, Monti D, Sansoni P, Fagiolo U, Castellani G, Bersani F, Londei M, Franceschi C: **CD45 isoforms expression on CD4$^+$ and CD8$^+$ T cells throughout life, from newborns to centenarians: implications for T cell memory.** *Mech Ageing Dev* 1996, **86:**173–195.
10. Vasto S, Colonna-Romano G, Larbi A, Wikby A, Caruso C, Pawelec G: **Role of persistent CMV infection in configuring T cell immunity in the elderly.** *Immun Ageing* 2007, **4:**2.
11. Pawelec G, Larbi A: **Immunity and ageing in man: annual review 2006/2007.** *Exp Gerontol* 2008, **43:**34–38.
12. Sauce D, Appay V: **Altered thymic activity in early life: how does it affect the immune system in young adults?** *Curr Opin Immunol* 2011, **23:**543–548.
13. Appay V, Fastenackels S, Katlama C, Ait-Mohand H, Schneider L, Guihot A, Keller M, Grubeck-Loebenstein B, Simon A, Lambotte O, Hunt PW, Deeks SC, Costagliola D, Autran B, Sauce D: **Old age and anti-cytomegalovirus immunity are associated with altered T-cell reconstitution in HIV-1-infected patients.** *AIDS* 2011, **25:**1813–1822.
14. Pawelec G, McElhaney JE, Aiello AE, Derhovanessian E: **The impact of CMV infection on survival in older humans.** *Curr Opin Immunol* 2012, **24:**1–5.
15. Colonna-Romano G, Cossarizza A, Aquino A, Scialabba G, Bulati M, Lio D, Candore G, Di Lorenzo G, Fradà G, Caruso C: **Age- and gender-related values of lymphocyte subsets in subjects from Northern and Southern Italy.** *Arch Gerontol Geriatr Suppl* 2002, **8:**99–107.
16. Colonna-Romano G, Bulati M, Aquino A, Scialabba G, Candore G, Lio D, Motta M, Malaguarnera M, Caruso C: **B cells in the aged: CD27, CD5, and CD40 expression.** *Mech Ageing Dev* 2003, **124:**389–393.
17. Shi Y, Yamazaki T, Okubo Y, Uehara Y, Sugane K, Agematsu K: **Regulation of aged humoral immune defense against pneumococcal bacteria by IgM memory B cell.** *J Immunol* 2005, **175:**3262–3267.
18. Veneri D, Ortolani R, Franchini M, Tridente G, Pizzolo G, Vella A: **Expression of CD27 and CD23 on peripheral blood B lymphocytes in humans of different ages.** *Blood Transfus* 2009, **7:**29–34.
19. Colonna-Romano G, Bulati M, Aquino A, Pellicanò M, Vitello S, Lio D, Candore G, Caruso C: **A double-negative (IgD$^-$CD27$^-$) B cell population is increased in the peripheral blood of elderly people.** *Mech Ageing Dev* 2009, **130:**681–690.
20. Buffa S, Bulati M, Pellicanò M, Dunn-Walters DK, Wu YC, Candore G, Vitello S, Caruso C, Colonna Romano G: **B cell immunosenescence: different features of naive and memory B cells in elderly.** *Biogerontology* 2011, **12:**473–483.
21. Listì F, Candore G, Modica MA, Russo M, Di Lorenzo G, Esposito-Pellitteri M, Colonna-Romano G, Aquino A, Bulati M, Lio D, Franceschi C, Caruso C: **A study of serum immunoglobulin levels in elderly persons that provides new insights into B cell immunosenescence.** *Ann N Y Acad Sci* 2006, **1089:**487–495.
22. Chong Y, Ikematsu H, Yamaji K, Nishimura M, Nabeshima S, Kashiwagi S, Hayashi J: **CD27(+) (memory) B cell decrease and apoptosis-resistant CD27(−) (naive) B cell increase in aged humans: implications for age-related peripheral B cell developmental disturbances.** *Int Immunol* 2005, **17:**383–390.
23. Frasca D, Riley RL, Blomberg BB: **Humoral immune response and B-cell functions including immunoglobulin class switch are downregulated in aged mice and humans.** *Semin Immunol* 2005, **17:**378–384.
24. Bulati M, Buffa S, Candore G, Caruso C, Dunn-Walters DK, Pellicanò M, Wu YC, Colonna Romano G: **B cells and immunosenescence: a focus on IgG$^+$IgD$^-$CD27$^-$ (DN) B cells in aged humans.** *Ageing Res Rev* 2011, **10:**274–284.
25. Frasca D, Diaz A, Romero M, Phillips M, Mendez NV, Landin AM, Blomberg BB: **Unique biomarkers for B-cell function predict the serum response to pandemic H1N1 influenza vaccine.** *Int Immunol* 2012, **24:**175–182.
26. Candore G, Di Lorenzo G, Mansueto P, Melluso M, Fradà G, Li Vecchi M, Esposito Pellitteri M, Drago A, Di Salvo A, Caruso C: **Prevalence of organ-specific and non organ-specific autoantibodies in healthy centenarians.** *Mech Ageing Dev* 1997, **94:**183–190.
27. Weksler ME, Szabo P: **The effect of age on the B-cell repertoire.** *J Clin Immunol* 2000, **20:**240–249.
28. Gibson KL, Wu YC, Barnett Y, Duggan O, Vaughan R, Kondeatis E, Nilsson BO, Wikby A, Kipling D, Dunn-Walters DK: **B-cell diversity decreases in old age and is correlated with poor health status.** *Ageing Cell* 2009, **8:**18–25.
29. Akbar AN, Henson SM: **Are senescence and exhaustion intertwined or unrelated processes that compromise immunity?** *Nat Rev Immunol* 2011, **11:**289–295.
30. Henson SM, Riddell NE, Akbar AN: **Properties of end-stage human T cells defined by CD45RA re-expression.** *Curr Opin Immunol* 2012, **24:**1–6.
31. Macaulay R, Akbar AN, Henson SM: **The role of the T cell in age-related inflammation.** *Age (Dordr)* 2012. doi:10.1007/s11357-012-9381-2.
32. Blomberg BB, Frasca D: **Quantity, not quality, of antibody response decreased in the elderly.** *J Clin Invest* 2011, **121:**2981–2983.
33. Wolf J, Weinberger B, Grubeck-Loebenstein B: **The immunoregulatory effects of CMV-infection in human fibroblasts and the impact on cellular senescence.** *Immun Ageing* 2012, **9:**1–6.
34. Herndler-Brandstetter D, Landgraf K, Tzankov A, Jenewein B, Brunauer R, Laschober GT, Parson W, Kloss F, Gassner R, Lepperdinger G, Grubeck-Loebenstein B: **The impact of aging on memory T cell phenotype and function in the human bone marrow.** *J Leukoc Biol* 2012, **91:**197–205.
35. Wikby A, Ferguson F, Forsey R, Thompson J, Strindhall J, Löfgren S, Nilsson BO, Ernerudh J, Pawelec G, Johansson B: **An immune risk phenotype, cognitive impairment, and survival in very late life: impact of allostatic load in Swedish octogenarian and nonagenarian humans.** *J Gerontol A Biol Sci Med Sci* 2005, **60:**556–565.
36. Strindhall J, Nilsson BO, Löfgren S, Ernerudh J, Pawelec G, Johansson B, Wikby A: **No immune risk profile among individuals who reach 100 years of age: findings from the Swedish NONA immune longitudinal study.** *Exp Gerontol* 2007, **42:**753–761.
37. Wikby A, Månsson IA, Johansson B, Strindhall J, Nilsson SE: **The immune risk profile is associated with age and gender: findings from three Swedish**

population studies of individuals 20–100 years of age. *Biogerontology* 2008, 9:299–308.
38. Vasto S, Caruso C: **Immunity & Ageing: a new journal looking at ageing from an immunological point of view.** *Immun Ageing* 2004, 1:1–4.
39. Krabbe KS, Pedersen M, Bruunsgaard H: **Inflammatory mediators in the elderly.** *Exp Gerontol* 2004, **39**:687–699.
40. Bruunsgaard H: **The clinical impact of systemic low-level inflammation in elderly populations. With special reference to cardiovascular disease, dementia and mortality.** *Dan MedBull* 2006, **53**:285–309.
41. De Martinis M, Franceschi C, Monti D, Ginaldi L: **Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity.** *FEBS Lett* 2005, **579**:2035–2039.
42. Spirig R, Tsui J, Shaw S: **The emerging role of TLR and innate immunity in cardiovascular disease.** *Cardiol Res Pract* 2012, **2012**:181394.
43. Gui T, Shimokado A, Sun Y, Akasaka T, Muragaki Y: **Diverse roles of macrophages in atherosclerosis: from inflammatory biology to biomarker discovery.** *Mediators Inflamm* 2012, **2012**:693083.
44. Coussens LM, Werb Z: **Inflammation and cancer.** *Nature* 2002, **420**:860–867.
45. Shacter E, Weitzman SA: **Chronic inflammation and cancer.** *Oncology* 2002, **16**:217–226.
46. Rubin DC, Shaker A, Levin MS: **Chronic intestinal inflammation: inflammatory bowel disease and colitis-associated colon cancer.** *Front Immunol* 2012, **3**:107. doi:10.3389/fimmu.2012.00107.
47. Yamauchi H, Woodward WA, Valero V, Alvarez RH, Lucci A, Buchholz TA, Iwamoto T, Krishnamurthy S, Yang W, Reuben JM, Hortobagyi GN, Ueno NT: **Inflammatory breast cancer: what we know and what we need to learn.** *Oncologist* 2012, **17**:891–899.
48. Wimo A, Jonsson L, Winblad B: **An estimate of the worldwide prevalence and direct costs of dementia in 2003.** *Dement Geriatr Cogn Disord* 2006, **21**:175–181.
49. Rubio-Perez JM, Morillas-Ruiz JM: **A review: inflammatory process in Alzheimer's disease, role of cytokines.** *ScientificWorldJournal* 2012, **2012**:756357.
50. Hebert LE, Scherr PA, Bienias JL, Bennett DA, Evans DA: **Alzheimer disease in the US population: prevalence estimates using the 2000 census.** *Arch Neurol* 2003, **60**:1119–1122.
51. Candore G, Balistreri CR, Colonna Romano G, Lio D, Listì F, Vasto S, Caruso C: **Gender-related immune-inflammatory factors, age-related diseases, and longevity.** *Rejuvenation Res* 2010, **13**:292–297.
52. Luciano M, Marioni RE, Gow AJ, Starr JM, Deary IJ: **Reverse causation in the association between C reactive protein and fibrinogen levels and cognitive abilities in an aging sample.** *Psychosom Med* 2009, **71**:404–409.
53. Henderson VW: **Estrogen-containing hormone therapy and Alzheimer's disease risk: understanding discrepant inferences from observational and experimental research.** *Neuroscience* 2006, **138**:1031–1039.
54. Müller UC, Zheng H: **Physiological functions of APP family proteins.** *Cold Spring Harb Perspect Med* 2012, **2**:a006288.
55. Braak H, Del Tredici K: **The pathological process underlying Alzheimer's disease in individuals under thirty.** *Acta Neuropathol* 2011, **121**:171–181.
56. Griffin WS, Mrak RE: **Interleukin-1 in the genesis and progression of and risk for development of neuronal degeneration in Alzheimer's disease.** *J Leukoc Biol* 2002, **72**:233–238.
57. Cacquevel M, Lebeurrier N, Cheenne S, Vivien D: **Cytokines in neuroinflammation and Alzheimer's disease.** *Curr Drug Targets* 2004, **5**:529–534.
58. Finch CE, Morgan TE: **Systemic inflammation, infection, ApoE alleles, and Alzheimer disease: a position paper.** *Curr Alzheimer Res* 2007, **4**:185–189.
59. Town T, Nikolic V, Tan J: **The microglial 'activation' continuum: from innate to adaptive responses.** *J Neuroinflammation* 2005, **2**:24–33.
60. Friedlander RM: **Apoptosis and caspases in neurodegenerative diseases.** *N Engl J Med* 2003, **348**:1365–1375.
61. Atwood CS, Obrenovich ME, Liu T: **Amyloid-β: a chameleon walking in two worlds: a review of the trophic and toxic properties of amyloid-β.** *Brain Res Rev* 2003, **43**:1–16.
62. Lindberg C, Hjorth E, Post C, Winblad B, Schultzberg M: **Cytokine production by a human microglial cell line: effects ofβ-amyloid andα-melanocyte-stimulating hormone.** *Neurotox Res* 2005, **8**:267–276.
63. Pellicanò M, Bulati M, Buffa S, Barbagallo M, Di Prima A, Misiano G, Picone P, Di Carlo M, Nuzzo D, Candore G, Vasto S, Lio D, Caruso C, Colonna-Romano G: **Systemic immune responses in Alzheimer's disease: in vitro mononuclear cell activation and cytokine production.** *J Alzheimers Dis* 2010, **21**:181–192.
64. Ferretti MT, Cuello AC: **Does a pro-inflammatory process precede Alzheimer's disease and mild cognitive impairment?** *Curr Alzheimer Res* 2011, **8**:164–174.
65. Strang F, Scheichl A, Chen YC, Wang X, Htun NM, Bassler N, Eisenhardt S, Habersberger J, Peter K: **Amyloid plaques dissociate pentameric to monomeric C-Reactive-Protein: a novel pathomechanism driving cortical inflammation in Alzheimer's disease?** *Brain Pathol* 2012, **22**:337–346.
66. Ji SR, Wu Y, Zhu L, Potempa LA, Sheng FL, Lu W, Zhao J: **Cell membranes and liposomes dissociate C-reactive protein (CRP) to form a new, biologically active structural intermediate: mCRP(m).** *FASEB J* 2007, **21**:284–294.
67. Eisenhardt SU, Habersberger J, Murphy A, Chen YC, Woollard KJ, Bassler N, *et al*: **Dissociation of pentameric to monomeric C-reactive protein on activated platelets localizes inflammation to atherosclerotic plaques.** *Circ Res* 2009, **105**:128–137.
68. Pepys MB, Hirschfield GM: **C-reactive protein: a critical update.** *J Clin Invest* 2003, **111**:1805–1812.
69. Bonotis K, Krikki E, Holeva V, Aggouridaki C, Costa V: **Systemic immune aberrations in Alzheimer's disease patients.** *J Neuroimmunol* 2008, **193**:183–187.
70. Miscia S, Ciccocioppo F, Lanuti P, Velluto L, Bascelli A: **Aβ(1–42) stimulated T cells express P-PKC-delta and P-PKC-zeta in Alzheimer disease.** *Neurobiol Aging* 2009, **30**:394–406.
71. Liu YJ, Guo DW, Tian L, Shang DS, Zhao WD: **Peripheral T cells derived from Alzheimer's disease patients overexpress CXCR2 contributing to its transendothelial migration, which is microglial TNF-α-dependent.** *Neurobiol Aging* 2010, **31**:175–188.
72. Monsonego A, Zota V, Karni A, Krieger JI, Bar-Or A, Bitan G, Budson AE, Sperling R, Selkoe DJ, Weiner HL: **Increased T cell reactivity to amyloid beta protein in older humans and patients with Alzheimer's disease.** *J Clin Invest* 2003, **112**:415–422.
73. Fiala M, Lin J, Ringman J, Kermani-Arab V, Tsao G, Patel A, Lossinsky AS, Graves MC, Gustavson A, Sayre J, Sofroni E, Suarez T, Chiappelli F, Bernard G: **Ineffective phagocytosis of amyloid-beta by macrophages of Alzheimer's disease patients.** *J Alzheimers Dis* 2005, **7**:221–232.
74. Britschgi M, Wyss-Coray T: **Systemic and acquired immune responses in Alzheimer's disease.** *Int Rev Neurobiol* 2007, **82**:205–233.
75. Pellicanò M, Larbi A, Goldeck D, Colonna Romano G, Buffa S, Bulati M, Rubino G, Iemolo F, Candore G, Caruso C, Derhovanessian E, Pawelec G: **Immune profiling of Alzheimer patients.** *J Neuroimmunol* 2012, **242**:52–59.
76. Sardi F, Fassina L, Venturini L, Inguscio M, Guerriero F, Rolfo E, Ricevuti G: **Alzheimer's disease, autoimmunity and inflammation. The good, the bad and the ugly.** *Autoimmun Rev* 2011, **11**:149–153.
77. Reale M, Iarlori C, Gambi F, Lucci I, Salvatore M, Gambi D: **Acetylcholinesterase inhibitors effects on oncostatin-M, interleukin-1 beta and interleukin-6 release from lymphocytes of Alzheimer's disease patients.** *Exp Gerontol* 2005, **40**:165–171.
78. Man SM, Ma YR, Shang DS, Zhao WD, Li B, Guo DW, Fang WG, Zhu L, Chen YH: **Peripheral T cells overexpress MIP-1α to enhance its transendothelial migration in Alzheimer's disease.** *Neurobiol Aging* 2007, **28**:485–496.
79. Larbi A, Pawelec G, Witkowski JM, Schipper HM, Derhovanessian E, Goldeck D, Fulop T: **Dramatic shifts in circulating CD4 but not CD8 T cell subsets in mild Alzheimer's disease.** *J Alzheimers Dis* 2009, **17**:91–103.
80. Sastre M, Richardson JC, Gentleman SM, Brooks DJ: **Inflammatory risk factors and pathologies associated with Alzheimer's disease.** *Curr Alzheimer Res* 2011, **8**:132–141.
81. Richartz-Salzburger E, Batra A, Stransky E, Laske C, Köhler N, Bartels M, Buchkremer G, Schott K: **Altered lymphocyte distribution in Alzheimer's disease.** *J Psychiatr Res* 2007, **41**:174–178.
82. Speciale L, Calabrese E, Saresella M, Tinelli C, Mariani C, Sanvito L, Longhi R, Ferrante P: **Lymphocyte subset patterns and cytokine production in Alzheimer's disease patients.** *Neurobiol Aging* 2007, **28**:1163–1169.
83. Xue SR, Xu DH, Yang XX, Dong WL: **Alterations in lymphocyte subset patterns and co-stimulatory molecules in patients with Alzheimer disease.** *Chin Med J (Engl)* 2009, **122**:1469–1472.
84. Jóźwik A, Landowski J, Bidzan L, Fülop T, Bryl E, Witkowski JM: **Beta-amyloid peptides enhance the proliferative response of activated CD4CD28 lymphocytes from Alzheimer disease patients and from healthy elderly.** *PLoS One* 2012, **7**:e33276.

85. Querfurth HW, LaFerla FM: Alzheimer's disease. *N Engl J Med* 2010, 362:329–344.
86. Teeling JL, Perry VH: Systemic infection and inflammation in acute CNS injury and chronic neurodegeneration: underlying mechanisms. *Neuroscience* 2009, 158:1062–1073.
87. Li M, Shang DS, Zhao WD, Tian L, Li B, Fang WG, Zhu L, Man SM, Chen YH: Amyloid beta interaction with receptor for advanced glycation end products up-regulates brain endothelial CCR5 expression and promotes T cells crossing the blood–brain barrier. *J Immunol* 2009, 182:5778–5788.
88. Reale M, Iarlori C, Feliciani C, Gambi D: Peripheral chemokine receptors, their ligands, cytokines and Alzheimer's disease. *J Alzheimers Dis* 2008, 14:147–159.
89. Iarlori C, Gambi D, Gambi F, Lucci I, Feliciani C, Salvatore M, Reale M: Expression and production of two selected beta-chemokines in peripheral blood mononuclear cells from patients with Alzheimer's disease. *Exp Gerontol* 2005, 40:605–611.
90. Stuart LM, Bell SA, Stewart CR, Silver JM, Richard J, Goss JL, Tseng AA, Zhang A, El Khoury JB, Moore KJ: CD36 signals to the actin cytoskeleton and regulates microglial migration via a p130Cas complex. *J Biol Chem* 2007, 282:27392–27401.
91. Schmidt AM, Sahagan B, Nelson RB, Selmer J, Rothlein R, Behr JM: The role of RAGE in amyloid-beta peptide-mediated pathology in Alzheimer's disease. *Curr Opin Investig Drugs* 2009, 10:677–680.
92. Buchanan MM, Hutchinson M, Watkins LR, Yin H: Toll-like receptor 4 in CNS pathologies. *J Neurochem* 2010, 114:13–27.
93. Gonzalez-Navajas JM, Fine S, Law J, Datta SK, Nguyen KP, Yu M, Corr M, Katakura K, Eckman L, Lee J, Raz E: TLR4 signaling in effector CD4+ T cells regulates TCR activation and experimental colitis in mice. *J Clin Invest* 2010, 120:570–581.

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CHAPTER 9

"Genetics of longevity. Data from the studies on Sicilian centenarians"

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Genetics of longevity. Data from the studies on Sicilian centenarians

Carmela R Balistreri[1*], Giuseppina Candore[1], Giulia Accardi[1], Manuela Bova[1], Silvio Buffa[1], Matteo Bulati[1], Giusi I Forte[1], Florinda Listì[1], Adriana Martorana[1], Marisa Palmeri[1], MariaValeria Pellicanò[1,2], Loredana Vaccarino[1], Letizia Scola[1], Domenico Lio[1] and Giuseppina Colonna-Romano[1]

Abstract

The demographic and social changes of the past decades have determined improvements in public health and longevity. So, the number of centenarians is increasing as a worldwide phenomenon. Scientists have focused their attention on centenarians as optimal model to address the biological mechanisms of "successful and unsuccessful ageing". They are equipped to reach the extreme limits of human life span and, most importantly, to show relatively good health, being able to perform their routine daily life and to escape fatal age-related diseases, such as cardiovascular diseases and cancer. Thus, particular attention has been centered on their genetic background and immune system. In this review, we report our data gathered for over 10 years in Sicilian centenarians. Based on results obtained, we suggest longevity as the result of an optimal performance of immune system and an over-expression of anti-inflammatory sequence variants of immune/inflammatory genes. However, as well known, genetic, epigenetic, stochastic and environmental factors seem to have a crucial role in ageing and longevity. Epigenetics is associated with ageing, as demonstrated in many studies. In particular, ageing is associated with a global loss of methylation state. Thus, the aim of future studies will be to analyze the weight of epigenetic changes in ageing and longevity.

Keywords: Immune system, Genetics, Pro/anti-inflammatory polymorphisms, Epigenomics

Introduction

Data from centenarian offspring

As well known, life expectancy is a familial trait and longevity is determined by different factors. In particular, the environmental milieu and genetic background play a central role. As demonstrated by many epidemiological studies, family members of long-lived subjects have a significant survival advantage compared to general population. In this context, the study of centenarian offspring (CO), a group of healthy elderly people with a familiar history of longevity, might help gerontologists to better identify the correlation between genetic profile and hope of a healthy ageing. Previous studies have reported that CO, like their centenarian parents, have genetic and immune system advantages, which reflect a minor risk to develop major age-related diseases, such as cardiovascular diseases, hypertension or diabetes mellitus as well as cancer [1,2]. The lower cardiovascular disease risk in CO suggests the probability that CO have some protective factors against atherosclerosis, such as a good lipid profile. Male CO have higher plasma HDL-C levels and lower plasma LDL-C levels. Since lipid profile is directly correlated to atherosclerotic cardiovascular diseases, this metabolic feature could preserve CO both to develop these diseases and, as consequence, to reach a healthy ageing and longer survival [3]. Furthermore, Rose et al. [4] reported that centenarians and CO show significantly higher levels of heteroplasmy in mtDNA control region than controls, a favorable condition for longevity.

In these last years, some researchers have speculated about the distinctive immunological profile of offspring enriched for longevity respect to the immunological features of coeval elderly. The cytomegalovirus (CMV) is one of the most common viruses that affect elderly people. Many evidences have shown that CMV infection

* Correspondence: carmelarita.balistreri@unipa.it
[1]Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Corso Tukory 211, Palermo 90134, Italy
Full list of author information is available at the end of the article

may influence the T cell subset distribution, having an essential role in immunosenescence [5-7]. CMV infection is strongly related to both a reduction of CD8$^+$CD45$^+$CCR7$^+$CD27$^+$CD28$^+$ naïve T cells and to a contemporarily increase of CD8$^+$CD45RA$^-$CCR7$^-$CD27$^-$CD28$^-$ late differentiated effector memory and CD45RA-re-expressing T cells. These parameters are considered typical of immunosenescence in elderly. Recently, it has been demonstrated that CMV-seropositive offspring of long-lived people don't show the age-associated decrease of naïve T cells. On the other hand, memory T cell subsets above described do not increase in offspring of long-lived families, differently from that observed in age-matched controls [8]. It has been also demonstrated that CMV-seropositive offspring of long-lived people have reduced levels of CD8+ T cells expressing CD57 and KLRG1, sometimes referred as "marker of senescence", when compared to their CMV-infected age-matched controls. The reduction of effector memory T cells lacking the expression of CD27 and CD28 and expressing CD57 and KLRG1, observed in CMV-infected offspring could explain their high proliferative response against CMV. The CMV-seropositive offspring have also shown significantly lower CRP levels compared to their CMV-seropositive age-matched controls that could be related to a lower pro-inflammatory status [8].

During ageing, B cell compartment also shows significant modifications in numbers and functions [9-12]. In fact, advanced age is per se a condition characterized by lack of B clonotypic immune response to new extracellular pathogens. In any event, data are suggesting that the loss of naive B cells could represent a hallmark of immunosenescence [13]. On the other hand, a B cell population lacking of both IgD and CD27 resulted increased in healthy elderly [14]. We have suggested that this IgD$^-$CD27$^-$ B cell subset is a population of memory B cells lacking CD27, a typical memory marker, likely considered a late memory exhausted B cell subset (Table 1) [14-16]. This population resulted also increased in active Lupus patients [17], in healthy subjects challenged with respiratory syncitial virus [18], and in HIV patients [19]. CO don't show the typical naïve/memory B cell shift observed in elderly. Although a decreased B cell count was observed in CO and their age-matched controls, it has been demonstrated that naïve B cells (IgD$^+$CD27$^-$) were more abundant and DN B cells (IgD$^-$CD27$^-$) were significantly decreased, as looked similarly in young people [20]. This B cells distribution in CO could suggest that antigenic load or inflammatory environment play a central role in exhaustion of the B cell branch. It is well documented that the quality and the size of the humoral immune response declines with age [15,21-26]. This change is characterized by lower antibody responses and decreased production of high affinity antibodies. The evaluation of IgM secreted in CO serum shows that the values are within the range of the levels observed in young subjects [20]. In this way, CO could have a bigger advantage to fight against new infections and appropriately respond to vaccinations, giving them a selective advantage for longevity in healthiness.

Table 1 Main modifications of B cells and B cells products in elderly human observed in our laboratory

B cells or B cells products	Changes	References
Total B cells (percentage)	↓	[9]
CD19$^+$CD5$^+$ B1 cells (percentage and absolute number)	↓	[10]
IgG, IgA	↑	[11]
IgM, IgD	↓	[11]
IgE	=	[11]
Autoantibodies	↑	[12]
Naive (IgD$^+$CD27$^-$)	↓	[13]
DN (IgD$^-$CD27$^-$)	↑	[14-16]

In conclusion, individuals genetically enriched for longevity possess immune different signatures respect to those of the general population (Table 2). This suggests the idea of the "familiar youth" of the immune system. In addition, the lower pro-inflammatory status in CMV-infected offspring of long-lived people might represent an optimal advantage for healthy longevity and against mortality associated to major age-related diseases.

Gender and longevity

A characteristic enigma of longevity is the gender and the social phenomenon of "*feminization of old age*". The demographic and social changes of the past decades, responsible for longevity and the improvements in public health, have created new and often very dissimilar realties for women and men. People are all aware that they differ in their anatomy and physiology, but also in more complex traits, such as lifespan (in Italy, 78.8 years for men and 84.1 years for women, respectively) and mortality [27-29]. No conclusive explanation for these new differences is actually demonstrated. An intricate interaction between environmental, social structural, behavioural (i.e. the complex pattern of roles and values that define what is thought as *masculine* and *feminine*) and genetic factors have been suggested as the more probable reason [30-32].

From a genetic prospective, our suggestion based on the studies in Sicilian population supports a female-specific gene-longevity association, by emphasizing the paradoxical role of socio-cultural habits in female longevity [33]. This concerns the HFE gene, the most telomeric HLA class I gene, codifying for a class I α chain, the HFE protein, which seemingly no longer participates

Table 2 Cellular and humoral immune modification in offspring from longevity families compared to their AM controls

T and B cell Phenotypes and Products	Changes	References
Naïve T cells ($CD3^+CD8^+CD45RA^+CCR7^+CD27^+CD28^+$)	Increase	[8]
Late differentiated effector memory T cells ($CD3^+CD8^+CD45RA^-CCR7^-CD27^-CD28^-$)	Decrease	[8]
TEMRA ($CD3^+CD8^+CD45RA^+CCR7^-CD27^-CD28^-$)	Decrease	[8]
Naïve B cells (IgD^+CD27^-)	Increase	[20]
Double Negative B cells ($IgG^+/IgA^+IgD^-CD27^-$)	Decrease	[20]
Serum IgM	Increase	[20]

in immunity. It has lost its ability to bind peptides due to a definitive closure of the antigen binding cleft that prevents peptide binding and presentation. The HFE protein, expressed on crypt enterocytes of the duodenum, regulates the iron uptake by intestinal cells, having acquired the ability to form complex with the receptor for iron-binding transferring. Mutations in HFE gene are associated with hereditary hemochromatosis, a disorder caused by excessive iron uptake [34,35]. Three common mutations, C282Y, H63D and S65C, have been identified in HFE gene. In particular, the C282Y mutation (a cysteine-to-tyrosine mutation at amino acid 282) destroys its ability to make up a heterodimer with β2-microglobulin. The defective HFE protein fails to associate to the transferring receptor, and the complex cannot be transported to the surface of the duodenal crypt cells. As a consequence, in homozygous people, two to three times the normal amount of iron is absorbed from food by the intestine, resulting in end-organ damage and reducing lifespan. Two other mutations, H63D (a histidine to aspartate at amino acid 63) and S65C (a serine to cysteine at amino acid 65), are associated with milder forms of this disease [34,35].

An association between C282Y mutation and longevity characterizes the Sicilian population studied [33]. In particular, women carriers of C282Y mutation had a higher frequency among the oldest old compared to control women (Table 3). Thus, the C282Y mutation may confer a selective advantage in terms of longevity in Sicilian women. Considering the historical and social context in which the generation of women under study lived, our data seem to propose that the possession of iron-sparing alleles significantly increases the possibility for women to reach longevity. For instance, in Sicily, many pregnancies and an iron-poor diet, consisting mainly in grains, vegetables, and fruits, were still the rule for women born at the beginning of last century. In fact, meat was available for men but not for women; this clearly explains how genetic background also interacts with culture habits [30,31,33].

Our data, showing the relevance of C282Y for women survival to late age, allow adding another piece of evidence to the complex puzzle of genetic and environmental factors involved in control of lifespan in humans. The complex interaction of environmental, historical and genetic factors, differently characterizing the various parts of a country, i.e. Italy, likely plays an important role in determining the gender-specific probability of attaining longevity [30,31,33,36].

Role of innate immunity genes in longevity: the paradigmatic case of TLR4, CCR5, COX-2 and 5-LO genes

According to evolutionary ageing theories, most of the parameters influencing immunosenescence appear to be under genetic control [32,37,38]. An example is given by the innate immune system, involved in neutralizing infectious agents [39]. It plays a beneficial role until the time of reproduction and parental care. In old age, a period largely not foreseen by evolution, it can determine an opposite and detrimental effect through chronic inflammatory responses ("antagonistic pleiotropy") [38,40]. Genetic pro-/anti-inflammatory variations in innate immune response are, indeed, thought to influence the susceptibility of age-related human diseases, by altering host response to environmental and endogenous stress [41]. Thus, they are able to determine a negative or positive control of inflammation, by affecting both interactions between host and microbes and survival of the individual and attainment of longevity. Furthermore, they appear both to be responsible, at least in large part, for different men and women strategies to achieve longevity, and to contribute to the preferential sex dimorphism of the age-related diseases [30,31,33].

From our investigations in Sicilian population, TLR4, CCR5, Cox2, 5-Lo genes can be considered good examples. They provide an ideal model to understand the different implications of their genetic variants in the risk of age-related diseases, i.e. atherosclerosis and prostate cancer (PC), and reciprocally in increased chance to attain longevity.

TLR4 gene (number accession of GenBank: NM-138554.1) codifies the best understood TLR member involved in recognition of LPS, the prototypic TLR4 ligand, and other exogenous and endogenous (i.e. HSPs, hyaluronic acid, β-defensin-2, ox-LDL, fibronectin and amyloid peptide) ligands. TLR4 activation implies a downstream signaling mediated by several intracellular adaptor molecules and the consequent activation of transcription

Table 3 Data from our investigations in Sicilian population

Gene	Alleles of genetic variants	Centenarians	Young controls (< 55 years)		P
		N = 35 females	N = 106 females		
HFE	C282	47 (84%)	132 (0%)		8.3×10^{-5} [35]
	282Y	9 (16%)	0 (0%)		
Genes	**Alleles of SNPs or genetic variants**	**Centenarians**	**Young controls (< 55 years)**	**MI patients (< 55 years)**	**P**
		N = 55 males	N = 127 males	N = 105 males	
TLR4	+896A	94 (85.4%)	239 (94.1%)	205 (97.6%)	< 0.001 [49]
	896 G	16 (14.6%)	15 (5.9%)	5 (2.4%)	
		N = 123 males	N = 136 males	N = 133 males	
CCR5	WT	221(89.8%)	252 (92.6%)	263 (98.8%)	0.00006 [48]
	Δ32	25 (10.2%)	20 (7.4%)	3 (1.2%)	
		N = 96 males	N = 170 males	N = 140 males	
Cox-2	-765 G	129 (63.5%)	240 (70.6%)	232(82.8%)	0.000007 [47]
	-765 C	70(36.5%)	100(29.4%)	48(17.2%)	
5-Lo	-1708 G	180 (93.7%)	302(88.8%)	224(80%)	0.00003 [47]
	-1708A	12(6.3%)	38(11.2%)	56(20%)	0.03
	21 C	176(91.7%)	299(88%)	225(80.4%)	
	21 T	16(8.3%)	41(12%)	55(19.6%)	
Genes	**Alleles of SNPs or genetic variants**	**Centenarians**	**Young controls (< 55 years)**	**PC patients (< 55 years)**	**P**
		N = 55 males	N = 125 males	N = 50 males	
TLR4	+896A	94 (85%)	235 (94%)	99 (99%)	0.001 [54]
	−896 G	16 (15%)	15 (6%)	1 (1%)	
Cox-2	-765 C	67 (61%)	176 (70%)	77 (77%)	0.03
5 Lo	765 C	43 (39%)	74 (30%)	23(23%)	0.0007
	-1708 G	104 (95%)	223 (89%)	77 (77%)	
	-1708A	6 (5%)	27 (11%)	23 (23%)	
		N = 53 males		N = 50 males	
CCR5	WT	95 (89.6%)		97 (97%)	0.03 [53]
	Δ32	11(10.4%)		3(3%)	
Gene	**Alleles of SNPs or genetic variants**	**Centenarians**	**Young controls (< 55 years)**	**BF patients (30-60 years)**	**P**
		N = 42 females	N = 42 females	N = 42 females	
TLR4	1896A	81 (96.4%)	78 (92.9%)	76 (90.4%)	0.003 [55]
	+ 896 G	3 (3.6%)	6 (7.1%)	8 (9.6%)	

factors, such as NF-kB. This determines the production of different pro/anti-inflammatory mediators. These lasts, such as IL-10, are produced by the parallel activation of anti-inflammatory pathways to limit the potential tissue damage from excessive activation of the innate immune system [42]. SNPs seem to modulate both TLR4 activity and function. In human, only two SNPs, +896A/G (Asp299Gly; rs4986790) and +1196 C/T (Thr399Ile; rs4986791), have a frequency > 5%. They induce a blunted response to LPS, as first suggested by Arbour et al., and are phenotypically associated to changes in the production of cytokines, principally those carrying the Asp299Gly mutation [43-45]. Accordingly, recent literature data suggest the ability of this SNP to modulate the risk of major age-related diseases [42].

The CCR5 gene (number accession of GenBank: NM-00579) codifies for a G protein-coupled chemokine receptor, which regulates trafficking and effector functions of memory/effector Th1 cells, macrophages, NK cells and immature dendritic cells. CCR5 and its ligands are important molecules in viral pathogenesis. Recent evidence has also demonstrated the role of CCR5 in a variety of human diseases, ranging from infectious and inflammatory age-related diseases to cancer. A notable variant of CCR5 gene is a 32 bp (Δ32) deletion, which causes a frame shift mutation in exon 4 (CCR5Δ32; rs333) and determines stop protein maturation and loss of expression of functional CCR5 receptor [46]. Accordingly, it seems to have a protective role against CVD and other age-related diseases, such as PC. It, indeed,

determines a slower progression of atherogenesis or cancerogenesis as a consequence of an attenuated inflammatory response.

COX-2 gene maps in the 1q25 chromosome and codifies for the Cox-2 enzyme involved in the conversion of arachidonic acid to prostaglandins. Polymorphisms regulate its expression and hence prostanoid biosynthesis. In particular, it has been identified a guanine to cytosine substitution at position -765 G/C, located within a putative binding site for the transcription factor Sp1, associated to a reduction in the risk of clinical cardiovascular events. COX-2 is expressed at low levels in most tissues, but its expression enhances under inflammatory stimuli and in inflammatory age-related processes, i.e. atherosclerosis, rheumatoid diseases and cancer [47].

The 5-LO gene maps in the chromosome 10q11.2 and codifies the 5-Lo enzyme involved in the synthesis of LTs. The 5-LO pathway has been associated to atherosclerosis in mouse and human histological studies. Several SNPs have been described. In particular, the - 1708 G/A, -1761 G/A and 21 C/T SNPs in promoter region and exon-1 of 5-LO gene modify the gene transcription or the putative protein [47].

An over-expression of anti-inflammatory CCR5Δ32 variant, +896 G (299Gly) TLR4 allele, -765 C Cox-2 allele, -1708 G and 21 C 5-Lo alleles characterizes male Sicilian centenarians (Table 3) [47-49]. So, male centenarians are people who seem genetically equipped for defeating major age-related diseases. They present SNPs in the immune system genome (i.e. SNPs or other genetic variations, located within the promoter regions of pro-inflammatory cytokines) which, regulating the immune-inflammatory responses, seem to be associated to longevity [30-32]. Furthermore, centenarians are characterized by marked delay or escape from age-associated diseases, responsible for the high mortality in earlier ages. In particular, it has been demonstrated that centenarian offspring have an increased likelihood of surviving to 100 years and show a reduced prevalence of age associated diseases, such as CVD, and lower prevalence of CVD risk factors [1,30-32,50] Thus, genes involved in CVD may play an opposite role in human male longevity. Our data in male Sicilian population confirm this suggestion and emphasize the role of antagonistic pleiotropy in ageing and longevity [51,52]. A high frequency of proinflammatory CCR5wt variant, +896A TLR4 allele, -765 G Cox-2 allele, 1708A and 21 T 5-Lo alleles characterizes male Sicilian patients affected by MI (Table 3) [47-49]. In a recent study, we also found a similar overexpression of these proinflammatory SNPs in male Sicilian patients affected by PC (Table 3). Opposite data were obtained in male centenarians [53,54].

In contrast, female Sicilian centenarians have a different frequency of the alleles of +896A/G TLR4 SNP than that observed in male Sicilian centenarians. In particular, female Sicilian centenarians show an over-expression of the pro-inflammatory +896A TLR4 allele respect to female patients affected by Boutonneuse fever and age-matched controls (Table 3) [55].

On the other hand, pro-inflammatory responses are evolutionary programmed to resist fatal infections. Thus, it is not surprising that the genetic background of people that survive to an advanced age may be protective against infections [55].

Based on our data, we suggest that Sicilian men and women may follow different trajectories to reach longevity. For men it might be more important to control atherogenesis and cancerogenesis, whereas for women it might be more important to control infectious diseases [30,31].

In order to confirm our suggestions on the biological effects of +896A/G TLR4 SNP and its role in the pathophysiology of age-related diseases studied (i.e. MI and PC) and longevity, we recently assessed the levels of IL-6, TNF-α, IL-10 and eicosanoids in LPS-stimulated whole blood samples from 50 young healthy Sicilians, screened for the presence of this SNP. Both pro-inflammatory cytokines and eicosanoids were significantly lower in carriers bearing the +896 G TLR4 allele, whereas the anti-inflammatory IL-10 values were higher [56]. This suggests the ability of the +896 G TLR4 allele to mediate a better control of inflammatory responses induced by chronic stimuli, so likely decreasing the effects of atherogenetic damage and prostate carcinogens.

On the basis of data reported herein, some suggestions can be drawn. First, pathogen load, by interacting with the host genotype, determines the type and intensity of inflammatory responses, according to the pro-inflammatory status and tissue injury, implicated in the patho-physiology of major age-related diseases. Second, adequate control of inflammatory response might reduce the risk of these diseases, and, reciprocally, might increase the chance of extended survival in an environment with reduced pathogen load. Accordingly, a higher frequency of the anti-inflammatory +896 G TLR4 allele has been observed in centenarians [49].

Cytokine profile: a biomarker for successful ageing

Cytokines are considered key players in maintaining lymphocyte homeostasis [57,58]. Their function is not limited to induce response after an immune insult, but they can modulate the nature of response (cytotoxic, humoral, cell mediated, inflammatory or allergic) or, in contrast, they may cause non-responsiveness and active immune suppression [58]. Furthermore, sequence variations in several cytokine genes, such as IFN-γ and IL-10 genes, have been demonstrated to be associated with successful ageing and longevity [58]. On the other hand, individual

changes in type and intensity of immune response affecting life span expectancy and health ageing seem to have a genetic component. A well-preserved immune function characterizing the successful ageing has been found in centenarians [38]. Recent evidence suggests that centenarians seem to be genetically equipped gene polymorphism for overcame the major age-related diseases and polymorphisms in immune system genes involved in regulation of immune responses have been found associated to longevity. In particular, associations between both cytokine gene polymorphisms and longevity, and differential gender longevity in males and females, and reciprocally to age-related diseases have been demonstrated [38,58,59].

Our data in Sicilian population confirm these associations and suggest that differences in the genetic regulation of immune inflammatory processes might explain the reason why some people but not others develop age-related diseases and why some develop a greater inflammatory response than others. In particular, this suggestion seems to be suitable for some SNPs in IFN-γ and IL-10 genes (Table 4) [60-63].

IFN-γ gene codifies for a cytokine involved in defense against viruses and intracellular pathogens, and in induction of immune mediated inflammatory responses. Its production is genetically regulated. A variable length CA repeat sequence in the first intron of IFN-γ gene has been described to be associated with high IFN-γ production. Furthermore, a SNP, T to A (+874 T/A), at 59 end of the CA repeat region has been described and T presence has been related to high-producing microsatellite allele 2. This SNP coincides with a putative NF-κB binding site, which might have functional consequences for transcription of IFN-γ gene. Thus, this SNP might directly influence IFN-γ production levels associated to CA microsatellite marker [60].

IL-10 gene codifies for IL-10 cytokine. IL-10 is produced by macrophages, T and B cells. It is one of the major immune-regulatory cytokines, usually considered to mediate potent down-regulation of inflammatory responses. IL-10 production, independently on interaction with other cytokine gene products, is generally controlled by several polymorphic elements in the 5' flanking region of IL-10 gene. Multiple SNPs have been identified in human IL-10 5' flanking region and some of these (i.e. -592, -819, -1082) combine with microsatellite alleles to form haplotype associated with differential IL-10 production. These three SNPs in the IL-10 proximal gene region (considered potential targets for transcription regulating factors) might be involved in genetic control of IL-10 production, even if contrasting literature data have been reported. In particular, the homozygous -1082GG genotype seems to be associated with higher IL-10 production respect to G/A heterozygous and AA homozygous genotypes. Furthermore, this SNP seems to be functionally relevant. It has been demonstrated that -1082 A carriers (low producers) seem likely develop a major number of chronic inflammatory diseases [61-63].

Our results demonstrated an increase of subjects carrying the -1082 G IL-10 allele in centenarian men [61-63]. This allele is associated to significantly increased IL-10 production. Conversely, we observed that the frequency of -1082A allele, associated to low IL-10 production, was significantly higher in MI patients (Table 4) [63]. Thus,

Table 4 Cytokine data from our studies in Sicilian population

Gene	Genotypes	Centenarians	Young controls (< 55 years)		P
		N = 31 males	N = 161 males		
IL-10	-1082GG	18 (58%)	55 (34%)		< 0.025 [61]
	-1083GA	9 (29%)	88 (55%)		
	-1082AA	4 (13%)	18 (11%)		
		N = 72 males	N = 115 males		
IL-10	-1082GG	33 (46%)	32(28%)		0.019 [62]
	1083GA	34(47%)	64 (56%)		
	-1082AA	5(7%)	19(17%)		
		Centenarians	Young controls (< 55 years)	MI patients (< 55 years)	P
		N 52 males	N 110 males	N 90 males	
IL-10	-1082GG	25 (48.1%)	26(23.6%)	17 (18.9%)	0.003 [63]
	-1083GA	23 (44.2%)	56 (50.9%)	29 (32.2%)	
	-1082AA	6(11.5%)	28 (25.5%)	44 (48.9%)	
Genes	**Alleles of SNP**	**Centenarians**	**Young controls (< 55 years)**		P
		N = 142 females	N = 90 females		
IFN-γ	-874 T	102 (35.9%)	85 (47.2%)		0.02 [60]
	− 874A	182 (64.1%)	95 (52.8%)		

high IL-10 production seems to be protective vs. MI and a possible biomarker for longevity. People with exceptional longevity have genetic factors (i.e. protective factors for CVD) that modulate ageing processes [63]. This supports the opinion that a genetic background protective against CVD is a component of longevity. On the other hand, our immune system has evolved to control pathogens and pro-inflammatory responses are likely programmed by evolution to resist fatal infections. From this prospective, low IL-10 production is correlated with increased resistance to pathogens. In older ages not evolutionally programmed, increased IL-10 levels might better control inflammatory responses induced by chronic vessel damage and reduce the risk for atherogenetic complications. These conditions might permit to achieve exceptional ages in an environmental with a reduced pathogen load [63].

In contrast, female Sicilian centenarians are characterized by an over-expression of +874 INF-γ allele (Table 4) [60]. The INF-γ production is also influenced by hormonal control fundamentally mediated by 17β extradiol. Hormonal regulation of this cytokine has been suggested to modulate, in part, the ability of estrogens to potentiate many types of immune responses and to influence the disproportionate susceptibility of women for immune-inflammatory diseases. Thus, gene variants representing genetic advantage for one gender might not be reciprocally relevant for the other gender in terms of successful or unsuccessful ageing [60].

The data from Sicilian investigation add another piece to complex puzzle of genetic and environmental factors involved in the control of life span expectancy in humans. Studies on cytokine gene SNPs may promise to individuate a complex network of trans-inactive genes able to influence the type and strength of immune responses to environmental stressors, and as final result, conditioning individual life expectancy [60-63]. On the other hand, we recently suggested the possibility to use cytokine profile as biomarker of successful ageing, by evaluating through Lumines technology cytokine serum levels in 44 Sicilian nonagenarians and 79 control subjects (aged between 30 and 50 years old) [64]. IFN-γ and IL-2 levels are unmodified, suggesting a substantial maintenance of relevant T functions. In addition, a significant increase of IL-12 serum levels was observed. This condition might be associated with the increase of NK cell function with ageing. Furthermore, an increase of IL-13 and a reduction of IL-4 were found. Thus, the maintenance of some effector's mechanisms of immune-response characterizes advanced ages. From a general point of view, our data firstly confirm the age-related remodeling of cytokine network. Furthermore, they underline the presence of unchanged levels of some crucial cytokines useful in preserving key immune function in long-living persons [64].

Future perspectives

The ageing process and longevity are multi-factorial events. Genetic, epigenetic, stochastic and environmental factors seem to have a crucial role in ageing and longevity. Epigenetic is associated to ageing, as shown in the major number of studies. In particular, ageing is associated to a global loss of methylation state [65]. In addition, tissue-dependent age-related hypermetylation of specific DNA regions have been observed. Thus, it can be concluded that epigenetic age-related modification are stochastic and no linked to specific DNA region, while epigenetic changes linked to specific environmental stimuli are limited in specific DNA region [66,67]. These observations have led to address the research on epigenomics and its implication in ageing and longevity.

Epigenomics is the systematic study of the global gene expression changes due to epigenetic processes, but not to DNA base sequence changes. Epigenetic processes consist in heritable modification that result in a selective gene expression or repression and consequently in phenotype changes [68]. These changes include nucleosome positioning, post-translation histone modifications, action of small RNAs, DNA replication timing, heterochromatinization and DNA methylation [69]. This last one consists in the addition of a methyl group (-CH3) in the carbon 5 of cytosines, particularly in the CpG dinucleotide. This condition particularly concerns the CpG islands (CpGIs), located at the regulatory site of gene promoter regions. Methylation rate is associated to transcriptional regulation. In particular, gene silencing is associated to increase of -CH3 groups on DNA, conversely hypometylation of CGIs is associated to an open chromatin state resulting in gene expression [70].

Although the association between ageing and epigenetic is a real evidence, processes involved are not clear. Certainly, the nutrition affects epigenetic modifications. Nutrients can be active on specific sites. For example, vitamin B12, vitamin B6, riboflavin, methionine, choline and betaine, well known as folates, regulate levels of S-adenosylmethionine and S-adenosylhomocysteine, donor of -CH3 group and methyltransferase inhibitor respectively [71]. Curcumin, resveratrol, polyphenols and flavonoids, phytoestrogen, and lycopene are also considered key nutritional factors both for regulation of enzyme involved in acetylation and deacetylation mechanism and for one-carbon metabolism [71,72]. A diet rich in vegetables and fruit, such as Mediterranean diet, may contain these nutrients. Sicilian centenarians are characterized to observe this kind of diet, as we reported [73]. Since genetic and environmental factors contribute to longevity, it may suggest that epigenetic events associated to the modifications diet-induced are very important for successful ageing processes. Furthermore, several literature data reported a possible link between epigenetic and several age-related diseases, such as cancer, metabolic

syndrome, diabetes and neurodegenerative disorders. Stable propagation of gene expression from cell to cell during disease pathogenesis is regulated by epigenetic mechanisms. For example, during the diabetes onset epigenetic changes act on insulin and insulin metabolism regulating the gene coding [74]. In particular, a recent study has demonstrated that human insulin gene and mouse insulin 2 gene expression are under control of epigenetic changes in CpGIs. Insulin non expressing cells are, indeed, methylated in the promoter region of insulin coding gene, while insulin expressing cells are completely demethylated in the same site resulting in insulin gene expression [75]. Another study on monozygotic twin has demonstrated that insulin resistance is also under control of DNA methylation [76]. Alterations in insulin pathway are known to be involved in metabolic disease, such as metabolic syndrome, insulin resistance and type 2 diabetes. Recent data also support the existence of a correlation between these alterations and Alzheimer's disease.

In the light of these observations, the purpose of our future studies will be to evaluate the weight of epigenetic changes in ageing and longevity, using centenarians as super-controls.

Abbreviations

AD: Alzheimer's disease; BF: Boutonneuse fever; CCR5: CC chemokine receptor 5; COX-2: Cyclo-oxygenase 2; CRP: C reactive protein; CVD: Cardiovascular disease; HSPs: Heat-shock proteins; INF-γ: Interferon-γ; IL-6: Interleukin-6; IL-10: Interleukin-10; 5-LO: 5-Lipoxygenase; LPS: Lipopolysaccharide; LTs: Leukotrienes; MI: Myocardial infarction; ox-LDL: Oxidized-Low Density Lipoproteins; PC: Prostate cancer; PGs: Prostaglandins; SNPs: Single nucleotide polymorphisms; TLR4: Toll-like-receptor-4; TNF-α: Tumor necrosis factor-α.

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Author details

[1]Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Corso Tukory 211, Palermo 90134, Italy. [2]Department of Internal Medicine II, Center for Medical Research, University of Tübingen, Tübingen Ageing and Tumor Immunology group, Waldhörnlestr. 22, Tübingen 72072, Germany.

Authors' contributions

CRB, CA, SB, MB, AM, and GCR wrote the first draft. Subsequent drafts were written by CRB, who had the overall supervision of the review processing. All authors edited the paper and approved its final version.

Competing interests

The authors declare that they have no competing interests.

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References

1. Terry DF, Wilcox MA, McCormick MA, Pennington JY, Schoenhofen EA, Andersen SL, Perls TT: Lower all-cause, cardiovascular, and cancer mortality in centenarians' offspring. *J Am Geriatr Soc* 2004, 52:2074-2076.
2. Terry DF, McCormick M, Andersen S, Pennington J, Schoenhofen E, Pazsina L, Bausero M, Ogawa K, Perls TT, Asea A: Cardiovascular disease delay in centenarian offspring: role of heat shock proteins. *Ann N Y Acad Sci* 2007, 1019:502-505.
3. Barzilai N, Gabriely I, Gabriely M, Iankowitz N, Sorkin JD: Offspring of centenarians have a favorable lipid profile. *J Am Geriatr Soc* 2001, 49:78-79.
4. Rose G, Passarino G, Scornaienchi V, Romeo G, Bonafè S, Bellizzi D, Marini V, Feraco E, Maletta R, Bruni A, Franceschi C, De Benedictis G: The mitochondrial DNA control region shows genetically correlated levels of heteroplasmy in leukocytes of centenarians and their offspring. *BMC Genomics* 2007, 8:293.
5. Pawelec G, Akbar A, Caruso C, Solana R, Grubeck-Loebenstein B, Wikby A: Human immunosenescence: is it infectious? *Immunol Rev* 2005, 205:257-268.
6. Chidrawar S, Khan N, Wei W, McLarnon A, Smith N, Nayak L, Moss P: Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals. *Clin Exp Immunol* 2009, 155:423-432.
7. Derhovanessian E, Vallo A, Pawelec G: Biomarkers of human immunosenescence: impact of Cytomegalovirus infection. *Curr Opin Immunol* 2009, 21:440-445.
8. Derhovanessian E, Maier AB, Beck R, Jahn G, Hähnel K, Slagboom PE, de Craen AJ, Westendorp RG, Pawelec G: Hallmark features of immunosenescence are absent in familial longevity. *Immunol* 2010, 185:4618-4627.
9. Colonna Romano G, Bulati M, Aquino A, Scialabba G, Candore G, Lio D, Motta M, Malaguarnera M, Caruso C: B cells in the aged: CD27, CD5 and CD40 expression. *Mech Ageing Dev* 2003, 124:389-393.
10. Colonna Romano G, Cossarizza A, Aquino A, Scialabba G, Bulati M, Lio D, Candore G, Di Lorenzo G, Fradà G, Caruso C: Age- and gender-related values of lymphocyte subsets in subjects from northern and southern Italy. *Arch Gerontol Geriatr (Suppl)* 2002, 8:99-107.
11. Listì T, Candore G, Modica MA, Russo MA, Di Lorenzo G, Esposito-Pellitteri M, Colonna Romano G, Aquino A, Bulati M, Lio D, Franceschi C, Caruso C: A study of serum immunoglobulin levels in elderly persons that provides new insights into B cell immunosenescence. *Ann N Y Acad Sci* 2006, 1089:487-495.
12. Candore G, Di Lorenzo C, Mansueto P, Melluso M, Fiada G, Di Vecchi M, Esposito Pellitteri M, Drago A, Di Salvo A, Caruso C: Prevalence of organ-specific and non organ-specific autoantibodies in healthy centenarians. *Mech Ageing Dev* 1997, 94:183-190.
13. Colonna-Romano G, Bulati M, Aquino A, Vitello S, Lio D, Candore G, Caruso C: B cell immunosenescence in the elderly and in centenarians. *Rejuvenation Res* 2008, 11:433-439.
14. Colonna Romano G, Bulati M, Aquino A, Pellicanò M, Vitello S, Lio D, Candore G, Caruso C: A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people. *Mech Ageing Dev* 2009, 130(10):681-690.
15. Bulati M, Buffa S, Candore G, Caruso C, Dunn-Walters DK, Pellicanò M, Wu YC, Colonna Romano G: B cells and immunosenescence: a focus on IgG+IgD-CD27- (DN) B cells in aged humans. *Ageing Res Rev* 2011, 10:274-284.
16. Buffa S, Bulati M, Pellicanò M, Dunn-Walters DK, Wu YC, Candore G, Vitello S, Caruso C, Colonna-Romano G: B cell immunosenescence: different features of naive and memory B cells in elderly. *Biogerontology* 2011, 12:473-483.
17. Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, Lee EH, Milner EC, Sanz I: 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* 2007, 178:6624-6633.
18. Sanz I, Wei C, Lee FE, Anolik J: Phenotypic and functional heterogeneity of human memory B cells. *Semin Immunol* 2008, 20(1):67-82.
19. Cagigi A, Du L, Dang LV, Grutzmeier S, Atlas A, Chiodi F, Pan-Hammarström Q, Nilsson A: CD27(-) B-cells produce class switched and somatically hyper-mutated antibodies during chronic HIV-1 infection. *PLoS One* 2009, 4(5):e5427.
20. Colonna Romano G, Buffa S, Bulati M, Candore G, Lio D, Pellicanò M, Vasto S, Caruso C: B cells compartment in centenarian offspring and old people. *Curr Pharm Des* 2010, 16:604-608.

21. Frasca D, Riley RL, Blomberg BB: **Humoral immune response and B-cell functions including immunoglobulin class switch are downregulated in aged mice and humans.** *Semin Immunol* 2005, **17(5)**:378-384.
22. Dunn-Walters DK, Ademokun AA: **B cell repertoire and ageing.** *Curr Opin Immunol* 2010, **22(4)**:514-520.
23. Cancro MP, Hao Y, Scholz JL, Riley RL, Frasca D, Dunn-Walters DK, Blomberg BB: **B cells and aging: molecules and mechanisms.** *Trends Immunol* 2009, **30(7)**:313-318.
24. Kumar R, Burns EA: **Age-related decline in immunity: implications for vaccine responsiveness.** *Expert Rev Vaccines* 2008, **7(4)**:467-479.
25. Gibson KL, Wu YC, Barnett Y, Duggan O, Vaughan R, Kondeatis E, Nilsson BO, Wikby A, Kipling D, Dunn-Walters DK: **B-cell diversity decreases in old age and is correlated with poor health status.** *Aging Cell* 2009, **8(1)**:18-25.
26. Grubeck-Loebenstein B, Della Bella S, Iorio AM, Michel JP, Pawelec G, Solana R: **Immunosenescence and vaccine failure in the elderly.** *Aging Clin Exp Res* 2009, **21(3)**:201-209.
27. Perrig-Chiello P, Hutchison S: **Health and well-being in old age: the pertinence of a gender mainstreaming approach in research.** *Gerontology* 2010, **56**:208-213.
28. May RC: **Gender, immunity and the regulation of longevity.** *Bioessays* 2007, **29(8)**:795-802.
29. [http://www.istat.it].
30. Candore G, Balistreri CR, Colonna Romano G, Lio D, Listì F, Vasto S, Caruso C: **Gender-related immune-inflammatory factors, age-related diseases, and longevity.** *Rejuvenation Res* 2010, **13**:292-729.
31. Candore G, Balistreri CR, Listì F, Grimaldi MP, Vasto S, Colonna Romano G, Franceschi C, Lio D, Caselli G, Caruso C: **Immunogenetics, gender, and longevity.** *Ann N Y Acad Sci* 2006, **1089**:516-537.
32. Capri M, Salvioli S, Monti D, Caruso C, Candore G, Vasto S, Olivieri F, Marchegiani F, Sansoni P, Baggio G, Mari D, Passarino G, De Benedictis G, Franceschi C: **Human longevity within an evolutionary perspective: The peculiar paradigm of a post-reproductive genetics.** *Exp Gerontol* 2008, **43**:53-60.
33. Lio D, Balistreri CR, Colonna-Romano G, Motta M, Franceschi C, Malaguarnera M, Candore G, Caruso C: **Association between the MHC class I gene HFE polymorphisms and longevity: a study in Sicilian population.** *Genes Immun* 2002, **3**:20-24.
34. Beutler E: **Iron storage disease: facts, fiction and progress.** *Blood Cells Mol Dis* 2007, **39**:140-147.
35. Pietrangelo A: **Hereditary hemochromatosis: pathogenesis, diagnosis, and treatment.** *Gastroenterology* 2010, **139**:393-408, 408.e1-2.
36. Franceschi C, Motta L, Motta M, Malaguarnera M, Capri M, Vasto S, Candore G, Caruso C: **IMUSCE. The extreme longevity: the state of the art in Italy.** *Exp Gerontol* 2008, **43**:45-52.
37. Troen BR: **The biology of aging.** *Mt Sinai J Med* 2003, **70**:3-22.
38. Ostan R, Bucci L, Capri M, Salvioli S, Scurti M, Pini L, Monti D, Franceschi C: **Immunosenescence and immunogenetics of human longevity.** *Neuroimmunomodulation* 2008, **15**:224-240.
39. Candore G, Colonna Romano G, Balistreri CR, Di Carlo D, Grimaldi MP, Listì F, Nuzzo D, Vasto S, Lio D, Caruso C: **Biology of longevity: role of the innate immune system.** *Rejuvenation Res* 2006, **9**:143-148.
40. Williams GC: **Pleiotropy, natural selection, and the evolution of senescence.** *Evolution* 1957, 398-411, 11.
41. Vasto S, Candore G, Balistreri CR, Caruso M, Colonna-Romano G, Grimaldi MP, Listì F, Nuzzo D, Lio D, Caruso C: **Inflammatory networks in ageing, age-related diseases and longevity.** *Mech Ageing Dev* 2007, **128**:83-91.
42. Balistreri CR, Colonna-Romano G, Lio D, Candore G, Caruso C: **TLR4 polymorphisms and ageing: implications for the pathophysiology of age-related diseases.** *J Clin Immunol* 2009, **29**:406-415.
43. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, Frees K, Watt JL, Schwartz DA: **TLR4 mutations are associated with endotoxin hyporesponsiveness in humans.** *Nat Genet* 2000, **25**:187-191.
44. Ferwerda B, McCall MB, Alonso S, Giamarellos-Bourboulis EJ, Mouktaroudi M, Izagirre N, Syafruddin D, Kibiki G, Cristea T, Hijmans A, Hamann L, Israel S, ElGhazali G, Troye-Blomberg M, Kumpf O, Maiga B, Dolo A, Doumbo O, Hermsen CC, Stalenhoef AF, van Crevel R, Brunner HG, Oh DY, Schumann RR, de la Rúa C, Sauerwein R, Kullberg BJ, van der Ven AJ, van der Meer JW, Netea MG: **TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans.** *Proc Natl Acad Sci USA* 2007, **104**:16645-16650.
45. Ferwerda B, McCall MB, Verheijen K, Kullberg BJ, van der Ven AJ, Van der Meer JW, Netea MG: **Functional consequences of toll-like receptor 4 polymorphisms.** *Mol Med* 2008, **14**:346-352.
46. Balistreri CR, Caruso C, Grimaldi MP, Listì F, Vasto S, Orlando V, Campagna AM, Lio D, Candore G: **CCR5 receptor: biologic and genetic implications in age-related diseases.** *Ann NY Acad Sci* 2007, **1100**:162-172.
47. Listì F, Caruso M, Incalcaterra E, Hoffmann E, Caimi G, Balistreri CR, Vasto S, Scafidi V, Caruso C, Candore G: **Pro-inflammatory gene variants in myocardial infarction and longevity: implications for pharmacogenomics.** *Curr Pharm Des* 2008, **14**:2678-2685.
48. Balistreri CR, Candore G, Caruso M, Incalcaterra E, Franceschi C, Caruso C: **Role of polymorphisms of CC-chemokine receptor-5 gene in acute myocardial infarction and biological implications for longevity.** *Haematologica* 2008, **93**:637-638.
49. Balistreri CR, Candore G, Colonna-Romano G, Lio D, Caruso M, Hoffmann E, Franceschi C, Caruso C: **Role of Toll-like receptor 4 in acute myocardial infarction and longevity.** *JAMA* 2004, **292**:2339-2340.
50. Perls T, Terry D: **Genetics of exceptional longevity.** *Exp Gerontol* 2003, **38**:725-730.
51. Candore G, Balistreri CR, Grimaldi MP, Listì F, Vasto S, Caruso M, Caimi G, Hoffmann E, Colonna Romano G, Lio D, Paolisso G, Franceschi C, Caruso C: **Opposite role of pro-inflammatory alleles in acute myocardial infarction and longevity: results of studies performed in a Sicilian population.** *Ann NY Acad Sci* 2006, **1067**:270-275.
52. Incalcaterra E, Caruso M, Balistreri CR, Candore G, Lo Presti R, Hoffmann E, Caimi G: **Role of genetic polymorphisms in myocardial infarction at young age.** *Clin Hemorheol Microcirc* 2010, **46**:291-298.
53. Balistreri CR, Carruba G, Calabrò M, Campisi I, Di Carlo D, Lio D, Colonna Romano G, Candore G, Caruso C: **CCR5 proinflammatory allele in prostate cancer risk: a pilot study in patients and centenarians from Sicily.** *Ann NY Acad Sci* 2009, **1155**:289-292.
54. Balistreri CR, Caruso C, Carruba G, Miceli V, Campisi I, Listì F, Lio D, Colonna-Romano G, Candore G: **A pilot study on prostate cancer risk and pro-inflammatory genotypes: pathophysiology and therapeutic implications.** *Curr Pharm Des* 2010, **16**:718-724.
55. Balistreri CR, Candore G, Lio D, Colonna-Romano G, Di Lorenzo G, Mansueto P, Rini G, Mansueto S, Cillari E, Franceschi C, Caruso C: **Role of TLR4 receptor polymorphisms in Boutonneuse fever.** *Int J Immunopathol Pharmacol* 2005, **18**:655-660.
56. Balistreri CR, Caruso C, Listì F, Colonna-Romano G, Lio D, Candore G: **LPS-mediated production of pro/anti-inflammatory cytokines and eicosanoids in whole blood samples: biological effects of +896A/G TLR4 polymorphism in a Sicilian population of healthy subjects.** *Mech Ageing Dev* 2011, **132**:86-92.
57. Sanjabi S, Zenewicz LA, Kamanaka M, Flavell RA: **Anti-inflammatory and pro-inflammatory roles of TGF-beta, IL-10, and IL-22 in immunity and autoimmunity.** *Curr Opin Pharmacol* 2009, **9**:447-453.
58. Iannitti T, Palmieri B: **Inflammation and genetics: an insight in the centenarian model.** *Hum Biol* 2011, **83**:531-559.
59. Caruso C, Candore G, Colonna Romano G, Lio D, Franceschi C: *Inflammation and life span. Science* 2005, **307**:208-209.
60. Lio D, Scola L, Crivello A, Colonna Romano G, Candore G, Bonafè M, Cavallone L, Franceschi C, Caruso C: **Gender-specific association between -1082 IL-10 promoter polymorphism and longevity.** *Genes Immun* 2002, **3**:30-33.
61. Lio D, Scola L, Crivello A, Colonna-Romano G, Candore G, Bonafè M, Cavallone L, Marchegiani F, Olivieri F, Franceschi C, Caruso C: **Inflammation, genetics, and longevity: further studies on the protective effects in men of IL-10-1082 promoter SNP and its interaction with TNF-alpha -308 promoter SNP.** *J Med Genet* 2003, **40**:296-299.
62. Lio D, Candore G, Crivello A, Scola L, Colonna-Romano G, Cavallone L, Hoffmann E, Caruso M, Licastro F, Caldarera CM, Branzi A, Franceschi C, Caruso C: **Opposite effects of interleukin 10 common gene polymorphisms in cardiovascular diseases and in successful ageing: genetic background of male centenarians is protective against coronary heart disease.** *J Med Genet* 2004, **41**:790-794.
63. Lio D, Scola L, Crivello A, Bonafè M, Franceschi C, Olivieri F, Colonna Romano G, Candore G, Caruso C: **Allele frequencies of +874 T- > A single**

nucleotide polymorphism at the first intron of interferon-gamma gene in a group of Italian centenarians. *Exp Gerontol* 2002, **37**:315-319.
64. Palmeri M, Misiano G, Malaguarnera M, Forte GI, Vaccarino L, Milano S, Scola L, Caruso C, Motta M, Mangeri D, Lio D: Cytokine serum profile in a group of sicilian nonagenarians. *J Immunoassay Immunochem* 2012, **33**:82-90.
65. Park LK, Friso S, Choi SW: Nutritional influences on epigenetics and age-related disease. *Proc Nutr Soc* 2012, **71**:75-83.
66. Portela A, Esteller M: Epigenetic modifications and human disease. *Nat Biotechnol* 2010, **28**:1057-1068.
67. Fuke C, Shimabukuro M, Petronis A, Sugimoto J, Oda T, Miura K, Miyazaki T, Ogura C, Okazaki Y, Jinno Y: Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study. *Ann Hum Genet* 2005, **69**(Pt 1):132.
68. Wu C, Morris JR: Genes, genetics, and epigenetics: a correspondence. *Science* 2001, **293**:1103-1105.
69. Antequera F, Bird A: *CpG Islands. NAS* 1993, **64**:169-185.
70. Decottignies A, d'Adda di Fagagna F: Epigenetic alterations associated with cellular senescence: a barrier against tumorigenesis or a red carpet for cancer? *Semin Cancer Biol* 2011, **21**:360-366.
71. Lim U, Song MA: Dietary and lifestyle factors of DNA methylation. *Methods Mol Biol* 2012, **863**:359-376.
72. Choi SW, Friso S: Epigenetics: A New Bridge between Nutrition and Health. *Adv Nutr* 2010, **1**:8-16.
73. Vasto S, Scapagnini G, Rizzo C, Monastero R, Marchese A, Caruso C: Mediterranean diet and longevity in Sicily: a survey in Sicani Mountains Population. *Rejuvenation Res*.
74. Miré E, Emanuelli C: Diabetes-induced epigenetic signature in vascular cells. *Endocr Metab Immune Disord Drug Targets* 2012.
75. Kuroda A, Rauch TA, Todorov I, Ku HT, Al Abdullah IH, Kandeel F, Mullen Y, Pfeifer GP, Ferreri K: Insulin gene expression is regulated by DNA methylation. *PLoS One* 2009, **4**:10.
76. Zhao J, Goldberg J, Bremner JD, Vaccarino V: Global DNA methylation is associated with insulin resistance: a monozygotic twin study. *Diabetes* 2012, **61**:542-546.

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CHAPTER 10

"Centenarian Offspring: a model for Understanding Longevity"

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Centenarian Offspring: a model for Understanding Longevity

Carmela Rita Balistreri, Giulia Accardi, Silvio Buffa, Matteo Bulati, Adriana Martorana, Giuseppina Candore, Giuseppina Colonna-Romano, Domenico Lio, Calogero Caruso

Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Palermo, Italy

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Author for correspondence:

Carmela Rita Balistreri, PhD
Gruppo di Studio sull'Immunosenescenza
Dipartimento di Biopatologia e di Biotecnologie Mediche e Forensi
Corso Tukory 211
90134 Palermo – Italy
carmelarita.balistreri@unipa.it

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Abstract

A main objective of current medical research is the improving of life quality of elderly people as priority of the continuous increase of *ageing population*. This phenomenon implies several medical, economic and social problems because of dramatic increase in number of not autonomous individuals affected by various pathologies. Accordingly, the research interest is focused on understanding the biological mechanisms involved in determining the positive ageing phenotype, i.e. *the centenarian phenotype*. In achieving this goal the choice of an appropriate study models is fundamental. Centenarians have been used as an optimal model for successful ageing. However, it is characterized by several limitations, i.e. the selection of appropriate controls for centenarians and the use itself of the centenarians as a suitable model for healthy ageing. Thus, the interest has been centered on centenarian offspring, healthy elderly people. They may represent a model for understanding exceptional longevity for the following reasons: to exhibit a protective genetic background, cardiovascular and immunological profile as well as a reduced rate of cognitive decline than age-matched people without centenarian relatives. Several of these aspects are summarized in this review based on the literature and the results of our studies.

Keywords: Ageing, genetic background, centenarians, centenarian offspring, memory decline, cardiovascular profile, immunosenescence.

INTRODUCTION

Medical research currently is influenced by the massive ageing in the developed countries. This trend implies a set of medical, economic and social problems. The cause is principally the increase in number of not autonomous elderly individuals affected by several pathologies. So, an improvement of human elderly health is required. Thus, the research interest should be focused on the so-called *positive biology*. It differs from well-known current biology that has as central focus the causes of chronic diseases, afflicting millions of people living today, such as cardiovascular diseases (CVD), cancer and Alzheimer disease (AD). In contrast, positive biology has as principal goal the identification of biological mechanisms of the positive phenotypes. In this context, the positive phenotype is represented by some individuals living long, showing relatively good health, able to perform their daily life tasks and to escape fatal age-related diseases [1,2]. Observation of exceptional longevity can represent an interesting study model of health and well-being. This new study approach might provide useful data. Their translation might consent to obtain beneficial biological effects, i.e. modulation of ageing rate, development of drugs or new lifestyle habits (i.e. a healthy diet, caloric uptake reduction, use of antioxidants, prebiotics and probiotics and increased physical exercise) able to enhance health and well-being, to retard ageing, and to prevent or reduce frailty and disability especially in individuals genetically predisposed. Furthermore, it might provide evidence, which would further strengthen the concepts of ageing theories [1.2].

From this point of view, centenarian offspring (CO), healthy elderly people with a family history of longevity, may represent a model for understanding exceptional longevity. Their selection is essentially based on current opinion of longevity as a familial trait, influenced principally by genetic factors and environmental conditions.Apart from differences in their genomes, development and life expectancy, a major difference between humans and laboratory animals is the quantity and quality of antigenic exposure. Typical laboratory organisms are usually housed in *artificially clean* environments. Thus, they are underexposed to pathogens, or even completely protected from them,

except for limited period of time required for experimental reasons. These animals are quite different from those living in the wild exposed to a plethora of different microorganisms, such as bacteria, viruses and parasites. Human beings have an environment, although controlled, not sterile, but rather full of microbes [3]. Some of them are normally hosted on skin and mucosal tissues, having a protective role against pathogenic microbes. In contrast, others are or can become frankly pathogenic and trigger infectious diseases that constituted a major life-threatening event throughout the natural history of animal species. Furthermore, lifelong antigenic load and the consequent inflammation are assumed as the major determinants of human ageing rate and unsuccessful ageing.

Other interesting characteristics, such as a protective genetic background, immunological profile and clinical history seem to be exhibited by CO when compared with age-matched people without centenarian relatives. Many of these aspects are summarized in this review based on the literature data and results of our studies.

FOCUS ON CENTENARIANS OFFSPRING: FAVORABLE CARDIOVASCULAR RISK PROFILE

Several and comparable epidemiological studies in different populations (Ashkenazi Jews, Americans from New England, Japanese from Okinawa, Icelanders, Mormons, Netherlanders from Leiden) have established a role for genetic component in attaining longevity [4-9]. Siblings and CO, but not their spouses, show an increased odd ratio (OR) between 4- and 17-fold for longevity compared with appropriate controls [4,5]. This supports the existence of strong genetic determinants for longevity. Furthermore, it also suggests that the favorable modulation not only of ageing processes, but also of disease susceptibility, is strongly inherited in families with exceptional longevity. Thus, it supposes a possible transmission from centenarians to their descendants of the capacity to escape and/or postpone the major age-related diseases. In order to assess this, the interest has been centered on CO. On the other hand, their evaluation bypasses some problems, such as to find appropriate controls for centenarians, to eliminate the doubts to consider the centenarians

a useful model for healthy ageing, since only few studies reported centenarians as healthier or well functioning during most of their lives compared with average populations [10-13].

The first effort based on CO was made by the group of Perls and Terry in a cross-sectional study [14]. It reported the prevalence of several age-related diseases in 177 CO and 166 age-matched controls without centenarian parents enrolled in the nationwide New England Centenarian Study. Like centenarians, their descendants had a markedly reduced risk for CVD (56%; OR 0.44, 95% CI 0.24-0.80), hypertension (66%; OR 0.34, 95% CI 0.21-0.55) and diabetes (59%; OR 0.41, 95% CI 0.15-1.12) compared with age-matched controls without centenarian parents. Furthermore, the same researchers reported a significant 62% reduction in all mortality causes and an 85% lower risk ofcoronary heart disease-specific mortality in CO [15]. In addition, median ages of onset for coronary heart disease, hypertension, diabetes and stroke are significantly delayed in this cohort by 5.0, 2.0, and 8.5 and 8.5 years, respectively, compared with age-matched controls [16].

Subsequently, same authors [15] also observed that CO had a 71% lower risk of dying from cancer when compared with age-matched controls. It is possible that, at younger ages (e.g. <70 years), CO have a lower risk of cancer than controls, but that this difference disappears as the birth cohort ages. Left behind are the survivors of the two groups, with similar susceptibility to cancer. The possible protective factors are not clear. However, it supposes that CO may have likely inherited genetic variations from their long-lived parents able to protect them from multiple mutations, thus resulting in lower cancer-specific mortality [15].

To further confirm these findings, a recent longitudinal study has been executed in a group of 440 CO, considered to be predisposed to healthy [4, 14-18] ageing, and 192 control subjects enrolled and followed for over 10 years by the New England Centenarian Study. The specific goal of this longitudinal analysis was to discern whether the differences in the presence of age-related diseases between CO and control subjects persist over time, or whether the differences disappear as the two groups get older. During the follow-up period, CO had a 78% lower risk of myocardial

infarction, 83% lower risk of stroke, and 86% lower risk of developing diabetes mellitus than the control cohort. There were no significant differences in new onset of other age-related diseases. Additionally, CO were 81% less likely to die than the controls during follow-up. These results suggest that CO retain some important cardiovascular advantages over time similarly to their parents. These findings reinforce the concept of longevity family history likely determined by physiological causes. CO indeed have a better cardiovascular health and a lower mortality than their peers [17].

Concordant results were obtained by Barzilai's group. In this study, the offspring of long lived parents from Ashkenazi Jewish population had significantly lower prevalence of hypertension (by 23%), diabetes mellitus (by 50%), heart attacks (by 60%), and strokes (no events reported) than several age-matched control groups [4].

In this context, it is also noteworthy to mention that centenarians and offspring of the Long Life Family Study (LLFS) cohort [18] were less likely to have diabetes mellitus, chronic pulmonary disease and peripheral artery disease than the Cardiovascular Health Study and Framingham Heart Study cohort members [16]. Measures of physical function and cardiovascular risk factors were more optimal in LLFS compared with the other groups [18].

This data concordance led to indentifying of the possible genetic and molecular pathways able to confer the protective cardiovascular profile in CO. Barzilai and colleagues [19,20]studied the lipid profiles amongAshkenazi Jewish centenarians, their children and thechildren's spouses (the controls in the study). Bothmale and female children had significantly higher high density lipoprotein-cholesterol (IIDL-C) levels comparedwith controls and the males also had significantly lower low density lipoprotein-cholesterol (LDL-C) levels. In order to focus candidate genes of lipoprotein metabolism, they also identified a markedly higher frequency of a functional variant (homozygosity for the 405 valine) of the gene codifying the cholesteryl ester transfer protein (CETP) in centenarians and their offspring. This genetic variant increases the particle size of both

HDL and LDL [21]. Consistent with this, Li and colleagues[22] also reported a larger HDL and LDL protein size in centenarians and their descendants compared with controls. Newman et al [18] found that the HDL-C levels were higher and triglycerides were lower in LLFS centenarians and CO. These features reflect the protective lipid and cardiovascular profile of the people studied. Furthermore, it has been recently evidenced a significant correlation between the genetic CETP variant and a lower prevalence of hypertension, a reduced systolic blood pressure, and a trend toward lower diastolic blood pressure [23].

In a more recent study, Barzilai group genotyped Ashkenazi Jewishcentenarians (n = 213), their offspring (n = 216) and an age-matched Ashkenazi control group (n = 258) for 66 polymorphisms in 36 genes related to CVD. They identified a higher prevalence of homozygosity for the -641C (rs2542052) allele inthe APOC3 promoter in centenarians (25%) and their offspring (20%) than controls (10%). This genotype is associated with significantly lower serum levels of APOC3 and afavorable pattern of lipoprotein levels and size. A lower prevalence of hypertension and greater insulinsensitivity in the -641C homozygotes was found, suggesting a protective effect against CVD and metabolic syndrome. Homozygosis for the APOC3 -641C allele is associated with a favorable lipoprotein profile, cardiovascular health, insulin sensitivity and longevity. These data show that genetic modulation of lipoproteins may be considered a pathway influencing lifespan [24].

Of interest are the concordant data on lower levels of 70-kd heat shock protein (Hsp70) observed by three research groups in centenarians and their offspring. Hsp70 is a highly conserved protein having protective and deleterious effects [25-27]. It is involved in the pathophysiology of major age-related diseases, particularly in atherosclerosis and its complications. Usually, its expression and release in vascular smooth muscle cells increases under oxidative stress induced by risk factors, such as smoking, hyperlipidemia, diabetes and hypertension. Thus, circulating Hsp70 levels predict the development of CVD. In contrast, in those individuals that reach an advanced age,

Hsp70 shows lower serum levels, conferring a beneficial and protective effect. Searching to explain the differences in serum Hsp70 levels between centenarians and their offspring and age-matched controls from the New England Centenarian Study and the Longevity Genes Project on Ashkenazi Jewish population, their genotyping for two functional tag polymorphisms, rs1043618 in HSP70-A1A and rs6457452 in HSP70-A1B genes, demonstrated no associations. However, serum Hsp70 levels may be considered as biomarkers of exceptional longevity [28].

Another possible and key pathway is that of insulin/IGF1. In 2007, Cohen and colleagues studied the level of IGF1 in Ashkenazi Jewish descendants. They showed higher levels of this factor related to the control population without a family history of longevity. In particular, they found 35% higher serum levels in female CO respect to age and gender-matched controls. In contrast, male offspring showed the same levels as controls [29].

These results suggest that phenotypic and genotypiccharacteristics associated with exceptional longevity aretransmitted in long lived families. In addition, it emphasizes the importance of cardiovascular health in achieving exceptional old age as well as the familial nature of longevity.

REDUCED RISK FOR ALZHEIMER'S DISEASE AND MEMORY DECLINE IN CENTENARIAN OFFSPRING

With advancing age, individuals can experience cognitive decline and develop several forms of dementia. Among these, AD represents one of the major health problems, afflicting millions of people today [30]. Centenarians are escaping and/or postponing this pathology [4, 21, 31]. No consistent data exist on the eventual advantage transmitted from centenarians to their offspring to maintain a high physical and cognitive function and sustained engagement in social and productive activities, and to outlive this disease. Longitudinal studies of CO and offspring of parents with the

average of life expectancy at birth for specific cohorts,using rigorous neuropsychological assessments are needed to clarify the influence of parental longevity on cognitive decline.

Recently, the Bronx Aging Study [32] provided the opportunity to apply this approach. The study enrolled community residing subjects (aged 75-85 years) from 1980 to 1983 and followed them for 23 years. The study included information on parental age at death and extensive annual clinical, neuropsychological and neurological evaluations to determine memory decline and the incidence of dementia during follow-up. Using the data from the Bronx Aging Study, the Barzilai group tested the hypothesis that parental longevity protects against memory decline and the onset of dementia. Indeed, the CO had a reduced incidence for AD (hazard ratio0.57; 95% CI: 0.35 – 0.93) than the control group. In particular,they were 36% less likely than thecontrol group to develop dementia. They also had a significantly reduced rate of memory decline on the Selective Reminding Test in comparison with the control group [32].

On the other hand, Barraland colleagues [33] observed in offspring generation from LLFS family members, a better performance on multiple tasks requiring attention, working memory and semantic processing when compared with individuals without a family history of exceptional survival. This suggests that cognitive performance may serve as an important endophenotype for longevity [33]. Hagberg and Samuelsson [34] also evidenced that hereditary factors, social relationships, marital status and personality did not contribute to survival prediction in this exceptional age group. In consequence, in very old age, stochastic determinants may dominate over programmed factors (for example family longevity) in the determining of survival. Yates and colleagues [35] found that those who survived to 90 years old had better late-life physical function (mean ± SD score [maximum 100], 73 ± 23 *vs* 62 ± 30, $p < 0.001$) and mental well-being (mean score, 84 ± 14 *vs* 81 ± 17, $p = 0.03$). More than 68% (*vs* 45%) rated their late-life health as excellent or very good and less than 8% (*vs* 22%) reported fair or poor health.

Based on these observations, CO develops dementia and AD at a significantly lower rate than controls. This result is not explained by demographic or medical confounders. Factors associated with longevity may protect against dementia and AD.

IMMUNOLOGICAL HALLMARKS IN CENTENARIAN OFFSPRING

The age-related decline of the immune system which accompanies the ageing with several modifications is called immunesenescence[36- 38]. A reduced amount of T, B and NK cells is observed in healthy elderly people [39-43]. Furthermore, the T cell compartment shows shrinkage in elderly people of a cellular repertoire caused by the filling of the immunological space characterized by memory-late differentiated (CD3+CD8+CD45RA-CD27-CD28-) CD8+ T cells [44]. Concerning the B cellular compartment, studies did not lead to a common consensus. Several contrasting data have been suggested on changes occurring in the naove/memory subsets. However, a serum increase of switched immunoglobulins and a reciprocal decrease of both serum IgM and IgD have been demonstrated in elderly people [45-47]. This last evidence seems to be in agreement with the reduced number of naive B cells and the increase of a Double negative, DN ($IgD^{-}CD27^{-}$) IgG^{+} B cell population observed in healthy elderly, as reported by our recent studies [43, 49, 50].

The interest in establishing the relevance of the immunological features in the centenarian phenotype led also to detect several immune parameters [47, 50-54].Therefore, it has been suggested that centenarians share some typical immunological parameters of young people and others characteristic of the elderly. Among these, the number of NK cells and their activity in centenarians are well conserved and comparable to those observed in young people, when compared with elderly people. In contrast, a decrease in B lymphocytes and naive T cells, a progressive increase of CD28-cytotoxic T cells, the expansion of clones of memory T cells, a shrinkage of the T cell repertoire characterise the centenarians, are typical changes of immunosenescence. Furthermore, they have no organ-specific auto-antibodies, often observed in healthy elderly

individuals, and present some signs of inflammation, including high plasma levels of pro-inflammatory cytokines, i.e. IL-6 and a lipoprotein, Lp(a), considered a CVD risk factor and genetically controlled, as well as coagulation factors [36, 37, 52, 55-57].

Siblings and offspring of long lived subjects may become a good model to analyse the immune profile and consequently to understand the advantages of centenarian phenotype [25, 58-60]. Until now, the literature has reported only on some aspects of the CO immune profile, i.e. data about the B cell branch. In this context, our recent data are of interest. Comparing the B cells from individuals of different age groups (young, elderly and CO), we noted that B cells from CO are more comparable to those from young people compared with those from healthy old subjects. Like young individuals, CO is characterised, indeed, by an increase of naïve (IgD^+CD27^-) B cells [61]. This is a remarkable observation, since the bone marrow ability to generate B cells is impaired with age [61]. Furthermore, our comparative analysis revealed that CO also has a decrease in percentage of DN B cells [43]. The analysis of serum IgM concentration in CO, also revealed the presence of higher levels in CO than their controls. Our supposition is that CO behaves as the young individuals. This might allow them to preserve themselves from new infections and to respond to vaccinations. All together, these data might be related to their healthier ageing [62].

ROLE OF VARIATIONS IN HUMAN TELOMERASE GENE AND MITOCONDRIAL DNA

A close relation between telomere length and life span in humans, including long lived humans, has been reported [63].However, interpretations of results are often confounded because of a lack of adequate controls [64,65]. Recently, the group of Barzilai employed a unique study design to overcome this inadequacy in a cohort of Ashkenazi Jewish individuals with exceptional longevity (centenarians), their offspring (approximate age of 70 years), and age- and gender-matched controls without a family history of longevity. Using this study's approach, the potential associations between telomere length and longevity, and age-related diseases were investigated. Furthermore,

the potential role of genetic variations in the human telomerase reverse transcriptase (hTERT) and human telomerase RNA component (hTERC) genes in the better maintenance of telomere length in the centenarians and their CO was also assessed[66].

Significantly longer telomeres were detected in centenarians and their offspring than controls [66]. In addition, CO did not show an appreciable age-related decline in telomere length as observed using unrelated controls or in cross-sectional studies[66].

Because shorter telomere length is associated with age-related disease, including hypertension, metabolic syndrome and dementia, the authors analyzed the association between telomere length, major age-related diseases and lipid profiles in centenarians, their offspring and controls [64,65,67]. They observed that longer telomeres are indeed associated with lower prevalence of hypertension, the metabolic syndrome, type 2 diabetes mellitus, and better cognitive function as well as with healthier lipid profiles. In addition, it was revealed that two synonymous (973 G > A (Ala-305 Ala) and 3097 C > T (His-1013 His)) and two intronic variants (IVS1–187 T > C andIVS16+99 C > T) and their haplotypes in hTERT gene are enriched in centenarians compared with controls. These variants are known to play a functional role in the regulation of gene expression through modulation of mRNA stability, mRNA secondary structure, alternative splicing or translational efficiency [66]. Thus, the telomerase genes may function as important genetic determinants of both human longevity and telomere length. Additionally, these data suggest that both telomere length and variants of telomerase genes may have a cumulative influence on lower disease prevalence and a favorable lipid profile in centenarians and their offspring [66, 67].

Mitochondrial DNA (mtDNA) is another important tool to understand longevity [68]. It encodes for 2rRNA, 22 tRNA and 13 polypeptides that take part to oxidative phosphorylation process [69]. It produces reactive oxygen species potentially dangerous for the mitochondrion itself [69]. It does not present non coding region except one, the D-loop, containing binding sites for the replication and transcription[69]. Iwata and colleagues [69] published a paper about

mtDNAmutations in Askhenazi Jews. In particular, blood leukocyte samples of mixed gender offspring and non-maternally related controls were analyzed to confirm the role of the C150T transition in longevity. This variant occurs in one of heavy chain replication origins shifting this origin from position 151 to 149. It was previously observed that this genetic variant is more frequently expressed in Italian centenarians [69]. The association with longevity was not confirmed in the Askhenazi population[69]. Thus, these data suggest that this mutation is population-dependent, such as shown in some studies in Finnish and Japanese populations [69]. In contrast, an ageing-related raise of the heteroplasmic T152C transition frequency was observed. However, other studies will be useful to confirm the role of these mutations in longevity [69].

CONCLUSIONS

In these last years, the gerontologists have focused their efforts in identifying a correct study model to understand the characteristics of successful ageing. CO seems to represent the appropriate model to analyze successful ageing. They show a favorable lipid, immunological and cardiovascular profile, a decreased cognitive decline and a protective genetic background (**Table1**).

Lower serum levels of APOC3, Hsp70 and LDL-C, and higher amount of HDL-C characterize their lipid and cardiovascular profile [18-22,24-28]. Cardiovascular health consents them to escape morbidity and mortality for adverse cardiac events, including atherothrombosis, myocardial infarction, stroke and heart failure. The CVD represents the first cause of death in Western countries. This condition implies serious social, medical and economic problems, as hospitalization and assistance of not autonomous and invalid individuals, and the existence of no appropriate therapies.

Furthermore, CO seem to show an optimal immune control. They like their parents present an immunological profile comparable in several aspects, i.e. number and activity of NK cells and B compartment, to that of young individuals [43,44]. Thus, CO behaves as the young people,

counteracting new infections and responding to vaccinations. At the same time, this condition determines an adequate control of inflammatory response and as consequence a concomitant reduction of the risk for major age-related diseases.

In addition, CO maintain a high physical and cognitive function and sustained engagement in social and productive activities, and they outlive several dementia forms like AD [4, 21, 32-35].

Another feature of CO is to have an advantageous genetic background characterized by homozygosity for the -641C (rs2542052) allele inthe APOC3 promoter, homozygosity for the 405 valine of CEPT, reduced telomere attrition associated with the presence of synonymous andintronic variants in h-TERT gene, and heteroplasmic T152C variant in mt-DNA [19-21, 23, 24, 66, 69].

The concomitant action of all factors described suggests that the positive ageing phenotype might be the result of an efficient physical performance, rather than an avoidance of death. This allows them to have a major chance to extend survival in health and well-being.

However, other studies are needed to confirm and extend these current data. For example, genomic, transcriptomic and epigenetic investigations may eventually lead to better understanding the molecular and cellular mechanisms associated with successful ageing. On the other hand, the first epigenetic data obtained by the group of Gentilini seem to be promising [70]. They recently demonstrated that a better preservation of DNA methylation status, a slower cell growing/metabolism, and a better control in signal transmission through epigenetic mechanisms may be involved in the process of human longevity [70].

In this future and perspective way of research, it could also be interesting to evaluate the protective role of TERT on mitochondria. It has been recently evidenced that mitochondrial TERT binds to and protects mitochondrial DNA and function from damage [71,72].This relatively new research area could be crucial to better understand the cross talk between nucleus and mitochondria and its age-related changes, including the possible impact on successful ageing.

Furthermore, these investigations might also consent likely to understand the biologic gender effect on longevity. A characteristic enigma of longevity is, indeed, the gender and the social phenomenon of "*feminization of old age*". The demographic and social changes of the past decades, responsible for longevity and the improvements in public health, have created new and often very dissimilar realties for women and men. They differ not only in their anatomy and physiology, but also in more complex traits, such as lifespan (in Italy, 78.8 years for men and 84.1 years for women, respectively) and mortality [73]. No conclusive explanation for these new differences is actually demonstrated. However, the group of Gentilini has recently investigated the role of age-related X chromosome inactivation (XCI) skewing in the lifespan of women. The data obtained showed that age-dependent skewing of XCI appears delayed in centenarians' offspring [74].

These study approach might permit the identification of targets to use as possible successful ageing biomarkers. Furthermore, it might consent to develop anti-ageing therapies, modulating ageing rate, developing drugs (i.e. antioxidants) or new lifestyle habits (i.e. a healthy diet, caloric uptake reduction, use of prebiotics and probiotics and increased physical exercise).

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REFERENCES

[1]Farrelly C. 'Positive biology' as a new paradigm for the medical sciences. Focusing on people who live long, happy, healthy lives might hold the key to improving human well-being. EMBO Rep 2012;13:186-8.

[2]Caruso C, Passarino G, Puca A, Scapagnini G. "Positive biology": the centenarian lesson. Immun Ageing 2012; 9 :5. [Epub ahead of print].

[3]O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Rep 2006; 7:688-93.

[4]Atzmon G, Schechter C, Greiner W, Davidson D, Rennert G, Barzilai N. Clinical phenotype of families with longevity. J Am GeriatrSoc 2004; 52: 274-7.

[5]Perls TT, Bubrick E, Wager CG, Vijg J, Kruglyak L. Siblings of centenarians live longer. Lancet 1998; 351:1560.

[6]Shimizu K, Takeda S, Noji H, *et al*. Dietary patterns and further survival in Japanese centenarians. J. Nutr. Sci. Vitam. (Tokyo) 2003; 49:133-38.

[7]Schoenmaker M, de Craen AJ, de Meijer PH, *et al*. Evidence of genetic enrichment for exceptional survival using a family approach: the Leiden Longevity Study. Eur J Hum Genet 2006; 14:79-84.

[8]Palsdottir A, Helgason A, Palsson S, *et al*. A drastic reduction in the life span of cystatin C L68Q carriers due to life-style changes during the last two centuries. PLoS Genet 2008;4:e1000099.

[9]Carmelli D, Andersen S. A longevity study of twins in the Mormon genealogy. ProgClinBiol Res 1981;69A:81-3

[10]Darviri C, Demakakos P, Tigani X, *et al*. Psychosocial dimensions of exceptional longevity: a qualitative exploration of centenarians' experiences, personality, and life strategies. Int J Aging Hum Dev 2009;69:101-18.

[11]Hitt R, Young-Xu Y, Silver M, Perls T. Centenarians: the older you get, the healthier you have been. Lancet 1999 ;354:652.

[12]Samuelsson SM, Alfredson BB, Hagberg B, *et al*. The Swedish Centenarian Study: a multidisciplinary study of five consecutive cohorts at the age of 100. Int J Aging Hum Dev 1997;45:223-53.

[13]Willcox DC, Willcox BJ, Shimajiri S, Kurechi S, Suzuki M. Ageing gracefully: a retrospective analysis of functional status in Okinawan centenarians. Am J Geriatr Psychiatry 2007;15:252-5

[14]Terry DF, Wilcox M, McCormick MA, Lawler E, Perls TT. Cardiovascular advantages among the offspring of centenarians. J Gerontol A BiolSci Med Sci 2003;58:M425-31.

[15]Terry DF, Wilcox MA, McCormick MA, Perls TT. Cardiovascular disease delay in centenarian offspring. J Gerontol A BiolSci Med Sci 2004;59:385-9.

[16]Terry DF, Wilcox MA, McCormick MA, *et al*. Lower all-cause, cardiovascular, and cancer mortality in centenarians' offspring. J Am GeriatrSoc 2004;52:2074-6.

[17]Adams ER, Nolan VG, Andersen SL, Perls TT, Terry DF. Centenarian offspring: start healthier and stay healthier. J Am GeriatrSoc 2008;56:2089-92.

[18]Newman A, Glynn N, Taylor C, *et al*. Health and function of participants in the Long Life Family Study: A comparison with other cohorts. Ageing (Albany NY) 2011; 3: 63-76.

[19]Barzilai N, Atzmon G, Schechter C, *et al*. Unique lipoprotein phenotype and genotype associated with exceptional longevity. J Am Med Assoc 2003; 290:2030-40.

[20]Barzilai N, Gabriely I, Gabriely M, Iankowitz N, Sorkin JD. Offspring of centenarians have a favorable lipid profile. J. Am. Geriatr. Soc 2001; 49: 76-9.

[21]Atzmon G, Rincon M, Rabizadeh P, Barzilai N. Biological evidence for inheritance of exceptional longevity. Mech Ageing Dev 2005;126:341-5.

[22]Li WW, Dammerman MM, Smith JD, *et al*. Common genetic variation in the promoter of the human apo CIII gene abolishes regulation by insulin and may contribute to hypertriglyceridemia. J Clin Invest 1995;96:2601-5

[23]Schechter CB, Barzilai N, Crandall JP, Atzmon G. Cholesteryl ester transfer protein (CETP) genotype and reduced CETP levels associated with decreased prevalence of hypertension. Mayo Clin Proc. 2010;85:522-6

[24]Atzmon G, Rincon M, Schechter CB, *et al*. Lipoprotein genotype and conserved pathway for exceptional longevity in humans. PLoS Biol. 2006;4:e113.

[25]Terry DF, McCormick M, Andersen S, *et al*. Cardiovascular disease delay in centenarian offspring: role of heat shock proteins. Ann N Y AcadSci 2004;1019:502-5.

[26]Rea IM, McNerlan S, Pockley AG. Serum heat shock protein and anti-heat shock protein antibody levels in aging. ExpGerontol 2001;36:341-52.

[27]Jin X, Wang R, Xiao C, *et al*. Serum and lymphocyte levels of heat shock protein 70 in aging: a study in the normal Chinese population. Cell Stress Chaperones 2004;9:69-75.

[28]Terry DF, Wyszynski DF, Nolan VG, *et al*. Serum heat shock protein 70 level as a biomarker of exceptional longevity. Mech Ageing Dev 2006;127:862-8.

[29]Suh Y, Atzmon G, Cho MO, et al. Functionally significant insulin-like growth factor I receptor mutations in centenarians. ProcNatlAcadSci U S A 2008;105:3438-42.

[30]Claeysen S, Cochet M, Donneger R, Dumuis A, Bockaert J, Giannoni P. Alzheimer culprits: Cellular crossroads and interplay. Cell Signal 2012;24:1831-40

[31]James BD, Wilson RS, Barnes LL, Bennett DA. Late-life social activity and cognitive decline in old age. J IntNeuropsycholSoc 2011;17:998-1005.

[32]Lipton RB, Hirsch J, Katz MJ, et al. Exceptional parental longevity associated with lower risk of Alzheimer's disease and memory decline. J Am GeriatrSoc 2010;58:1043-9.

[33]Barral S, Cosentino S, Costa R, *et al*. Cognitive function in families with exceptional survival Neurobiol Ageing. 2012;33:619.e1-7

[34]Hagberg B, Samuelsson G. Survival after 100 years of age: a multivariate model of exceptional survival in Swedish centenarians. J Gerontol A BiolSci Med Sci 2008;63:1219-26.

[35]Yates LB, Djoussé L, Kurth T, Buring JE, Gaziano JM. Exceptional longevity in men: modifiable factors associated with survival and function to age 90 years. Arch Intern Med. 2008;168:284-90

[36]Franceschi C, Monti D, Sansoni P, Cossarizza A. The immunology of exceptional individuals: the lesson of centenarians. Immunol Today 1995a; 16: 12-6.

[37]Franceschi C, Monti D, Barbieri D, *et al*. Immunosenescence in humans: deterioration or remodelling? Int Rev Immunol 1995b; 12: 57-74.

[38]Pawelec G, Akbar A, Caruso C, Solana R, Grubeck-Loebenstein B, Wikby A. Human immunosenescence: is it infectious? Immunol Rev 2005; 205:257-68.

[39]Solana R, Pawelec G, Tarazona R. Aging and innate immunity. Immunity 2006;24:491-4.

[40]Sridharan A, Esposo M, Kaushal K, *et al*. Age-associated impaired plasmacytoid dendritic cell functions lead to decreased CD4 and CD8 T cell immunity. Age (Dordr) 2011; 33:363-76.

[41]Gupta S, Bi R, Su K, Yel L, Chiplunkar S, Gollapudi S. Characterization of naïve, memory and effector CD8+ T cells: effect of age. ExpGerontol 2004;39:545-50.

[42]Chong Y, Ikematsu H, Yamaji K, *et al*. CD27(+) (memory) B cell decrease and apoptosis-resistant-CD27(-) (naive) B cell increase in aged humans: implications for age-related peripheral B cell developmental disturbances. Int Immunol 2005;17:383-90.

[43]Colonna-Romano G, Bulati M, Aquino A, *et al*. A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people. Mech Ageing Develop 2009; 130: 681-90.

[44]Derhovanessian E, Maier AB, Beck R, *et al*.: Hallmark features of immunosenescence are absent in familial longevity. Immunol 2010, 185:4618-24.

[45]Frasca D, Landin AM, Lechner SC, Ryan JG, *et al*. Aging down-regulates the transcription factor E2A, activation-induced cytidinedeaminase, and Ig class switch in human B cells. J Immunol 2008; 15;180:5283-90.

[46]Shi Y, Yamazaki T, Okubo Y, Uehara Y, Sugane K, Agematsu K. Regulation of aged humoral immune defense against pneumococcal bacteria by IgM memory B cell. J Immunol 2005;175:3262-7.

[47]Listi` F, Candore G, Modica MA, *et al*. Study of Serum Immunoglobulin Levels in Elderly Persons That Provides New Insights into B CellImmunosenescence. Ann NY AcadSci 2006; 1089: 487-95.

[48]Buffa S, Bulati M, Pellicanò M, *et al*. B cell immunosenescence: different features of naive and memory B cells in elderly. Biogerontol 2011; 12:473-83.

[49]Bulati M, Buffa S, Candore G, *et al*. B cells and immunosenescence: a focus on IgG+IgD-CD27- (DN) B cells in aged humans. Ageing Res Rev 2011; 10: 274-84.

[50]Mariotti S, Sansoni P, Barbesino G, *et al*. Thyroid and other organ-specific autoantibodies in healthy centenarians. Lancet 1992; 339: 1506-8.

[51]Sansoni P, Cossarizza A, Brianti V, *et al*. Lymphocyte subsets and natural killer cell activity in healthy old people and centenarians. Blood 1993; 82: 2767-73.

[52]Cossarizza A, Ortolani C, Paganelli R, *et al*. CD45 isoforms expression on CD4+ and CD8+ T cells throughout life, from newborns to centenarians: implications for T cell memory. Mech Ageing Dev 1996; 86:173-95.

[53]Candore G, Di Lorenzo G, Mansueto P, *et al*. Prevalence of organ-specific and non organ-specific autoantibodies in healthy centenarians. Mech Ageing Dev 1997; 94: 183-90.

[54]Bagnara GP, Bonsi L, Strippoli P, *et al*. Hemopoiesis in healthy old people and centenarians: well-maintained responsiveness of CD34+ cells to hemopoietic growth factors and remodelling of cytokine network. J Gerontol A Biol Sci Med Sci 2000; 55: B61-6.

[55]Baggio G, Donazzan S, Monti D, *et al*. Lipoprotein(a) and lipoprotein profile in healthy centenarians: a reappraisal of vascular risk factors. FASEB J 1998;12:433-7.

[56]Passeri G, Pini G, Troiano L, *et al*. Low vitamin D status, high bone turnover, and bone fractures in centenarians. J Clin Endocrinol Metab 2003; 88: 5109-15.

[57]Coppola R, Mari D, Lattuada A, Franceschi C. Von Willebrand factor in Italian centenarians. Haematologica 2003; 88: 39-43.

[58]Capri M, Salvioli S, Monti D, *et al*. Human longevity within an evolutionary perspective: The peculiar paradigm of a postreproductive genetics. ExpGerontol 2008; 43:53-60.

[59]Rea SL, Wu D, Cypser JR, Vaupel JW, Johnson TE. A stress-sensitive reporter predicts longevity in isogenic populations of Caenorhabditiselegans. Nat Genet 2005; 37: 894-8.

[60]Cevenini E, Invidia L, Lescai F, *et al*. Human models of aging and longevity. Expert OpinBiolTher 2008; 8: 1393-1405.

[61]Colonna-Romano G, Buffa S, Bulati M, *et al*. B cells compartment in centenarian offspring and old people. Curr Pharm Des 2010; 16:604-8.

[62]Cancro MP, Hao Y, Scholz JL, *et al*. B cells and ageing: molecules and mechanisms. Trends Immunol 2009; 30: 313-8.

[63]Finch CE. The Biology of Aging (Academic Press, Burlington, MA), 2007.

[64]Oeseburg H, de Boer RA, van Gilst WH, van der Harst P. Telomere biology in healthy aging and disease. Pflugers Arch 2010;459:259-68.

[65]Zhu H, Belcher M, van der Harst P. Healthy aging and disease: role for telomere biology? ClinSci (Lond) 2011;120:427-40.

[66]Atzmon G, Cho M, Cawthon RM, *et al*. Evolution in health and medicine Sackler colloquium: Genetic variation in human telomerase is associated with telomere length in Ashkenazi centenarians. ProcNatlAcadSci U S A 2010;107Suppl 1:1710-7.

[67]Calado R, Young N. Telomeres in disease. F1000 Med Rep. 2012;4:8.

[68]Rose G, Passarino G, Scornaienchi V, *et al*. The mitochondrial DNA control region shows genetically correlated levels of heteroplasmyinleukocytes of centenarians and their offspring. BMC Genomics. 2007;8:293.

[69]Iwata N, Zhang J, Atzmon G, *et al*. Aging-related occurrence in Ashkenazi Jews of leukocyte heteroplasmicmtDNA mutation adjacent to replication origin frequently remodeled in Italian centenarians. Mitochondrion. 2007;7:267-72.

[70]Gentilini D, Mari D, Castaldi D, *et al* Role of epigenetics in human aging and longevity: genome-wide DNA methylation profile in centenarians and centenarians' offspring. Age (Dordr). 2012 Aug 25.

[71]Haendeler J, Dröse S, Büchner N, *et al*. Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage. ArterioscIerThrombVasc Biol. 2009;29:929-35.

[72]Sharma NK, Reyes A, Green P, *et al*. Human telomerase acts as ahTR-independent reverse transcriptase in mitochondria. Nucleic Acids Res. 2012;40:712-25.

[73]Balistreri CR, Candore G, Accardi G, *et al*. Genetics of longevity. data from the studies on Sicilian centenarians. Immun Ageing. 2012;9:8

[74]Gentilini D, Castaldi D, Mari D, *et al*. Age-dependent skewing of X chromosome inactivation appears delayed in centenarians' offspring. Is there a role for allelic imbalance in healthy aging and longevity? Aging Cell. 2012;11:277-83.

Table 1. Molecular and phenotypical features of CO

Lipid and Cardiovascular Profile	**Changes**	**References**
Serum level of APOC3	Decrease	[24]
Serum level HSP70	Decrease	[25-28]
Level and larger size LDL-C	Decrease	[18-22]
Level and larger size HDL-C	Increased	[18-22]
Risk for hypertension	Decrease	[4, 14-18]
Risk for type II diabetes	Decrease	[4, 14-18]
Risk of stroke	Decrease	[4, 14-18]
Risk myocardial infarction	Decrease	[4, 14-18]
Coronary heart disease	Decrease	[4, 14-18]
Cognitive Profile	**Changes**	**References**
Development of dementia	Reduced	[21,32-35]
Development of Alzheimer's disease	Reduced	[4, 21]
Favorable Immunological Profile	**Changes**	**References**
Naïve T cells ($CD3^+CD8^+CD45RA^+CCR7^+CD27^+CD28^+$)	Increase	[43]
Late differentiated effector memory T cells ($CD3^+CD8^+CD45RA^-CCR7^-CD27^-CD28^-$)	Decrease	[43]
TEMRA ($CD3^+CD8^+CD45RA^+CCR7^-CD27^-CD28^-$)	Decrease	[43]
Naïve B cells (IgD^+CD27^-)	Increase	[44]
Double Negative B cells ($IgG^+/IgA^-IgD^-CD27^-$)	Decrease	[44]
Serum IgM	Increase	[44]
Genetic Profile	**Changes**	**References**
Homozygosis for the -641C (rs2542052) allele inthe APOC3 promoter	Increase	[24]
Homozygosis for the 405 valine of CEPT	Increase	[19-21, 23]
Telomere attrition	Decrease	[66]
Presence of synonymous andintronic variants in h-TERT gene	Increase	[66]
Heteroplasmic T152C variant in mt-DNA	Increase	[69]

ABBREVIATIONS

Alzheimer disease =AD

Apolipoprotein C-III= APOC3

Cardiovascular diseases = CVD

Centenarian offspring = CO

Cholesteryl ester transfer protein= CETP

Double negative=DN

Heat shock protein= Hsp70

High density Lipoprotein-cholesterol= HDL-C

Insulin-like growth factor 1= IFG1

Lipoprotein= Lp(a)

Long Life Family Study= LLFS

Low density lipoprotein-cholesterol= LDL-C

CHAPTER 11

Summary and General discussion

11. Summary and general discussion

The aging of the immune system is a gradual and dynamic process that modifies some immunological functions. These changes are known as "immunosenescence" (Franceschi C et al., 1995; Pawelec G et al., 2005) that have a great impact on immune performance in late life, contributing to the decreased ability of the elderly people to respond to emerging pathogens and to the decreased responsiveness to vaccinations (Grubeck-Loebenstein B et al., 2009; Thompson WW et al., 2003; Fulop T et al., 2013). Both the innate and the adaptive immune functions are affected in the aged. In particular, with aging, the acquired compartment of the immune system shows significant modifications in both T and B cell branches. Lifelong and chronic antigenic load are the major driving forces of this process, resulting in the reduction of the number of virgin antigen-non experienced T cells (Franceschi C et al., 2007) and, contemporarily, in the filling of the immunological space with expanded clones of memory and effector, experienced cells (Franceschi C et al., 2000; Miller JP, and Cancro MP, 2007).

It is known that the adaptive immune response of elderly people is qualitatively and quantitatively reduced when compared with the immune response of young people. Many studies have focused on T cells but B cell compartment is also affected with age. It has been demonstrated that, although the number of B cells is diminished, the levels of serum IgG, IgA and, to a lesser extent IgE increase while IgM and IgD are decreased (Listì F et al., 2006). Thus, it is suggested the possible shift from naïve toward memory compartment (Gupta S et al., 2005; Listì F et al., 2006; Colonna-Romano G et al., 2008).

A typical feature of aging is a chronic low grade of pro-inflammatory status, named “Inflamm-aging”, observed in the old people. This phenomenon, under genetic controls, is associated with a general increase in the production of pro-inflammatory cytokines and inflammatory markers (Cevenini E et al., 2010; Cevenini E 2013, Fulop T et al 2004, 2013) that render elderly people prone to frailty (Balistreri CR et al., 2008; Franceschi C et al., 2005).

It has been suggested that chronic antigenic stimuli, such as herpes virus infections (e.g.CMV), play a relevant role in exhaustion of the immune system, accelerating immune aging (Pawelec G et al., 2005; Akbar AN and Fletcher JM, 2005). Furthermore, the continuous attrition caused by chronic antigenic load results in the generation of inflammatory responses involved in age-related diseases (Pawelec G et al., 2005; Vasto S et al., 2007).

A typical pathology, correlated to aging, is Alzheimer’s disease (AD). It is considered the most common form of age-related cognitive failure. Extracellular deposits of amyloid beta peptide trigger inflammatory reaction in the brain (Weiner et al., 2006). Moreover, changes in lymphocytes distribution and in cytokine levels in the plasma of AD patients have been reported (Richartz-Salzburger E et al., 2007; Speciale L et al., 2007; Larbi A et al., 2009; Pellicanò M et al., 2011).

On the other hand, centenarians represent an example of successful aging because they have delayed diseases that normally cause mortality in the general population (Franceschi C, Bonafè M, 2003). In addition, centenarian offspring (CO) have a genetic advantage that could predispose them for longevity as they are preserved from age-associated

diseases, in particular cardiovascular diseases, stroke, myocardial infarction, and diabetes mellitus (Terry DF et al., 2004 a,b).

The aim of this thesis is to study changes in immune system with age, also focusing on people genetically advantaged for healthy ageing (Centenarian Offspring) or unsuccessfully aged patients (Alzheimer's Disease) affected paying attention principally on the naïve/memory B cell compartments.

In **chapter 3**, we have shown our recent work about the naïve/memory B cells characterization based on two developmentally regulated markers, CD38 and CD24. We discussed about a new population of late memory B cells, CD19+CD38-CD24-, that is increased in elderly people compared to young and contemporarily reduced in centenarian offspring when compared to age-matched controls. We evaluated the production of pro- and anti-inflammatory cytokines by CD38/CD24 B cell subsets, confirming data in literature which affirm that $CD19^{+}CD38^{hi}CD24^{hi}$ (Breg) is the main IL-10-producing B cell population. We have also observed a high production levels of TNF by $CD19^{+}CD38\text{-}CD24^{+}$ and $CD19^{+}CD38^{-}CD24^{-}$ B cells. Despite the increase of the number of markers using for characterization, we confirmed that centenarian offspring (CO) have the B cell branch similar to young people but different to their healthy age-matched controls. The immunological profile of centenarian offspring could guarantee them a protection against the risk of infection and the inflammatory processes of ageing.

In **chapter 4** we show our data obtained in a recent study on the T-cell immune profile of centenarian offspring (CO), their age-matched (AM) controls, healthy elderly and young subjects. We have studied the

distribution of $CD4^+$ and $CD8^+$ T cells within $CD3^+$ T cell population. Here, we observed that CO have a slight increase of percentage $CD4^+$ and a lower percentage of $CD8^+$ T cells, compared to AM controls, so the CD4:CD8 results higher than AM subjects. Then, it was performed the analysis of costimulatory molecules, CD27 and CD28 on $CD4^+$ and $CD8^+$ T cells, to identify the stage of differentiation of T cell branch. We have shown that, young subjects have the higher percentage and absolute number of early differentiated $CD4^+CD27^+$ lymphocytes when compared to any of older groups (CO, AM and elderly donors). However, CO have the higher but not significant percentage and absolute number of these two cell subsets within elderly population. A similar pattern was observed within $CD8^+$ T cells. Indeed, in young donors there were the highest percentage of $CD27^+$ and $CD28^+$ cells compared the other donors analyzed; differently, the mean of percentage of $CD27^+$ T cells and the percentage and absolute number of $CD28^+$ were higher in CO than AM controls. Moreover, we have also identified the naïve/memory distribution within $CD4^+$ and $CD8^+$ populations. Our results show the most frequent percentage and absolute number of $CD27^+CD28^+CD45RA^+CD45RO^-$ naïve cells within the $CD4^+$ and $CD8^+$ subsets in centenarian offspring, compared with their age-matched controls. Moreover, we have reported the reduction of $CD28^-CD27^-CD45RA^+CD45RO^+$ late differentiated memory T cells in CO when compared with age-matched controls and old people. Next, we have also demonstrated that CO have a lower levels of potentially senescent $CD8^+CD57^+$ cells when compared with AM and elderly people. It is possible conclude that CO seems to have a similar phenotype to the young

subjects than that of AM controls. Thus, T cell arm appears resistant to major infections and might contribute to reach the extreme limits of life.

In **chapter 5** we have studied the expression of some chemokine receptors in peripheral naïve/memory B cell, from young and elderly, to evaluate whether the inflammatory milieu could influence B cell subsets trafficking. We have observed that naïve and memory unswitched B cells have a "lymph node phenotype", while memory switched B cells express molecules useful to leave the lymphoid organs. Indeed, our data show a different expression of these chemokine receptors on peripheral naïve and memory B cells between the two groups studied. In particular, in young donors, naïve B cells express CCR7, CCR6 and CXCR4 allowing B cells to circulate. Thereafter, both memory unswitched and memory switched modulate the expression of CD62L and CXCR5 necessary to cooperate with cells in lymphoid organs. Memory unswitched and DN B cells from young subjects show also high levels of CXCR3, a chemokine receptor that consent to reach the inflammatory sites. Thus, 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 the expression of CXCR3, memory unswitched B cells retain the capacity to migrate to the sites of inflammation, while DN B cells lose the expression of this molecule, but express CCR6 and CCR7, that are also implicated in the migration to the inflammatory sites. Further, we investigated also whether total B cells and DN memory B lymphocytes are able to act as granzyme B (GrB) producing cells. We have shown that, after stimulation, both in young and elderly people, total

B cells produce GrB when compared to the not stimulated cells without differences between the two groups. Next, we focused on DN B cells, observing that also this particular memory population, under the same conditions, shows a GrB producing ability without differences between the two age groups studied. We hypothesize that the inflammatory environment, typical of aging, in some ways changes the trafficking ability of B cells and renders them more sensitive both to the cytokines and to the pro-inflammatory molecules which are over-produced in the elderly. Moreover, these data suggest that DN B cells, which are increased in old subjects and show a different pro-inflammatory trafficking profiles, in young and elderly subjects are able, if properly stimulated, to migrate into inflammatory sites, to cooperate with other immune cells(i.e. memory unswitched B cells), to produce GrB.

In **chapter 6** we show our data (manuscript in preparation) about the expression of two inhibitory receptors (CD307d and CD22) on naïve/memory B cell subsets from young and elderly donors. We have observed that, both in young and elderly subjects, CD307d is mainly expressed on memory unswitched B cells and it is significantly reduced on naïve B cells and lost by memory switched and DN B cells. Interestingly, memory unswitched B cells from old donors, show significantly high levels of this molecule compared to memory unswitched B cells from young donors. Differently, CD22 is expressed at high level in all B cell subsets, independently from the age of donors and no significant differences were observed between young and elderly subjects. Thus, we conclude that DN B cells do not express a unique pattern of inhibitory receptors. Moreover, we have stimulated B cells *in*

vitro with the TLR9 ligand CpG alone, or with α-IgG/CD40, that engages the adaptive receptor BCR or with the contemporary engagement of adaptive and innate immune receptors (BCR and TLR9). We have reported that, in young subjects, total $CD19^+$ cells and DN B lymphocytes respond to all stimuli used, albeit at different levels. Indeed, they proliferate at high levels with the triggering of BCR/TLR9, and at lower levels engaging BCR alone or TLR9. In old donors, the situation is different, because $CD19^+$ lymphocytes and DN B cells do not proliferate after physiological stimuli (CpG alone, nor α-IgGCD40) but proliferate with the double stimulation (CpGα-IgGCD40), although al lower levels than those observed in young donors. This could reflect that, the simultaneous BCR and TLR stimulation might activate some pathway involved in immune function. Furthermore, we have evaluated the relative telomerase activity (RTA) in young and in different groups of elderly subjects (CO, AM and elderly donors) to verify whether the ability to respond to the stimulation with CpG, α-IgG and CD40 could modify telomerase expression in DN B cells. We have reported that, the contemporary innate and adaptive stimulation induces the activation of telomerase in DN B cells from old and young. Moreover, we have also evaluated the induction of telomerase activity in groups of CO and AD subjects. Here, again, the contemporary engagement of innate and adaptive immune receptors activate telomerase although at different levels in the different groups. Indeed, old subjects show a reduced RTA when compared with young donors and CO subjects. So, we confirm that CO behave halfway between young and elderly subjects. Differently, severe AD patients show very low levels of RTA. This findings

demonstrated that, DN B cells can proliferate with appropriate stimulation and also that healthy aging does not annul the ability to induce telomerase activity in DN B cells but influences the level of expression of this enzyme.

In **chapter 7** we illustrate data from a manuscript in preparation, about the evaluation of naïve/memory B cell distribution, identified with the combination of the "core" IgD and CD27 surface markers (Kaminski et al., 2012), in the two different groups of AD patients object of the study (Severe and Mild), comparing them each other and with their age-matched healthy controls. We have confirmed (Pellicanò et al., 2009) a significant decrease, both in percentage and absolute number, in total B cell population in severe AD subjects, compared to healthy elderly, but no significant difference between elderly and mild AD patients. Moreover, we have found a significant decrease both in percentage and absolute number of ($IgD^{+}CD27^{-}$) naïve B cells, and a marked increase in percentage of Double Negative ($IgD^{-}CD27^{-}$) B cells, compared to age-matched healthy donors. Differently, Mild AD patients seem behave as healthy elderly subjects. Further, we have assessed the expression of CXCR3, CXCR4, CXCR5, CCR6, and CCR7, chemokine receptors, on DN B lymphocytes in healthy elderly donors, severe and mild AD patients. We have demonstrated that DN B cells from AD patients do not express CXCR4 and CXCR5. Concerning CXCR3, we have observed high expression of this receptor in DN B cell in all groups studied, with no significant differences between them. On the other hand, CCR6 is a significantly increased both in mild and severe AD patients, while CCR7 expression is significantly increased in severe AD patients, but not in mild

AD subjects. Thus, we conclude that severe AD status might affects the distribution of specific naïve and DN B cells and that might also influence the pro-inflammatory profile of trafficking receptor in DN B cells, driving these lymphocytes to inflamed brain tissue.

In **chapter 8**, we have discussed about the literature and previous data from our group, regarding the involvement of immunological modifications in Alzheimer's disease (AD). We conclude that changes in innate and acquired immune system, genetic background and other risk factors contribute to the AD pathogenesis. Indeed, it is suggested that Aβ peptides induce a persistent stimulation of the immune system that causes chronic T and B cells inflammatory responses and the release of inflammatory mediators. These events seem to contribute to neurodegeneration. On the other hand, genetic background, such as polymorphisms of genes involved in the immune-inflammation, might play a role in the generation of AD. Beside, other risk factors, such as hormones, levels of education and environmental events could be related to this age-related disease. In conclusion, it is considered relevant to identify a new method that allows the use of peripheral blood from AD patients to identify prognostic or disease markers. Until now, different factors, that might be useful at this purpose, has been identified although there are many discordant results.

In **chapter 9** we have reviewed our and literature data on Sicilian centenarians. We conclude that both a well preserved immune function and genetic background have a crucial role in aging and longevity and that centenarians represent the optimal model to understand successful and

unsuccessful aging. Indeed, it has been demonstrated that people genetically enriched for longevity possess immune different signatures respect to those general population. This suggests the idea of “familiar youth”. At this regard, centenarian offspring (CO) do not show a typical T naïve/memory shift observed in their age-matched (AM) controls. Moreover, also the B cell compartment of CO results similar to that young subjects than elderly donors. Indeed, although a decrease in B cell count was observed in centenarian offspring and their age-matched controls, it has been verified that naïve B cells ($IgD^{+}CD27^{-}$) were more abundant and double negative (DN) B cells ($IgD^{-}CD27^{-}$) were significantly decreased when compared with in young people. In this review, we suggest that there are other factors that contribute to longevity, such as, gender, environmental conditions, the low pro-inflammatory status, diet and genetics variants in TLR4, CCR5 Cox2 genes and also in cytokine (e.g.INF-γ, IL-10) genes that, regulating the immune response, seem to increase the chances to attain longevity.

In **chapter 10** we review literature data and the results of our studies about centenarian offspring (CO). We discussed about the biological mechanisms involved in determining the long-lived phenotype and the role that these investigations might have in developing of anti-aging therapies, drugs and modulating aging rate. We conclude that, CO may represent a model for understanding exceptional longevity. Indeed, they have a protective genetic background and may have inherited genetic variations, from their long-lived parents that protect them from ageing processes and hypertension, cancer, diabetes, cardiovascular disease (CVD). Moreover, CO show favorable lipid (i.e. high levels of HDL-C,

low levels of LDL-C), cardiovascular and an optimal immune profile (i.e. high levels of naïve B cells, decrease of DN B cells and high levels of serum IgM.). Furthermore, centenarians and their offspring, also, escape and/or postpone cognitive decline, dementia and have a reduced incidence for AD when compared to their AM controls. These data suggest that, the exceptional longevity might be the result of a concomitant action of phenotypic and genotypic characteristics, transmitted in long-lived families.

11.1 Conclusions

It is known that the lifelong exposure to new and persistent infections (Fulop T et al., 2013; Saule P et al., 2006), typical of aging, affects the adaptive immune reactions. Thus, modifications in both T and B cell compartments has been demonstrated (Pawelec et al., 2002; 2008; 2013; Miller and Cancro, 2007; Colonna-Romano G et al., 2009). Regarding T cell branch, these age-related changes result in progressive decrease of circulating $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes and in the accumulation of late stage differentiated $CD8^+$ T cell subset in elderly people (Pawelec G et al., 2002). Particularly, several studies suggested the increase of the number of highly differentiated $CD28^-$ T cells, especially within the $CD8^+$ T-cell subset in aged subjects (Effros RB et al., 1994**;** Pawelec G et al., 2008). These ($CD28\text{-}CD8^+$) T cells exhibit reduced antigen receptor diversity, defective antigen-induced proliferation and a shorter replicative lifespan while showing enhanced cytotoxicity regulatory functions (Oviedo-Orta et al., 2013; Weng NP, et al., 2009) and resistance to apoptosis (Pawelec G et al., 2008). Another

interesting memory T cell population increased with aging is TEMRA ($CCR7^-$, $CD27^-$, $CD28^-$, $CD45RA^+$). It has been suggested that many of these TEMRA cells are not able to produce cytokines, to mediate cytotoxic activity and also they show senescence-related proliferative defects (Effross RB, 2011; Fulop T et al.,2013). However, it has been recently demonstrated that, in some circumstances, these cells might secrete cytokines and express high levels of granzyme B and perforin (Libri VR et al., 2011). So, they may be important for protection against certain infections *in vivo* (Bruns H et al., 2009; Di Mitri D et al., 2011).

As previously described, it has been also reported the impairment of the B cell branch with aging (Colonna-Romano G et al., 2003; Miller JP and CancroMP, 2007; Frasca D et al., 2008). Indeed, the time-enduring stimulation seems induce in elderly people, the decrease of naïve B cells (Gupta s et al., 2005; Colonna-Romano G et al., 2008), although other authors have observed the increase of percentage but not in the absolute number with age of these last cells (Shi Y et al., 2005; Frasca et al., 2008). Concerning memory B cell population, Frasca et al., (2012) have shown a decrease of both percentage and absolute number of switched memory B lymphocytes, suggesting a defect to undergo to class switch. Unlike the above-mentioned data, our group (Colonna-Romano G et al., 2003, 2009) has reported no significant changes of memory B cells identified as $CD19^+CD27^+$ in the elderly whereas a population of DN ($CD19^+IgG^+IgD^-CD27^-$) B cells is increased in the elderly (Colonna-Romano G et al., 2009). Beside, the levels of total and specific serum Ig isotypes are modified. Indeed, although the number of total B lymphocytes is reduced the amount of IgG, IgA and, to a lesser extent

IgE, is augmented. In a different way, the levels of IgM decrease or not change, while those of IgD decline (Listì F et al., 2006; Frasca D et al., 2010).

Most of scientific research on immunosenescence has focused on the mechanisms that could lead to longevity. Thus, it is important to study the complex process of aging of the immune system in the elderly, in successful and unsuccessful models aging, to identify biomarkers of human healthy aging that could be potentially useful for the evaluation of anti-aging treatments or to improve the quality of life of the growing elderly population. Generally, centenarian offspring (CO), that are genetically advantaged for longevity, represent a model for understanding longevity, while Alzheimer's disease patients are useful to study unsuccessful aging (Terry et al., 2004a, 2004b; Derhovanessian et al., 2010). Our studies show that CO have a T cell branch more similar to that of young people than in their age-matched (AM) controls. Accordingly, in these subjects, also the naïve/memory B cell compartment is more similar to that of young people than elderly donors. Indeed, naïve B cells are increased and DN B cells are reduced when compared to their AM controls. Thus, these interesting data indicate that naïve B lymphocytes and DN B cells could represent two hallmarks of aging process. The relevant role of DN B cells, as biomarker of human life, also results from studies conducted on immune system of Alzheimer's disease patients. Indeed, we have demonstrated the decline of naïve B cells and an increase of percentage of Double Negative B cells in severe AD subjects, compared to age-matched healthy donors; whereas, mild AD patients seem behave as healthy elderly subjects. Therefore, it is possible to

suppose that the evaluation of the percentage of DN B cells could indicate both the chronological age and the biological age of immune system and also the performance of immune response against pathogens. Indeed, it has been reported that DN B pool is enlarged in chronically stimulated individuals, such as healthy subjects challenged with respiratory syncityal virus (RSV) (Sanz I et al., 2008), active Lupus patients (Wei C et al., 2007) and in HIV patients (Cagigi A et al., 2009). Thus, the increased of these cells could be the result of persistent stimulation of immune system. Furthermore, we have evaluated whether DN B cells represent only a marker of aging or whether they are related to systemic inflammation because it is known that both elderly and AD patients are characterized by an inflammatory milieu that is not typical of CO (Terry DF et al., 2006; Salvioli S et al., 2013). For this purpose, we have studied the expression of some chemokine receptors that drive the cells to inflamed tissues, in peripheral naïve/memory B cell from young and elderly. These experiments have suggested that the inflammatory milieu could influence B cell subsets trafficking. Indeed, naïve and memory B cell subsets express different pattern of chemokine receptors. The most important difference is represented by DN B lymphocytes. Indeed, DN B cells from young subjects show high levels of CXCR3, a chemokine receptor that consent to reach the inflammatory sites. Differently, in aged people, DN B cells lose the expression of this molecule, but show high levels of CCR6 and CCR7, that are also implicated in the migration to the inflammatory sites. In addition, the same analysis has been realized in severe and mild AD and it has demonstrated that in patients with severe status of Alzheimer's disease, DN B cells show significant high expression of

CCR6 when compared with mild AD subjects, whereas the CCR7 expression is significantly increased in severe AD patients, but not in mild AD subjects. Thus, it seems that the increase of expression of CCR6 depends on the severity of the pathology. Recently it has been observed an increase of CCR6 also on T cells obtained from AD patients (Goldeck et al., 2013). A similar behaviour is mirrored in total B cells, suggesting that the expression of these chemokines receptors could be influenced by the pro-inflammatory milieu of AD patients, and that, CCR6 and, probably, CCR7 could drive B cells to inflamed brain tissue. Until now, it is known that DN B cells might migrate into tissues with inflammation, although their role in these sites is unidentified. Nevertheless, it has been demonstrated that, with adequate stimulation, DN B cells secrete immunoglobulins against tetanus toxoid and influenza virus (Wirths and Lanzavecchia et al., 2005). Moreover, it has been demonstrated that both total B cells and DN B lymphocytes, might exert cytotoxic functions. Indeed, these cells, when stimulated with α-IgG and IL-21, that is increased in aged people, become GrB producing cells that might play a significant role in early antiviral immune responses, in the regulation of autoimmune diseases and in cancer immunosorvelliance (Hagn M et al., 2009; Kurschus FC et al., 2004; Gross C et al., 2003). Additionally, we have demonstrated that both total B cells and DN B cell become able to produce granzyme B, after adequate stimulation, without no differences between young and elderly people. Thus, we conclude that DN B cells, that show a pro-inflammatory trafficking profile, in young and old subjects, if properly stimulated, might migrate into inflammatory sites

and, cooperating with other immune cells (e.g. memory unswitched) might produce GrB (Bulati M et al., 2014).

Furthermore, it is known that DN B cells of the elderly donors have very short telomeres compared to the same subpopulation of young donors and that are not responder to CpG stimulation, although can be activated with $F(ab')_2$ (anti-BCR) (Colonna-Romano et al., 2009). We have demonstrated that the engagement of BCR and of TLR9 induces the proliferation of DN B cells and also the reactivation of telomerase in DN B cells from young and, progressively lesser, in CO, old people and AD subjects. This process permits the maintenance of length of telomeres or induces replications.

In conclusion, in the present thesis I show data obtained studying some aspects of immune system in young and elderly donors, comparing the data obtained from these subjects with the data obtained studying a cohort of "genetically advantaged" for long-life subjects (CO) and elderly people affected by AD. The presented data suggest that the study of naïve/memory B and T cell compartments may be relevant in the evaluation of biological ageing of the immune system.

CHAPTER 12

Sommario e discussione generale

12. Sommario e discussione generale

I cambiamenti progressivi e cumulativi del sistema immunitario con l'età, conosciuti con il termine di "Immunosenescenza" (Franceschi C et al., 1995; Pawelec G et al., 2005), hanno un grande impatto sulle performance del sistema immune nella tarda età, contribuendo alla ridotta abilità degli anziani di rispondere in maniera adeguata nei confronti di nuovi patogeni e vaccini (Grubeck-Loebenstein B et al., 2009; Thompson WW et al., 2003; Fulop T et al., 2013). Durante tale processo, sia le risposte innate che quelle adattative subiscono delle modificazioni. In particolar modo, con l'invecchiamento, il compartimento acquisito del sistema immunitario presenta evidenti variazioni e sia nella branca B che in quella T. La stimolazione antigenica cronica rappresenta la causa principale di tale processo, che comporta la riduzione del numero di cellule T vergini che non hanno mai incontrato l'antigene (Franceschi C et al., 2007) e, contemporaneamente, il riempimento dello spazio immunologico con cloni espansi di cellule memoria ed effettrici che hanno già incontrato l'antigene (Franceschi C et al., 2000; Miller JP, Cancro MP, 2007).

La risposta umorale delle persone anziane è qualitativamente e quantitativamente ridotta rispetto a quella presente nei giovani. Molti studi si sono concentrati sulle disfunzioni dei linfociti T e B, che risultano essere compromessi con l'invecchiamento. E' stato dimostrato che, sebbene il numero di cellule B si riduca, i livelli sierici di IgG, IgA e, in misura minore di IgE aumentano, invece quelli di IgM e IgD diminuiscono (Listì F et al., 2006). Pertanto, è stato suggerito un possibile

passaggio dalle cellule vergini verso quelle memoria (Gupta S et al., 2005; Listì F et al., 2006; Colonna-Romano G et al., 2008).

Un elemento caratteristico dell'invecchiamento è rappresentato dallo stato di infiammazione cronica basale, definito "Inflamm-aging", osservato negli anziani. Tale processo, che è sotto controllo genico, è correlato con un aumento generale della produzione di citochine pro-infiammatorie e marcatori infiammatori (Cevenini E et al., 2010; Cevenini E 2013, Fulop T et al 2004, 2013) che predispone gli anziani alla fragilità (Balistreri CR et al., 2008; Franceschi C et al., 2005).

E' stato ipotizzato che stimoli antigenici cronici, come l'infezione da parte di virus erpetici (es. CMV), svolgono un ruolo rilevante nel rendere il sistema immunitario "esausto", accelerando l'invecchiamento immunologico (Pawelec G et al., 2005; Akbar AN and Fletcher JM, 2005). Inoltre, il logoramento continuo del sistema immunitario, causato dalla stimolazione antigenica cronica, genera risposte di tipo infiammatorio che sono implicate nelle malattie età-correlate (Pawelec G et al., 2005; Vasto S et al., 2007).

Una tipica patologia, correlata all'età, è la malattia di Alzheimer (AD), che è considerata la causa più comune dei problemi cognitivi negli anziani. Depositi extracellulari di beta amiloide innescano una reazione infiammatoria nel cervello (Weiner et al., 2006). Inoltre, nei pazienti con AD sono stati osservati dei cambiamenti nella distribuzione linfocitaria e nei livelli di citochine plasmatiche (Richartz-Salzburger E et al., 2007; Speciale L et al., 2007; Larbi A et al., 2009; Pellicanò M et al., 2011).

Diversamente, i centenari rappresentano un esempio di invecchiamento con successo, poiché sono sopravvissuti alle malattie che

normalmente causano mortalità nella popolazione generale (Franceschi C, Bonafè M, 2003). Inoltre, i figli di centenari (CO) hanno un background genetico che potrebbe predisporli alla longevità, poiché sembrano essere protetti contro le principali malattie associate all'età, come per esempio patologie cardiovascolari, infarto, infarto del miocardio e diabete mellito (Terry DF et al.,2004 a,b).

L'obiettivo di questa tesi è di studiare i cambiamenti del sistema immunitario durante l'invecchiamento, focalizzando l'attenzione su quegli individui che sono "geneticamente avvantaggiati" per l'invecchiamento in buono stato di salute (Centenarian Offspring) oppure nei confronti di soggetti che mostrano un invecchiamento senza successo (Alzheimer's disease patients), prestando attenzione principalmente ai linfociti B naive e memoria.

Nel **capitolo 3**, abbiamo presentato un nostro lavoro recente sulla caratterizzazione dei linfociti B vergini/memoria basata sull'espressione di due marcatori di sviluppo, CD38 e CD24. Abbiamo discusso di una nuova popolazione di linfociti B della memoria tardivi, $CD19^{+}CD38^{-}CD24^{-}$, che è incrementata negli anziani rispetto ai giovani ed è, contemporaneamente, ridotta nei figli dei centenari, quando confrontati con i controlli della stessa età. Abbiamo valutato la produzione di citochine pro- e anti-infiammatorie da parte delle popolazioni CD38/CD24, confermando i dati presenti in letteratura, i quali affermano che, la popolazione $CD19^{+}CD38^{hi}CD24^{hi}$ (Breg) è la principale popolazione B responsabile della produzione di IL-10. Abbiamo anche osservato che le popolazioni $CD19^{+}CD38^{-}CD24^{+}$ e $CD19^{+}CD38^{-}CD24^{-}$ sono le principali cellule responsabili della produzione di TNF.

Nonostante, l'incremento del numero di marcatori utilizzati per la caratterizzazione, confermiamo che i figli di centenari (CO) hanno una branca B linfocitaria simile a quella dei giovani ma differente da quella dei loro controlli sani della stessa età. Quindi, il profilo immunologico dei figli dei centenari potrebbe garantire loro una maggiore protezione contro il rischio di infezioni e dai processi infiammatori tipici dei soggetti anziani.

Nel **capitolo 4** abbiamo mostrato i nostri dati ottenuti studiando il profilo immunologico dei linfociti T dei figli di centenari (CO), dei loro controlli coetanei (AM), dei soggetti anziani sani e dei soggetti giovani. Abbiamo studiato la distribuzione dei linfociti T $CD4^+$ e $CD8^+$ dentro la popolazione dei linfociti $CD3^+$. In tale studio, abbiamo osservato che i CO hanno un debole incremento della percentuale di $CD4^+$ ed una ridotta percentuale di cellule T $CD8^+$, per cui il rapporto $CD4^+$:$CD8^+$ risulta essere maggiore di quello osservato nei controlli di pari età. Inoltre, è stata effettuata l'analisi delle molecole costimolatorie CD27 e CD28 sui linfociti $CD4^+$ e $CD8^+$, per identificare lo stadio di differenziamento dei linfociti T. Abbiamo dimostrato che, i soggetti giovani mostrano una percentuale ed un numero assoluto di cellule $CD4^+CD27^+$ precocemente differenziate maggiore rispetto a quella osservata negli altri gruppi studiati (CO, AM e anziani). Tuttavia, i CO esprimono una percentuale, anche se non significativa, e un numero assoluto di queste due sottopopolazioni cellulari più elevati rispetto agli AM e anziani sani.

Un pattern simile è stato osservato nella popolazione dei linfociti T $CD8^+$. Infatti, i donatori giovani mostravano una percentuale di $CD27^+$ e $CD28^+$ maggiore rispetto a tutti gli altri soggetti studiati; diversamente, la

percentuale media di linfociti T $CD27^+$ e sia la percentuale che numero assoluto di $CD28^+$ erano maggiori nei CO rispetto gli AM. Inoltre, abbiamo anche identificato la distribuzione di cellule naive/memoria all'interno delle popolazioni di $CD4^+$ e $CD8^+$. I nostri risultati mostrano che i figli di centenari hanno, sia nell'ambito dei $CD4^+$ che dei $CD8^+$, i valori percentuali e numero assoluto delle cellule T naive $CD27^+CD28^+CD45RA^+CD45RO^-$ maggiore rispetto agli AM. Per di più, i CO presentano una riduzione delle cellule T $CD28^-CD27^-$ $CD45RA^+CD45RO^+$ (late differentiated memory) rispetto sia i controlli di pari età che gli anziani sani. Oltre a ciò, abbiamo dimostrato che i figli dei centenari hanno un livello basso di cellule potenzialmente senescenti $CD8^+CD57^+$, rispetto a quello osservato negli AM e nelle persone anziane. Quindi, è possibile concludere che i CO presentano un fenotipo più simile a quello dei soggetti giovani rispetto ai controlli coetanei. Così, il compartimento cellulare T sembra essere più resistente alle principali infezioni e potrebbe contribuire al raggiungimento degli estremi limiti della vita.

Nel **chapter 5** abbiamo studiato l'espressione di alcuni recettori chemochinici in cellule B naïve/memoria di sangue periferico, di soggetti giovani ed anziani, per valutare se l'ambiente infiammatorio influenza il traffico cellulare. Abbiamo osservato che i linfociti B naive e memoria unswitched hanno un fenotipo linfonodale, mentre le cellule memoria switched esprimono molecole utili per lasciare gli organi linfonodali. Infatti, i nostri dati dimostrano una espressione diversa di questi recettori chemochinici sulle cellule B naive e memoria tra i due gruppi studiati. In particolar modo, nei donatori giovani, le cellule B naïve esprimono il

CCR7, CCR6 e CXCR4 che consentono ai linfociti B di ricircolare. Inoltre, sia le cellule memoria unswitched che quelle switched modulano l'espressione del CD62L e CXCR5, necessari a cooperare con le cellule negli organi linfoidi. Le cellule B memoria unswitched e le cellule doppio negative (DN) dei soggetti giovani mostrano anche livelli elevati di espressione del CXCR3, un recettore chemochinico che consente di raggiungere i siti infiammatori. Quindi, sembra che sia le cellule memoria che le DN siano in grado di migrare nei siti infiammati in una reazione flogistica comune. Con l'invecchiamento, le cellule B naive/memoria mostrano alcune modificazioni nell'espressione dei recettori studiati. Infatti, a causa dell'espressione di CXCR3, le cellule memoria B unswitched conservano la capacità di migrare nei siti di infiammazione, mentre le cellule B DN perdono l'espressione di questa molecola, ma esprimono CCR6 e CCR7, che a loro volta sono anche coinvolti nella migrazione ai siti con infiammazione. Successivamente, abbiamo cercato di capire se le cellule B totali e le cellule B DN fossero in grado di agire come cellule produttrici di granzima B. A questo scopo, abbiamo stimolato *in vitro* le cellule di soggetti giovani ed anziani, con IL-21, che è incrementata nelle persone anziane, e con anti-IgG. Abbiamo dimostrato che, dopo la stimolazione, le cellule B totali di persone giovani e anziane, producono GrB, senza notare alcuna differenza tra i due gruppi. In seguito, abbiamo focalizzato la nostra attenzione sulle cellule B DN, osservando che anche questa popolazione particolare di cellule memoria, nelle stesse condizioni, produce granzima B, senza alcuna differenza tra i due gruppi studiati. Abbiamo ipotizzato che l'ambiente infiammatorio, tipico dell'invecchiamento, in qualche modo cambia le abilità di

ricircolare (trafficking) delle cellule, rendendole più sensibili sia alle citochine e alle molecole pro-infiammatorie che sono over-prodotte nell'anziano. Inoltre, questi dati suggeriscono che le cellule B DN, che sono aumentate nei soggetti anziani e mostrano un profilo di trafficking pro-infiammatorio, in soggetti giovani ed anziani che consente loro, con la stimolazione adeguata, di migrare nei siti infiammati, per cooperare con le altre cellule del sistema immunitario (es. le cellule memoria B unswitched), per produrre il GrB.

Nel **capitolo 6** abbiamo studiato l'espressione di due recettori inibitori (CD307d and CD22) sulle cellule B naïve/memory in soggetti giovani e anziani. Abbiamo osservato che, sia nei giovani che negli anziani, il CD307d è principalmente espresso dalle cellule B memoria unswitched, ridotto in modo significativo nelle cellule B naïve B e non espresso dalle cellule B memoria switched e anche dalle DN. Un dato molto interessante è rappresentato dalle cellule B memoria unswitched dei soggetti anziani, le quali mostrano livelli elevati e significativi di questa molecola rispetto alle cellule B memoria unswitched nei soggetti giovani. Diversamente, il CD22 è espresso a livelli elevati da tutte le sottopopolazioni cellulari B, in modo indipendente dall'età dei donatori e senza differenze significative tra soggetti giovani ed anziani. Quindi, possiamo concludere che le cellule B DN non esprimono un unico pattern di recettori di inibizione. Inoltre, abbiamo stimolato le cellule B *in vitro* con il CpG, ligando del TLR9, o con α-IgG/CD40, per stimolare il recettore BCR oppure abbiamo stimolato contemporaneamente i recettori dell'immunità acquisita e innata (BCR e TLR9). Abbiamo riportato che, nei soggetti giovani, le cellule $CD19^{+}$ totali e i linfociti B DN rispondono a tutti gli stimoli

utilizzati, sebbene a differenti livelli. Infatti, queste cellule proliferano a livelli elevati stimolando sia il BCR che il TLR9, e a bassi livelli stimolando solamente il BCR oppure il TLR9. Nei donatori anziani, la situazione è differente, perchè i linfociti $CD19^{+}$ tot e quelli DN non proliferano dopo stimolazione fisiologica (CpG o α-IgGCD40) ma proliferano con la doppia stimolazione (CpGα-IgGCD40), sebbene a livelli inferiori rispetto a quelli osservati nei donatori giovani. Questo potrebbe indicare che, stimolando contemporaneamente il BCR e il TLR, potrebbe essere attivato qualche pathway coinvolto nelle funzioni immunologiche. Inoltre, abbiamo anche saggiato l'attività relativa della telomerasi (RTA) in giovani e anche in diversi gruppi di soggetti anziani (CO, AM e anziani sani) per verificare se la capacità di rispondere alla stimolazione con CpG, α-IgG e CD40, potesse essere in grado di modificare l'espressione della telomerasi nelle cellule B DN. Abbiamo dimostrato che, utilizzando simultaneamente uno stimolo innato ed uno specifico si induce l'attivazione delle telomerasi nelle cellule B DN di soggetti giovani e anziani. Per di più, abbiamo anche saggiato l'attività della telomerasi nei figli di centenari e nei soggetti con malattia di Alzheimer. Anche in questi altri due gruppi di individui, la stimolazione di recettori dell'immunità innata ed acquisita attivano la telomerasi anche se a differenti livelli nei diversi gruppi. Infatti, i soggetti anziani mostrano una RTA ridotta quando paragonati con i giovani e i figli di centenari. Di conseguenza, confermiamo che i CO hanno un comportamento immunologico intermedio tra quello dei giovani e degli anziani. Invece, i soggetti con AD in forma grave mostrano bassi livelli di RTA. Questi risultati dimostrano che, le cellule B DN possono proliferare con una

stimolazione appropriata e anche che il processo di invecchiamento non annulla l'abilità di indurre l'attività della telomerasi nelle cellule B DN ma influenza i livelli di espressione di questo enzima.

Nel **capitolo 7** abbiamo riportato i dati, inseriti in un lavoro in preparazione, in merito alla valutazione della distribuzione delle cellule B naïve e memoria, identificate con una combinazione di due marcatori di superficie, IgD e CD27 (Kaminski et al., 2012), in due diversi gruppi di pazienti con malattia di Alzheimer (gravi e lievi), paragonandoli l'un l'altro e con i soggetti sani di controllo loro coetanei. Abbiamo confermato (Pellicanò et al., 2009) una riduzione significativa, sia della percentuale che del numero assoluto delle cellule B totali nei soggetti con AD in forma grave, rispetto agli anziani sani, senza trovare alcuna differenza tra gli anziani e i pazienti con AD in forma lieve. Inoltre, abbiamo trovato una riduzione significativa sia della percentuale che del numero assoluto delle cellule B naive ($IgD^{+}CD27^{-}$), ed un significativo incremento della percentuale delle cellule B DN ($IgD^{-}CD27^{-}$) rispetto i controlli sani di pari età. Diversamente, i pazienti con AD lieve sembrano comportarsi in modo simile agli anziani sani. Inoltre, abbiamo saggiato l'espressione di alcuni recettori chemochinici, quali CXCR3, CXCR4, CXCR5, CCR6, e CCR7 da parte delle cellule B DN in soggetti anziani sani e pazienti con AD grave o lieve. Abbiamo dimostrato che le cellule B DN dei soggetti con AD in forma grave non esprimono CXCR4 e CXCR5. Per quanto riguarda il CXCR3, abbiamo dimostrato un'espressione elevata di questo recettore nelle cellule B DN di tutti i gruppi studiati, senza osservare alcuna differenza significativa tra loro. Sorprendentemente, il CCR6 è aumentato in modo significativo sia nei

pazienti con AD in forma grave che in quelli con forma live, invece il CCR7 è aumentato in modo significativo nei pazienti con AD in forma grave, ma non in quelli con forma lieve. Quindi, concludiamo che la gravità della malattia di Alzheimer potrebbe influenzare la distribuzione delle cellule naïve e delle cellule B DN, ed anche il profilo dei recettori di trafficking pro-infiammatorio nelle cellule B DN, guidando questi linfociti nel tessuto cerebrale infiammato.

Nel **capitolo 8**, abbiamo discusso alcuni dati nostri ed altri presenti in letteratura riguardanti il coinvolgimento delle modifiche del sistema immunitario nella malattia di Alzheimer (AD). Dall'esame di questi, abbiamo dedotto che i cambiamenti nel sistema immunitario innato e acquisito, il background genetico ed altri fattori di rischio, contribuiscono alla patogenesi di questa patologia. Infatti, è stato suggerito che il peptide Aβ induce una stimolazione persistente del sistema immunitario che causa risposte infiammatorie croniche da parte delle cellule T e B ed anche il rilascio di mediatori infiammatori. Questi eventi sembrano contribuire alla neurodegenerazione. Per quanto riguarda il background genetico, è stato dimostrato che i polimorfismi dei geni coinvolti nel processo di infiammazione del sistema immunitario, potrebbero avere un ruolo nella generazione dell'AD. Inoltre, altri fattori di rischio, come ormoni, il livello di istruzione e fattori ambientali potrebbero essere associati a questa malattia età-correlata. In conclusione, è stato considerato di importanza rilevante, identificare un metodo nuovo che consenta l'uso del sangue periferico di pazienti con AD per identificare marcatori prognostici o associati alla malattia. Sino ad oggi, sono stati identificati

diversi fattori che possono essere utili a tale scopo, sebbene ci siano molti risultati discordanti.

Nel **capitolo 9** abbiamo commentato nostri dati e alcuni di letteratura riguardanti i centenari siciliani. Dalla riflessione su questi dati, è possibile concludere che, sia una funzione immunitaria ben preservata che il background genetico svolgono un ruolo cruciale nell'invecchiamento e nella longevità ed anche che, i centenari rappresentano il modello migliore per comprendere l'invecchiamento con e senza successo. Infatti, è stato dimostrato che le persone avvantaggiate geneticamente per la longevità possiedono delle caratteristiche immunologiche differenti rispetto quelle della popolazione generale. Questo suggerisce l'ipotesi della "giovinezza familiare". A tal proposito, i figli dei centenari (CO) non possiedono il tipico profilo delle cellule T naive e memoria osservato nei controlli di pari età (AM). Inoltre, anche il compartimento cellulare dei linfociti B dei CO risulta essere più simile a quello osservato nei soggetti giovani rispetto a quello degli anziani. Infatti, sebbene nei figli dei centenari e nei loro controlli di pari età sia stato osservata una riduzione nel numero delle cellule B, è stato dimostrato che le cellule B naïve ($IgD^{+}CD27^{-}$) aumentano e che le cellule B DN ($IgD^{-}CD27^{-}$) si riducono in modo significativo quando paragonate con le persone giovani. In questo lavoro, noi ipotizziamo che esistono altri fattori che contribuiscono alla longevità, come il genere, condizioni ambientali, lo stato pro-infiammatorio, la dieta e le mutazioni nei geni per il TLR4, CCR5 Cox2 ed anche in quelli per le citochine (es. INF-γ, IL-10) che regolano la risposta immunitaria. Tali fattori aumentare le possibilità per il raggiungimento della longevità.

Nel **capitolo 10** abbiamo esaminato i dati presenti in letteratura insieme ai risultati dei nostri studi riguardanti i figli dei centenari (CO). Abbiamo discusso dei meccanismi biologici che contribuiscono al raggiungimento di un fenotipo longevo ed anche il ruolo che queste ricerche potrebbero avere nello sviluppo di terapie anti-invecchiamento, farmaci e nel controllo della velocità del processo di invecchiamento. Concludiamo che, i figli dei centenari (CO) potrebbero rappresentare un modello di invecchiamento per comprendere la longevità. Infatti, essi hanno un background genetico protettivo e potrebbero avere ereditato delle varianti geniche, dai loro genitori longevi, che li proteggono dal processo di invecchiamento e dall'ipertensione, cancro, diabete, patologie cardiovascolari (CVD). Inoltre, i CO mostrano un profilo cardiovascolare e lipidico favorevoli (es. alti livelli di HDL-C, bassi livelli di LDL-C) ed un profilo immunologico ottimale (es. alti livelli di cellule B naïve, diminuzione di cellule B DN ed alti livelli di IgM sieriche). Inoltre, i centenari ed i loro figli, sfuggono o posticipano il declino cognitivo, la demenza ed hanno una ridotta incidenza per l'AD rispetto ai loro controlli di pari età (AM). Questi dati suggeriscono che, l'eccezionale longevità potrebbe essere il risultato di un'azione concomitante di caratteristiche fenotipiche e genotipiche, trasmesse nelle famiglie longeve.

12.1 Conclusioni

E' noto che l'esposizione duratura alle infezioni nuove e persistenti (Fulop T et al., 2013; Saule P et al., 2006), tipiche degli anziani, influenzano le reazioni immunitarie acquisite. Infatti sono state dimostrate modifiche nel compartimento cellulare sia dei linfociti T che

in quello dei B (Pawelec et al., 2002; 2008; 2013; Miller and Cancro, 2007; Colonna-Romano G et al., 2009). Per quanto concerne la branca cellulare dei linfociti T, i cambiamenti correlati all'età risultano in una riduzione progressiva dei linfociti T $CD3^+$, $CD4^+$ e $CD8^+$ T circolanti e nell'incremento delle cellule T $CD8^+$ terminalmente differenziate (late stage differentiated) nelle persone anziane (Pawelec G et al., 2002). In particolar modo, diversi studi hanno suggerito l'incremento del numero di cellule T $CD28^-$ altamente differenziate (highly differentiated), specialmente nell'ambito dei linfociti T $CD8^+$ nei soggetti anziani (Effros RB et al., 1994**;** Pawelec G et al., 2008). Queste cellule T ($CD28^-CD8^+$) mostrano una ridotta diversità recettoriale, proliferazione difettiva indotta dall'antigene, breve vita replicativa e un'aumentata funzione citotossica (Oviedo-Orta et al., 2013; Weng NP, et al., 2009) e resistenza all'apoptosi (Pawelec G et al., 2008). Un'altra popolazione di linfociti T memoria interessante, che incrementa con l'età, è rappresentata dalle cellule T memoria effettrici terminalmente differenziate, TEMRA ($CCR7^–$, $CD27^–$, $CD28^–$, $CD45RA^+$). E' stato suggerito che molte di queste cellule TEMRA non siano in grado di produrre citochine, di mediare attività citotossiche, e sembrano mostrare difetti proliferativi associati alla senescenza (Effross RB, 2011; Fulop T et al.,2013). Tuttavia, di recente, è stato dimostrato che in alcune circostanze queste cellule possono secernere citochine ed esprimere alti livelli granzima B e perforina (Libri VR et al., 2011). Quindi, queste cellule potrebbero essere importanti per la protezione contro alcune infezioni *in vivo* (Bruns H et al., 2009; Di Mitri D et al., 2011). Come discusso precedentemente, nel compartimento cellulare dei linfociti B sono stati dimostrati dei cambiamenti correlati

all'età (Colonna-Romano et al., 2003; Miller JP and Cancro MP, 2007; Frasca D et al., 2008). Infatti, la stimolazione persistente sembra indurre nelle persone anziane, la diminuzione delle cellule B $CD19^+$ totali (Colonna-Romano et al., 2003; Frasca D et al., 2008; Veneri D et al., 2009) e delle cellule naive (Gupta s et al., 2005; Colonna-Romano G et al., 2008), sebbene altri gruppi di ricerca abbiano osservato l'incremento della percentuale ma non del numero assoluto di queste ultime con l'età (Shi Y et al., 2005; Frasca et al., 2008). Per quanto riguarda la popolazione di cellule B memoria, il gruppo di ricerca della Frasca (Frasca et al.,, 2012) ha dimostrato una diminuzione sia della percentuale che del numero assoluto dei linfociti B memoria switched, suggerendo un difetto delle cellule nel compiere lo switch di classe. Diversamente da quanto riportato prima, il nostro gruppo (Colonna-Romano G et al., 2003, 2009) non ha riportato nessun cambiamento significativo nelle cellule B memoria, identificate come $CD19^+CD27^+$ nell'anziano, ma un incremento significativo della popolazione di linfociti B DN($CD19^+IgG^+IgD^-CD27^-$) in tali soggetti (Colonna-Romano G et al., 2009). Inoltre, i livelli sierici di immunoglobuline totali e degli isotipi specifici subiscono modifiche con l'età. Infatti, sebbene il numero dei linfociti B totali si reduca con l'età, i livelli di IgG, IgA , e in misura minore di IgE, sono aumentati. Diversamente, i livelli di IgM diminuiscono oppure non cambiano, mentre quelli di IgD si riducono (Listì F et al., 2006; Frasca D et al., 2010).

La maggior parte delle ricerche scientifiche sull'immunosenescenza hanno focalizzato l'attenzione sui meccanismi che potrebbero portare alla longevità. Per cui, si ritiene importante studiare il complesso processo dell'invecchiamento del sistema immunitario negli anziani ed anche in

modelli di studio dell'invecchiamento con e senza successo, per identificare biomarcatori di invecchiamento in buona salute, che potrebbero essere utili per valutare i trattamenti di anti-invecchiamento oppure migliorare la qualità della vita della popolazione anziana che è in aumento. In generale, i figli dei centenari (CO), che sono avvantaggiati geneticamente per la longevità, rappresentano un modello per comprendere la longevità, invece i pazienti con malattia di Alzheimer sono utili per studiare l'invecchiamento senza successo (Terry et al., 2004a, 2004b; Derhovanessian et al., 2010). I nostri studi dimostrano che i CO hanno un compartimento cellulare dei linfociti T molto più simile a quello dei soggetti giovani che ai loro controlli di pari età (AM). Inoltre, in questi soggetti, anche il compartimento cellulare dei linfociti B naïve e memoria è più simile quello delle persone giovani rispetto a quello delle persone anziane. Infatti, le cellule B naive sono aumentate e i linfociti B DN sono ridotti rispetto ai loro controlli AM. Di conseguenza, questi dati interessanti indicano che i linfociti B naïve e le cellule B DN potrebbero rappresentare due marcatori del processo di invecchiamento. Il ruolo rilevante delle cellule B DN, come biomarcatori di vita umana, risulta anche da studi condotti sul sistema immunitario dei pazienti con malattia di Alzheimer. Difatti, abbiamo dimostrato il declino delle cellule B naïve e un incremento della percentuale di cellule B DN in soggetti con AD grave, rispetto ai donatori coetanei in buone condizioni di salute; invece, i pazienti con AD lieve sembrano comportarsi come gli anziani sani. Quindi, è possibile supporre che la valutazione della percentuale delle cellule B DN potrebbe indicare sia l'età cronologica che quella biologica del sistema immunitario ed anche le prestazioni delle risposte immunitarie

contro i patogeni. Infatti, è stato riportato che il pool di cellule B DN è espanso in individui stimolati cronicamente, come soggetti sani infettati con virus respiratorio sinciziale (RSV) (Sanz I et al., 2008), pazienti con Lupus (Wei C et al., 2007) ed in soggetti con HIV (Cagigi A et al., 2009). Di conseguenza, l'incremento di queste cellule potrebbe essere il risultato di una stimolazione persistente del sistema immunitario. Inoltre, abbiamo anche cercato di comprendere se le cellule B DN possono essere considerate un marcatore di invecchiamento oppure se queste cellule sono correlate all'infiammazione sistemica, poiché è noto che sia gli anziani che i pazienti con AD sono caratterizzati da un'infiammazione sistemica che non è tipica dei CO (Terry DF et al., 2006; Salvioli S et al., 2013). A tale scopo, abbiamo studiato l'espressione di alcuni recettori chemochinici che guidano le cellule verso i tessuti infiammati, nelle cellule B naive e memoria di sangue periferico di soggetti giovani e anziani. Questi esperimenti hanno suggerito che l'ambiente infiammatorio potrebbe influenzare il traffico delle cellule B. Infatti, le sottopopolazioni B naive e memoria esprimono differenti pattern di recettori chemochinici. La differenza più importante è rappresentata dai linfociti B DN. In particolar modo, le cellule B DN dei soggetti giovani esprimono livelli elevati di CXCR3, un recettore chemochinico che consente di raggiungere i siti infiammati. Differentemente, nelle persone anziane, le cellule B DN perdono l'espressione di questa molecola, ma mostrano livelli elevati di CCR6 e di CCR7, che sono anche implicati nella migrazione ai siti infiammati. Inoltre, la stessa analisi ha è stata realizzata in soggetti con AD grave o lievi. E' stato dimostrato che nei pazienti con la forma grave di Alzheimer, le cellule B DN esprimono

elevati e significativi livelli di CCR6 rispetto i soggetti con AD lieve, mentre l'espressione del CCR7 è aumentata in modo significativo nei pazienti con AD in forma grave, ma non in quelli con la forma lieve della malattia. Quindi, sembra che l'incremento dell'espressione del CCR6 dipenda dalla gravità della patologia. Di recente, è stato osservato un incremento di espressione del CCR6 anche da parte delle cellule T ottenute da pazienti con AD (Goldeck et al., 2013). Un comportamento simile è effettuato anche da parte delle cellule B totali, suggerendo che l'espressione di questi recettori chemochinici potrebbe essere influenzato dall'ambiente pro-infiammatorio dei pazienti con AD, e che, il CCR6 e, probabilmente, il CCR7 potrebbero giudare le cellule B verso il tessuto cerebrale infiammato. Ad oggi, è noto che le cellule B DN potrebbero migrare nei tessuti con infiammazione, sebbene il loro ruolo in questi siti non si conosca. Tuttavia, è stato dimostrato che, con un'adeguata stimolazione, le cellule B DN secernono immunoglobuline contro la tossina tetanica ed il virus influenzale (Wirths and Lanzavecchia et al., 2005). Per di più, è stato dimostrato che sia le cellule B totali che i linfociti B DN, possono esercitare funzioni citotossiche. Infatti, queste cellule, quando stimolate con α-IgG e IL-21, che è aumentata nelle persone anziane, producono il granzima B (GrB) che può svolgere un ruolo significativo nelle fasi precoci della risposta immunitaria antivirale, nella regolazione delle patologie autoimmuni e nell'immunosorveglianza nel cancro (Hagn M et al., 2009; Kurschus FC et al., 2004; Gross C et al., 2003). Inoltre, abbiamo dimostrato che sia le cellule B tot che le B DN, dopo adeguata stimolazione, diventano abili a produrre granzima B, senza nessuna differenza tra soggetti giovani e anziani. Quindi, concludiamo

che le cellule B DN, che mostrano un profilo di trafficking di tipo pro-infiammatorio, in soggetti giovani e anziani, se adeguatamente stimolate, potrebbero migrare verso i siti infiammatori, cooperando con altre cellule del sistema immunitario (es.le cellule memoria unswitched), potrebbero produrre il GrB (Bulati M et al., 2014).

Inoltre, è noto che le cellule B DN degli anziani hanno i telomeri corti rispetto la stessa sottopopolazione dei soggetti giovani e che non rispondono alla stimolazione con CpG, sebbene possano essere attivate con il $F(ab')_2$ (anti-BCR) (Colonna-Romano et al., 2009). Abbiamo dimostrato che la stimolazione del BCR e del TLR9 induce la proliferazione delle cellule B DN ed anche la riattivazione delle telomerasi nelle cellule B DN B di soggetti giovani, anziani, CO e soggetti con AD. Questo processo permette di mantenere la lunghezza dei telomeri o di indurre la replicazione.

In conclusione, nella presente tesi ho mostrato i dati ottenuti studiando alcuni aspetti del sistema immunitario in soggetti giovani e anziani, paragonando i dati ottenuti da questi soggetti con quelli ottenuti studiando una coorte di soggetti "geneticamente avvantaggiati" per la longevità (CO) e persone anziane affette da AD. I dati riportati suggeriscono che lo studio dei compartimenti cellulari dei linfociti T e B naïve/memoria potrebbero essere rilevanti nella valutazione dell'invecchiamento biologico del sistema immunitario.

References

13. REFERENCES

- Agematsu K, Hokibara S, Nagumo H, Komiyama A. CD27: a memory B-cell marker.Immunol Today. 2000 May;21(5):204-6.
- Agrawal N, Gupta SK. Birth weight, early weight gain, and risk of type 1 diabetes. Natl Med J India. 2010 Sep-Oct;23(5):289-90.
- Akbar AN, HensonSM. Are senescence and exhaustion inter-twined or unrelated processes that compromise immunity? Nat RevImmunol (2011) 11:289–95.
- Akbar AN, Fletcher JM. Memory T cell homeostasis and senescence during aging. Curr Opin Immunol. 17(5):480-485 (2005)
- Akiyama H, Arai T, Kondo H, Tanno E, Haga C, Ikeda K. Cell mediators of inflammation in the Alzheimer disease brain. Alzheimer Dis Assoc Disord. Suppl 1:S47-53. (2000) Review.
- Akushevich I, Veremeyeva G, Kravchenko J, Ukraintseva S, Arbeev K, Akleyev AV, Yashin AI. New stochastic carcinogenesis model with covariates: an approach involving intracellular barrier mechanisms. Math Biosci. 2012 Mar;236(1):16-30.
- Almanzar G, Schwaiger S, Jenewein B, Keller M, Herndler-Brandstetter D, Würzner R, Schönitzer D, Grubeck-Loebenstein B. Long-term cytomegalovirus infection leads to significant changes in the composition of the $CD8^+$ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. *J Virol.* 79:3675-3683 (2005)
- Anderson G, Jenkinson EJ. Lymphostromal interactions in thymic development and function. Nat Rev Immunol (2001) 1:31–40.

- Anolik JH, Barnard J, Cappione A, Pugh-Bernard AE, Felgar RE, Looney RJ, Sanz I. Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus. Arthritis Rheumat. 40:3538-3590 (2004)
- Aw D, Palmer DB. It's not all equal: a multi phasic theory of thymic involution. Biogerontology (2012) 13:77–81.
- Baggio G, Donazzan S, Monti D, Mari D, Martini S, Gabelli C, Dalla Vestra M, Previato L, Guido M, Pigozzo S, Cortella I, Crepaldi G, Franceschi C. Lipoprotein(a) and lipoprotein profile in healthy centenarians: a reappraisal of vascular risk factors. FASEB J. 1998 Apr;12(6):433-7.
- Balistreri CR, Candore G, Caruso M, Incalcaterra E, Franceschi C, Caruso C. Role of polymorphisms of CC-chemokine receptor-5 gene in acute myocardial infarction and biological implications for longevity. *Haematologica.* 93:637-638 (2008)
- Bartlett DB, Firth CM, Phillips AC, Moss P, Baylis D, Syddall H, Sayer AA, Cooper C, Lord JM.The age-related increase in low-grade systemic inflammation (Inflammaging) is not driven by cytomegalovirus infection.Aging Cell. 2012 Oct;11(5):912-5.
- Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, He B, Biermann K, Lange JF, van der Burg M, van Dongen JJ, van Zelm MC. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood. 118(8):2150-2158 (2011)
- Bertho JM, Demarquay C, Moulain N, Van Der Meeren A, Berrih-Aknin S, Gourmelon P. Phenotypic and immunohistological analyses

of the human adult thymus: evidence for an active thymus during adult life. Cell Immunol (1997) 179:30–40.

- Beyer I, Mets T, Bautmans I. Chronic low-grade inflammation and age-related sarcopenia. Curr Opin Clin Nutr Metab Care. 2012 Jan;15(1):12-22.
- Bird TD. Genetic factors in Alzheimer's disease.N Engl J Med. 352(9):862-4 (2005).
- Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, Mauri C. 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):129-40 (2010)
- Blink EJ, Light A, Kallies A, Nutt SL, Hodgkin PD, Tarlinton DM. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. J Exp Med. 2005 Feb 21;201(4):545-54.
- Bouaziz JD, Calbo S, Maho-Vaillant M, Saussine A, Bagot M, Bensussan A, Musette P. IL-10 produced by activated human B cells regulates CD4(+) T-cell activation *in vitro*. Eur J Immunol. 2010 Oct;40(10):2686-91.
- Britschgi M and Wyss-Coray T Systemic and acquired immune responses in Alzheimer's disease. Int Rev Neurobiol 82, 205-33 (2007).
- Bruns H, Meinken C, Schauenberg P, Härter G, Kern P, Modlin RL, Antoni C, Stenger S. Anti-TNF immunotherapy reduces $CD8^+$ T cell-

mediated antimicrobial activity against Mycobacterium tuberculosis in humans. J Clin Invest. 2009 May;119(5):1167-77.

- Bruunsgaard H., “A high plasma concentration of tnf-α is associated with dementia in centenarians,” Journals of Gerontology. Series A, vol. 54, no. 7, pp. M357–M364, 1999.
- Buffa S, Bulati M, Pellicanò M, Dunn-Walters DK, Wu YC, Candore G, Vitello S, Caruso C, Colonna-Romano G. B cell immunosenescence: different features of naive and memory B cells in elderly. Biogerontology. 2011 Oct;12(5):473-83.
- Bulati M, Buffa S, Candore G, Caruso C, Dunn-Walters DK, Pellicanò M, Wu YC, Colonna Romano G. B cells and immunosenescence: a focus on $IgG^{+}IgD^{-}CD27^{-}$ (DN) B cells in aged humans. Ageing Res Rev. 2011 Apr;10(2):274-84.
- Cagigi A, Du L, Dang LV, Grutzmeir S, Atlas A, Chiodi F, Pan-Hammarstrom Q, Nilsson A. CD27(-) B-cells produce class switched and somatically hyper-mutated antibodies during chronic HIV-1 infection. PLos One. 4(5):e5427 (2009)
- Campisi J. Aging and cancer cell biology, 2007. Aging Cell. 2007 Jun;6(3):261-3. Review.
- Cancro MP, Hao Y, Scholz JL, Riley RL, Frasca D, Dunn-Walters DK, Blomberg BB. B cells and ageing: molecules and mechanisms. Trends Immunol. 30:313-318 (2009)
- Candore G, Caruso C, Colonna-Romano G. Inflammation, genetic background and longevity. Biogerontology. 2010 Oct;11(5):565-73.
- Candore G, Balistreri CR, Listì F, Grimaldi MP, Vasto S, Colonna-Romano G, Franceschi C, Lio D, Caselli G, Caruso C.

Immunogenetics, gender, and longevity. Ann N Y Acad Sci. 1089:516-37. (2006) Review b.

- Candore G, Balistreri CR, Grimaldi MP, Vasto S, Listì F, Chiappelli M, Licastro F, Lio D, Caruso C. Age-related inflammatory diseases: role of genetics and gender in the pathophysiology of Alzheimer's disease. Ann N Y Acad Sci. 1089:472-86. (2006) Review a.
- Carsetti R. Characterization of B-cell maturation in the peripheral immunesystem. Methods Mol Biol. 2004;271:25-35.
- Cevenini E, Monti D, Franceschi C. Inflamm-ageing. Curr Opin Clin Nutr Metab Care. 2013 Jan;16(1):14-20. Review.
- Cevenini E, Caruso C, Candore G, Capri M, Nuzzo D, Duro G, Rizzo C, Colonna-Romano G, Lio D, Di Carlo D, Palmas MG, Scurti M, Pini E, Franceschi C, Vasto S. Age-related inflammation: the contribution of different organs, tissues and systems. How to face it for therapeutic approaches. Curr Pharm Des. 16:609-618 (2010)
- Cevenini E, Invidia L, Lescai F, Salvioli S, Tieri P, Castellani G, Franceschi C. Human models of aging and longevity. Expert Opin Biol Ther. 2008 Sep;8(9):1393-405.
- Challen GA, Boles NC, Chambers SM, Goodell MA. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. Cell Stem Cell. 2010 Mar 5;6(3):265-78.
- Chambers SM, Shaw CA, Gatza C, Fisk CJ, Donehower LA, Goodell MA. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. PLoS Biol. 2007 Aug;5(8):e201.
- Chidrawar S, Khan N, Wei W, McLarnon A, Smith N, Nayak L, Moss P. Cytomegalovirus-seropositivity has a profound influence on the

magnitude of major lymphoid subsets within healthy individuals. *Clin Exp Immunol.* 155:423-432 (2009)

- Chinn IK, Blackburn CC, Manley NR, Sempowski GD. Changes in primary lymphoid organs with aging. Semin Immunol (2012) 24:309–20.
- Colonna-Romano G, Bulati M, Aquino A, Pellicanò M, Vitello S, Lio D, Candore G, Caruso C. A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people. Mech Ageing Develop. 130(10):681-690 (2009)
- Colonna-Romano G, Malavolta M, Lio D, Nuzzo D, Mocchegiani E, Di Bona D, Caruso C. Inflammation, genes and zinc in Alzheimer's disease. Brain Res Rev. 58:96-105 (2008)
- Colonna-Romano G, Bulati M, Aquino A, Scialabba G, Candore G, Lio D, Motta M, Malaguarnera M, Caruso C. B cells in the aged: CD27, CD5 and CD40 expression. Mech Ageing Dev. 124:389-393 (2003)
- 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.
- Cossarizza A, Ortolani C, Paganelli R, Barbieri D, Monti D, Sansoni P et al. CD45 isoforms expression on $CD4^+$ and $CD8^+$ T cell

throughout life, from newborns to centenarians: implications for T cell memory. Mech Ageing Dev. 86:173-195 (1996)

- Crestini A, Napolitano M, Piscopo P, Confaloni A, Bravo E. Changes in cholesterol metabolism are associated with PS1 and PS2 gene regulation in SK-N-BE. J Mol Neurosci. 30(3):311-322 (2006)
- De Benedictis G, Franceschi C. The unusual genetics of human longevity. Sci Aging Knowledge Environ. 2006 Jun 28;2006(10):pe20.
- De Martinis M., Franceschi C., Monti D., Ginaldi L.: Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. FEBS Lett 579,2035-2039 (2005)
- Derhovanessian E, Maier AB, Beck R, Jahn G, Hahnel K, Slagboom PE, de Craen AJ, Westendorp RG, Pawelec G. Hallmark features of immunosenescence are absent in familial longevity. J Immunol. 185:4618-4624 (2010)
- Derhovanessian E, Maier AB, Hähnel K, Beck R, de Craen AJ, Slagboom EP, Westendorp RG, Pawelec G. Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated $CD4^+$ and $CD8^+$ T-cells in humans. J Gen Virol. 2011 Dec;92(Pt 12):2746-56.
- Dewan SK, Zheng SB, Xia SJ, Bill K. Senescent remodeling of the immune system and its contribution to the predisposition of the elderly to infections. Chin Med J (Engl). 2012 Sep;125(18):3325-31.
- Di Mitri D, Azevedo RI, Henson SM, Libri V, Riddell NE, Macaulay R, Kipling D, Soares MV, Battistini L, Akbar AN. Reversible

senescence in human $CD4^+CD45RA^+CD27$- memory T cells. J Immunol. 2011 Sep 1;187(5):2093-100.

- Dixit VD. Thymic fatness and approaches to enhance thymopoietic fitness in aging. Curr Opin Immunol (2010) 22:521–8.
- Duddy ME, Niino M, Adatia F, Hebert S, Freedman M, Atkins H, Kim HJ, Bar-Or A. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. J Immunol. 178:6092-6099 (2007)
- Duddy ME, Alter A, Bar-Or A. Distinct profiles of human B cell effector cytokines: a role in immune regulation? J Immunol. 172(6):3422-3427 (2004)
- Dunn-Walters DK, Ademokun AA. B cell repertoire and ageing. Curr Opin Immunol. Curr Opin Immunol. 2010 Aug;22(4):514-20.
- Efferth T.Adenosine triphosphate-binding cassette transporter genes in ageing and age-related diseases. Ageing Res Rev. 2003 Jan;2(1):11-24.
- Effross RB. Telomere/Telomerase dynamics within the human immune system: effect of chronic infection and stress. Exp Gerontol 2011. 46:135-40.
- Effros RB, Boucher N, Porter V, Zhu X, Spaulding C, Walford RL, Kronenberg M, Cohen D, Schachter F: Decline in $CD28^+$ T cells in centenarians and in long-term T cell cultures: a possible cause for both *in vivo* and *in vitro* immunosenescence. Exp Gerontol 1994, 29:601-9.
- Fagnoni FF, Vescovini R, Passeri G, Bologna G, Pedrazzoni M, Lavagetto G, Casti A, Franceschi C, Passeri M and Sansoni P.

Shortage of circulating naive $CD8^+$ T cells provide new insights of immunodeficiency in aging. Blood. 95:2860-2868 (2000)

- Fassbender K, Masters C, Beyreuther K Alzheimer's disease: an inflammatory disease?Neurobiol Aging. 21(3):433-6 (2000)
- Fecteau JF, Côté G, Néron S. 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 (2006)
- Fiala M, Lin J, Ringman J, Kermani-Arab V, Tsao G, Patel A, Lossinsky AS, Graves MC, Gustavson A, Sayre J, Sofroni E, Suarez T, Chiappelli F, Bernard G.Ineffective phagocytosis of amyloid-beta by macrophages of Alzheimer's disease patients. J Alzheimers Dis. 7(3):221-32 (2005)
- Fox NC, Crum WR, Scahill RI, Stevens JM, Janssen JC, Rossor MN. Imaging of onset and progression of Alzheimer's disease with voxel-compression mapping of serial magnetic resonance images. Lancet. 2001 Jul 21;358(9277):201-5.
- Franceschi C, Motta L, Motta M, Malaguarnera M, Capri M, Vasto S, Candore G, Caruso C; IMUSCE. The extreme longevity: the state of the art in Italy. Exp Gerontol. 2008 Feb;43(2):45-52.
- Franceschi C, Capri M, Monti D, Giunta S, Olivieri F, Sevini F, Panourgia MP, Invidia L, Celani L, Scurti M, Cevenini E, Castellani GC, Salvioli S. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. Mech Ageing Dev. 2007 Jan;128(1):92-105.
- Franceschi C, Olivieri F, Marchegiani F, Cardelli M, Cavallone L, Capri M, Salvioli S, Valensin S, De Benedictis G, Di Iorio A, Caruso

C, Paolisso G, Monti D. Genes involved in immune response/inflammation, IGF1/insulin pathway and response to oxidative stress play a major role in the genetics of human longevity: the lesson of centenarians. *Mech Ageing Dev.* 126:351-361 (2005)

- Franceschi C, Bonafè M. Centenarians as a model for healthy aging. *Biochem Soc Trans.* 31:457-461 (2003)
- Franceschi C, Valensin S, Bonafè M, Paolisso G, Yashin AI, Monti D, De Benedictis G. The network and the remodeling theories of aging: historical background and new perspectives. Exp Gerontol. 35(6-7):879-96. Review (2000) b
- Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, De Benedictis G. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci. Jun;908:244-54. Review 2000a
- Franceschi C, Monti D, Barbieri D, Grassilli E, Troiano L, Salvioli S, Negro P, Capri M, Guido M, Azzi R et al. Immunosenescence in humans: deterioration or remodelling? Int Rev Immunol. 12(1):57-74 (1995)
- Frasca D, Diaz A, Romero M, Phillips M, Mendez NV, Landin AM, Blomberg BB. Unique biomarkers for B-cell function predict the serum response to pandemic H1N1 influenza vaccine. Int Immunol. 2012 Mar;24(3):175-82.
- Frasca D, Blomberg BB. Aging affects human B cell responses. J Clin Immunol. 31(3):430-435 (2011)
- Frasca D, Diaz A, Romero M, Landin AM, Phillips M, Lechner SC, Ryan JG, Blomberg BB. Intrinsic defects in B cell response to

seasonal influenza vaccination in elderly humans. Vaccine. 2010 Nov 29;28(51):8077-84.

- Frasca D, Landi AM, Lechner SC, Ryan JG, Schwartz R, Riley RL, Blomberg BB. Ageing down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. J Immunol. 180:5283-5290 (2008)
- Frasca D, Riley RL, Blomberg BB. Humoral immune response and B-cell functions including immunoglobulin class switch are downregulated in aged mice and humans. Sem Immunol. 17:378-384 (2005)
- Fülöp T, Larbi A, Pawelec G. Human T Cell Aging and the Impact of Persistent Viral Infections. Front Immunol. 2013 Sep 13;4:271. Review.
- Gangemi S, Basile G, Monti D, Merendino RA, Di Pasquale G, Bisignano U, Nicita-Mauro V, Franceschi C. Age-related modifications in circulating IL-15 levels in humans. Mediators Inflamm. 2005 Aug 31;2005(4):245-7.
- George AJ, Ritter MA. Thymic involution with ageing: obsolescence or good housekeeping? Immunol Today (1996) 17:267–72.
- Gerli R, Monti D, Bistoni O, Mazzone AM, Peri G, Cossarizza A, Di Gioacchino M, Cesarotti ME, Doni A, Mantovani A, Franceschi C, Paganelli R.Chemokines, sTNF-Rs and sCD30 serum levels in healthy aged people and centenarians. Mech Ageing Dev. 2000 Dec 20;121(1-3):37-46.
- Gibson KL, Wu YC, Barnett Y, Duggan O, Vaughan R, Kondeatis E, Nilsson BO, Wikby A, Kipling D, Dunn-Walters DK. B-cell diversity

decreases in old age and is correlated with poor health status. Ageing cell. 8:18-25 (2009)

- Ginaldi L., Loreto M. F.,. Corsi M. P, Modesti M., and De Martinis M., “Immunosenescence and infectious diseases,”Microbes and Infection, vol. 3, no. 10, pp. 851–857, 2001.
- Globerson A, Effros RB. Ageing of lymphocytes and lymphocytes in the aged. Immunol Today. 2000 Oct;21(10):515-21.
- Goldberger A.L., Peng C.K., Lipsitz L.A. What is physiologic complexity and how does it change with aging and disease? Neurobiol Aging. 23, 23-6. (2002)
- Goldeck D, Larbi A, Pellicanó M, Alam I, Zerr I, Schmidt C, Fulop T, Pawelec G. Enhanced Chemokine Receptor Expression on Leukocytes of Patients with Alzheimer's Disease. 2013. PLoS One, 8 (6).
- Gomez CR, Karavitis J, Palmer JL, Faunce DE, Ramirez L, Nomellini V, Kovacs EJ. Interleukin-6 contributes to age-related alteration of cytokine production by macrophages.Mediators Inflamm. 2010;2010:475139
- Goto M. Inflammaging (inflammation + aging): A driving force for human aging based on an evolutionarily antagonistic pleiotropy theory? BioScience Trends. 2(6):218-230 (2008)
- Griffin WS, Mrak RE. Interleukin-1 in the genesis and progression of and risk for development of neuronal degeneration in Alzheimer's disease. J Leukoc Biol. 72(2):233-8. (2002) Review.
- Griffiths PD. CMV as a cofactor enhancing progression of AIDS. J Clin Virol. 35:489-492 (2006)

- Grimm MO, Rothhaar TL, Hartmann T. The role of APP proteolytic processing in lipid metabolism. Exp Brain Res. DOI: 10.1007/s00221-011-2975-6 (2011)
- Gross C, Koelch W, DeMaio A, Arispe N, Multhoff G. Cell surface-bound heat shock protein 70 (Hsp70) mediates perforin-independent apoptosis by specific binding and uptake of granzyme B. J Biol Chem. 2003 Oct 17;278(42):41173-81.
- Grubeck-Loebenstein B, Della Bella S, Iorio AM, Michel JP, Pawelec G, Solana R. Immunosenescence and vaccine failure in the elderly. Aging Clin Exp Res. 2009
- Jun;21(3):201-9.
- Guerrettaz LM, Johnson SA, Cambier JC. Acquired hematopoietic stem cell defects determine B-cell repertoire changes associated with aging. Proc Natl Acad Sci USA. 105:11898-11902 (2008)
- Gui J, Zhu X, Dohkan J, Cheng L, Barnes PF, Su D-M. The aged thymus shows normal recruitment of lymphohematopoietic progenitors but has defects in thymic epithelial cells. Int Immunol (2007) 19:1201–11.
- Gupta S, Su H, Bi R, Agrawal S, Gollapudi S. Life and death of lymphocytes: a role of immunosenescence. Immun Ageing. 23:2-12 (2005)
- Hagn M, Schwesinger E, Ebel V, Sontheimer K, Maier J, Beyer T, Syrovets T, Laumonnier Y, Fabricius D, Simmet T, Jahrsdörfer B. Human B cells secrete granzyme B when recognizing viral antigens in the context of the acute phase cytokine IL-21. J Immunol. 2009 Aug 1;183(3):1838-45.

- Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, van Lier RA. Phenotipic and functional separation of memory and effector human CD8+ T cells. J Exp Med. 186:1407-1418 (1997)
- Harris DP, Ortiz F, Adler FM, Yu K, Maines ML, Barba D, Viggiani SI, Wolf SM, Fitten LJ, Chodosh J, Vickrey BG. Challenges to screening and evaluation of memory impairment among Hispanic elders in a primary care safety net facility. Int J Geriatr Psychiatry. 2011 Mar;26(3):268-76.
- Heron MP, Smith BL. Deaths: leading causes for 2003. Natl Vital Stat Rep. 55:1-92 (2007).
- Inamine A, Takahashi Y, Baba N, Miyake K, Tokuhisa T, Takemori T, Abe R. Two waves of memory B-cell generation in the primary immune response. Int Immunol. 2005 May;17(5):581-9.
- Kaech SM, Wherry EJ, Ahmed R: Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol, 2:251-262 (2002)
- 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.
- Klein, U., Rajewsky, K., Küppers, R. Human immunoglobulin (Ig)M^+IgD^+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J. Exp. Med. 188, 1679-89 (1998)

- Kogut I, Scholz JL, Cancro MP, Cambier JC. B cell maintenance and function in aging. Semin Immunol. 2012 Oct;24(5):342-9.
- Kohler S, Wagner U, Pierer M, Kimmig S, Oppmann B, Möwes B, Jülke K, Romagnani C, Thiel A. Post thymic *in vivo* proliferation of näive CD4+ T cells constrains the TCR repertoire in healthy human adults. Eur J Immunol 35: 1987 (2005)
- Kolibab K, Smithson SL, Rabquer B, Khuder S, Westerink MA. Immune response to pneumococcal polysaccharides 4 and 14 in elderly and young adults: analysis of the variable heavy chain repertoire. Infect Immun. 2005 Nov;73(11):7465-76.
- Krabbe K., Pedersen M., Bruunsgaard H.: Inflammatory mediators in the elderly. Exp Gerontol 39, 687-699 (2004)
- Krawinkel MB: Interaction of nutrition and infections globally:an overview. Ann Nutr Metab 2012, 61(Suppl 1):39-45.
- Kunkel, E.J., Butcher, E.C., 2003. Plasma-cell homing. Nat. Rev. Immunol. 3, 822-829.
- Kurschus FC, Bruno R, Fellows E, Falk CS, Jenne DE. Membrane receptors are not required to deliver granzyme B during killer cell attack. Blood. 2005 Mar 1;105(5):2049-58.
- Larbi A, Pawelec G, Witkowski JM, Schipper HM, Derhovanessian E, Goldeck D, Fulop T. Dramatic shifts in circulating CD4 but not CD8 T cell subsets in mild Alzheimer's disease. J Alzheimers Dis. 17(1):91-103 (2009)
- Lee JS, Lee WW, Kim SH et al.: Age-associated alteration in naive and memory Th17 cell response in humans. Clin Immunol 2011, 140:84-91.

- Leuner K, Muller WE and Reichert AS (2012). From mitochondrial dysfunction to amyloid beta formation: novel insights into the pathogenesis of Alzheimer's disease. Mol Neurobiol, 46: 186-193
- Li L, Hsu HC, Grizzle WE, Stockard CR, Ho KJ, Lott P, et al. Cellular mechanism of thymic involution. Scand J Immunol (2003) 57:410–42.
- Li M, Shang DS, Zhao WD, Tian L, Li B, Fang WG, Zhu L, Man SM, Chen YH. Amyloid beta interaction with receptor for advanced glycation end products up-regulates brain endothelial CCR5 expression and promotes T cells crossing the blood-brain barrier. J Immunol. 2009 May 1;182(9):5778-88.
- Libri V, Azevedo RI, Jackson SE, Di Mitri D, Lachmann R, Fuhrmann S, Vukmanovic-Stejic M, Yong K, Battistini L, Kern F, Soares MV, Akbar AN. Cytomegalovirus infection induces the accumulation of short-lived, multifunctional CD4+CD45RA+CD27+ T cells: the potential involvement of interleukin-7 in this process. Immunology. 2011 Mar;132(3):326-39.
- Licastro F, Candore G, Lio D, Porcellini E, Colonna-Romano G, Franceschi C, Caruso C. Innate immunity and inflammation in ageing: a key for understanding age-related diseases. Immun Ageing. 18;2:8(2005)
- Listì F, Candore G, Modica MA, Russo MA, Di Lorenzo G, Esposito-Pellitteri M, Colonna-Romano G, Aquino A, Bulati M, Lio D, Franceschi C, Caruso C. A study of serum immunoglobulin levels in elderly persons that provides new insights into B cell immonosenescence. Ann N Y Acad Sci. 1089:487-495 (2006)

- Looney RJ, Falsey A, Campbell D, Torres A, Kolassa J, Brower C, McCann R, Menegus M, McCormick K, Frampton M, Hall W, Abraham GN. Role of cytomegalovirus in the T cell changes seen in elderly individuals. Clin Immunol. 1999 Feb;90(2):213-9.
- Lund FE. Cytokine-producing B lymphocytes-key regulators of immunity. Curr Opin Immunol. 20:332-338 (2008)
- Lynch HE, Goldberg GL, Chidgey A, Van den Brink MR, Boyd R, Sempowski GD. Thymic involution and immune reconstitution. Trends Immunol (2009)30:366–73.
- Ma D, Wei Y, Liu F. Regulatory mechanisms of thymus and T cell development. Dev Comp Immunol (2013) 39:91–102.
- Macallan DC, Wallace DL, Zhang Y, Ghattas H, Asquith B, de Lara C, Worth A, Panayiotakopoulos G, Griffin GE, Tough DF, Beverley PC. B-cell kinetics in humans: rapid turnover of peripheral blood memory cells. Blood. 2005 May 1;105(9):3633-40.
- Man SM, Ma YR, Shang DS, Zhao WD, Li B, Guo DW, Fang WG, Zhu L, Chen YH Peripheral T cells overexpress MIP-1alpha to enhance its transendothelial migration in Alzheimer's disease. Neurobiol Aging. 28(4):485-96. (2007)
- Martin F, Chan AC. B cell immunobiology in disease: evolving concepts from the clinic. Annu Rev Immunol. 2006;24:467-96.
- Martin Prince RB, Cleusa Ferri (2011) World Alzheimer Report 2011. Vol. 2013, Alzheimer’s Disease International, London
- Miller JP, Cancro MP. B cells and aging: balancing the homeostatic equation. Exp Gerontol. 2007 May;42(5):396-9. Epub 2007 Feb 4.

- Mitchell WA, Lang PO, Aspinall R. Tracing thymic output in older individuals. Clin ExpImmunol (2010) 161:497–503.
- Monsonego A, Zota V, Karni A, Krieger JI, Bar-Or A, Bitan G, Budson AE, Sperling R, Selkoe DJ, Weiner HL Increased T cell reactivity to amyloid beta protein in older humans and patients with Alzheimers disease. J Clin Invest 112, 415-22 (2003)
- Moore SE, Fulford AJ, Darboe MK, Jobarteh ML, Jarjou LM,Prentice AM: A randomized trial to investigate the effects of pre-natal and infant nutritional supplementation on infant immune development in rural Gambia: the ENID trial: Early Nutrition and Immune Development. BMC Pregnancy Childbirth 2012, 12:107.
- Nasi M, Troiano L, Lugli E, Pinti M, Ferraresi R, Monterastelli E, et al. Thymic output and functionality of the IL-7/IL-7 receptor systemin centenarians: implications for the neo lympho- genesis at the limit of human life. AgingCell (2006) 5:167–75.
- Naylor K, Li G, Vallejo AN, Lee WW, Koetz K, Bryl E, Witkowsky J, Fulbright J, Weyand CM, Goronzy JJ. The influence of age on T cell generation and TCR diversity. J Immunol. (2005) 174:7446-7452
- Nicholson KG, Kent J, Hammersley V, Cancio E. Acute viral infections of upper respiratory tract in elderly people living in the community: comparative, prospective, population based study of disease burden. BMJ. 315:1060-1064 (1997)
- Nociari MM, Telford W, Russo C. Postthymic development of CD28-CD8+ T cell subset: age-associated expansion and shift from memory to naive phenotype. J Immunol. 162(6):3327-3335 (1999)

- Nussbaum RL, Ellis CE.Alzheimer's disease and Parkinson's disease. N Engl J Med. 348(14):1356-64 (2003)
- Ouyang Q, Wagner WM, Voehringer D, Wikby A, Klatt T, Walter S, Müller CA, Pircher H, Pawelec G. Age-associated accumulation of CMV-specific CD8+ T cells expressing the inhibitory killer cell lectin-like receptor G1 (KLRG1). Exp Gerontol. 38(8):911-20 (2003)
- Oviedo-Orta E, Li CK, Rappuoli R.Perspectives on vaccine development for the elderly. Curr Opin Immunol. 2013 Aug;25(4):529-34
- Palanichamy A, Barnard J, Zheng B, Owen T, Quach T, Wei C, Looney RJ, Sanz I, and Anolik H. Novel human Transitional B cell Populations Revealed by B cell Depletion Theraphy. J Immunol. 182:5982-5993 (2009)
- Palmer DB.The effect of age on thymic function. Front. Immunol., 2013 Oct 7;4:316.
- Pan-Hammarström Q, Zhao Y, Hammarström L. Class switch recombination: a comparison between mouse and human. Adv Immunol. 2007;93:1-61.
- Pavlidis N, Stanta G, Audisio RA. Cancer prevalence and mortality in centenarians: a systematic review. Crit Rev Oncol Hematol. 2012 Jul;83(1):145-52
- Pawelec G. Immunosenescence: Role of cytomegalovirus. Exp Gerontol. 2013 Nov 28. doi :10.1016/j.exger.2013.11.010.
- Pawelec G and Larbi A. Immunity and ageing in man: Annual Review 2006/2007. Exp Gerontol. 43:34-38 (2008)

- Pawelec G, Akbar A, Caruso C, Solana R, Grubeck-Loebenstein B, Wikby A. Human immunosenescence: is it infectious? Immunol Rev. 205:257-268 (2005)
- Pawelec G, Barnett Y, Forsey R, Frasca D, Globerson A, McLeod J et al. T cell and ageing. Front Biosci. 7:d1056-d1183 (2002)
- Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nat Rev Endocrinol. 2012 Apr 3;8(8):457-65.
- Pellicanò M, Bulati M, Buffa S, Barbagallo M, Di Prima A, Misiano G, Picone P, Di Carlo M, Nuzzo D, Candore G, Vasto S, Lio D, Caruso C, Colonna-Romano G. Systemic immune responses in Alzheimer's disease: *in vitro* mononuclear cell activation and cytokine production. J Alzheimers Dis. 21(1):181-192 (2010)
- Pellicanò M, Larbi A, Goldeck D, Colonna-Romano G, Buffa S, Bulati M, Rubino G, Iemolo F, Candore G, Caruso C, Derhovanessian E, Pawelec G. Immune profiling of Alzheimer patients. J Neuroimmunol. in press (2011)
- Priller C, Bauer T, Mitteregger G, Krebs B, Kretzschmar HA, Herms J. Synapse formation and function is modulated by the amyloid precursor protein.J Neurosci. 26(27):7212-21(2006)
- Rattan SI. Increased molecular damage and heterogeneity as the basis of aging. Biol Chem. 2008 Mar;389(3):267-72.
- Reale M, Iarlori C, Feliciani C, Gambi D.Peripheral chemokine receptors, their ligands, cytokines and Alzheimer's disease. J Alzheimers Dis. 14(2):147-59 (2008)

- Reiley WW, Wittmer ST, Ryan LM, Eaton SM, Haynes L, Winslow GM, et al. Maintenance of peripheral T cell responses during Mycobacterium tuberculosisinfection. J Immunol (2012) 189:4451–8.
- Richartz-Salzburger E, Batra A, Stransky E, Laske C, Köhler N, Bartels M, Buchkremer G, Schott K. Altered lymphocyte distribution in Alzheimer's disease. J Psychiatr Res. 41(1-2):174-8 (2007)
- Rieger A, Bar-Or A. B-cell-derived interleukin-10 in autoimmune disease: regulating the regulators. Nat Rev Immunol. 2008 Jun;8(6):486-7.
- Rodier F, Campisi J. Four faces of cellular senescence. J Cell Biol. 2011 Feb 21;192(4):547-56.
- Rogers J, Luber-Narod J, Styren SD, Civin WH Expression of immune system associated antigens by cells of the human central nervous system: Relationship to the pathology of Alzheimer’s disease. Neurobiol Aging 9, 339-349(1998)
- Roman BE, Beli E, Duriancik DM, Gardner EM: Short-term supplementation with active hexose correlated compound improves the antibody response to influenza B vaccine. Nutr Res 2013, 33:12-17.
- Rossi DJ, Bryder D, Zahn JM, Ahlenius H, Sonu R, Wagers AJ, Weissman IL. Cell intrinsic alterations underlie hematopoietic stem cell aging. Proc Natl Acad Sci U S A. 2005 Jun 28;102(26):9194-9. Epub 2005 Jun 20.
- Rossi MI, Yokota T, Medina KL, Garrett KP, Comp PC, Schipul Jr AH, Kincade PW. B lymphopoiesis is active throughout human life,

but there are developmental age-related chances. Blood. 101(2):576-584 (2003)

- Roush W. Live long and prosper? Science 273(5271):42-6 (1996)
- Salvioli S, Monti D, Lanzarini C, Conte M, Pirazzini C, Bacalini MG, Garagnani P, Giuliani C, Fontanesi E, Ostan R, Bucci L, Sevini F, Yani SL, 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. Immune system, cell senescence, aging and longevity--inflamm-aging reappraised. Curr Pharm Des. 2013;19(9):1675-9.
- Salvioli S, Olivieri F, Marchegiani F, Cardelli M, Santoro A, Bellavista E, Mishto M, Invidia L, Capri M, Valensin S, Sevini F, Cevenini E, Celani L, Lescai F, Gonos E, Caruso C, Paolisso G, De Benedictis G, Monti D, Franceschi C. Genes, ageing and longevity in humans: problems, advantages and perspectives. Free Radic Res. 2006 Dec;40(12):1303-23.
- Sansone P, Storci G, Tavolari S, Guarnieri T, Giovannini C, Taffurelli M,Ceccarelli C, Santini D, Paterini P, Marcu KB, Chieco P, Bonafè M. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. J Clin Invest. 2007 Dec;117(12):3988-4002. PubMed PMID: 18060036;
- Sanz I, Anolik JH, Looney RJ. B cell depletion therapy in autoimmune diseases. Front Biosci. 12:2546-2567 (2007)
- Sanz I, Wei C, Lee FE, Anolik J. Phenotypic and functional heterogeneity of human memory B cells. Semin Immunol. 20(1):67-82 (2008)

- Sauce D, Appay V. Altered thymic activity in early life: how does it affect the immune system in young adults? Curr Opin Immunol (2011) 23:543–8.
- Saule P, Trauet J, Dutriez V, Lekeux V, Dessaint JP, Labalette M. Accumulation of memory T cells from childhood to old age: central and effector memory cells in CD4(+) versus effector memory and terminally differentiated memory cells in CD8(+) compartment. Mech Ageing Dev. 2006 Mar;127(3):274-81.
- Saurwein-Teissl M, Blasko I, Zisterer K, Neuman B, Lang B, Grubeck-Loebenstein B. An imbalance between pro- and anti-inflammatory cytokines, a characteristic feature of old age. Cytokine. 2000 Jul;12(7):1160-1.
- Schmaltz HN, Fried LP, Xue QL, Walston J, Leng SX, Semba RD. Chronic cytomegalovirus infection and inflammation are associated with prevalent frailty in community-dwelling older women. J Am Geriatr Soc. 53:747-754 (2005)
- Scholz JL, Diaz A, Riley RL, Cancro MP, Frasca D. A comparative review of aging and B cell function in mice and humans. Curr Opin Immunol. 2013 Aug;25(4):504-10.
- Schutyser, E., Struyf, S., Van Damme, J., 2003. The CC chemokine CCL20 and its receptor CCR6. Cytokine Growth Factor. Rev. 14(5), 409-426.
- Selkoe DJ.Alzheimer's disease results from the cerebral accumulation and cytotoxicity of amyloid beta-protein.J Alzheimers Dis. 3(1):75-80 (2001)

- Shi Y, Yamazaki T, Okubo Y, Uehara Y, Sugane K, Agematsu K. Regulation of aged humoral immune defense against pneumococcal bacteria by IgM memory B cell. J Immunol. 175:3262-3267 (2005)
- Shi Y., Wei C., Lee F., Anolik J.: Functional analysis of human memory B-cell subpopulations: IgD+CD27+ B cells are crucial in secondary immune response by producing high affinity IgM. Clinical Immunol 108, 128-137 (2003)
- Shurin GV, Yurkovetsky ZR, Chatta GS, Tourkova IL, Shurin MR, Lokshin AE. Dynamic alteration of soluble serum biomarkers in healthy aging. Cytokine. 39(2):123-9 (2007)
- Simone R, Zicca A, Saverino D: The frequency of regulatory CD3+CD8+CD28SCD25+ T lymphocytes in human peripheral blood increases with age. J Leuk Biol 2008, 84:1454-1461.
- Singh T, Newman AB. Inflammatory markers in population studies of aging. Ageing Res Rev. 2011
- Speciale L, Calabrese E, Saresella M, Tinelli C, Mariani C, Sanvito L, Longhi R, Ferrante P Lymphocyte subset patterns and cytokine production in Alzheimer's disease patients. Neurobiol Aging 28, 1163-1169 (2007)
- Studebaker AW, Storci G, Werbeck JL, Sansone P, Sasser AK, Tavolari S, Huang T, Chan MW, Marini FC, Rosol TJ, Bonafé M, Hall BM. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. Cancer Res. 2008 Nov 1;68(21):9087-95. beyer

- Sultana R, Baglioni M, Cecchetti R, Cai J, Klein JB, Bastiani P, Ruggiero C, Mecocci P, Butterfield DA. Lymphocyte mitochondria: toward identification of peripheral biomarkers in the progression of Alzheimer disease. Free Radic Biol Med. 2013 Dec;65:595-606.
- Taub DD, Longo DL. Insights into thymic aging and regeneration. Immunol Rev(2005) 205:72–93.
- Terry DF, Wyszynski DF, Nolan VG, et al. Serum heat shock protein 70 level as a biomarker of exceptional longevity. Mech Ageing Dev 2006; 127:862 8.
- Terry DF, Wilcox MA, McCormix MA, Pennington JY, Schoenhofen EA, Andersen SL et al. Cardiovascular advantages among the offspring of centenarians. J Am Geriatr Soc. 52:2074-2076 (2004b)
- Terry DF, Wilcox MA, McCormix MA, Perls TT. Cardiovascular disease delay in centenarian offspring. J Gerontol A Biol Sci Med Sci. 59:385-389 (2004a)
- Terry DF, Wilcox M, McCormick MA, Lawler E, Perls TT.Cardiovascular advantages among the offspring of centenarians.J Gerontol A Biol Sci Med Sci. 2003 May;58(5):M425-31.
- Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, Fukuda K. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA. 2003 Jan 8;289(2):179-86.
- Trans. 2003 Apr;31(2):457-61. Review. PubMed PMID: 12653662.
- Troen B.R. The biology of aging Mt Sinai J Med. 70(1):3-22 (2003)

- Trzonkowski P., My´sliwska J., Godlewska B.et al., “Immune consequences of the spontaneous pro-inflammatory status in depressed elderly patients,” Brain, Behavior, and Immunity, vol. 18, no. 2, pp. 135–148, 2004.
- Vasto S, Candore G, Listì F, Balistreri CR, Colonna-Romano G, Malavolta M, Lio D, Nuzzo D, Mocchegiani E, Di Bona D, Caruso C. Inflammation, genes and zinc in Alzheimer’s disease. Brain Res Rev. 58:96-105 (2008)
- Vasto S., Candore G., Balistreri CR, Caruso M., Colonna-Romano G., Grimaldi MP: Inflammatory networks in ageing, age-related diseases and longevity. Mech Aging Dev 128, 83-91 (2007)
- Veneri D, Ortolani R, Franchini M, Tridente G, Pizzolo G, Vella A. Expression of CD27 and CD23 on peripheral blood b lymphocytes in humans of different ages. Blood Transfus. 7:29-34 (2009)
- Vescovini R, Biasini C, Telera AR, Basaglia M, Stella A, Magalini F, Bucci L, Monti D, Lazzarotto T, Dal Monte P, Pedrazzoni M, Medici MC, Chezzi C, Franceschi C, Fagnoni FF, Sansoni P. Intense antiextracellular adaptive immune response to human ytomegalovirus in very old subjects with impaired health and cognitive and functional status. J Immunol. 2010 Mar 15;184(6):3242-9.
- Wang GC, Kao WH, Murakami P, Xue QL, Chiou RB, Detrick B, McDyer JF, Semba RD, Casolaro V, Walston JD, Fried LP. Cytomegalovirus infection and the risk of mortality and frailty in older women: a prospective observational cohort study. Am J Epidemiol. 171:1144-1152 (2010)

- Warren LA, Rossi DJ. Stem cells and aging in the hematopoietic system. Mech Ageing Dev. 130(1-2):46-53 (2009)
- Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, Lee EH, Milner EC, Sanz I. 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 (2007)
- Wei C, Jung J, Sanz I. OMIP-003: phenotypic analysis of human memory B cells. Cytometry A. 2011 Nov;79(11):894-6.
- Weiner HL, Frenkel D. Immunology and immunotherapy of Alzheimer's disease. NatRev Immunol. 2006 May;6(5):404-16. Review. Erratum in: Nat Rev Immunol. 2006 Jun;6(6):490.
- 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.
- Weng NP, Akbar AN, Goronzy J: CD28(S) T cells: their role in the age-associated decline of immune function. Trends Immunol 2009, 30:306-312.
- Weng NP. Telomere and adaptive immunity. Mech Ageing Dev. 129(1-2):60-6 (2008)
- Wherry EJ, Teichgraber V, Becker TC, Masopust D, Kaech SM, Antia R, von Andrian UH, Ahmed R. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol 4:225-234 (2003)

- Wick G, Jansen-Durr P, Berger P, Blasko I, Grubeck-Loebenstein B. Diseases of aging. Vaccine. 18:1567-1583 (2000)
- Wikby A, Johansson B, Ferguson F, Olsson J. Age-related changes in immune parameters in a very old population of Swedish people: a longitudinal study. Exp Gerontol. 1994 Sep-Oct;29(5):531-41.
- Wikby A, Johansson B, Olsson J, Löfgren S, Nilsson BO, Ferguson F. Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with Cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. Exp Gerontol. 2002 Jan-Mar;37(2-3):445-53.
- William J, Euler C, Christensen S, Shlomchik MJ. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. Science. 297:2066-2070 (2002)
- 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.

Curriculum Vitae

CURRICULUM VITAE

Adriana Martorana was born on 8th June 1982 in Palermo, Italy.

In 2000 she got a socio-psycho-pedagogy Diploma.

In 2009 she graduated in Biotechnology for industrial and scientific research at the University of Palermo.

In 2009 she qualified also as a professional biologist.

In January 2011, she started her doctorate under the supervision of Prof. G. Colonna Romano purchasing advanced specialized studies in Immunology with particular attention on mechanisms of aging.