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sempre al mio fianco

A Veronica, compagna di un lungo
viaggio chiamato "vita"

Ad Asia ed alle infinite giornate di
studio in sua compagnia

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CHAPTER 1

Introduction

The features of aging

Aging is a multidimensional process that involves physical, psychological and social changes. In humans, aging has been viewed as the declining function of most body systems as the result either of the progressive accumulation of damaged tissue and substances or the progressive loss of normal tissue and substances either by intrinsic or extrinsic mechanisms, already after 30 years old. This phenomenon is gradual and progressive, although variable for each individual. Elderly people are characterized by the impairment of homeostatic processes, that leads to an increased susceptibility and vulnerability to stressing events and diseases. The less homeostatic ability depends of:

1. Reduced functional reserve of each organ and system by aging (intrinsic aging process), living place and lifestyle (extrinsic aging process);

2. Functional loss of major integration systems (nervous, endocrine and immune system) which play a coordination role of homeostatic functions.

A major age-related health issue is the increasing prevalence and severity of infectious diseases, which are the fourth most common cause of death in the elderly (Heron

MP et al., 2007). Indeed, aging is associated with an increased incidence of infections such as viral influenza, respiratory syncitial virus (RSV) and pneumococcal pneumonia (Nicholson KG et al., 1997). Elderly people also have an increased incidence of bacterial infection in lungs, urinary tract, skin and other tissues and a higher incidence of tuberculosis and herpes zoster reactivation (Ginaldi L et al., 2001; Bulati M et al., 2011). Vaccination has only limited success since aging is also associated with poor protective antibody response to vaccines, indeed, it has been demonstrated that the currently vaccines protect only a small proportion of the elderly population (Weinberger B et al., 2008).

The increase in the elderly population is an important phenomenon in our society strictly dependent on health care progress, antibiotics vaccination and improving life standards caused a dramatic increase in lifespan and a consequent reduction of the overall morbidity and mortality (Troen BR, 2003). By 2050, almost 40% of the European and US population is predicted to be older than 60 years old (Lutz W. et al., 1997). Nonetheless, the extreme limit of life is not changed.

The immune system is a dynamic system that is highly dependent on the regenerative ability of hematopoietic

precursor cells that is constantly challenged by external and internal forces threatening the homeostasis of the system. It is not surprising that the immune system undergoes dramatic changes with age. These changes occur in all leukocytes and accordingly, affect innate and adaptive immune functions. Innate immune responses are a first step toward the development of adaptive immune responses, and age-related deficits in innate immune functions might therefore alter both cell-mediated and humoral adaptive immune reactions (Mills KHG, 2009). The progressive and cumulative modifications of immune system over the lifespan, known as "Immunosenescence" (Franceschi C et al., 1995; Pawelec G et al., 2005), have a major impact on the capacity to respond to immune challenge. Several years ago, Franceschi C et al. proposed the "remodelling theory of ageing" according to which, during aging, clonotypical immunity deteriorates, while ancestral innate/natural immunity is largely conserved or even up-regulated with age. The peculiar remodeling of immunosenescence, probably, expresses the balance between chronic antigenic stress faced by the organism, and its intrinsic ability to respond to it as well as other stressor (e.g. free radicals). In a recent paper, it was shown as some functions are impaired while others remain unchanged or even increased (Bürkel A et al., 2007). However,

Immunosenescence is primarily responsible for the diminished ability of older individuals to overcome infections (Globerson A and Effros RB, 2000). Indeed, it was demonstrated that, although elderly people are able to mount anamnestic responses, don't show good ability to establish a primary type response against new antigens (Fagnoni FF et al., 2000).

A typical feature of aging is a chronic, low-grade pro-inflammatory status observed in the old people that, is potentially linked to the most important age-related diseases. So, aging increases risk of disabilities and chronic diseases, such as cardiovascular diseases, arthrosis, arthritis, neurodegeneration, cancer, atherosclerosis, metabolic syndrome, osteoporosis, sarcopenia and frailty (Gao HM and Hong HS, 2008; Ginaldi L et al., 2005; Florez H et al., 2006; Rajala MV et al., 2003). In this context, elderly people are characterized by a general increase in the production of pro-inflammatory cytokines and inflammatory markers (Cevenini E et al., 2010). CRP and fibrinogen, the major clinical markers of inflammation, have been associated with coronary disease, myocardial ischemia and myocardial infarction, in association with IL-1, IL-1ra, IL-6, IL-6ra, IL-18, TNF-α , serum amyloid A and ICAM-1 (Chung HY et al., 2006; Van Den Biggelaar AH et al., 2004; Ferrucci L et

al., 2005). Some investigators identified an age-related increase of IL-6 and TNF-α, defining them as markers of functional disability and as predictors of disability and mortality among the elderly (Bruunsgaard H et al., 2003a, b, c). Moreover, IL-6, defined as "cytokine for gerontologists" (Krabbe KS et al., 2004; Fulop T et al., 2006), may negatively impact hematopoiesis either by inhibition of erythropoietin production or interaction with erythropoietin receptors (Eisenstaedt R et al., 2006). In contrast, TNF-α has been found to induce production of the Alzheimer Amyloid beta peptide, increase its toxicity and upregulate smooth muscle cell proliferation during atherosclerosis development (Wick G et al., 2000; Saurwin-Teissl M et al., 2000). Another group demonstrated an increase in serum interferon-γ-inducible chemokines (MIG and IP-10), eotaxin, a chemoattractant for eosinophils, and TNFR-II with the advanced age. On the other hand, serum levels of EGFR and EGF, important regulators of cell growth, proliferation and differentiation, decreased with aging (Shurin GV et al., 2007). The consequence of this situation is that age-dependent up-regulation of the inflammatory response, termed "Inflamm-aging" (inflammation and aging) (Franceschi C et al., 2000), render elderly prone to frailty (Balistreri CR et al., 2008; Franceschi C et al., 2005; Lio D

et al., 2004; Pes et al., 2004). Anyway, the inflamm-aging theory fails in separating true physiological aging and true pathological aging during natural aging process (Goto M, 2008). The concept of inflamm-aging coincides with antagonistic pleiotropy theory of aging postulating that aging is the late deleterious effect of genes (pro- and anti-inflammatory), that are beneficial at an earlier stage of life for the development and maintenance of body integrity against infectious agents and stressors (Goto M, 2008). The physiological aging process and many age-related diseases may due to the continuous exposure to stressors, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), that activate NFκB, which has a central position in the inflammatory reaction, controlling expression of different inflammatory and oncogenic genes (Chung HY et al., 2006; Dinarello CA, 2006). Several studies have shown the strong link between inflammation and chronic infections with the initiation and progression of atherosclerosis. Exposition to Clamydia Pneumoniae and Helicobacter Pilory have been associated to atherosclerotic tissues in humans, suggesting like these microorganisms may act stimulating the vessel-associated leukocytes or inducing the transformation of vascular muscles or vascular endothelial cells (Libby P et al., 1997). Latent cytomegalovirus (CMV)

infection appears to accelerate immune aging (Pawelec G et al., 2005; Akbar AN and Fletcher JM, 2005). The relationship between chronic CMV infection and adverse health outcomes was evaluated in the Swedish OCTO/NONA studies, in which anti-CMV IgG seropositivity was included as one of the very important immunological parameters defining an immunological risk profile (IRP) predicting 2, 4 and 6 years of mortality (Pawelec G et al., 2005). IRP was defined by a cluster of immunological parameters: inverted CD4:CD8 ratio, poor T cell proliferative response to mitogens, low interleukin-2 production, accumulation of late-stage differentiated CD8+ T cells, decreased B cell count. In addition, other studies reported associations between CMV seropositivity or anti-CMV IgG titres and higher levels of IL-6 as predictor of incident frailty and mortality (Schmaltz HN et al., 2005; Wang GC et al., 2010). Moreover, detectable CMV DNA rather than anti-CMV IgG titres was associated with high serum levels of neopterin, an immune activation marker produced by monocytes and macrophages, which, in turn, is associated with frailty in the elderly (Leng SX et al., 2011a, b). CMV is considered a cofactor that enhances progression to AIDS (Griffiths PD, 2006) and infection with this herpes virus, has been associated with poor clinical response to

influenza vaccination in the elderly (Trzonkowski P et al., 2003). Increased levels of inflammatory mediators are associated with dementia, like Parkinson's and Alzheimer's disease. Inflammation clearly occurs in pathologically vulnerable regions of the Alzheimer's disease (AD) brain, where damaged neurons and neurites and highly insoluble amyloid beta peptide deposits and neurofibrillary tangles provide obvious stimuli for inflammation (Akiyama H et al., 2000).

Modification of the immune system during aging

The thymus is the primary lymphoid organ where T cells mature; it undergoes dramatic structural changes with age as thymopoietic niches disappear, while the thymic perivascular space increases. These changes are associated with a loss of thymic epithelial cells and thymocytes and with the predominance of adipocytes that infiltrate the perivascular space (Dixit VD, 2010). These age-related modifications result in a gradually and progressive thymus

output abatement; the thymus activity becomes insufficient to replace the naïve T cells lost in the periphery and to maintain the size of the T cell repertoire (Kohler S et al., 2005; Naylor K et al., 2005). The loss of naïve T cells is due to cumulative exposures to foreign pathogens and environmental antigens that promotes the accumulation of memory and effector T cells with age (Saule P et al., 2006). One of the most evident change in the memory T cell population during aging is the clonally expansion of CD8+CD28- T cells. These appear as senescent cells as they have short telomeres, produce Tumor Necrosis Factor α (TNF- α), are resistent to apoptosis and show reduced proliferative capacity (Pawelec G et al., 2008; Globerson A and Effros RB, 2000). During aging, T cells lose the ability to produce and respond to IL-2 resulting in a great impairment in T cell function (Desai A et al., 2010). Many authors have associated the reduced expression of IL-2 by human T lymphocytes, with the defective activation of transcription factors AP-1 and NF-AT during aging with (Haynes I et al., 1999; Nagel JE et al., 1988; Whisler RL et al., 1996).

The percentage and the absolute number of circulating CD3+ T lymphocytes and of CD4+ and CD8+ T cell subsets decreases with age (Pawelec G et al., 2002; Cossarizza A et

al., 1996). The natural reduction of naïve T cells, coupled with the narrowing of the T cell repertoire, has profound consequences for immune function, rendering elderly people less responsive to immune stimulation and vaccination, as well as predisposing them to cancer (Hakim FT, Gress RE, 2007). Memory and naïve human T cells have been distinguished for long time on the basis of CD45 family members on their surface. Indeed, CD45RA antigen is expressed primarily on naïve T lymphocytes, while CD45RO is present on the surface of memory T cells. Upon contact with antigen, some activated T lymphocytes become effector after asymmetric division, while others form clones of memory cells. Both subpopulations are CD45RO+, but phenotypes apparently "naïve" (CD45RA+) were also found among the memory T cell pool. Indeed, CD45RA re-expression by late-differentiated memory T lymphocytes, makes this marker not fully useful to discriminate between naïve and memory cells (Hamann D et al., 1997). Hence, the need to use more T cell surface markers for a better classification, including: CD28/CTLA4, CD27, CD62L, CD95, CD95L, CD7, CD11a, CD103, CCR7. Naïve T lymphocytes are characterized by the absence of CD95 and by the presence of CD45RA, CD62L, CD27 and CD28 (Gupta S et al., 2005). Moreover, memory T cells also

exhibit adhesion molecules, like CD62L, and the chemokine receptor CCR7, that allow them to adhere to the endothelial wall, migrate and pass through vessels to reach the peripheral sites. The deep characterization of memory T lymphocytes has revealed the existence of distinct populations of memory: the central memory (TCM) and effector memory (TEM) cells characterized by distinct homing capacity and effector function. The former, identified as CD45RA-CCR7+CD62L+, principally located in the lymph nodes, is a population of memory T cell less differentiated, (Nociari MM, Telford W, Russo C, 1999); the latter, CD45RA-CCR7-CD62L-, is a highly mature T cell population located in extranodal tissues and mucous membranes; it has been shown to be responsible for the tissue damage which characterizes many autoimmune diseases, as itcan rich sites of inflammation in non-lymphoid tissues, while not participating in the process of lymphoid recirculation carried out by most other lymphocytes. Once divided into TCM and TEM, according to the expression of CCR7 and CD62L, these populations have been further classified by the presence/absence of the costimulatory molecules CD27 and CD28 (Gupta S et al., 2004). Some "reverted" memory T cells express again CD45RA, so they become terminally differentiated and functionally anergic

(TEMRA), with a characteristic phenotype: CD45RA+CCR7-CD28-CD27-CD62L- (Gupta S et al., 2005). Another classification is based on the expression of two inhibitory receptors expressed by late-stage differentiated cells, CD57 and KLRG1, sometimes referred to as markers of "senescence". CD57 is expressed on the surface of NK cells and late-stage CD8+ T cells, while on CD4+ its expression is reduced. On the other hand, KLRG1 is largely expressed on CD4+ as well as CD8+ T lymphocytes and NK cells (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. So, 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 antigens stimuli (Goto M, 2008) . Moreover the cytokines and chemokines play a central role in the generation of the "inflammed" milieu, typical of ageing. Ageing is also associated with a shift from the Th1 to the Th2 cytokine profile in response to immune stimulation. The overproduction of Th2 cytokines could increase B cell mediated autoimmune disorders by enhancing the production of autoreactive antibodies. In recent years, an important role of CD4+CD25+Fox3+ regulatory T cells (Treg) in the maintenance of immune homeostasis has been described (Wing K et al., 2005; Mills KHG, 2009). Aged individuals show an increase of Treg cells, but the lack of IL-7 receptor (CD127) expression on the surface of these cells results in their functional impairment. It has been suggested that a decrease in Treg cell numbers or function could result in autoimmune diseases or rejection of a transplant, while an excess of Treg lymphocytes might contribute to poor responses to infectious diseases, vaccines and cancer (Rouse BT et al., 2006).

Most researchers have studied the impairment of cell-mediated immune response during aging, although B cell function is also modified in the elderly (Cancro MP et al., 2009; Frasca D, Blomberg BB, 2011; Buffa S et al., 2011). Moreover, the humoral immune response of elderly people is

qualitatively and quantitatively diminished when compared with the immune response of young people. Age-associated changes in the composition of peripheral B cells reflect both increased B cell longevity and decreased B cell generation (Kline GH et al., 1999). Many authors have described a decrease both in percentage and absolute number of total CD19+ B lymphocytes (Paganelli R et al., 1992; Colonna Romano G et al., 2003; Shi Y et al., 2005; Frasca D et al., 2008; Faria AM et al., 2008; Veneri D et al., 2009). In addition, defects in hematopoietic stem cells (HSC), that influence B lymphopoiesis, have been described in aging. These defects consist in: failure in telomere maintenance (Warren LA et al., 2009; Weng NP, 2008), epigenetic modifications (Issa JP, 2003), decrease in lymphopoiesis and contemporary increase in myelopoiesis process (Kim M et al., 2003; Cho RH et al., 2008), altered development in bone marrow (Mehr R et al., 2003), shrinkage of B cell repertoire (Guerretaz LM et al., 2008). Indeed, it has been demonstrated an age-related reduction of the absolute number of B cell precursors in the bone marrow (BM) (Kline GH et al., 1999); however, B lymphopoiesis persists throughout adult life (Rossi MI et al., 2003). Reduced B cell generation in aged BM is also due to decreased IL-17 production (Tsuboi I et al., 2004) and impaired V-DJ

rearrangement (Gibson KL et al., 2009). In aged people, it has been described a change in the B cell repertoire, particularly in the heavy chain of BCR. In elderly people, the extended survival of memory B cells and the clonal expansion contribute to the limited repertoire and to the collapse in B cell diversity that are correlated with the impairment of antibody response and poor health status (Miller JP, Cancro MP, 2007; Kumar R et al., 2008; Gibson KL et al., 2009; Dunn-Walters DK et al., 2010; Ademokun A et al., 2011). Moreover, the amount of the different Ig isotypes changes with age: in fact, although the number of B cells is decreased, the serum concentration of IgG, IgA and, to a lesser extent, IgE is increased. On the contrary, both IgM and IgD are decreased (Listì F et al., 2006; Frasca D et al., 2008). So the immunoglobulins produced during an anamnestic response are preserved, whereas the Igs typically produced by naïve B cells are reduced.

Naïve B cells express surface IgD, whereas "classical" memory B cells carry switched Igs (IgG, IgA and IgE). On the other side memory B cells are characterized by the presence of somatic hypermutation in their Ig variable gene sequences (Klein U et al., 1998) and most of them are CD27+, so, CD27 has been considered the typical marker of

memory B cells (Agematsu K et al., 2000) and IgD the typical marker of naïve B cells.

Nevertheless, the description of an IgD+ subset expressing somatically mutated Ig genes failed to use this marker to unequivocally distinguish between naïve and memory B lymphocytes (Klein U et al., 1998).

On these basis the combination of IgD and CD27 surface markers, allowed to characterize three distinguished B cell subpopulations :

1) IgD+CD27- Naïve B cells
2) IgD+CD27+ Unswitched memory B cells
3) IgD- (IgG+ or IgA+)CD27+ Switched memory B cells

By using these two markers it has been demonstrated that naïve B cells are significantly reduced in the elderly (Gupta S et al., 2005; Colonna-Romano G et al., 2008), although other authors have described the increase of naïve B cells in the elderly (Shi Y et al., 2005; Frasca D et al., 2008). Moreover, Frasca D et al. (2008) have report no changes of the absolute number of these cells in the old people.

During last years, many authors have demonstrated the presence in the blood of a memory B cell subset that lack

CD27 (Anolik JA et al., 2004; Fecteau JF et al., 2006; Wei C et a., 2007; Colonna-Romano G et al., 2009; Cagigi A et al., 2009). The expansion of this IgD-CD27- (DN) memory B cell population has been demonstrated in healthy elderly (Colonna-Romano G et al., 2009), in active Lupus patients (Wei C et al., 2007), in healthy subjects challenged with respiratory syncitial virus (RSV) (Sanz I et al., 2008) and in HIV patients (Cagigi A et al., 2009). So, this phenomenon is probably the consequence of a persistent stimulation of the immune system. So, these data suggest that also CD27 cannot be used as an unequivocal marker useful to distinguish naïve from memory B cells. The complexity of the memory B cell pool and the discrediting of the "dogma": IgD and IgM=naïve, switched Igs=memory, has been further demonstrated by the evidence of a IgM memory B cell population identified as IgM+IgD+CD27+ and of the "IgM only" memory B cells identified as IgD-IgM+CD27+.

It was suggested that IgM memory B cells are generated in the spleen and control *S. Pneumoniae* infections (Kruetzmann S et al., 2003; Weller S et al., 2004; Shi Y et al., 2005). Moreover, the decline of IgM memory B cell pool in the elderly, could be involved in defective immune

responses against infections by encapsulated bacteria (Shi Y et al., 2005; Buffa S et al., 2011).

Recently, two developmentally regulated markers, CD38 and CD24, were used for a better discrimination between naïve and memory B subpopulations. In this way, in the human B cell compartment, two naïve and six memory B cell subsets were defined (Fecteau JF et al., 2006; Palanichamy A et al., 2009; Blair PA et al., 2010; Berkowska AM et al., 2011):

1) CD38hiCD24hi Transitional B cells
2) IgM+CD27- Naïve mature
3) IgD+IgM+CD27+ Natural effector or IgM memory
4) IgD-IgM+CD27+ IgM-only
5) IgG+/IgA+CD27+ "Classical" Switched memory
6) IgD- IgG+/IgA+CD27- DN B cells

Human peripheral blood CD19+CD38hiCD24hi B cells, identified as immature transitional B cells have regulatory capacity and they are also known as Breg (Blair PA et al., 2010). Indeed, these cells suppress the differentiation of T helper 1 cells, in part via the provision of interleukin-10 (IL-10). These cells are expanded in different patients suffering of lymphoma and autoimmune diseases, such as SLE and rheumatoid arthritis (RA) (Palanichamy A et al.,

2009; Blair PA et al., 2010). Blair et al (2010) has observed also 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.

In the DN (IgG+IgD-CD27-) B population of elderly people, the rate of mutations evaluated in the V region of IgG genes is dramatically reduced when compared with young (Buffa S et al., 2011). 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).

In literature, naïve and memory B cells have been distinguished also by producing different pro- and anti-inflammatory cytokines. Moreover, naïve B cells produce principally 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). On this regard, 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.

Successful Aging

The increase in life expectancy was the most characteristic demographic process during the second half of the last century. Improved child survival, reduced mortality rates and decreasing fertility rates worldwide, is resulting in a rapid aging of the world's population, particularly marked in all the industrialized countries. These result in modifications in the types of existing elderly groups, with considerable socio-economic implications, which are difficult to evaluate, and which will certainly influence the evolution of the human society (Golini A, 1997). The increase of the human lifespan is not simply due to the improved economic and cultural conditions and social/health cares, but also to the interaction of these new conditions with the genetic variability present in human populations. The term ''successful aging'' has been widely used since 1961, when it appeared in the first issue of the journal ''The Gerontologist'' (Havighurst, 1961). In 1998, Rowe & Kahn defined the term successful aging as "avoidance of disease

and disability, maintenance of high physical and cognitive function and sustained engagement in social and productive activities".

Centenarians may represent the prototypes of successful aging, indeed, they have escaped major age-related diseases, reaching the extreme limit of human life in good clinical condition. In most cases, histories of centenarians reveal them to be free of cancer, dementia, diabetes and cardiovascular diseases. The interest in centenarians as a model for healthy aging is driven by the desire to identify key factors associated with exceptional longevity in humans. Centenarians are equipped with well-preserved and efficient immunological defense mechanisms, and optimal combinations of an appropriate lifestyle and genetic background. Looking at immune system, some parameters, such as NK cell number and activity, were remarkably conserved in centenarians and quite similar to those found in young people: NK cells are so considered as "markers of successful aging". On the other hand centenarians show a decrease of B lymphocytes and naïve T cells, a progressive increase of CD28-cytotoxic T cells and the accumulation of expanded clones of memory T cells, a shrinkage of the T cell repertoire as not successfully aged people. An age-related increase in adhesion molecules on the lymphocytes surface

and a complex reshaping of the cytokine network was also demonstrated in centenarians (Cossarizza A et al., 1996). Unexpectedly, centenarians have high plasma level of IL-6 that, in combination with CMV positivity, significantly increased the odds ratio for frailty (Schmaltz HN et al., 2005). The inflammation is not per se a negative phenomenon: it is the response of the immune system to the aggression of viruses or bacteria. It is an emergent evidence that polymorphic alleles of inflammatory cytokines, involved in high cytokine production, are related to unsuccessful aging as atherosclerosis and Alzheimer's disease; on the contrary, the control of the inflammatory status may allow to us better attain successful aging (Candore G et al., 2006). It must be mentioned that Centenarian studies opened the debate on the choose of "the right control" to be used in the experiments.

Certainly , unlike their parents, centenarian offspring (CO) have an appropriate control group, i.e. age-matched healthy elderly, who haven't a familiar history of longevity. Centenarian offspring, like their centenarian parents, have genetic and functional advantages that predispose them to healthy aging and longer survival. Some evidences suggest that these longevity advantages are associated with lower risk of cardiovascular disease, stroke, myocardial infarction,

and diabetes mellitus. The prevalence of other age-related diseases, like cancer, dementia, osteoporosis, Parkinson's disease are similar between CO and their controls (Terry DF et al., 2004 a,b).

Focusing on B cell branch of the immune system, centenarian offspring don't show the typical naïve/memory shift observed in elderly. Though a decreased B cell count was observed both in CO and in their age-matched controls, in the former, naïve B cells (IgD+CD27-) were more abundant whereas exhausted memory cells (IgD-CD27-) were significantly decreased, looking similar to the young (Colonna-Romano et al., 2010). It is well documented that the quality and the size of the humoral immune response declines with age (Frasca D et al., 2005; Cancro MP, 2007; Kumar R et al., 2008; Gibson KL et al., 2009; Dunn-Walters DK et al., 2010; Bulati M et al., 2011). This change is characterized 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 hypothesis that centenarian offspring have a good bone marrow cell reservoir that give them a big 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.

An example of unsuccessful aging: Alzheimer's disease

Alzheimer'disease (AD), the most common form of dementia among elderly people, is one of the major example of unsuccessful aging. Initially, AD manifests itself as a progressive dementia characterized by amnesia and other cognitive deficits. As the disease progresses, people affected show defective instrumental functions mediated by the association cortex and they may therefore present aphasia, apraxia, up to present interneurological disorders. Anyway, diagnosis can be made with firmness only upon brain autopsy. Pathological changes in the AD brain, observed by post-mortem analysis include neurological loss, extracellular amyloid plaques, due to the deposit of amyloid beta peptide, and intracellular neurofibrillary tangles, consisting of phosphorylated tau-proteins. APP molecule, amyloid beta peptide precursor, is a transmembrane glycoprotein expressed in a ubiquitous way that, once synthesized in the

endoplasmatic reticulum, plays an important role for neuronal growth, dendritic extension and synapse formation (Priller C et al., 2006). The physiological pathway involves the α-secretase enzyme which cuts within the APP domain, generating two fragments, P3 and AICD, two γ-secretase substrates; the pathogenic or amyloidogenic 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.

The disease occurs predominantly after the age of sixty, although there are rarer cases of onset between thirty-fifty years. It's possible to make a distinction between sporadic and familial AD. Most cases of Alzheimer's disease are not hereditary. However, there is a small subset of cases that have an earlier age of onset and have a strong genetic element. To date, mutations on APP, Presenilin-1 (PS1) and Presenilin-2 (PS2), enzymes/cofactors 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). In addition, other genes have been identified (e.g. Apolipoprotein E gene ε4 allele) that increase the risk of disease. Some studies 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).

Age is the major risk factor in Alzheimer's disease. 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 neurodegeneration. The female gender could be another risk factor, regardless of the greater longevity of women: over eighty years, women's risk of developing AD is 20-30% higher than in males. Furthermore, high levels of homocysteine may be related with the onset of AD. The causes of hyper-homocysteinemia include genetic enzyme defects and acquired conditions, quite frequent in the elderly, such as vitamin deficiency that altered the methylation process. These conditions can promote the development of dementia through endothelial modifications, oxidative stress, neurotoxicity and neuronal apoptosis. 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). Different studies have shown an increase in chemokine 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 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).

Patients suffering from Alzheimer's disease show systemic changes at the immunological level. An evident decrease of B and T cell numbers it has been demonstrated, while the number of natural killer doesn't change (Richartz-Salzburger et al., 2007). Looking at T cell branch, major changes were seen within CD4+ compartment, where AD patients show a significant reduction of naïve (CD45RA+CCR7+) and a contemporary increase of effector

memory (CD45RA-CCR7-) and TEMRA (CD45RA+CCR7-) T cells, when compared to age-matched controls (Larbi A et al., 2009). In our recent paper we have seen a higher frequency of activated T cells (CD4+CD25+FoxP3-) in AD patients compared to old controls (Pellicanò M et al., 2011).

OUTLINE OF THE THESIS

The gradual increase in life expectancy, already observed from the last century, has had a great appeal to the scientific community that concentrated its efforts trying to understand the mechanisms that could lead to longevity.

Aging is a natural process that occurs in all cells, tissues and organs. It is modulated by both genetic and environmental factors. One of the most important characteristic of aging is the progressive deregulation of immune responses, resulting in an increased susceptibility to infectious diseases and pathological conditions relating to inflammation and the onset of autoimmune diseases. The modifications of the immune system in the elderly, known as "immunosenescence", is a complex process that involves both the innate and adaptive immune compartment. Lifelong and chronic antigenic load are the major driving force of

immunosenescence. The characteristic consequence of aging is the progressive filling of the immunological system 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, contribute to the limited repertoire and the collapse of cell diversity that is frequently correlated with poor health status .

Anyway, some people reach the extreme limit of human life in good clinical condition, escaping major age-related diseases. Centenarians may represent the prototypes of successful aging. Centenarian studies pose the challenge of whom to use as control. Unlike their parents, centenarian offspring (CO) have an appropriate control group, i.e. common elderly, who haven't a familiar history of longevity. Centenarian offspring have a genetic background that could predispose them to healthy aging and longer survival.

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 it exists a correlation between their genetic advantage and the immune system, focusing our attention in particular to the B cell branch.

Moreover we have also focused our attention on the T cell branch of patient affected by Alzheimer's disease (a model of unsuccessful aging). Recent studies have shown as 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). In this thesis we will also show the results of our study about the immunological changes related to AD, analyzing the T cell branch in patients compared to healthy age-matched and young controls.

In **chapter 2** we present a short report about the modifications of the immune system with age (immunosenenscence). We focus on the impact of CMV infection and we discuss about IRP. Moreover, we analyze B cell branch and the collapse of diversity in old age.

In **chapter 3** we compare some B cell parameters between centenarian offspring and their age-matched controls to evaluate whether any differences exists that could be related with the increase lifespan expectancy of people genetically advantaged for longevity.

In **chapter 4** we examine the systemic signs of immune-inflammatory response in AD. We analyze lymphocyte subsets and activation markers after in vitro activation of

PBMC of AD patients with rAβ42. Moreover, we evaluate the production of cytokines and chemokines after in vitro activation. We compare all the data with those obtained by age-matched controls.

In **chapter 5** we present a review that show the current knowledge about B cell immunosenescence focusing our attention on memory B cells and a particular subset, $IgG^{+}IgD^{-}CD27^{-}$, that we have demonstrated is increased in healthy elderly.

In **chapter 6** we analyze the modifications of naïve/memory B cell compartment in the elderly, evaluating also the production of pro- and anti-inflammatory cytokines after in vitro activation with different stimuli. In addition, we study the somatic hypermutation on memory B cell subsets.

In **chapter 7** we analyze the T cell branch of AD patients comparing results with those obtained by the analysis of their age-matched controls. In particular, the greatest differences were observed in the $CD4^{+}$ rather than the $CD8^{+}$ T cell compartment.

In **chapter 8** 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 production induced by in vitro activation with CpG/PMA/Ionomycin.

Finally, in **chapter 9**, a summary and general discussion of the results are presented.

CHAPTER 2

Mechanisms of immunosenescence

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Short report

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Mechanisms of immunosenescence

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Abstract

On April 7,8, 2009 a Symposium entitled "Pathophysiology of Successful and Unsuccessful Ageing" took place in Palermo, Italy. Here, the lectures of G. Pawelec, D. Dunn-Walters and. G. Colonna-Romano on T and B immunosenescence are summarized. In the elderly, many alterations of both innate and acquired immunity have been described. Alterations to the immune system in the older person are generally viewed as a deterioration of immunity, leading to the use of the catch-all term immunosenescence. Indeed, many immunological parameters are often markedly different in elderly compared to young people, and some, mostly circumstantial, evidence suggests that retained function of both innate and acquired immunity in the elderly is correlated with health status. What is often not clear from studies is how far immune dysfunction is a cause or an effect. A better understanding of immunosenescence and mechanisms responsible for proven deleterious changes is needed to maintain a healthy state in later life and to design possible therapeutic interventions.

Background

The immune system of older people is usually perceived as declining in fidelity and efficiency with age, resulting in an increased susceptibility to infectious diseases and pathological conditions relating to inflammation (e.g. cardiovascular disease, Alzheimer's disease) or autoreactivity (e.g. rheumatoid arthritis). This overall change in immunity is loosely termed "immunosenescence". The individual contributing factors to immunosenescence are many and varied, due to the multi-factorial complexity of the immune system. It is often difficult to determine whether changes in a particular cell type are intrinsic to that cell, or caused by environmental changes, or both. This is particularly the case for lymphocytes, where the interplay between B cells and T cells is crucial for effective responses, so if one subset is affected it will change the function of the other one [1-3].

Impact of Cytomegalovirus (CMV) infection on immunosenescence

Anecdotally, the clinical relevance of immunosenescence is well-documented, but exact detailed information is, in fact, hard to come by. Immunosenescence is a very vaguely-defined descriptive term covering the deleterious

age-associated changes to immunity observed in all mammals studied so far. As immunity almost certainly evolved to protect against infectious disease, which is a major cause of reduced lifespan, correlations between immune function and longevity have been sought for many years. Early studies indicated that responses to mitogens predicted mortality to some degree, and since then many studies have probed associations between survival and parameters of both innate and acquired immunity. An emerging consensus suggests that maintenance of appropriate immunity is essential for exceptional longevity and, by implication, also for "normal" longevity [4]. While all components of innate and acquired immunity are changed with age, the clinical impact of these changes is not clear, and mechanisms of and markers for immunosenescence are controversial. In humans, cross-sectional study design raises many difficulties – potentially confounding the interpretation of the published data. Examining immune status in the current elderly is rather like an astronomer examining the far-away cosmos: we are seeing the results of events that happened a long time ago, when circumstances were different from those applicable nowadays. These differences, which cannot be controlled for, include genetics, environment, nutrition, developmental variables and pathogen load [5]. The advantages and disadvantages of longitudinal studies on the same individuals emerged in the context of the pioneering OCTO/NONA studies of people >85 yr of age, which resulted in the definition of an "immune risk profile" (IRP) [5-8]. Although this is a concept which has become increasingly accepted of late, it should be emphasized that the IRP has only been shown to predict mortality in very elderly Swedes, on the basis of very limited data. These studies must be repeated and performed in other populations too. On the basis of even less data, we can say that it seems that the IRP is not predictive of excess mortality at 55 yr baseline, but might start to become so at 65 yr (on 10-yr follow-up). Because one very strong influence on the IRP is infection with CMV, it will be extremely important to test whether immune signatures like the IRP are informative under other circumstances, in different populations, and whether polypathogenicity has an additive effect. There is some epidemiological evidence for excess mortality in CMV-positive populations, which is further increased in those co-infected with hepatitis A and B as well. There is also some emerging evidence that CMV antibody titer may also be informative in this regard: individuals in the upper quartile had significantly reduced survival times compared to those in the lower quartile. The marked influence of CMV on immune signatures is illustrated in the finding that cross-sectional studies on several different European populations clearly indicate that the consensus view of T cell immunosenescence (that the fraction of naïve CD8 cells decreases in the elderly and the fraction of late-differentiated memory cells increases) does indeed hold true – but only for people who are infected with CMV. Such individuals also have higher levels of C-reactive protein, indicating that they are more likely to suffer "inflammaging", itself linked with increased occurrence of diabetes and other inflammatory diseases, as well as general frailty and increased mortality. This too may therefore be markedly influenced by CMV. Infection with other persistent herpesviruses, at least EBV, HSV and VSV, does not appear to have any similar effect [5,9]. The uniqueness of CMV in this context remains enigmatic. We propose that there may have been some advantage in early life to being CMV-positive – possibly precisely because of the enhanced pro-inflammatory status in infected people which might have had a protective effect against infection with other pathogens under conditions in the wild. It is thus concluded that immune signatures are indeed informative for "immunosenescence", which predicts mortality, but that these immune signatures are materially influenced by CMV infection [10]. Any immunogerontological study must therefore take CMV status into account. Although immunosenescence is clearly not caused by CMV, if only because not all elderly people are CMV-positive, this infectious agent seems to have a large impact on immune parameters in later life and may contribute to increased morbidity and eventual mortality. If truly carrying a benefit in early life, this would be yet another example of "antagonistic pleiotropy" which seems almost to constitute one of the few general laws of ageing [11].

B immunosenescence

Literature on immunosenescence has focused mainly on T cell impairment, but the B cell compartment is also affected in aged. The quality of the antibody response is substantially impaired. Until recently it was considered that the most likely cause of B cell failure was a lack of effective T cell help in a T-dependent reaction. Thymic involution is well known, and there is a substantial literature on the functional decline of T cells with age. However, there are T-independent functions of B cells, such as the polysaccharide responses that are crucial for anti-bacterial protection, which also appear to be lacking in later life. Additionally, there is emerging evidence to suggest that B cells are important antigen presenting cells in their own right and can be key regulators of T cell development, leading us to speculate that some of the failures of T cell function may yet be blamed on insufficient help from B cells! Changes in B cell number and repertoire have been described, and decreased IgM and IgD levels in the elderly suggest a shift from the naïve (CD27-) compartment of the B cell branch towards the memory (CD27+) compartment. However, these data are controversial since not all studies have shown this [12-14].

Circulating B cells can be divided on the basis of their expression of IgD and CD27 into different functional subsets. In the aged, a double-negative (DN) IgD-CD27- B cell subset is significantly increased [14]. Most of these cells are IgG+. Preliminary data on telomere length and expression of the ABCB1 transporter and anti-apoptotic molecule, Bcl2, suggest that DN cells have the markers of memory B cells. Furthermore, these cells do not seem to act as antigen presenting cells, nor do they express significant levels of the CD40 molecule necessary to interact with T lymphocytes through the ligand, CD154. Hence, these expanded cells may be late memory or exhausted cells that have down-modulated the expression of CD27 and filled the immunologic space in the elderly. These cells might be the age-related manifestation of time-enduring stimulation or dysregulation of the immune system [14]. Interestingly this DN B cell population is increased also in patients affected by Lupus [15] and in healthy subjects challenged with respiratory syncitial virus (RSV) [16].

Of interest, B naive lymphocytes are increased in the offspring of healthy centenarians [17]. It is well known that older offspring of centenarians, who are in their 70 s and 80 s, have a survival advantage when compared with control subjects of the same age range whose parents died at an average life expectancy [18]. The main lymphocyte differences observed between the two groups concern B cells. Indeed naive B cells are more abundant in centenarian offspring. These data are similar to that found in previous studies on younger subjects. So, the B cell compartment of the older offspring of centenarians seems to have more in common with that of younger controls than with control subjects of a similar age [17].

Human B cell repertoire diversity in old age

It is clear that the humoral immune response to challenge is impaired in later life, since the titre and affinity of antibodies raised by vaccination are consistently lower for a variety of different vaccine challenges [19]. The actual number of B cells does not appear to change with age in proportion to the decrease in vaccine efficiency. In fact, in mice, there is little evidence for any change in overall B cell numbers. Yet there are reports of perturbations in the germinal centre (GC) reaction, which is key to the development of effective B cells in a T-dependent response. Some groups studying mice have reported a decrease in the size and number of GCs responding to challenge [20]. Other studies in humans have not shown any difference in GC number [21], but have shown more subtle differences in the dynamics of the response within the individual GCs [22]. The strength of selection in the affinity maturation process within the germinal centre was shown to decrease with age in the germinal centres of Peyer's patches in the gut. This was not seen in those of the spleen, so there are tissue-specific differences occurring that are not always easy to elucidate in humans [22]. A decrease in function of GCs in the gut could be due to a number of factors. Availability of T cell help is an obvious one, although we did not see any differences in the numbers of CD4+ T cells present in these follicles [21].

Diversity of the available pool of B cells is another possible factor. It has been shown in mice that the lymphoid lineage is decreased at the expense of the myeloid lineage and the bone marrow B cell output decreases with age [23]. Although this has not been shown in humans it is a strong possibility. A decreased output of naive B cells, coupled with the accumulation of memory cells from previous immune challenges along the lifecourse, may well result in a reduction of B cell diversity. This would be especially true in the mucosal immune system where the antigenic challenge is frequent. Loss of diversity in a population of B cells would theoretically impair the B cell response to new challenges as the available repertoire from which to find an effective responder would be decreased. This would be particularly important if, as suggested, a larger proportion of the population are memory cells that had already been through the affinity maturation process [14]. Their B cell receptors would be more specific for a particular antigen and may therefore have lost the flexibility of their antigen binding site that might have otherwise allowed them to accommodate a broader range of antigens. B cell diversity in the peripheral blood of participants from the Swedish "NONA" longitudinal study using a method of B cell spectratyping was recently explored. This takes advantage of the fact that the CDR3 region of the immunoglobulin heavy chain is extremely diverse, so that when the region is PCR amplified the resultant fragment sizes follow a Gaussian distribution, providing that the starting material contains over 300 B cells. A change in diversity of the population can be detected by deviation from the normal distribution. Most spectratypes of a control group aged under 50 years show little variation. However, about a third of the older group, aged 86 to 94, has significant deviation from normal, indicating a loss of diversity of the B cell population in the peripheral blood. This loss of diversity correlated strongly with the health status of the individual, a loss of diversity being associated with those classified as "frail" [24]. Further studies will have to be undertaken to determine the exact relationship between loss of diversity and frailty, and whether the loss of B cell diversity substantially affects the response to antigen challenge. However it is becoming clearer that the B cell population is substantially altered in old age and that this has a significant contribution to immunosenescence.

Conclusion

In the elderly, many alterations of both innate and acquired immunity have been described. These alterations are generally viewed as a deterioration of immunity, leading to the use of the term immunosenescence. This process is also characterized by chronic inflammatory status. Hence, immunosenescence is responsible for the increased susceptibility of elderly to infectious diseases as well as being at the root of the biological mechanisms responsible for inflammatory age-related diseases [1-4]. A long life in a healthy, vigorous, youthful body has always been one of humanity's greatest dreams. Hence a better understanding of immunosenescence, and the development of new strategies to counteract it, are essential, not only for anti-ageing strategies aiming at rejuvenation, but, more importantly, with the aim of prolonging healthy life by preventing infectious diseases and thereby improving the quality of life in later years [25,26].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All the Authors drafted the manuscript and approved the final manuscript.

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CHAPTER 3

B cells compartment in Centenarian Offspring and Old People

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B Cells Compartment in Centenarian Offspring and Old People

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Abstract: Immunosenescence is considered a major contributory factor to the increased frequency of morbidity and mortality among elderly. On the other hand centenarians are considered the best example of successful ageing. To gain insight into mechanisms of immunosenescence and its clinical relevance, a possible model is represented by centenarians and/or their offspring. Nowadays centenarians are not more a curiosity, but in Europe are 1/8000 inhabitants and it has been demonstrated that the centenarian offspring, who are typically in their 70s and 80s, have a survival advantage when compared with age-matched controls whose parents died at an average life expectancy. Then again, studies on immunosenescence focus mainly on T cell impairment, although B cells are also affected. So, in the present preliminary report, we have studied B cell compartment in two classes of individuals, old people and centenarian offspring. B cell compartment was analysed using anti-IgD and CD27 antibodies which characterize naïve B cells (IgD^+CD27^-), memory unswitched B cells (IgD^+CD27^+), memory switched B cells(IgD^-CD27^+) and double negative B cells (DN) (IgD^-CD27^-), i.e. exhausted memory cells. As expected, in both cohorts we observed a decreased B cell count. However, in centenarian offspring, naïve B cells are more abundant whereas exhausted memory cells (DN B cells, IgD^-CD27^-) do not show the increase that we have previously demonstrated in healthy elderly donors. These data are similar to that found in previously experiments on young subjects. So, our preliminary results show that centenarian offspring do not have the typical trend of memory/naïve B cell subsets observed in elderly people and this is in agreement with the higher levels of IgM in the serum of centenarian offspring in comparison with data obtained in age-matched controls. This reservoir of naïve B cell might be one of the causes that make centenarian offspring able to keep fighting off new infections, hence prolonging their life. So, B cell subset changes could represent a hallmark of successful or unsuccessful ageing and could be used as a biomarker of human life span, potentially useful for the evaluation of anti-ageing treatment.

Keywords: B lymphocyte, centenarian, immunosenescence, longevity.

INTRODUCTION

Immunosenescence is considered a major contributory factor to the increased frequency of morbidity and mortality among elderly. It renders elderly increasingly susceptible to infectious diseases, leads to resurgence of latent infections, and also to infection by opportunistic organisms. These infections contribute significantly to morbidity in this age-group, and frequently lead to irreversible frailty and dependency. In addition, there is a decline in the protective effect of vaccination in the elderly [1-7]. Lifelong and chronic antigenic load seem to be the major driving force of immunosenescence, which impacts on human lifespan by reducing the number of virgin antigen-non experienced cells, and, simultaneously, filling the immunological space with expanded clones of memory and effector, antigen-experienced cells [7-9]. On the other hand, it has been demonstrated that centenarians escape the main diseases typical of aged and show well preserved immune functions. In fact immunosenescence is a complex process in which different immunological functions are remodeled and centenarians are an impressive demonstration of this phenomenon [10,11].

Prolongation of life expectancy has represented one of the humanity's greatest triumph in the 20th century. This unprecedented success is now one of society's greatest challenges. Improved child survival, reduced mortality rates, and decreasing fertility rates worldwide, is resulting in a rapid ageing of the world's population. This ageing is evident worldwide, and particularly evident in developing countries where the elderly population is predicted to quadruple over the next 25 years at which time it will represent over 25% of the total population. In particular, around the 60's in all the industrialized countries the progressive decline of the mortality (-2% year) in individuals over-80 years old has risen up of about twenty times the number of oldest old people. This has increased the number of centenarians that nowadays are not more a curiosity, but in Europe are 1/8000 inhabitants [10,12,13].

Furthermore, centenarians are considered the best example of successful ageing [10,11]. To gain insight into mechanisms of immunosenescence and its clinical relevance, a possible model is represented by centenarians and/or their offspring. In fact, it has been demonstrated that the centenarian offspring, who are typically in their 70s and 80s, have a survival advantage when compared with age-matched controls whose parents died at an average life expectancy. Centenarian offspring, like their centenarian parent(s), have genetic and functional advantages associated with lower cardiovascular disease risk [14,15]. These findings support the hypothesis that centenarian offspring are predisposed to healthy aging and longer survival, making them a suitable target of ageing studies, because, unlikely of centenarians, they have an appropriate control group, i.e. common elderly.

Literature of immunosenescence has focused mainly on T cells, although B cell compartment is also affected in aged. Changes in B cell number and repertoire have been described and data in literature demonstrate that elderly frequently do not have protective antibody concentrations against recall antigens so suggesting the impairment of B cell branch [16,17]. The aim of this study was to compare some B cell parameters between healthy centenarian offspring and healthy age-matched controls to evaluate whether any difference exists in the naïve/memory B cell compartment that might explain the familiar increased lifespan expectancy of centenarian offspring. So, in the present preliminary report, we have studied B cell compartment in two classes of individuals, old people and centenarian offspring. B cell compartment was analyzed using anti-IgD and CD27 antibodies which characterize naïve B cells (IgD^+CD27^-), memory unswitched B cells (IgD^+CD27^+), memory switched B cells (IgD^-CD27^+) and double negative B cells (DN) (IgD^-CD27^-), i.e. exhausted memory cells [18,19]. In fact we have recently demonstrated an increase of late (exhausted) memory B cells in elderly [20] also accompanied by a decrease of serum IgM, so confirming the loss of naïve cell/products (i.e. antibodies) in the elderly [21]. Here we show that centenarian offspring have an

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increased amount of naïve B cells and IgM when compared to their controls, whereas we do not observe the increase of DN B cells shown in healthy elderly people.

MATERIALS AND METHODS

Twenty-nine Sicilian centenarian offspring (CO, age range 59-83, mean 73.4 ± 7 years), with almost one of their parents centenarian (>99 years), whose age had been confirmed from records at the city hall and/or church registries, were studied. A total of 25 age-matched Sicilian controls (A-M) (age range 60-85, mean 78.6 ± 4.7 years) were also included in the study. All subjects were in good health according to their clinical history and blood tests (complete blood cell count, erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, C reactive protein, liver function tests, iron, proteins). The study received approval from local ethic committee and all participants gave their informed consent.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by density gradient centrifugation on Ficoll-Lympholyte (Cedarlane Laboratories Limited, Ontario, Canada). PBMCs were adjusted to 1 x 10^6 /ml in RPMI 1640 medium (Euroclone, Devon, UK) supplemented with 10% heat-inactivated fetal calf serum (Euroclone), 1% penicillin/streptomicin, 10 mM HEPES, and 1 mM L-glutamine.

In order to evaluate the lymphocytes subsets, total PBMCs were stained with different combinations of the following monoclonal antibodies: anti-CD3, anti-CD4, anti-CD8, anti-CD16, anti-CD19. All the antibodies were directly coupled either to fluorescein isothiocyanate (FITC), phycoerytrin (PE) or PE-Cy7 (Pharmingen, BD Bioscience, Mountain View, CA, USA). To analyze B cell subsets, PBMCs were stained with the following monoclonal antibodies combination: anti-$CD19_{FITC}$, anti-IgD_{PE} and anti-$CD27_{APC}$ (Pharmingen).

All measurements were made with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with the same instrument setting. At least 10^5 cells were analyzed using CellQuest Pro (Becton Dickinson, San Jose, CA, USA) software.

For IgG, IgA and IgM assay, the serum of all subjects was stored in aliquots at -80°C until analysis and the immunoglobulin concentrations were determined by Integra 800 (Roche Diagnostics, Milan, Italy) according to manufacturer instructions.

Values, given as the mean ± SD, were compared using one-way analysis of variance (ANOVA). Differences were considered significant when a p value < 0.05 was obtained by comparison between the different groups.

RESULTS

In Table **1** we report the percentage and absolute number of lymphocyte subpopulations evaluated in 29 centenarian offspring (age range 59-83 years, mean age 73.4 ± 7 years) and 25 healthy age-matched controls (age range 60-85, mean 78.6 ± 4.7). As shown no significant differences have been observed between the lymphocyte values in the two groups studied.

So, concerning these lymphocyte subsets, centenarian offspring behave as the common elderly population.

Regarding naïve/memory B cell subsets, we analysed circulating $CD19^+$ lymphocytes obtained by peripheral blood of centenarian offspring and their age-matched controls on the basis of the expression of IgD and CD27 into different functional subsets. These markers allow to divide blood B cells in four subsets, naïve B cells (IgD^+CD27^-), memory unswitched B cells (IgD^+CD27^+), memory switched B cells(IgD^-CD27^+) and double negative B cells (DN) (IgD^-CD27^-), i.e. exhausted memory cells [20] (Fig. (**1**) shows a characteristic plot).

In Table **2** we report the percentage and absolute number of these B lymphocyte subpopulations. As shown, the percentage and the absolute values of IgD^+CD27^- naïve B cells are significantly increased in centenarian offspring when compared to age-matched controls. Instead, the percentages (but not the absolute values) of both IgD^+CD27^+ memory unswitched B cells and double negative IgD^-CD27^- B cells, i.e. exhausted memory cells, are significantly reduced in centenarian offspring. So, concerning these B cell subsets, centenarian offspring behave as the young population [20].

This observation is strengthened by serum immunoglobulin measurement. In fact, as displayed in Fig. (**2**), the concentration of IgM, a marker of the primary response, shows significant higher levels in centenarian offspring when compared to age-matched controls, whereas IgG and IgA levels are not significantly different between the two groups.

DISCUSSION

Ageing is a natural process that occurs in all cells, tissues, organs and organisms. It is modulated by both genetic and environmental factors. One of the most important characteristics of ageing is immunosenescence, that is the consequence of the continuous attrition caused by chronic antigenic load. The antigenic load results in the progressive generation of inflammatory responses involved in age-related diseases [8,9,22]. In the elderly many alterations of both innate and acquired immunity, have been described. The acquired compartment of immune system shows significant modifications in the elderly, in fact both T and B

Table 1. Lymphocyte Subpopulations in 29 Centenarian Offspring (CO, Mean Age 73.4 Years, Age Range 59-83) and 25 Age-Matched Controls (A-M, Mean Age 78,6 Years, Age Range 60-85). Data are Expressed as Mean ± SD of Absolute Numbers and Percentages. Significance has been Evaluated by ANOVA Test

Lymphocyte subpopulation	CO (29) % (MEAN±SD)	A-M (25) % (MEAN±SD)	p	CO (29) Absolute Number	A-M (25) Absolute Number	P
CD3+	68.4±12.2	64.9±10.8	0.1	1483±494	1352±360	0.3
CD4+	46.2±11.4	44.1±10.1	0.2	952±421	875±224	0.4
CD8+	20.8±7.0	18.8± 7.2	0.3	402±190	429±228	0.6
CD19+	7.7±3.8	10.2± 6.1	0.3	152±107	106±97	0.1
CD3-CD16+	12.1±7.3	12.6± 5.7	0.3	277±184	267±149	0.8

p= values of CO vs. A-M.

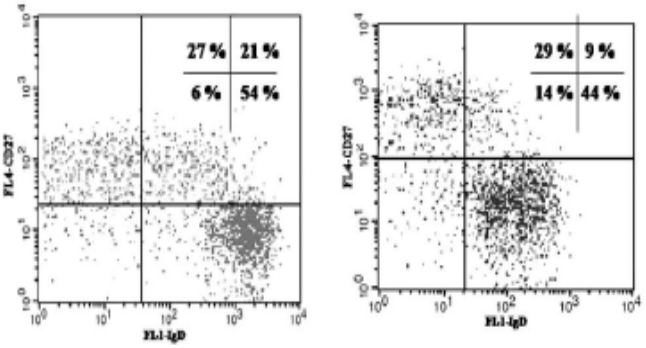

Fig. (1). Density Plot of memory/naïve B cells distribution. B subpopulations are identified by the expression of IgD and CD27 in centenarian offspring (A) and age-matched controls (B). the analysis of Figure refers to cells gated as CD19+.

Table 2. B Cell Subsets of 29 CO Subjects, 25 A-M Controls as Analyzed According to the Expression of IgD and CD27. Data are Expressed as Mean ± SD of Absolute Numbers and Percentage. Significance has been Evaluated by ANOVA Test

B Lymphocytes subpopulations	CO (29) % (MEAN±SD)	A-M (25) % (MEAN±SD)	p	CO (29) Absolute Number	A-M (25) Absolute Number	P
IgD^+CD27^-	49.5±18.3	36.1±17.8	**0.01**	79.5±75.4	43.0±41.0	**0.04**
IgD^+CD27^+	15.6±10.5	24.1±14.7	**0.02**	23.7±20.3	28.3±32.2	0.5
IgD^-CD27^+	26.0±15.4	22.9±10.9	0.4	37.2±27.3	24.1±25.9	0.1
IgD^-CD27^-	8.3±5.1	15.0±9.2	**0.003**	10.3±7.0	18.6±28.6	0.1

p= values of CO vs. A-M.

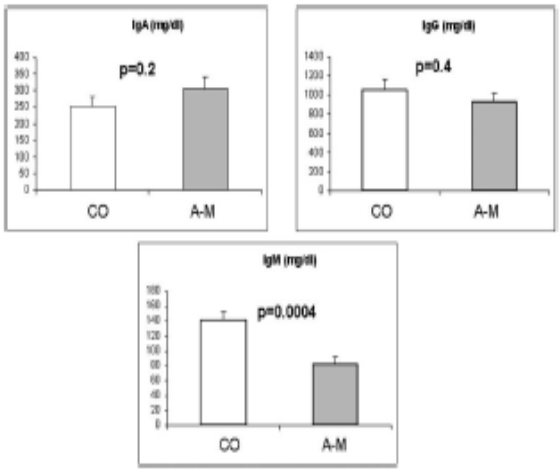

Fig. (2). Analysis of IgG, IgA and IgM serum concentrations in centenarian offspring (CO) and age-matched controls (A-M).

lymphocytes are reduced and, in T cell compartment, memory T cells are increased [1,8,9,19,23]. Furthermore, the study of centenarian immune systems has revealed that several immune parameters are well conserved, suggesting that a complex remodelling of most immune parameters occurs with age [10,11].

In our paper, we have gained insight into the B cell compartment of centenarian offspring and in particular into the memory/naïve B cell branch, comparing our data to those obtained in healthy age-matched controls. We believe that our data, though preliminary, are of some interest as in centenarian offspring we do not observe the typical naïve-memory shift observed in elderly [20]. Moreover, comparing these data with data recently published by our group we observe that CO behave as young controls [20]. Also 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 as published by our group few years ago [21]. So, on the whole naïve B cells are well represented in CO compared with the AM controls, suggesting a good bone marrow cell reservoir. This is an interesting observation, as it has been recently reviewed [24] the bone marrow ability to generate B cells is impaired with age. The mirror image of this is the reduced amount of the primed/memory pool in centenarian offspring. In particular, here we report that in centenarian offspring we do not observe the increase of DN (IgD-CD27-) B cells. As we and others have recently suggested [20,25], these are memory B cells that lack the typical memory marker CD27, and are so considered to be late-memory B cells, i.e. exhausted ones. It is interesting to observe that these cells are expanded both in elderly [20] and in patients suffering of chronic immune inflammation as SLE [25,26], so suggesting that the antigenic load or the "inflammatory" environment play a central role in the exhaustion of the B cell branch too.

It is known that memory and naive B cells produce different cytokines. Naive and memory B cells also express different Toll-like receptors and so they have a regulatory role in the chronic infections against viruses and bacteria also producing cytokines and chemokines. This suggests that the immune-inflammation is also related to the B cell branch of the immune system in aged people since they are able to produce different chemokines and cytokines [27-30]. Hence, these studies are relevant both to instructive immunity and inflammation-related diseases typical of aged people.

All together these data support the hypothesis of a "familiar youth" of the immune system that can be a big advantage both to fight the main age-related diseases and to properly respond to vaccinations. In particular, the reservoir of naive B cell might be one of the causes that make centenarian offspring able to keep fighting off new infections, hence prolonging their life. So, B cell subset changes could represent an hallmark of successful or unsuccessful ageing and could be used as a biomarker of human life span, potentially useful for the evaluation of anti-ageing treatment [31,32].

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ABBREVIATIONS

A-M	=	Age-Matched Sicilian controls
APC	=	Allo-Phyco-Cyanin
CO	=	Centenarian Offspring
DN	=	Double Negative
PBMCs	=	Peripheral Blood Mononuclear Cells

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CHAPTER 4

Systemic immune responses in Alzheimer's disease: in vitro mononuclear cell activation and cytokine production.

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Systemic Immune Responses in Alzheimer's Disease: In Vitro Mononuclear Cell Activation and Cytokine Production

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Abstract. To investigate the systemic signs of immune-inflammatory responses in Alzheimer's disease (AD), in the present study we have analyzed blood lymphocyte subsets and the expression of activation markers on peripheral blood mononuclear cells (PBMCs) from AD patients and age-matched healthy controls (HC) activated *in vitro* by recombinant amyloid-β peptide ($rA\beta_{42}$). Our study of AD lymphocyte subpopulations confirms the already described decrease of the absolute number and percentage of B cells when compared to HC lymphocytes, whereas the other subsets are not significantly different in patients and controls. We report the increased expression of the activation marker CD69 and of the chemokine receptors CCR2 and CCR5 on T cells but no changes of CD25 after activation. B cells are also activated by $rA\beta_{42}$ as demonstrated by the enhanced expression of CCR5. Moreover, $rA\beta_{42}$ induces an increased expression of the scavenger receptor CD36 on monocytes. Some activation markers and chemokine receptors are overexpressed in unstimulated AD cells when compared to controls. This is evidence of the pro-inflammatory status of AD. Stimulation by $rA\beta_{42}$ also induces the production of the pro-inflammatory cytokines IL-1β, IL-6, IFN-γ, and TNF-α, and of the anti-inflammatory cytokines IL-10 and IL-1Ra. The chemokines RANTES, MIP-1β, and eotaxin as well as some growth factors (GM-CSF, G-CSF) are also overproduced by AD-derived PBMC activated by $rA\beta_{42}$. These results support the involvement of systemic immunity in AD patients. However, our study is an observational one so we cannot draw a conclusion about its contribution to the pathophysiology of the disease.

Keywords: Alzheimer's disease, chemokine, cytokine, PBMC, $rA\beta_{42}$

INTRODUCTION

Alzheimer's disease (AD) is a heterogeneous and progressive neurodegenerative disease that in Western societies accounts for the majority of clinical senile dementia. Since no early peripheral and reasonable signs of the disease have been identified so far, diagnosis can be made with firmness only upon brain autopsy. Moreover, no treatment is yet available to end or turn round the disease; existing drugs are only able to relieve symptoms for some time [1,2].

Pathological changes in the AD brain include neuronal loss, senile plaques, and neurofibrillary tangles. Together with the presence of intraneural tangles, con-

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sisting of phosphorylated tau-proteins, senile plaques are the hallmark of the pathological diagnosis of AD. However, the increased production of amyloid-β ($A\beta$) from the amyloid-β protein precursor and its brain accumulation and deposit in the senile plaques lead to inflammation and neuronal damage [3]. Depending on whether the 40th or the 42nd amino acid in C-terminus is the last of the $A\beta$ protein, it consists of two major forms, $A\beta_{40}$ and $A\beta_{42}$. The latter tends to cluster into oligomers, forming $A\beta$ fibrils that deposit as microstructures, amyloid plaques, and it is the predominant form that accumulates in the brain parenchyma of AD patients. Senile plaques result from the accumulation of several other proteins and an inflammatory reaction around deposits of amyloid. In addition, plaques contain dystrophic neurites, activated microglia, and reactive astrocytes. Aggregated amyloid fibrils and inflammatory mediators secreted by microglial and astrocytic cells contribute to neuronal dystrophy [3,4].

Inflammation clearly occurs in pathologically vulnerable regions of the AD brain, and it does so with the full complexity of local peripheral inflammatory responses [5]. 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. Furthermore, in experimental animals chronic systemic inflammatory response induced by lipopolysaccharide administration also induces glial activation. After activation, the microglia cells modify their morphology and become tissue macrophages producing inflammatory mediators [3,6]. Accordingly, case-control studies clearly demonstrate the role of inflammatory genes in AD [3,7,8]. In particular, two recent meta-analyses by our group have underscored the role of cytokine polymorphisms in AD susceptibility, hence indicating the role of immune-inflammatory responses in AD [9,10].

$A\beta$ induces a local immune response involving glial cells and astrocytes. The innate immunity tries to clear $A\beta$ and induces the production of inflammatory proteins such as complement factors, acute-phase proteins, pro-inflammatory cytokines, and chemokines that will be chronically produced and can induce neurotoxicity [5,11].

Although much evidence suggests the involvement of a systemic immune response in AD, it is poorly characterized [12]. Indeed, blood derived cells seem to accumulate in the AD brain [13], while other studies have shown changes in the distribution and reactivity of immune cells in the blood [12,14–16]. As reviewed by Britschgi and Wyss-Coray [12], many studies have shown that there is communication between central and systemic immune responses. In particular neuroinflammation induces the efflux of central nervous system (CNS) proteins, such as $A\beta$, or inflammatory mediators across the blood-brain-barrier (BBB); this may cause systemic immune reaction and recruitment of myeloid or lymphocytic cells into the CNS [12]. Thus, communication between the CNS and immune system in AD could influence both the lymphocyte distribution in the blood and the production of immune mediators [12]. Indeed peripheral blood mononuclear cells (PBMCs) from AD patients produce higher levels of some cytokines, such as interleukin (IL)-1β and IL-6 compared to PBMC from control subjects [17]. Other studies have shown that $A\beta$ stimulates macrophage inflammatory protein (MIP)-1α overexpression on peripheral T cells and its receptor CCR5 expression on brain endothelial cells for T cells crossing BBB [18].

In addition, the production of Regulated on Activation, Normal T Expressed and Secreted (RANTES) by AD PBMCs seems to increase after stimulation by $A\beta$ [19]. Moreover, an immune disregulation was recently documented as dramatic alterations on CD4+ subsets in patients with mild AD have been reported. In particular, decreased percentages of naïve cells and an increase of memory cells, an increased number of CD4+ lymphocytes that lack the co-stimulatory molecule CD28, and a reduction of CD4+CD25^{high} T regulatory cells (Treg) have been observed [20].

These data suggest a significant involvement of both the innate and acquired immunity in AD patients, although there are not enough data to determine if these are the causes or the consequences of the disease. To gain insight into this topic, we have investigated some basic immune parameters in patients with AD, and we have performed *in vitro* activation studies of AD PBMCs with recombinant $A\beta(_1A\beta_{42})$ to evaluate cellular reactivity to the peptide and the cytokine and chemokine production.

MATERIALS AND METHODS

Subjects

Diagnosis of probable AD was according to standard clinical procedures and followed the NINCDS/ADRDA and DSM-III-R criteria [21,22]. Cognitive perfor-

mance and alterations were measured according to the Mini Mental State Examination (MMSE) and the Global Deterioration Scale. All AD cases were defined as sporadic because their family history did not mention any first-degree relative with dementia. The population of AD consisted of 40 patients from Sicily (27 women and 13 men; age range: 64–86 years; mean age: 78.4 ± 7.6). AD patients included in the study did not present major co-morbidity such as cancer, symptomatic (present or previous) cardiovascular diseases, and major inflammatory diseases such as autoimmunity and infections. Eighteen of them were under treatment for hypertension and/or diabetes with drugs not known to affect the immune system. According to MMSE, 16 patients were affected by severe dementia (< 17), whereas the remaining 24 were affected by moderate grade of dementia (> 17 < 24). Patients with vascular dementia were not included in the study. Since treatment with acetyl-cholinesterase inhibitors may modulate cytokine expression [23], patients were included before starting that therapy. Healthy controls (HC) were 25 unrelated individuals (15 women and 10 men; age range: 63–85; mean age 77.5 ± 7.2) randomly selected from a retirement home. These subjects 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, C reactive protein, liver function tests, iron, proteins, cholesterol, triglycerides). The controls were collected from the same population as the patient cohort. 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. Lymphocyte subsets were analysed in 40 AD and 25 HC. Cell cultures for activation and cytokine production were performed on PBMCs from 18 AD and 15 HC randomly selected, taking into account gender, age range, and the ratio between severe and moderate AD. In fact, we studied 11 patients affected by moderate AD (4 men and 7 women, mean age 79.6 ± 1.3), 7 patients affected by severe AD (2 men and 5 women, mean age 81.0 ± 1.5), and 15 HC (6 men and 9 women, mean age 78.5 ± 1.2).

Preparation and characterization of rAβ_{42}

Isolation and purification of rAβ_{42} were performed as described by Carrotta et al. [24]. To obtain small oligomers, rAβ_{42} was dissolved in double-distilled H_2O (ddH_2O) to a final concentration of 1.4 mM and incubated at 37°C for 96 h. Immediately after dissolution (time 0), and after incubation, aliquots were taken and stained adding thioflavin-T (ThT) at a final concentration of 70 μM and applied to microscope slides [25]. The presence of aggregates with dimensions in the range of micrometers was visualized with the fluorescence optics of an Axioscop 2 microscope (Zeiss, USA). The images were captured using an Axiocam digital camera interfaced with a computer. Moreover, rAβ_{42} at time 0, and after incubation at 37°C for 96 h, was analyzed by Western blot. Analysis of the samples was carried out using SDS-PAGE, and after running, the gel was transferred to nitrocellulose and the filter was incubated with anti-Histidine (anti-His) 1:5000 (Pierce, Rockford, IL, USA) [24].

Cell cultures

PBMCs were separated by centrifugation with Ficoll-Lympholyte (Cederlane Laboratories Limited, Ontario, Canada) and washed twice in PBS. Viability was assessed by trypan blue dye exclusion. The viable cells (95–98% of the preparation) were re-suspended at the concentration of 1×10^6/μl in complete medium composed of RPMI 1640 (Euroclone, Devon, UK) supplemented with 10% heat-inactivated fetal calf serum (Euroclone), 1% penicillin/streptomycin, 10 mM HEPES, and 1 mM L-glutamine.

Cells were cultured in 24-well flat-bottom plates at 1.5×10^6 cells per well in RPMI medium. The PBMCs were either unstimulated or stimulated by oligomeric rAβ_{42} (10 μg/ml) obtained as described above. For the chemokine receptor expression study, the PBMCs were either unstimulated or stimulated by phytohaemagglutinin (PHA) (3 μg/ml), anti-CD3 (α-CD3) (1 μg/ml), rAβ_{42} (10 μg/ml), for 48 h at 37°C in a humidified 5% CO_2 atmosphere. The cells were then collected and used for flow cytometry.

Flow cytometry

In order to evaluate the T lymphocyte subsets in both AD patients and HC, fresh samples were stained using the following monoclonal antibodies: anti-CD3, anti-CD4, anti-CD8, anti-CD16, anti-CD19, anti-TCR $\gamma\delta$.

In order to evaluate the activation of PBMC obtained from AD patients and HC, the *in vitro* cultured cells were stained as follows: anti-CD25$_{FITC}$/anti-CD3$_{PE}$/anti-CD19$_{PECy5}$, anti-CD69$_{FITC}$/anti-CD3$_{PE}$/anti-

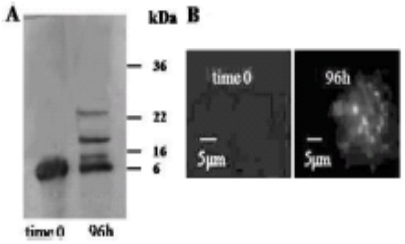

Fig. 1. Characterization of $rA\beta_{42}$. A) Western blot of $rA\beta_{42}$ incubated with anti-His, immediately after dissolution in ddH_2O (time 0) and after incubation for 96 h at 37°C. On the right, molecular weight standards are indicated. B) Staining with ThT. Fluorescence images of representative areas of the observation field at time 0 and at 96 h.

$CD19_{PBCy5}$, anti-$HLADR_{FITC}$/anti-$CD19_{PE}$/anti-$CD80_{PE-Cy5}$ and anti-$CD36_{FITC}$/anti-$CD14^{+}_{PE}$/$CD80_{PE-Cy5}$. The activation-induced expression of chemokine receptors was evaluated by staining cultured cells with the following monoclonal antibodies: anti-CD192 $(CCR2)_{Alexa^{Fluor}\text{®}647}$, anti-$(CD193\ (CCR3)_{PE}$ and anti-$CD195\ (CCR5)_{PE-Cy5}$ on B ($CD19^{+}$) and T ($CD3^{+}$) cells. All monoclonal antibodies were directly coupled either to fluorescein isothiocyanate (FITC), R-phycoerythrin (R-PE), phycoerytrin-Cyanine 5 (PE-Cy5), or Alexa Fluor® 647 (Pharmingen, BD Bioscience, Mountain View, CA, USA).

All the 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.

Cytokine production assay

The supernatants of the cultured PBMCs were collected and the following cytokines and chemokines were evaluated using a Bio-Plex kit (BioRad, Munich, Germany): IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, basic fibroblast growth factor (BFGF), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon(IFN)-γ, IFN-γ-inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, MIP-1α, MIP-1β, platelet-derived growth factor-bb (PDGF-bb), RANTES, tumor necrosis factor (TNF-α), and vascular endothelial growth factor (VEGF). 50 μl per sample were analyzed on the Luminex 100 (BioRad) according to manufacturer's instructions.

Statistical analysis

Values (percentage or MFI), given as the mean ± SEM, were compared using one-way analysis of variance (ANOVA). Differences were considered significant when a p value < 0.05 was obtained by comparison of the two different groups.

RESULTS

Characterization of $rA\beta_{42}$

To confirm that $rA\beta_{42}$, prepared as previously described, forms oligomers *in vitro*, an aliquot of the samples at time 0 and at 96 h obtained as described in the previous section was incubated with anti-His. As shown in Fig. 1A, on the basis of the molecular weight of the detected bands, we established that only monomers were present in the sample at time 0, and different small oligomers (ranging from 12 to 24 kDa) in the sample at 96 h. Furthermore, after staining with ThT no visible structures in the sample at time 0 were detectable, whereas, small structures of dimensions up to 2 μm in the 96 h sample were present (Fig. 1B). Therefore, the preparations used in our experiments were made up of oligomers.

Lymphocyte subsets

Table 1 shows the percentage and the absolute number of the main lymphocyte subpopulations evaluated in 25 HC and 40 AD patients. As shown, and as previously reported by others [26–28], we report a significant decrease in the percentage and absolute number

Table 1
Lymphocyte subpopulations in 40 AD subjects and 25 HC. Data are expressed as Mean ± SD of the percentage and as absolute number

Subpopulations	HC % (Mean ± SD)	HC (Absolute number/μL.)	AD % (Mean ± SD)	AD (Absolute number/μL.)
$CD3^+$	67.3 ± 11.8	1196 ± 444	68.4 ± 9.2	1260 ± 420
$CD4^+$	44.3 ± 9.6	801 ± 310	45.7 ± 8.9	845.6 ± 340
$CD8^+$	20.0 ± 10.5	373 ± 214	21.0 ± 8.9	361 ± 160
$CD19^+$	9.8 ± 5.3	184 ± 128	7.0 ± 3.7*	130 ± 54*
$CD3^-CD16^+$	17.2 ± 9	350 ± 195	15.7 ± 6.7	319 ± 165
$\gamma\delta^+$	3.0 ± 3.3	45 ± 55	2.5 ± 1.8	54 ± 39

Significance has been evaluated by ANOVA test. AD vs. HC* $p = 0.01$.

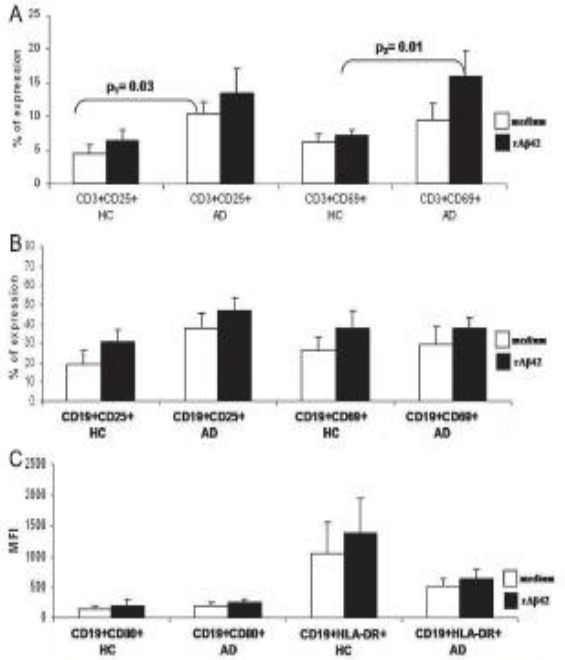

Fig. 2. Percentage (Mean ± SEM) of expression of early activation markers CD25 and CD69 on T ($CD3^+$) (panel A) and B ($CD19^+$) (panel B) cells of HC ($n = 15$) and AD subjects ($n = 18$) cultured in medium (white) or with $rA\beta_{42}$ (black). Panel C shows the MFI (Mean ± SEM) of expression of CD80 and HLA-DR on B cells of HC ($n = 15$) and AD subjects ($n = 18$) cultured in medium (white) or with $rA\beta_{42}$ (black). Statistical analysis was performed by ANOVA test. Significant values are indicated, p_1 = CD25 expression on unstimulated (medium) AD cells vs. unstimulated HC cells; p_2 = CD69 expression on $rA\beta_{42}$-stimulated cells from AD vs. $rA\beta_{42}$-stimulated cells from HC. Concerning B cells, no significant differences were observed for CD25 expression on $rA\beta_{42}$-stimulated cells from AD vs. $rA\beta_{42}$-stimulated cells from HC, for CD69 expression on unstimulated cells (medium) AD vs. unstimulated HC cells, nor for both CD80 and HLA-DR between the groups studied.

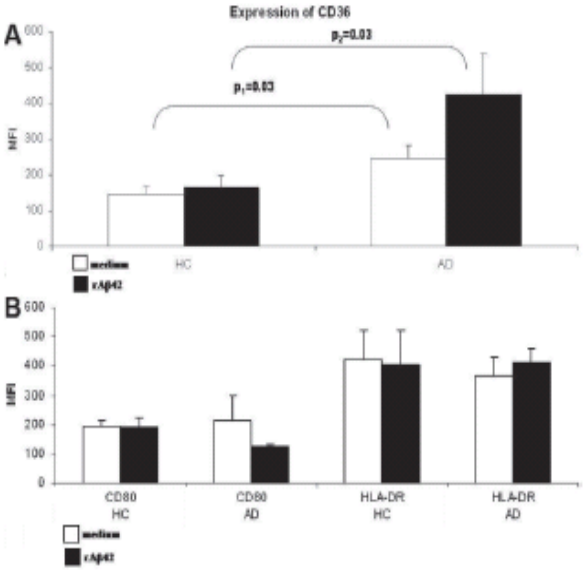

Fig. 3. MFI (Mean ± SEM) of expression of CD36 (panel A) and CD80 and HLA-DR (panel B) on monocytes (CD14+) of HC (n = 15) and AD (n = 18) subjects cultured in medium (white) or with rAβ_{42} (black). Statistical analysis was performed by ANOVA test. Significant values are indicated, p_1 = unstimulated (medium) AD cells vs. unstimulated HC cells, p_2 = rAβ_{42}-stimulated cells from AD vs. rAβ_{42}-stimulated cells from HC. No significant differences were observed for CD36 expression on unstimulated cells (medium) HC vs. stimulated HC cells, and on unstimulated (medium) AD cells vs. stimulated AD cells nor for the expression of CD80 and HLA-DR in the groups studied.

of circulating B lymphocytes of AD patients compared to HC. No significant differences have been instead observed between the two groups concerning the other lymphocyte subsets.

PBMC activation

To study the response to Aβ, we stimulated PBMCs from 18 AD and 15 HC (see Materials and Methods) with the oligomeric form of rAβ_{42} and analyzed the expression of the activation markers CD25 and CD69 on T and B lymphocytes, and of CD80 and HLA-DR on B lymphocytes.

We report (Fig. 2a) a significantly higher expression of CD25 on unstimulated T cells from AD patients compared to unstimulated T cells from HC, whereas the stimulation by rAβ_{42} does not induce significant differences between the two groups. On the contrary, CD69 is not significantly higher in unstimulated T cells from AD patients and is significantly overexpressed after stimulation by rAβ_{42}. The expression of CD25, CD69, HLA-DR, and CD80 on B cells showed no significant differences, after stimulation, between the two groups studied. We again observed that lymphocytes of AD patients showed a basically higher, but not significant, expression of some of these activation markers (Fig. 2b, c).

We have also evaluated the activation of monocytes after stimulation by rAβ42. We have observed a higher basal expression of CD36, a class B scavenger receptor that interacts with fibrillar Aβ [25,29], and a significant different expression of this receptor after stimula-

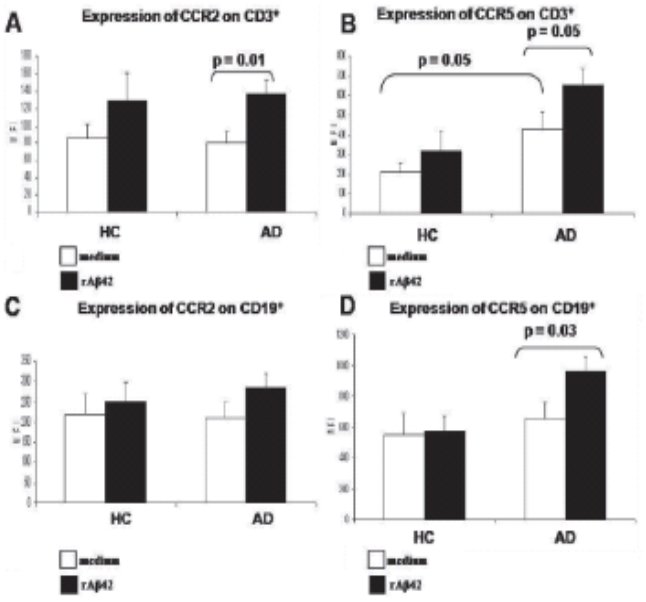

Fig. 4. MFI (Mean ± SEM) expression of CCR2 and CCR5 on CD3+ T cells (panels a, b) and CD19 B (panels c, d) peripheral lymphocytes obtained from 15 HC subjects and 18 AD patients and cultured for 48 h in presence (black) or absence (white) of rAβ_{42}. Statistical analysis was performed by ANOVA test. P values are calculated comparing the MFI ± SEM of positive cells obtained from unstimulated and rAβ_{42}-stimulated cells. Comparing unstimulated vs. stimulated cells we obtain significant values in: a) CCR2 expression on T cells from AD patients, (unstimulated vs. stimulated $p = 0.01$); b) CCR5 expression on T cells from AD patients, (unstimulated vs. stimulated $p = 0.05$); c) CCR2 expression on B cells from AD patients, (unstimulated vs. stimulated not significant); d) CCR5 expression on B cells from AD patients, (unstimulated vs. stimulated $p = 0.03$). Concerning the unstimulated PBMCs significant difference are observed comparing CCR5 expression on T cells from HC and AD. Indeed there is a higher basal expression of CCR5 on AD patients (HC vs. AD $p = 0.05$).

tion by rAβ_{42} between patients and controls (Fig. 3a). In contrast, analyzing the expression of CD80 and of HLA-DR on monocytes, we have not seen any variation between the groups studied (Fig. 3b).

In a further set of experiments, we have evaluated the expression of CCR2 (MCP-1 receptor), CCR3 (RANTES and eotaxin receptor) and of CCR5 (MIP-1α, MIP-1β, and RANTES receptor) on T ($CD3^+$) and B ($CD19^+$) lymphocytes.

As shown (Fig. 4a-d), CCR2 (Fig. 4a) and CCR5 (Fig. 4b) are overexpressed on T cells stimulated by rAβ_{42}, in both groups, although the significance was attained only in AD patients. In addition, the basal expression of CCR5 on the $CD3^+$ cells is significantly higher in AD patients. The evaluation of the same receptors on B cells, after activation by rAβ_{42}, shows a significantly greater expression of CCR5 in AD patients, and not in controls. No differences were observed for CCR2 expression on $CD19^+$ cells (Fig. 4c, d) as well as for CCR3 expression on T and B cells (data not shown).

The stimulation of cells from HC and AD by PHA or α-CD3, used as further control, does not induce the expression of these chemokine receptors either in HC or AD patients. Figure 5 shows a typical experiment that compares the response of cells from AD pa-

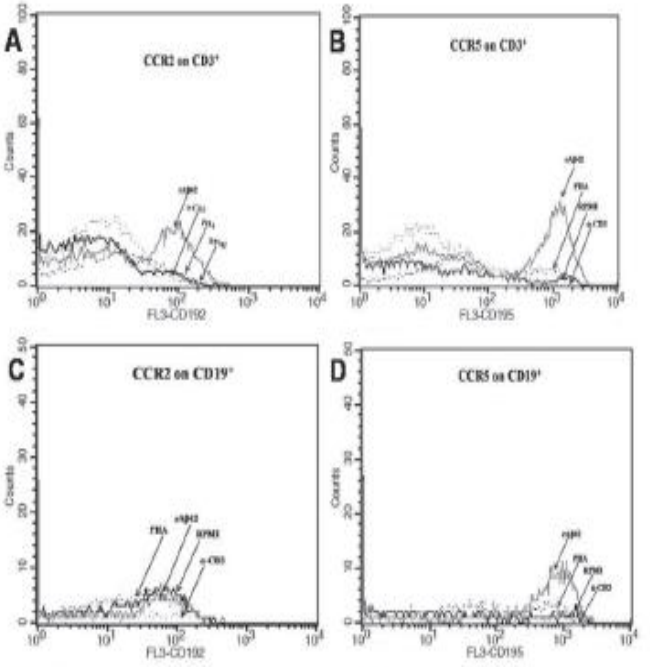

Fig. 5. Representative expression of CCR2 and CCR5 on CD3+ T cells (panels a, b) and CD19 B cells (panels c, d) obtained from one AD patient, and cultured for 48 h in presence of PHA (.....), α-CD3 (.....), rAβ_{42} (—), or none (RPMI) (—).

tients to the different stimuli used (PHA, α-CD3, and rAβ_{42}). As shown (Fig. 5a, b), CD3 cells overexpress CCR2 (panel A) and CCR5 (panel B) when stimulated by rAβ_{42}, whereas the stimulation by either PHA or α-CD3 gives a fluorescence intensity like that of unstimulated T cells. Panel C and panel D depict the results obtained stimulating CD19 B cells. As shown, no changes are observed in the expression of CCR2, using rAβ_{42}, PHA or α-CD3. Activation by rAβ_{42} causes an increased of expression of CCR5 on B cells, whereas the stimulation by either PHA or α-CD3 gives a fluorescence intensity like that of unstimulated B cells.

AD patients ($N = 18$) of this subgroup showed the same changes in lymphocyte populations (i.e., B lymphocyte decrease) as well as in the initial group (7.0 ± 2.2 versus 7.0 ± 3.7). None of these results were affected by the gender and the MMSE of AD patients; in particular no differences have been observed comparing data obtained from subjects with severe (< 17) versus moderate AD.

Cytokine production

We have analyzed the production of cytokines, growth factors, and chemokines in supernatants obtained from AD- and HC-PBMC cultured for 48 h in the presence/absence of the oligomeric form of rAβ_{42}. As shown in Table 2, after stimulation by rAβ_{42}, PBMC obtained from AD patients significantly increased the production of both pro-inflammatory cytokines (IL-1β, IL-6, IFN-γ, TNF-α) and anti-inflammatory cytokines (IL-10, IL-1ra) as well as some growth factors (GM-

Table 2

Percent of increase of cytokines, growth factors, and chemokines secretion by PBMCs of 15 HC and 18 AD patients in presence/absence of rAβ_{42}. The percentage was calculated using the following *formula*: stimulated-unstimulated/unstimulated x 100. In bracket % of increase of p25–p75 values. Significance has been evaluated by ANOVA test. n.s. = not significant

Cytokines	rAβ_{42} HC vs. medium HC Increase (%) (median and p-25–p75 values)	rAβ_{42} AD vs. medium AD Increase (%) (median and p-25–p75 values)	p_1	p_2
IL-1β	4 (0–22)	211 (133–285)	n.s.	0.01
IL-1ra	66 (0–258)	202 (152–288)	n.s.	0.006
IL-6	0 (0–151)	410 (280–532)	n.s.	0.01
IFN-γ	0 (0–52)	106 (84–134)	n.s.	0.002
TNF-α	0 (0–168)	63 (35–104)	n.s.	0.02
IL-10	0 (0–0)	236 (100–542)	n.s.	0.03
Growth Factors				
GM-CSF	0 (0–90)	77 (62–100)	n.s.	0.003
VEGF	0 (0–322)	32 (10–82)	n.s.	n.s.
PDGF bb	57 (0–283)	14 (0–26)	n.s.	n.s.
G-CSF	(0–280)	547 (367–1367)	n.s.	0.01
Chemokines				
Eotaxin	0 (0–128)	114 (98–141)	n.s.	0.003
MIP-1α	188 (0–577)	1710 (163–8032)	n.s.	n.s.
MIP-1β	74 (0–377)	465 (152–639)	n.s.	0.02
RANTES	275 (0–955)	123 (57–206)	n.s.	0.05
MCP-1(MCAF)	0 (0–0)	0 (0–0)	n.s.	n.s.

p_1 = Significance of differences between Medium and rAβ_{42} of HC; p_2 = Significance of differences between Medium and rAβ_{42} of AD.

CSF, G-CSF) and some chemokines (eotaxin, MIP-1β, RANTES) when compared to the cells cultured in medium. On the contrary, cells obtained from HC do not show any significant increase in the production of these cytokines.

By analyzing data according both to gender and to AD severity, no differences were observed. As previously stated, AD patients of this subgroup showed the same changes in lymphocyte populations (i.e., B lymphocyte decrease) as well as in the initial group.

DISCUSSION

Many studies have reported alterations of the immune system in AD and the involvement of both the innate and acquired branches of the immune system [3, 12]. In this paper, we report data obtained studying cells and factors involved in immune response in AD patients.

The "peripheral lymphocytes" topic has been examined by different groups with conflicting results [12, 20,26–28], and currently there is no general consensus on the modifications of lymphocyte subsets in AD patients [12]. Our study of AD lymphocyte subpopulations confirms the already described decrease of the absolute number and percentage of B cells when compared to HC lymphocytes [26–28], whereas the other subsets are not significantly different in patients and controls. This decreased number of B cells is already present in elderly when compared to young people [30]. Therefore, it suggests an exacerbation in AD of this feature linked to a reduced output of B cells from bone marrow [30]. This might be due to the well known systemic pro-inflammatory status of AD patients [3, 6–10,31] and present results. On the T cell side, the results reported in different studies are discordant with each other. In fact, whereas our data are in agreement with Speciale et al. [26], Xue et al. [28] show a significant reduction of CD3+, CD4+ and CD8+; Richartz-Salzburger et al. [29] confirm the decrease of CD3+ T cells and CD8+ T cells, but showed a slightly increase of CD4+. On the other hand, as previously stated, dramatic alterations on CD4+ subsets in patients with mild AD have been reported by Larbi et al. [20]. These differences might be the result of methodological differences among the different studies, including inclusion criteria of both AD patients and HC.

However, the finding that the B cell compartment is modified in AD patients compared to HC, together with the increased expression of the chemokine receptor CCR5 on B cells, after stimulation by rAβ_{42}, suggests the involvement of B cells in the complex cellular interactions active in AD patients. On the other hand, rAβ_{42} stimulation does not change the expression of the activation markers CD25 and CD69 on B cells: it is an unexpected result since B cells can produce antibodies against Aβ [32].

On the other hand, we report a good response of T cells to Aβ; in fact the CD69 activation marker is overexpressed in rAβ_{42}-stimulated AD cells when compared to HC stimulated cells. Furthermore, the chemokine receptors CCR2 and CCR5 are overexpressed in AD cells after *in vitro* stimulation by rAβ_{42}. This is an interesting result since the reactivity and ability of T cells to respond to the chemokines that can be produced locally in the brain might explain the migration of T cells across the brain microvascular endothelial cells occurring in AD [12]. It is noteworthy that some activation markers and chemokine receptors are overexpressed in unstimulated AD cells when compared to controls. This is evidence of the pro-inflammatory status of AD [3,6–10,31]. The increase of CD25+ T lymphocytes in cells from AD patients cultured in medium only might suggest an increase of Treg ($CD4^+CD25^{high}$). However, using as marker of Treg FOXP3, Rosenkranz and colleagues [33] did not report their increase in PBMC just collected from AD patients when compared to elderly donors. On the contrary, Larbi and coworkers [20] show that $CD4^+CD25^{high}$ are reduced in PBMC just collected from AD when compared with both young and old donors. Our experimental conditions are different as we observe an increase of basal expression of CD25 on total CD3 cultured for 48 h with medium only. So, in our opinion, the increase of CD3+CD25+ cells in AD patients might be the consequence of the basal inflammatory milieu of AD patients [3,6–10,31].

The increased expression of the scavenger receptor CD36 on monocytes from AD subjects in unstimulated and stimulated cultures is also an intriguing result as circulating monocytes might efficiently bind plasmatic Aβ. That causes the production of cytokines, chemokines, and reactive oxygen species, hence activating the signaling cascade useful for cellular migration, adhesion, and phagocytosis. In addition, the engagement of monocytes might render these cells more efficient in T cell activation [34].

Our data show that, in addition to expressing chemokine receptors, PBMC *in vitro* stimulated by rAβ_{42} are able to produce different chemokines and cytokines, rendering these cells active players in the inflammatory response in AD patients. The study of *in vitro* production of cytokines shows a significantly higher production of inflammatory cytokines IL-1β, IL-6, and TNF-α and of IFN-γ by AD *in vitro* stimulated PBMC. This is not a surprising result as inflammation is a characteristic of AD [3] and a high responder cytokine profile is associated with AD [9,10,32]. We also report an increase of anti-inflammatory cytokines IL-10 and IL-1ra, and we hypothesize that this increase *in vitro* production should balance the higher *in vitro* production of pro-inflammatory cytokines. However, as previously stated, there is an efflux of amyloid from CNS that can prime lymphocytes. Some authors have demonstrated a reduction of both pro- 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 [12,18,19,26]. Methodological differences (mitogen or Aβ stimulation) among the different studies, including inclusion criteria of both AD patients and healthy controls, might explain the great variability of data. Since monocytes are the main source of IL-6 and TNF-α and they possibly bind efficiently Aβ_{42} via CD36, the pattern of cytokine production observed in the present paper is that to be expected.

It has been demonstrated that peripheral T lymphocytes of AD patients produce higher MIP-1α levels than age-matched controls [18]. This observation together with the expression of the MIP-1α receptor CCR5 on the human brain microvascular endothelial cells (HBMEC), might explain the migration of T cells across the blood-brain barrier. 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 [12,18,35–37]. Here we show a higher expression of CCR5 on T and B lymphocytes, and of CCR2 on T cells stimulated *in vitro* by rAβ_{42}, participating in a vicious circle in the brain.

In contrast to these data, in our system we do not observe any significant overproduction of MIP-1α in PBMC *in vitro* stimulated by rAβ_{42}. This discrepancy might be due to the different experimental system since the production-binding of MIP-1α "*in vitro*" or "*in vivo*" was assessed using HBMEC [18]. As previously described by others [38], our AD patients show increased production of RANTES binding CCR5 also, when compared to unstimulated cells of the same donors. Finally, the results of MCP-1 production are very unpredictable in the different individuals of the two subject cohorts, so it is impossible to draw any conclusion.

A higher level of chemokines after stimulation with PHA has been demonstrated by other authors [38]. We have not evaluated the production of cytokines and chemokines from cells stimulated with αCD3 or PHA, but in our system the stimulation with mitogens does

not change the expression of CCR5 and CCR2 on T and B cells.

Finally, cytokine, growth factor, and chemokine production was obtained in a bulk culture preparation as we are interested in an *ex-vivo* model of interaction between different cells and factors. Furthermore, no proliferation assay was performed, because in our system no changes in IL-2 production were detectable after stimulation by r$A\beta_{42}$ in both AD and HC (data not shown).

On the whole, our data demonstrate that immune-inflammatory parameters are modified in PBMC obtained from AD patients and stimulated *in vitro* by r$A\beta_{42}$. Data on involvement of the immune system in AD are controversial and difficult to fully understand. The results obtained in our study can be the mirror of T and microglial abilities in AD patients. Therefore, these data on the activation of peripheral lymphocytes by r$A\beta_{42}$ support the occurrence in AD of the systemic activation of immune-inflammation [31,39,40], and, on the other hand demonstrated by the high level of basal activation. We know that the brain is not an immunologically privileged site since peripheral immune cells can influence local reactions and can be influenced by local immune-inflammatory responses [12].

We contribute new information about soluble factors and cellular ligands that might be involved in the pathogenesis or in the tissue damage observed in AD patients. However, our study is an observational one; hence we cannot draw a conclusion on its contribution to the pathophysiology of the disease. The knowledge that senile plaques, a hallmark of AD (together with neurofibrillary tangles) are formed by Aβ, has suggested the design of immunotherapies with the aim of removing or reducing the senile plaques from the brain. Unfortunately clinical trials of Aβ vaccination of AD patients show an unacceptable rate of meningoencephalitis [41]. On the other hand, intravenous immunoglobulin (IVIg) has been proposed as a potential agent for AD immunotherapy because it contains antibodies against Aβ. Accordingly, a retrospective case-control analysis demonstrated that previous treatment with IVIg is associated with a reduced risk of developing AD [42]. Thus, this kind of study might be useful to obtain biomarkers of AD for monitoring the effectiveness of therapeutic interventions [43].

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=354).

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CHAPTER 5

B cells and immunosenescence: a focus on IgG+IgD-CD27- (DN) B cells in aged humans.

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Review

B cells and immunosenescence: A focus on IgG$^+$IgD$^-$CD27$^-$ (DN) B cells in aged humans

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ABSTRACT

Immunosenescence contributes to the decreased ability of the elderly to control infectious diseases, which is also reflected in their generally poor response to new antigens and vaccination. It is known that the T cell branch of the immune system is impaired in the elderly mainly due to expansion of memory/effector cells that renders the immune system less able to respond to new antigens. B lymphocytes are also impaired in the elderly in terms of their response to new antigens. In this paper we review recent work on B cell immunosenescence focusing our attention on memory B cells and a subset of memory B cells (namely IgG$^+$IgD$^-$CD27$^-$) that we have demonstrated is increased in healthy elderly.

1. Introduction

In human ageing the ability to respond to vaccines and new infectious agents is impaired, mainly because of changes in adaptive immunity mediated by T and B cells. This phenomenon called 'immunosenescence' is influenced by environmental and genetic factors, but also by the antigenic load to which individuals are exposed throughout life, and this has an impact on immune performance in late life (Pawelec and Larbi, 2008; van Baarle et al., 2005).

Immunosenescence materially contributes to the decreased ability of the elderly to respond to new antigens and vaccinations and to control infectious diseases (Gardner et al., 2006; Genton et al., 2006). Indeed, ageing is associated with an increased incidence of infections such as viral influenza, respiratory syncitial virus (RSV) and pneumococcal pneumonia (Nicholson et al., 1997). Elderly people also have an increased incidence of bacterial infections in lungs, urinary tract, skin and other tissues and a higher incidence of tuberculosis and herpes zoster reactivation (Ginaldi et al., 2001). Vaccinations are powerful tools in order to prevent morbidity and mortality from infections in people over the age of 65 years, however, because of the impairment of immune functions with ageing, the currently available vaccines protect only a small proportion of the elderly population (Weinberger et al., 2008).

Although most of the literature on immunosenescence have focused on T cell impairment, B cell compartment is also defective in the elderly: indeed humoral immune response is modified in the elderly both in the quality and quantity of the antibodies produced, and the number of circulating B cells is reduced in the aged (Cancro et al., 2009; Frasca et al., 2010b).

In addition it is known that, B cells have effector and regulatory functions other than antibody production (Sanz et al., 2007; Martin and Chan, 2006; Harris et al., 2000) and memory and naïve B cells can produce different cytokines and chemokines; in particular memory B cells produce high levels of the proinflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α so suggesting that B cells might take part in the generation or in the maintenance of the inflammatory environment of the elderly (Agrawal and Gupta, 2010). Indeed a typical feature of ageing is the pro-inflammatory status observed in the elderly, related to chronic inflammatory diseases. So, ageing increases risk of disability and chronic diseases, with many older adults experiencing multiple chronic conditions in old age. Some evidence exists to support theories that increased health risks in old age are the result of environmental stressors that accumulate over time. These stressors potentially can disrupt the regulation of biological systems, however, not all individuals or population groups seem to be equally susceptible to the effects of stress. Questions remain as which factors mediate biological

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responses to stress or genetic predisposition, lifestyle, or other factors. For example, it is believed that in order to achieve an advanced age, centenarians should be equipped with well preserved and efficient defense mechanisms, optimal combination of an appropriate genetic background and lifestyle (Franceschi et al., 1995). In these subjects, a higher frequency of genetic markers (polymorphisms) associated with a reduced pro-inflammatory ability seems to work against the onset of the main age-related disorders (cancer, dementia, diabetes and cardiovascular diseases) (Franceschi et al., 2007). On this basis, it might be an advantage to have centenarian parents; indeed it has been demonstrated that offspring of centenarians, who are in their 70s and 80s, have a survival advantage when compared to age-matched controls whose parents died at an average life expectancy. Centenarian offspring, like their parents, have genetic and functional advantages associated with lower cardiovascular disease risk (Terry et al., 2004a, 2004b). These findings support the hypothesis that centenarian offspring are inclined to healthy ageing and longer survival, making them a suitable target of ageing studies. Accordingly, a recent paper (Derhovanessian et al., 2010) has demonstrated that the typical hallmarks of immunosenescence are not present in subjects with familiar longevity.

Some diseases such as tumors, autoimmune phenomena, atherosclerosis, heart disease and Alzheimer's disease, are frequent in the late phase of the life and involve the dysregulation of immune parameters and/or chronic inflammation in their pathology (Candore et al., 2008; Vasto and Caruso, 2004). In fact, in frail elderly (i.e. Alzheimer's disease, AD, patients), two recent meta-analyses by our group have highlighted the role of cytokine polymorphisms in AD susceptibility, indicating the role of immune-inflammatory responses in AD (Di Bona et al., 2009, 2008). Furthermore, much evidences suggest the involvement of a systemic immune response in the pathogenesis of AD (Britschgi and Wyss-Coray, 2007; Speciale et al., 2007). Indeed, blood derived cells seem to accumulate in the AD brain (Rogers et al., 1998), while other studies have shown changes in the distribution and reactivity of immune cells in the blood (Pellicanò et al., 2010; Britschgi and Wyss-Coray, 2007; Monsonego et al., 2003; Weksler et al., 2002).

On these basis in this paper we discuss the literature data on B cell immunosenescence and B cell memory focusing our attention on a subset of memory B cells (namely $IgG^+IgD^-CD27^-$) that we have demonstrated is increased in healthy elderly (Colonna Romano et al., 2009), but not in centenarian offsprings (Colonna Romano et al., 2010) and Alzheimer's disease patients (*preliminary observations, see below*).

2. Immune system and ageing

2.1. Inflamm-ageing and T lymphocytes

A typical feature of ageing 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). Indeed, elevated plasma concentrations of IL-6, IL-1β, and TNF-α have been described in elderly populations and were postulated as predictive markers of functional disability, frailty and mortality (Bruunsgaard et al., 2003; Ershler and Keller, 2000; O'Mahony et al., 1998) and it has been suggested that chronic inflammation supports the development and progression of age-related diseases, such as osteoporosis, neurodegeneration and atherosclerosis (Gao and Hong, 2008; Ginaldi et al., 2005; Libby, 2002). Subclinical inflammation may be caused by chronic stimulation of the innate immune system by degradation products and/or by the partial inability of the aged immune system to eliminate certain pathogens (Weinberger et al., 2009), this inflammatory status may slowly damage one or several organs, especially when unfavorable genetic polymorphisms and epigenetic alterations are concomitant, leading to an increased risk of frailty together with the onset of age-related chronic diseases (reviewed by Cevenini et al., 2010 and Vasto et al., 2007). The age-dependent up-regulation of the inflammatory response has been termed "inflamm-ageing" (Franceschi et al., 2000b, 2000c), due to both the chronic antigenic stimulation and the genetic background that render elderly prone to frailty (Balistreri et al., 2008, 2007; Franceschi et al., 2005; Lio et al., 2004; Pes et al., 2004).

T cell immunosenescence has been extensively studied and many details have been clarified (see reviews by McElhaney and Effros, 2009 and Pawelec and Larbi, 2008): the percentage and the absolute numbers of circulating $CD3^+$ T lymphocytes and of $CD4^+$ and $CD8^+$ T cell subsets decreases (Pawelec et al., 2002; Cossarizza et al., 1996; Sansoni et al., 1993) and there is a gradual shift from naïve $CD45RA^+$ to more activated or memory $CD45RO^+$ cells (Pawelec and Larbi, 2008; Pawelec et al., 2002) and it is believed that the decrease in naïve T cell numbers with age is the result of thymic involution in combination with ongoing differentiation of naïve T cells into antigen-experienced memory or effector cells (Appay et al., 2010).

One of the most remarkable qualitative changes in the memory T cell population during ageing is the appearance of clonally expanded $CD8^+CD28^-$ T cells. Analysis of T cell receptor clonotypes, CD28 expression, telomere length and proliferative capacity has suggested that these cytotoxic T cells have reached replicative senescence (Pawelec and Larbi, 2008; Globerson and Effros, 2000). The presence of high proportions of senescent $CD8^+CD28^-$ T cells would impact homeostatic mechanisms regulating the amount of memory and naïve T-cell subsets and hence would reduce the T cells available for an effective antiviral response (Pawelec and Larbi, 2008; Globerson and Effros, 2000; Fagnoni et al., 1996). These features are caused by persistent life-long antigenic stress that leads to the marked shrinkage of T cell repertoire diversity with age (Pawelec and Larbi, 2008; Pawelec et al., 2002; Wack et al., 1998). Moreover, it has been recently observed that the evaluation of the number of $CD8^+CD28^-$ T cells is not merely an "immunological curio" as it correlates with frailty (Semba et al., 2005) and with an impaired response to influenza vaccination in the elderly (Weng et al., 2009). It has been proposed that CMV infection leads to the described changes of CD8 T cells in the elderly, causing both the shrinkage of the TCR repertoire and the accumulation of $CD8^+CD28^-$ effector T cells. These cells can further stimulate the inflammatory processes by IFNγ production as suggested (Almanzar et al., 2004) although others (Ouyang et al., 2003) have demonstrated that these cells are dysfunctional. The reduction of the costimulatory molecule CD28 has also been reported on CD4 T cells that show a defect in CD154 (CD40L) expression too, this in turn causes the reduced ability of CD4 T cells to provide help to B cells for both proliferation and Ig production.

Other authors have described a significant decrease of $CD8^+$ T cells with no significant changes in $CD4^+$ T cells with ageing, leading to an unexpected increase in the CD4:CD8 ratio (Gruver et al., 2007; Yan et al., 2010).

2.2. B lymphocytes

Although T cell alterations play a significant role in age-related immune changes, alterations in B cells also occur both in human and mice, indeed advanced age is accompanied by substantial changes in all B cell compartments and, consequently, humoral immune function. These changes include shifts in the magnitude of all B cell compartments, specificity repertoire changes, modified peripheral B cell dynamics, and weakened humoral responses (Miller and Cancro, 2007).

Before leaving the bone marrow, B cells pass through several stages of development and, any change in the different steps will result in a change in overall repertoire. In mice there is a decline in bone marrow output and impairments in haematopoietic stem cell (HSC) commitment to the B lineage (Guerrettaz et al., 2008; Miller and Allman, 2003), which is manifested as a deficit in the numbers of early B cell populations such as the pro, pre, transitional and mature naïve B cells. Others have suggested that it is not the bone marrow output by itself that is crucially impaired but rather the ability of naïve B cells from older mice to colonise peripheral compartments that is reduced (Johnson et al., 2002). Even though the reduced influx of fresh naïve cells into the periphery, the total numbers of peripheral B cells in mice remain the same (Miller and Cancro, 2007). There are several mechanisms that can explain this anomaly. It has been demonstrated that splenic B cells in old mice have a reduced turnover as compared to splenic B cells from young mice, and so live longer (Kline et al., 1999), this increased longevity may signal to the newly made B cells that the niche is full and requires no more cells (Minges Wols et al., 2009).

In contrast to mouse model, in humans there is a decrease both in percentage and absolute number of total $CD19^+$ B lymphocytes (Veneri et al., 2009; Faria et al., 2008; Frasca et al., 2008; Shi et al., 2005; Chong et al., 2005; Colonna Romano et al., 2003, 2002; Breithart et al., 2002; Huppert et al., 1998; Wikby et al., 1994; Paganelli et al., 1992). Moreover, it has been demonstrated that absolute number of B cell precursors in the bone marrow declines with age and particularly during adolescence (McKenna et al., 2001), however, B lymphopoiesis persists throughout adult life (Rossi et al., 2003). In particular, in mice ratios between precursors and immature B cells within the bone marrow and mitotic activity were found to be remarkably constant during ageing (Riley et al., 1991).

As mentioned above, in elderly people there is a shrinkage of the T cell repertoire due to the chronic stimulation of the immune system by antigens and persistent infection by herpetic viruses such as CMV (Pawelec and Larbi, 2008; Vasto et al., 2007). On the B cell side, changes in the B cell repertoire have also been described and in old mice it has been reported a different usage of both V_H and V_L gene families that those used by young animals (Nicoletti et al., 1991). Old mice have also been shown to have altered repertoires in response to challenge with the hapten phosphorylcholine (Nicoletti et al., 1993, 1991) and, as in humans (see below), the response in young mice was oligoclonal, dominated by use of one particular immunoglobulin heavy chain variable region (IGHV) gene, whereas older mice had a more diverse response. Moreover, experiments using young and old donors to reconstitute immune-deficit SCID mice, suggest that these differences arise from B cell intrinsic factors, although the cytokine environment might also play a role (Shriner et al., 2006). In murine models it has also been reported the impaired expression of the surrogate λ chain and the increased usage of the VhS107 family that can also have consequences in the formation of the B cell repertoire (Alter-Wolf et al., 2009a). Some reports on B cell repertoire indicated that older mice showed evidence of non-malignant clonal expansion (LeMaoult et al., 1997), which in view of the homeostatic mechanisms that maintain the total numbers of B lymphocytes would lead to a decrease in diversity.

In humans after pneumococcal immunization the analysis of the V_H chain repertoire has shown differences between young and elderly donors, a loss of oligoclonality and a reduced frequency of somatic mutation in the elderly (Kolibab et al., 2005). The same group also analysed the V_L chain repertoire in response to the same immunization and has demonstrated significant differences in V_L gene usage between young and old subjects: in fact the elderly preferentially use Vk4 in response to pneumococcal polysaccharide 4 and 14, while young donors use predominantly Vλ3 and/or Vλ1 genes, moreover oligoclonality of light chains was not so regular in the elderly as it has been demonstrated in the heavy gene usage and no detectable changes in the frequency of mutations have been reported for the L genes studied (Smithson et al., 2005). However the authors observe that these might be recall responses as adults have significant levels of antibodies against pneumococcal antigens before the treatment with the vaccine (Kolibab et al., 2005; Smithson et al., 2005).

Previous studies, concentrating on IGHV gene use, have not always shown significant age-related differences in the pre-immune B cell repertoire (Kolar et al., 2006; Wang and Stollar, 1999), indeed Banerjee et al. (2002), have shown that the somatic hypermutation process occurs at the same rate in young and old humans, and so the increased levels of mutations in Ig genes found in older people by others (Chong et al., 2003) is more likely a consequence of accumulation rather than altered rate. The consequence might be the collapse in B cell diversity in some elderly individuals that, as it has been recently demonstrated using the CDR3 spectratyping, is correlated with poor health status in the elderly (Gibson et al., 2009). A schematic illustration is shown in Fig. 1.

On the whole, it seems that the antibodies generated in old humans are less protective compared to the antibodies generated in young ones, as shown by their reduced ability to opsonize in vitro after vaccination with bacteria-derived polysaccharides (Schenkein et al., 2008). Moreover the anti-influenza response is reduced in the elderly after vaccination (Weinberger et al., 2008; Murasko et al., 2002) and there are also diminished recirculating long-lived antibody-secreting plasma cells in the bone marrow (Zheng et al., 1997; Manz et al., 1997). This might be related to a poor persistence of effective antibodies after vaccination in the elderly although care needs to be taken in interpretation here since the current data is limited and is from different vaccine formulations such as live-attenuated vaccines (e.g. against polio and measles) or killed microorganisms or toxoids (e.g. against tetanus) which may affect data on persistence.

In mice, some reports suggest that, with ageing, the levels of total and specific serum IgG and IgM increase (Koga et al., 2000), while other authors found a reduction in specific IgG and IgM in aged animals (Faria et al., 1998). Speziali et al. (2009), have recently shown that basal levels of total serum immunoglobulins, IgG and IgA were increased while antigen-specific immunoglobulins, IgG and IgA were reduced in aged mice compared to young ones. This divergence between total and specific levels of immunoglobulins, IgG and IgA may reflect the biased repertoire of B cells in aged mice (Koga et al., 2000) and their inability to mount specific immune responses to new antigens. Moreover it has been shown that ageing is followed by an increased production of auto-antibodies in parallel with a diminished production of antibodies against foreign antigens (Weksler and Szabo, 2000). The skewed repertoire of B and T cells in aged animals probably result from the expansion of a limited number of memory cells that compensate for the reduction in the output of naïve B and T cells from bone marrow and thymus. The increased levels of non-specific immunoglobulins in aged mice seems to result from activation of memory cells already selected by previous encounters with antigens (Speziali et al., 2009). On other hand, both basal levels and antigen-specific IgM were not altered in old mice (Speziali et al., 2009) suggesting that class-switch-independent IgM responses, being low affinity reactions, are less affected by repertoire limitations. Concerning the effects of ageing on the number of immunoglobulin-producing cells in the bone marrow, Speziali et al. (2009) have shown that IgG, IgM and IgA immunoglobulin-producing cells were increased in aged mice and these results are coherent with the levels of serum immunoglobulins found, except for IgM that were unaltered in old mice. Frasca et al. (2004, 2007), have shown that in vitro stimulated splenic B cells from senescent mice are deficient in

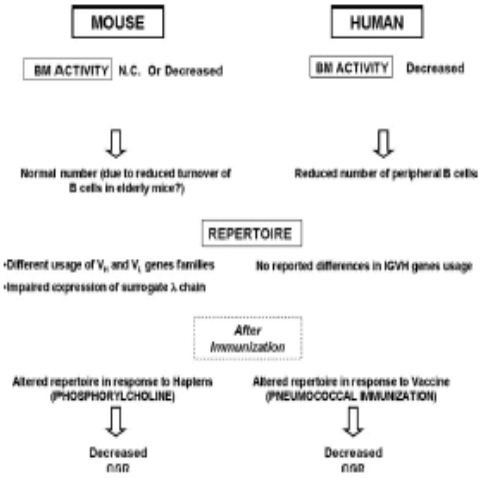

Fig. 1. Bone marrow activity and B cell repertoire in elderly mice and humans compared with young individuals. N.C.: no change; and CSR: class switch recombination.

the production of multiple class switch isotypes and that deficiency is correlated with decreased induction of transcription factor E47, poor activation-induced cytidine deaminase (AID) expression and consequent down-regulation of class switch recombination. As others (Speziali et al., 2009) have reported increased levels of post-switch immunoglobulin isotypes as well as a rise in the number of IgA- and IgG-producing cells in aged mice, it could be speculate that this augment may be due to the proliferation of memory B cells, rather than activation of naïve cells. Accordingly Frasca et al. (2004), have demonstrated that B cell proliferation seems not to be affected by ageing. Increase in proliferation of already activated B cells may also explain why total but not antigen-specific IgG and IgA levels were found higher in older animals.

Also in aged humans a paradox exists in B cell biology; although there is a reduced number of peripheral B cells, and a reduced ability to produce antibodies "in vitro" (Frasca et al., 2008), the amount of "switched" Ig in the serum of elderly and centenarians is increased (Listi et al., 2006; Paganelli et al., 1992). Indeed, there is an age-related increase of IgG and IgA serum levels, whereas IgM level decrease or remain unchanged and IgD decrease with age (Listi et al., 2006; Paganelli et al., 1992). However, in vitro stimulation of human peripheral blood B cells by anti-CD40 and IL-4 induces a lower production of IgG from elderly donors, correlated to a reduction of the transcription factor E47 and AID in old people (Frasca et al., 2008). Moreover the same authors have recently demonstrated that the level of AID in response to polyclonal stimulation by CpG can predict the size of the response to influenza vaccination (Frasca et al., 2010a).

There are still many contradictory observations on B cells and antibodies with age, as shown in Table 1.

3. Markers of memory B lymphocytes

Memory lymphocytes are crucial cells in the immune system: facilitating a recall (anamnestic) response to previously encountered antigens. The "memory topic" is an interesting field for immunologists involved in ageing studies, as it has been suggested that the filling of the immunological space with memory T cells renders the immune system less able to respond to new antigens (Franceschi et al., 2000a). On the other side, the ability to produce memory cells is essential for effective vaccination (Weinberger et al., 2008).

Memory B cells can be discriminated from naïve ones by the presence of somatic hypermutations in the variable gene sequences of their Ig (Klein et al., 1998). Moreover, to easily discriminate between naïve and memory B cells, phenotypic markers as surface immunoglobulins (IgD, IgM, IgG, IgA) and CD27 are currently used. Previously, IgD^+ cells were classified as naïve cells and IgD^- cells as memory B cells (Black et al., 1978), however, the description of an IgD^+ subset expressing somatic hypermutations of the Ig genes discontinued its use for the unequivocal identification of naïve and memory B cells (Klein et al., 1998).

Also CD27 was commonly used as a marker of human memory B cells, because its expression is correlated with the presence of somatic hypermutations in Ig genes (Agematsu et al., 2000), in spite of this, many authors have recently demonstrated the presence in the blood of memory B cells that lack CD27 (Colonna Romano et al., 2009; Frasca et al., 2008; Wei et al., 2007; Fecteau et al., 2006; Anolik et al., 2004). The disconnected expression of CD27 and IgG has been demonstrated by Fecteau et al. (2006), and we found the same results in our samples evaluating the expression of CD27 on gated IgG^+ magnetically sorted B cells, or the expression of IgG on gated $CD27^+$ or $CD27^-$ magnetically sorted B lymphocytes. Indeed, not all IgG^+ cells were $CD27^+$, in addition the $CD27^-$ B population also contains a small proportion of IgG-switched B cells (unpublished observations).

So naïve B cells are identified as $IgG^-IgA^-IgD^+CD27^-$, whereas the memory B cell population seems to be very heterogeneous, comprising three types: "IgM memory" cells that are $IgD^+IgM^+CD27^+$ (Klein et al., 1998), also identified as IgD^+CD27^+ "unswitched memory" by Shi et al. (2005), "classical" switched memory IgG^+/IgA^+CD27^+, and the IgG^+/IgA^+CD27^- B cells (Fecteau

Table 1
Main modifications of B cells and B cells products in elderly human.

B cells or B cells products	Changes	References
Total B cells (percentage)	Decrease	Faria et al. (2008) (dramatic decrease in over 75 years old), Franca et al. (2008), Colonna Romano et al. (2003), Wikby et al. (1994), Paganelli et al. (1992)
Total B cells (absolute number)	Decrease	Veneri et al. (2009), Franca et al. (2008), Shi et al. (2005), Chong et al. (2005), Breitfuart et al. (2002), Huppert et al. (1998), Wikby et al. (1994), Paganelli et al. (1992)
$CD19^+CD5^+$ B1 cells (percentage and absolute number)	Increase Decrease	Weksler (2000) Colonna Romano et al. (2002)
Total Ig [after specific immunization]	No change [Decrease]	Listl et al. (2006), Chong et al. (2005), Globerson and Effros (2000), Weksler (2000), Weksler and Szabo (2000) [Weksler, 2000; Franca et al., 2005]
IgG (/ml) [after specific immunization]	No change Increase [Decrease]	Chong et al. (2005) Franca et al. (2008), Listl et al. (2006), Paganelli et al. (1992) [Franca et al., 2005, 2008]
IgA	No change Increase	Chong et al. (2005) Listl et al. (2006), Paganelli et al. (1992)
IgM [after specific immunization]	Decrease No change [Decrease]	Listl et al. (2006), Chong et al. (2005) Paganelli et al. (1992) [Shi et al., 2005]
IgG [after specific immunization]	No change [Decrease]	Listl et al. (2006), Chong et al. (2005) [Franca et al., 2005]
IgD	Decrease	Listl et al. (2006)
Autoantibodies	Increase	Globerson and Effros (2000), Weksler (2000), Weksler and Szabo (2000), Candore et al. (1997)

et al., 2006), called also double negative (DN) B cells due to the lack of both IgD and CD27 (Colonna Romano et al., 2009).

Following antigenic challenge naïve B cells can differentiate into low-affinity Ig-secreting cells or mature within a germinal center into high-affinity memory cells expressing different Ig isotypes (Wolniak et al., 2004). Classically T cell help is required for memory B cell generation, germinal center formation, isotype switching and somatic hypermutation, and helping is, mainly provided following the binding of CD154 (CD40L) expressed on T cells with CD40 expressed on B cells (Wolniak et al., 2004). The importance of CD40 stimulation in these events has been demonstrated in X-linked hyper IgM syndrome patients, in which CD40–CD154 interaction is defective (Lougaris et al., 2005). In these patients, germinal center formation and the memory B cell response are strictly impaired, although a peripheral B cell fraction expressing CD27 persists (Weller et al., 2001), supporting the hypothesis that this marker does not exclusively identify classical germinal center-derived memory B cells: this $CD27^+$ (also IgM^+IgD^+) compartment was recognized as the peripheral counterpart of splenic marginal zone (MZ) B cells (Weller et al., 2005). Although expressing mutated Ig genes, this subset differs from classical memory B cells, since hypermutation can occur in absence of CD40–CD154 interactions and the two populations have a different Ig gene repertoire (Weller et al., 2005; Wu et al., 2010). Indeed, unlike classical memory B cells, the $IgM^+IgD^+CD27^+$ subset is dedicated to immune responses against T-independent antigens (Weller et al., 2004; Kruetzmann et al., 2003; Spencer et al., 1998) during which they act as innate effectors in the first line of defense but not as memory cells (Pillai et al., 2005, 2004; Weller et al., 2005, 2004).

On the other hand, the requirement for CD27 expression on classical memory B cells has never been firmly established in humans (Tangye and Hodgkin, 2004). Indeed, as reported by Fecteau and Néron (2003), prolonged CD40 stimulation triggered naïve B cells to switch to IgG and to express CD27 even in absence of somatic hypermutation, suggesting that these events could be independent. The CD27 molecule in mice, rather than a memory marker, appears to be expressed in recent B cell activation and is not absolutely necessary for secondary responses (Xiao et al., 2004). These results suggest that CD27 is not "the" marker of memory B cells and that the identification of naïve and memory B cells is not strictly related to this marker.

It has been reported (Anolik et al., 2004), that patients with systemic lupus erythematosus (SLE) show an increase of DN B cells that is correlated with the disease activity index (Wei et al., 2007). The Authors report that these B cells, that are IgG or IgA positive, show a degree of hypermutation of V_H gene comparable to $CD27^+$ unswitched memory cells. Moreover they suggest that these $CD27^-$ memory B cells could represent either progenitors, or the progeny, of $CD27^+$ memory cells that fail to go through a productive germinal center reaction. Thus the hypothesis that $CD27^-$ memory B cells might develop outside the germinal center, perhaps in extrafollicular reactions capable of supporting hypermutation as was demonstrated in mice (William et al., 2002). Interestingly, dendritic cells have been shown to activate extrafollicular B cells and are also known to induce isotype switching in a CD40-independent way through BLys–BAFF-R interaction (Qi et al., 2006) so this could be also the case for $CD27^-$ memory B cells. In our previous paper (Colonna Romano et al., 2009), we report that DN cells show low expression of the CD40 molecule, so these might not cooperate with T cells. Indeed, the finding that IgG^+ memory B cells in mice can be generated following T cell-independent responses corroborates the likelihood that this mechanism is also active in humans (Obukhanych and Nussenzweig, 2006).

Alternatively, $CD27^-$ cells might represent activated follicular cells that initiate the germinal center reaction after receiving early CD154-mediated T cell help, but fail to progress through this pathway, so explaining their failure to acquire CD27 and their lower rate of somatic hypermutation as compared with $CD27^+$ classical memory B cells. Given that CD27 interacts with CD70 on activated T cells, it is believable that the absence of CD27 might impair the ability of these cells to receive the complete and persistent degree of T cell help required to complete a germinal center reaction (Toellner et al., 2002). Interestingly, somatic hypermutation in the absence of CD27 is present in two different B cell tumors as Waldenstrom's macroglobulinemia and hairy cell leukemia, for which an extra-germinal center derivation has been proposed (Kriangkum et al., 2004; Forconi et al., 2004).

Moreover, to investigate whether IgG^+CD27^- cells corresponded to post-germinal center cells or not, Fecteau et al. (2006)

evaluated their frequency of somatic hypermutation compared to IgG$^+$CD27$^+$ classical memory B cells. IgG$^+$CD27$^-$ had significantly lower mutation levels than did IgG$^+$CD27$^+$ cells. The low frequency of somatic mutations in IgG$^+$CD27$^-$ cells may support the theory that this population could emerge independently from T cell help or from CD40–CD154 interaction in humans (Weller et al., 2001).

4. Naïve and double negative B lymphocytes in healthy aged, centenarian offsprings and Alzheimer's disease patients

Humoral memory is generally viewed as supported by two cellular compartments: the effector memory compartment, represented by antibody-producing plasma cells and the central memory one, represented by memory B cells that are precursors capable of generating and replenishing the plasma cell compartment. However, 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 et al., 2007; Martin and Chan, 2006; Harris et al., 2000) indeed it has been demonstrated that memory B cells produce great amounts of proinflammatory cytokines and so might play a role in the generation or in the maintenance of the inflammatory environment of the elderly (Agrawal and Gupta, 2010). In consideration of this and also in the light of the inflammatory environment in successful and unsuccessful ageing, here we focus on literature data of the naïve/memory compartment in the elderly. In previous papers we (Colonna Romano et al., 2009, 2003) and others (Gupta et al., 2005) have demonstrated a reduction of naïve CD27$^-$ B cells in the elderly and no significant reciprocal increase in CD27$^+$ memory B cells (Klein et al., 1998; Agematsu et al., 2000). On the other hand, Shi et al. (2005) have shown that CD27$^+$ memory B cells, particularly the IgD$^+$IgM$^+$CD27$^+$ "IgM memory" B cells, decline dramatically in elderly subjects. These data are in agreement with reduced output of B cells. Besides, as it has been proposed that IgM memory B cells are involved in the response to pneumococcal infection, this reduction is considered responsible for the increased susceptibility to bacterial infections. Furthermore, other groups (Frasca et al., 2008; Shi et al., 2005) have demonstrated the increase of naïve B cells in the elderly. Frasca et al. (2008), described peripheral blood naïve B cells [CD19$^+$CD27$^-$IgG$^-$IgA$^-$ (presumably IgM$^+$)] by lack of switched receptor rather than by the presence of IgD; they report the increase of the percentage and no changes of the absolute number of these cells in the elderly. As we have reported (Colonna Romano et al., 2009) the decrease of naïve B cells in the elderly (identified as IgD$^+$CD27$^-$) and the increase of IgD$^-$CD27$^-$, and in order to better characterize the DN B cells (IgD$^-$CD27$^-$), we are studying the expression on these cells of IgG and IgM. Our preliminary results show that, in our samples, the percentage of IgG$^-$IgM$^+$ B cells is a very small fraction (~5%) of total IgD$^-$CD27$^-$ DN B cells. However, further analysis (e.g. the expression of IgA) and the specific study of these cells in the elderly are necessary to better understand how this very small fraction changes with ageing, and whether the change of these cells might influence the amount of "naïve" B cells identified as IgG$^-$IgA$^-$CD27$^-$. In the same paper (Frasca et al., 2008) and in a following published review (Frasca et al., 2010b) they report the decrease of both percentage and absolute number of switched memory B cells (IgG$^+$/IgA$^+$/CD27$^+$ or CD27$^-$). We never found this result evaluating B memory cells as IgD$^-$CD27$^+$ or CD27$^-$ although, due the reduced number of total B cells in the elderly, the absolute number of memory B cells is reduced also in our old subjects.

Centenarian offspring (CO) can be considered people who might be genetically advantaged for successful ageing, and Alzheimer's disease patients as a model of unsuccessfully aged (Terry et al., 2004a, 2004b; Derhovanessian et al., 2010; Di Bona et al., 2009, 2008).

Table 2
Analysis of IgA, IgG and IgM serum concentrations in centenarian offspring (n = 29, age range 59–83) and age-matched controls (n = 25, age range 60–85). The serum of all subjects was stored in aliquots at −80 °C until analysis and the immunoglobulin concentrations were determined by Integra 800 (Roche Diagnostics, Milan, Italy) as described (Colonna Romano et al., 2010). Data are expressed as mean ± SD. Significance has been evaluated by ANOVA Test (values statistically significant when $p < 0.05$).

Serum immunoglobulin concentrations (mg/dl)	Centenarian offspring (n = 29) [mean ± SD]	Age matched controls (n = 25) [mean ± SD]	p
IgA	250 ± 25	280 ± 25	0.2
IgG	1080 ± 120	950 ± 115	0.4
IgM	150 ± 20	80 ± 20	0.0004

We do not show any significant changes, neither in percentage nor in absolute number of B cells, between centenarian offspring and their "normal" age-matched controls (Colonna Romano et al., 2010). However, Alzheimer's disease patients show a significant reduction in B cells (both in percentage and absolute number) when compared with healthy elderly (Pellicanò et al., 2010; Speciale et al., 2007). As mentioned looking at different B cell subsets, we have shown a significant decrease of the percentage of IgD$^+$CD27$^-$ naïve B cells in old compared to young donors (42.2 ± 3.6 in young, 33.3 ± 2.7 in elderly, $p < 0.05$; data are expressed as mean ± SEM), no differences in unswitched (IgD$^+$CD27$^+$) and switched (IgD$^-$ CD27$^+$) memory compartment and a significant increase of the percentage of IgD$^-$CD27$^-$ DN B cells (6 ± 0.6 in young, 15.8 ± 1.4 in elderly, $p < 0.0001$) (Colonna Romano et al., 2009). Our data in centenarian subjects shows the same trend of elderly people of naïve (47.6 ± 8.4) and IgD$^-$ CD27$^-$ (11.1 ± 4.7) B lymphocytes. In order to gain insight in the biological relevance of B cell subset age-related changes, we have compared (Colonna Romano et al., 2010; Bulati et al., 2008) memory/naïve B cell populations in centenarian offsprings to those in old age-matched control. In the former, we do not observe the typical naïve-memory shift observed in the elderly and in centenarians. Indeed, as previous demonstrated, IgD$^+$CD27$^-$ naïve B cells are significantly increased (50.3 ± 3.3, $p = 0.05$) in CO when compared to age-matched controls, while the IgD$^-$ CD27$^-$ DN B cells are significantly reduced (7.4 ± 0.9, $p = 0.003$). So, B cell subsets of CO are similar to those observed in young subjects previously described (Colonna Romano et al., 2009). This observation is strengthened by serum immunoglobulin measurement. In fact, the concentration of IgM, a marker of the primary response, shows significant higher levels in centenarian offspring when compared to age-matched controls, whereas IgG and IgA levels are not significantly different (Table 2) (Colonna Romano et al., 2010). Also in this case, the values are within the range of the level observed in young subjects (Listi et al., 2006) supporting the "youth" of B cell branch in these subjects.

Concerning the model of unsuccessful ageing, we are studying the distribution of B lymphocytes subsets in AD patients, comparing the results to data obtained in age-matched healthy controls. Our preliminary results show that no significant differences in the IgD$^+$CD27$^-$ naïve B cells compartment between the two groups, as well as in unswitched (IgD$^+$CD27$^+$) and switched (IgD$^-$ CD27$^+$) memory pool, are observed while there is a significant decrease of IgD$^-$ CD27$^-$ DN B cells in AD compared to age-matched controls (6.1 ± 0.7 vs. 15.8 ± 1.4, $p < 0.0001$). The finding that this population is not increased in AD subjects, as in age matched healthy controls, together with the observation that all others B cell subsets are unmodified, although both the percentage and the absolute number of B cells are decreased (Pellicanò et al., 2010), need further

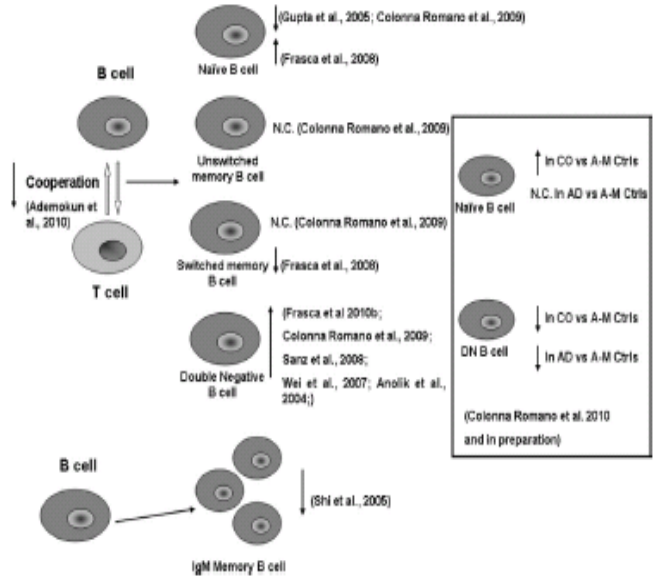

Fig. 2. Naïve and memory B cell compartment in humans as reported by different authors N.C.: no change; CO: centenarian offspring; AD: Alzheimer's disease patients; and A-M Ctrls: age-matched controls.

investigation. Moreover, this is an interesting result, as Speciale et al. (2007) have shown a reduction of $CD8^+CD28^-$ T cells in patients affected by AD. These cells, as mentioned, are increased in elderly and are considered to be exhausted late memory effector cells (Pawelec and Larbi, 2008). So, this might support the idea that DN B cells are also exhausted cells. The reported data are summarized in Fig. 2.

5. Conclusions

It has been shown that both T cell-mediated cellular and B cell-mediated humoral immune responses decrease in aged humans (Cancro et al., 2009; Gibson et al., 2009; Pawelec and Larbi, 2008; Pawelec et al., 2005, 2004; Linton and Dorshkind, 2004). This leads to increased frequency and severity of infectious diseases and reduces the protective effect of vaccinations. Despite intensive research work, many of the basic mechanisms of age-related immune dysfunction have not yet been clarified. However, because the number of elderly people is increasing in the world, identification of the causes and impact of immunosenescence may offer the possibility to improve prevention or delay some infectious diseases and improve the quality of life in our ageing population. Although B cells may suffer from lack of adequate T cell help in ageing (Ademokun et al., 2010), it is now clear that intrinsic changes in B cells also occur and have a significant impact on antibody production. Indeed, with ageing, in the B cell lineage there are reduction in the functional capacities of B cells and their progenitors, changes in the sizes of different subsets and shift in the diversity and clonotypic composition of the antigen-responsive repertoire (Cancro et al., 2009).

A memory B cell subpopulation lacking CD27 and IgD expression and IgG^+ is expanded in elderly people (Colonna Romano et al., 2009). This result, although without statistical significance has also been reported by others (Frasca et al., 2010b). So, the question remains: Where do these $IgG^+IgD^-CD27^-$ B cells come from and why are increased in healthy elderly? One hypothesis is that these cells could be senescent memory B cells that have down-modulated their expression of CD27, as demonstrated for T cells against chronic antigenic stimuli, e.g. herpetic viruses that fill the immunological space with exhausted antigen-specific CD8 memory T cells (Appay et al., 2002; Pawelec et al., 2004, 2005; Akbar and Fletcher, 2005) This could be also the case in the B cell compartment. On the other hand, it has been demonstrated that chronic stimulation of the immune system, as in patients with lupus (Anolik et al., 2004), is related to the expansion of these cells and that the amount of double negative B cells correlates positively with the clinical manifestations of SLE (Wei et al., 2007). Increased IgD^-CD27^- DN B cells has been also described in healthy subjects challenged with RSV (Sanz et al., 2008), and this feature may confirm the important role of the chronic antigenic load. So, the expansion of these memory B cell populations might be the manifestation of a physiologic modification time-related (elderly people) or a pathologic deregulation (SLE patients) of the immune system. Furthermore, it is known that elderly produce increased levels of antibodies to autologous antigens often accompanied by autoimmune phenomena (Candore et al., 2007, 1997; Banerjee et al., 2002) and are less able to make high-affinity antibodies to foreign antigens. Indeed, increased $CD5^+$ B lymphocytes, that, as known, play a key role as producers of autoantibodies (Dalloul, 2009; Youinou et al., 1988), have been demonstrated in old humans (Weksler, 2000) and mice

(Alter-Wolf et al., 2009b), in lupus mouse models (Zhong et al., 2009) and in human rheumatoid arthritis (Nakiri et al., 2007). However in our previous paper (Colonna Romano et al., 2003) we found an age-related decrease of CD5+ B cells, The intriguing aspect is that, in our elderly population, there is an increase of the IgD−CD27− DN B cells, and one hypothesis might be that these cells could be responsible for production of autoantibodies or cytokines that lead to an imbalance of the mechanism controlling the immune response against self antigens. Accordingly to their deregulatory feature, IgD−CD27− B cell were found increased also in hairy cell leukemia patients (Forconi et al., 2004).

Another hypothesis about the origin of these memory CD27− B cells is that supported by Wei et al. (2007). They suggested that these cells fail to go through a productive germinal center reaction. So they might develop outside the germinal center in extrafollicular reactions capable of supporting somatic hypermutation and isotype switching in a CD40-independent manner. In agreement, it has been demonstrated that ageing negatively affects germinal center formation in secondary lymphoid tissues (Frasca et al., 2005). So it could be postulated that the increase of the double negative memory B cell population observed in the elderly, but also in successfully aged centenarians, is due to an atypical formation of these cells in extrafollicular reactions. In particular DN B cells obtained from healthy elderly people, show a lower rate of somatic hypermutation (manuscript in preparation), so the reduction of the rate of mutation might be due to a total disconnected generation of these cells, in elderly, from either germinal centers or T cell help. In view of the lack of CD27 expression and the low rate of somatic mutation in IgG+CD27− B cells, here shown and reported by others (Anolik et al., 2004; Fecteau et al., 2006), it has been hypothesized that IgG+CD27− B cells might be a first wave of memory B cells (Blink et al., 2005; Inamine et al., 2005) or a pool of short-lived memory B cells (Dorner and Radbruch, 2005), in contrast to the IgG+CD27+ B cells that could be more related to long-lived memory B cells. Alternatively DN B cells might represent activated follicular cells that initiate germinal center reaction, after receiving early CD154-mediated T cell help, but fail to progress through this pathway. This could explain their failure to acquire CD27 and their low rate of somatic hypermutation as compared with classical CD27+ memory B lymphocytes (Fecteau et al., 2006; our manuscript in preparation).

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. In particular, the reservoir of naïve B cell might be one of the causes that make centenarian offspring able to keep fighting off new infections, hence prolonging their life (Colonna Romano et al., 2010). So, on the whole, naïve B cells are well preserved in centenarian offspring, suggesting a good bone marrow cell reservoir. This is an interesting observation, as it has been recently reviewed (Cancro et al., 2009) the bone marrow ability to generate B cells is impaired with age. This could be the reason why in these subjects there are less IgD/CD27 double negative B cells than their age-related counterpart that are not genetically advantaged. Becoming centenarians, their immune system, that allowed them to reach longevity, also proceeds versus exhaustion.

We also show that in old patients affected by Alzheimer's disease, the DN B cells are not increased as in their age-matched controls. The explanation of this is not obvious, but others have reported the decrease of effector CD8+CD28− T cells in AD patients (Speciale et al., 2007). Together these data support the hypothesis that DN B cells might be exhausted/terminally differentiated B memory cells. In fact they behave as CD8+CD28− T cells, i.e. are increased in elderly and centenarians, not increased in centenarian offspring (manuscript in preparation) and in AD patients (Speciale et al., 2007). The reduction on total B cells and the increased expression of the chemokine receptor CCR5 after stimulation with Aβ42 on B cells from AD patients, suggest us the participation of B cells in the complex cellular interactions active in AD patients (Pellicanò et al., 2010). Most of studies on frailty have focused on T cells, Semba et al. (2005) have demonstrated that the increase of CD8 T cells that lack CD28 is related to frailty, the same has been reported in the impaired response to vaccines (Weng et al., 2009). Moreover De Fanis et al. (2008) have related the amount of CCR5+ T cells with frailty, and suggest that this correlation might be due to the proinflammatory properties of these cells and the well known link between frailty and chronic inflammation. On the other side it has been demonstrated that B cell repertoire diversity decreases in the elderly with poor health status and this is, to the best of our knowledge the sole report on frailty and B cells in the elderly.

As it is known that memory and naïve B cells produce different cytokines and express different Toll-like receptors, they might have a regulatory role in the chronic infections against viruses and bacteria also producing cytokines and chemokines. This suggests that the immune-inflammation is also related to the B cell branch of the immune system in aged people (Pellicanò et al., 2010; Pasare and Medzhitob, 2005; Duddy et al., 2004; Schultze et al., 1999; Pistoia, 1997). So further studies are necessary to evaluate whether DN B cells in elderly might have some immunomodulatory role as cytokines production.

All together data obtained evaluating DN B cells in healthy elderly, centenarians, centenarian offspring and AD patients might be relevant in the field of immunosenescence and in studies of successful and unsuccessful ageing.

Hence it is important to study the complex process of ageing of the immune system and characterize the changes in both innate and adaptive immunity in order to elaborate better vaccination strategies for the prevention of infectious diseases in the elderly. So, B cell subset changes, associated also to their different regulatory role, could represent a hallmark of successful or unsuccessful ageing and could be used as a biomarker of human life span, potentially useful for the evaluation of anti-ageing treatment to improve the quality of life of the growing elderly population.

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CHAPTER 6

B cell immunosenescence: different features of naive and memory B cells in elderly.

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RESEARCH PAPER

B cell immunosenescence: different features of naive and memory B cells in elderly

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Abstract Elderly people show a reduced protection against new infections and a decreased response to vaccines as a consequence of impairment of both cellular and humoral immunity. In this paper we have studied memory/naïve B cells in the elderly, evaluating surface immunoglobulin expression, production of the pro- and anti-inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-10, and presence of somatic hypermutation, focusing on the $IgG^+IgD^-CD27^-$ double negative (DN) B cells that are expanded in the elderly. Our results show that naïve B cells from young donors need a sufficiently strong stimulus to be activated "in vitro", while naïve B cells from old subjects are able to produce IL-10 and TNF-α when stimulated "physiologically" (α-CD40/IL-4), suggesting that these cells might play a role in the control of the immuno-inflammatory environment in the elderly. In addition, in the elderly there is an accumulation of DN B cells with a reduced rate of somatic hypermutation. Thus, DN B lymphocytes may be exhausted cells that are expanded and accumulate as a by-product of persistent stimulation or impaired germinal center formation.

Keywords Cytokines · Elderly · Hypermutation · Inflammation · Memory B cells

Introduction

During ageing the humoral immune response is both quantitatively and qualitatively diminished compared with the immune response in young people. Many authors have reported a reduced antibody specificity, affinity, and isotype switch in the elderly (Weksler 2000; Frasca et al. 2004; Schenkein et al. 2008; Cancro et al. 2009; Frasca et al. 2011). As a consequence, old people show reduced protection against new infectious agents and a decreased response to vaccines (Frasca et al. 2010; Frasca and Blomberg 2011). The generation and maintenance of memory lymphocytes is a crucial event in the immune response; in fact, these cells are essential for effective vaccination and facilitate a recall

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(anamnestic) response to previously encountered antigens (Weinberger et al. 2008). Studies on T cell memory have suggested that the filling of the immunological space with memory T cells renders the immune system less able to respond to new antigens (Franceschi et al. 2000a, b) contributing to immunosenescence.

Memory B lymphocytes may also be important in the older immune system, they can be distinguished from naïve B cells by the expression of switched immunoglobulins and by the expression of the CD27 marker, although these markers don't discriminate unequivocally between naïve and memory B cells (Tangye and Hodgkin 2004; Fecteau et al. 2006; Bulati et al. 2011). Subsets of memory B cells can be easily, but not exhaustively, identified by the different expression of surface IgD and CD27 (Shi et al. 2003). In addition, memory B cells are characterized by the presence of somatic hypermutation in the variable gene sequences of the immunoglobulins (Klein et al. 1998).

In a previous paper we have reported the increase in a population of double negative, $IgD^{-}CD27^{-}$, (DN) B cells in the elderly that might be an exhausted pool of memory B cells that fill the immunological B cell space (Colonna-Romano et al. 2009), thus reducing the availability of naïve B cells that is crucial for a response to new antigens. Moreover, naïve and memory B cells produce different pro- and anti-inflammatory cytokines (Duddy et al. 2004; Duddy et al. 2007; Sanz et al. 2007, 2008; Lund 2008) and might play a role in the generation of the inflammatory environment typical of the elderly (Licastro et al. 2005; Vasto et al. 2007).

In aged people a change in the B cell repertoire has also been described, particularly in the heavy chain of BCR. 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). The consequence is a collapse in B cell diversity in elderly people which is correlated with poor health status in these subjects (Gibson et al. 2009).

In this report, we have analyzed some characteristics of DN B cells to evaluate whether these cells, that are expanded in the elderly, play any role in the ageing of the immune system and in the generation of the immune-inflammatory environment of the elderly.

Materials and methods

Subjects

Fifty-four healthy Sicilian subjects were studied, 25 young (age range 20–45 years) and 29 elderly (age range 70–86). None of the selected subjects had neoplastic, infectious, autoimmune diseases, or received any medications influencing immune function at the time of the study. All subjects gave informed consent according to Italian law. We have performed the evaluation of surface immunoglobulin expression on separated B cells of all subjects in the study, but we have selected ten young and ten elderly donors for B cell activation and cytokine detection, and three samples of each age class for assessment of somatic hypermutation.

Cell preparation and B cell enrichment

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised venous blood by density gradient centrifugation on Ficoll-Lympholyte (Cedarlane Laboratories Limited, Ontario, Canada). PBMCs were adjusted to 1×10^7/ml in RPMI 1640 medium (Euroclone, Devon, UK) supplemented with 10% heat-inactivated fetal calf serum (Euroclone), 1% penicillin/streptomicin, 10 mM HEPES, and 1 mM L-Glutamin. B lymphocytes were separated from PBMCs by immunomagnetic sorting, as described by Miltenyi et al. (1990) using anti-CD19 magnetic microbeads (MACS CD19 MultisortMicrobeads; Miltenyi Biotec, Auburn, CA, USA). Cells obtained from immunomagnetic sorting were >98% $CD19^+$ lymphocytes, as determined by flow cytometry analysis.

Immunoglobulin expression on B cell surface

Purified B cells were stained with different combinations of the following monoclonal antibodies (BD, Pharmingen): anti-IgD_{FITC} or anti-IgD_{PE}, anti-IgG_{FITC} or anti-IgG_{PECy7}, anti-IgM_{PE}, anti-IgA_{APC}, anti-$CD27_{PE}$ or anti-$CD27_{APC}$. 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.

B cell activation and intracellular cytokine detection

Stimulation of B cells with anti-CD40 and interleukin (IL)-4 for intracellular IL-10 and tumor necrosis factor (TNF)-α cytokine detection

Separated B cells (1×10^5/200 µl) were cultured in 96-well round-bottom plates and activated with 1 µg/ml of anti-human CD40 (BD, Pharmingen) and 10 ng/ml of human recombinant IL-4 (BD, Pharmingen) with or without 2 µg/ml of anti-BCR [F(ab')$_2$] (Jackson ImmunoResearch Laboratories, Inc, Philadelphia) for 48 h, at 37°C (CO_2 5%). Cells were harvested, washed and stained with anti-IgD$_{FITC}$ or anti-IgD$_{PE}$, (BD, Pharmingen) and anti-CD27$_{APC}$ (BD, Pharmingen). After two washes, cells were fixed with 100 µl of Perm & Fix Solution A (Caltag Burlingame), washed and permeabilized with 100 µl of Perm & Fix Solution B (Caltag Burlingame). Finally cells were stained with anti TNF-α$_{FITC}$ (BD, Pharmingen) or anti IL-10$_{PE}$ (BD, Pharmingen), washed and analyzed.

Stimulation of PBMCs with CpG/phorbol myristate acetate (PMA)/ionomycin for B cell IL-10 production

For intracellular IL-10 analysis, PBMCs (10^6 cells/ml) were suspended in complete medium with or without CpG-B 2006 (3 µg/ml, Tib Molhiol), PMA (50 ng/mL), ionomycin (1 mM) and monensin (2 mM; eBioscience) in 24-well flat-bottom plates for 5 h, at 37°C (CO_2 5%) (Bouaziz et al. 2010). Cells were harvested, washed and stained with anti-IgD$_{FITC}$, anti-CD19$_{PerCP}$ and anti-CD27$_{APC}$ (BD, Pharmingen). Then cells were fixed with 100 µl of Perm & Fix Solution A (Caltag Burlingame), washed twice and permeabilized with 100 µl of Perm & Fix Solution B (Caltag Burlingame). Finally cells were stained with anti IL-10$_{PE}$ (BD, Pharmingen), washed and analyzed.

Somatic hypermutation assay

B cells, separated by MACS (1×10^5), were stained with 20 µl of anti IgG$_{FITC}$, anti-IgD$_{PE}$ and anti-CD27$_{APC}$ (BD, Pharmingen), then washed and resuspended in 1 ml of PBS/BSA (0,5%). After defining the sorting region gate ($IgG^+IgD^-CD27^-$ or $IgG^+IgD^-CD27^+$) we optimized the sample concentration, verifying the event rate and the sort rate to maximize the efficiency of cell separation. cDNA was synthesized from $IgG^+IgD^-CD27^+$ and $IgG^+IgD^-CD27^-$ cells. Immunoglobulin IgG genes were isolated by semi-nested PCR and sequenced using the Roche Titanium platform as previously reported (Wu et al. 2010). Briefly: a 25-µl PCR1 reaction mix contained 6.25 µl of cDNA, 0.625 U Phusion DNA polymerase (NEB), 200 µM each dNTPs, 41.75 nM 5′ primer (mix of IGHV gene family 1–6 primers) and 250 nM constant region Cgamma primer. A 20 µl of PCR2 reaction mix comprising 0.5U Phusion DNA polymerase, 200 µM each dNTPs, 41.75 nM each 5′ primer mix (multiplex-identifier (MID)-linked IGHV family 1–6 primers) and 250 nM MID-linked Cgamma primer was used to amplify 2 µl of PCR1 products. PCR thermalcycling conditions are as follows: 98°C for (30 s), 15 (PCR1) or 20 (PCR2) cycles of 98°C (10 s); 58°C (15 s); 72°C (30 s), and 1 cycle of 72°C (5 min). Purification of PCR products in sufficient quantity for sequencing and the downstream data processing pipeline are as previously published (Wu et al. 2010). Mutational analysis of VH IgG transcripts was done by comparison with germline sequences from the ImMuno-GeneTics (IMGT) database (available at http://imgt.cines.fr_). The number of mutated nucleotides was determined for each transcript after their alignment with the germline gene.

Statistical analysis

Values (percentage or MFI), given as the mean ± SD or SE, were compared using one-way analysis of variance (ANOVA). Differences were considered significant when a P value <0.05 was obtained by comparison between the different groups.

Results

Surface immunoglobulin of DN cells

As we are interested in B cell memory, and particularly in DN (IgD^-CD27^-) B cells, we have evaluated the immunoglobulin expression (IgM, IgG or IgA) on B cells, gated on the basis of the presence/absence of IgD and CD27. Concerning naïve (IgD^+CD27^-) and memory unswitched (IgD^+

$CD27^+$) B cells, we found that all these cells are IgM^+ (not shown). Most of the DN cells are IgG^+ (more than 60%), others (more than 20%) are IgA and few of them (less than 10%) are IgM^+ (Fig. 1a). We did not find any age-related differences in the relative expression of IgM, IgG or IgA isotypes in memory DN B cells (IgD^-CD27^-) (Table 1). In contrast, the proportion of IgG, IgA and IgM memory B cells in the classical switched memory compartment (IgD^-CD27^+) is different between young and old (Fig. 1b), with a significant decrease of IgM^+ IgD^-CD27^+ memory B cells in old subjects (Table 2). This is in agreement with previous data that have demonstrated a reduction of IgM "only" memory B cells in the elderly (Shi et al. 2005). We show a slight increase in the expression of IgG and IgA on switched memory B cells but this did not reach significance, although the relative proportions of the different isotypes of memory cells mirrors the relative proportion of serum immunoglobulin of the different isotypes (Paganelli et al. 1992; Listi et al. 2006).

Intracellular cytokine production

In order to assess whether the modifications of the B cell subpopulations described in the elderly might in turn affect the cytokine environment of the aged we have evaluated TNF-α (pro-inflammatory) and IL-10 (anti-inflammatory) production by naïve and memory B cells. To this purpose magnetically sorted B lymphocytes from the two age groups of donors were stimulated by anti-CD40/IL-4 (Frasca et al. 2008). We analyzed the production of IL-10 and TNF-α by $CD27^+$ or $CD27^-$ B cells from young and old donors. As shown in Fig. 2, there is a different pattern of cytokine production between the two age groups: in fact, $CD27^-$ B cells from the elderly produce significantly higher levels of both IL-10 and TNF-α when compared to $CD27^-$ B cells from the young, whereas in the $CD27^+$ B cell subset the young donors produce the higher levels of these cytokines.

Evaluating the specific contribution of each B cell subset to IL-10 and TNF-α production (Table 3), most IL-10 (a) and TNF-α (b) production is from unswitched memory B cells in both young and elderly subjects. Interestingly, naïve B cells from old donors produce a large amount of these cytokines, significantly higher than those produced by naïve B cells from young donors.

In a recent paper by Bonaziz et al. (2010), the authors tested the ability of blood B cells to produce IL-10 after a short stimulation with CpG, PMA and Ionomycin, showing that the combined action of these stimuli was the most potent inducer of IL-10 production. We have performed this kind of analysis in our young and elderly subjects and we show that, unlike the CD40 stimulation, the main IL-10 producing cells are IgD^+CD27^+ memory unswitched B cells and IgD^+CD27^- naïve B cells in both young and elderly people with no significant differences between the two groups (Table 4). So the non-physiological stimulus activates more naïve cells than the physiological stimulus in the young donors. Neither the classical switched memory nor the double negative B cells are activated by the non-physiological "strong stimulus" at any age.

Somatic hypermutation in DN ($CD19^+IgG^+$ IgD^-CD27^-) cells

In order to evaluate the effect of ageing on B cell receptor hypermutation in memory B cell subsets, we have evaluated the number of somatic mutations in the IgG VH regions of $CD27^+$ and $CD27^-$ B cells in young and elderly donors. Our results show that, both in young and in elderly donors, DN memory B cells have significantly fewer mutations than $CD19^+IgG^+IgD^-CD27^+$ classical switched memory B lymphocytes (Fig. 3a), although the rate of mutations observed in the DN $CD19^+IgG^+$ IgD^-CD27^- B cell subset of old people is significantly reduced when compared to the rate of mutations observed in DN cells from young subjects (Fig. 3b). Moreover, there was no significant difference between the two age groups in the $CD27^+$ memory B cell compartment.

Discussion

The ageing of the immune system involves both cell-mediated and humoral immunity as well as aspects of innate immunity. Most researchers have studied the impairment of cell-mediated immune response in the elderly, although B cell function is also modified with age (Cancro et al. 2009; Bulati et al. 2011; Frasca and Blomberg 2011).

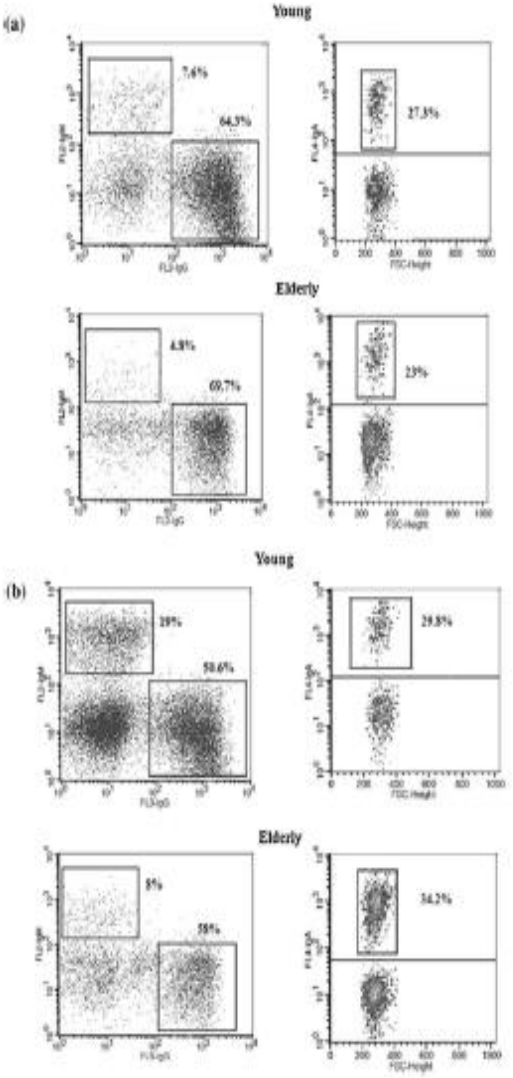


Fig. 1 a A typical experiment showing IgM, IgG or IgA expression on gated IgD$^-$CD27$^-$ (DN) B cells in young and elderly donors. No difference in expression was observed between the two groups analyzed. b A typical experiment showing IgM, IgG or IgA expression on gated IgD$^-$CD27$^+$ (memory switched) B cells in young and elderly donors. We show significant decrease of IgM$^-$IgD$^-$CD27$^+$ memory B cells in old subjects studied when compared to young

Table 1 IgM, IgG and IgA expression on gated IgD$^-$CD27$^-$ (DN) B cells from young and old donors

DN (IgD$^-$CD27$^-$) B cells	Young ($n = 25$) (% Mean±SD)	Elderly ($n = 29$) (% Mean±SD)	*P* value
IgM$^+$	6.64 ± 2.6	5.8 ± 3.1	0.7
IgG$^+$	63.7 ± 16.6	68.2 ± 17.3	0.9
IgA$^+$	23.8 ± 9.6	20.8 ± 10.9	0.8

No significant differences have been observed between the two groups studied

Table 2 IgM, IgG and IgA expression on gated IgD$^-$CD27$^+$ (memory switched) B cells from young and old donors

Memory switched (IgD$^-$CD27$^+$) B cells	Young ($n = 25$) (% Mean±SD)	Elderly ($n = 29$) (% Mean±SD)	*P* value
IgM$^+$	18.6 ± 4.7	8.1 ± 3.8	0.03
IgG$^+$	49.6 ± 8.1	57.2 ± 6.7	0.6
IgA$^+$	37.3 ± 7.7	41.5 ± 8.9	0.8

P value < 0.05, obtained by one way ANOVA nonparametric test, is considered significant

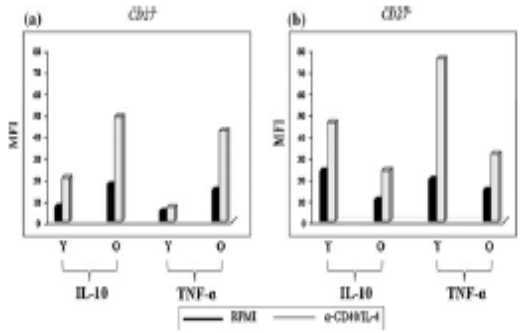


Fig. 2 Evaluation of pro- and anti-inflammatory cytokines production by CD27$^+$ and CD27$^-$ B lymphocytes in 10 young (Y) and 10 old (O) donors with or without IL-4/CD40 activation. **a** CD27$^-$ B cells from elderly subjects produce significantly higher levels of TNF- α and IL-10, both at basal level (TNF-α *P* value = 0.003, IL-10 *P* value = 0.02) and after activation (TNF-α *P* value = 0.002, IL-10 *P* value = 0.002), when compared with young donors. **b** *Viceversa*, CD27$^+$ B cells from young subjects produce higher levels of studied cytokines, when compared with old donors: we found, at basal level, significant differences between two age groups only for IL-10 (*P* value = 0.01), while, after activation, both cytokines have significant differences (TNF-α *P* value = 0.04; IL-10 *P* value = 0.01). (*P* value < 0.05, obtained by one way ANOVA nonparametric test, is considered significant)

In a previous paper (Colonna-Romano et al. 2009) we have demonstrated the expansion of a B memory subpopulation lacking the classic memory marker CD27 in elderly people, namely double negative (DN, IgG$^+$IgD$^-$CD27$^-$) CD19$^+$ cells. This kind of memory B cell has also been described by Fecteau et al. (2006) in healthy donors, Wei et al. (2007) in active Lupus patients and by Sanz et al. (2008) in healthy subjects challenged with respiratory syncitial virus (RSV). These cells are probably the consequence of a persistent stimulation of the immune system, for example in patients with lupus (Anolik et al. 2004) where the expansion of these cells correlates positively with the clinical manifestations

Table 3 IL-10 and TNF-α production among B cell subsets in 10 young (Y) vs 10 old (O) donors after CD40/IL-4 activation

	Percentage of IL-10 producing B cells		
	CD40/IL-4		*P*
	Y	O	
(a)			
Naive	15.1 ± 7	40.8 ± 5.1	**0.04**
Memory unswitched	79.6 ± 9.8	58.4 ± 4.9	0.2
Memory switched	3.1 ± 1.8	0.5 ± 0.2	0.2
DN	2 ± 1	0.2 ± 0.1	0.1
	Percentage of TNF-α producing B cells		
	CD40/IL-4		*P*
	Y	O	
(b)			
Naive	7 ± 3	24.9 ± 1.9	**0.01**
Memory unswitched	87.8 ± 4.8	72.3 ± 9	0.2
Memory switched	4.6 ± 3	1.6 ± 0.9	0.4
DN	0.5 ± 0.1	1.1 ± 0.8	0.5

In table (a) and (b) it is shown as the major contribute on the production of the two studied cytokines is given by unswitched memory B cells in both young and elderly subjects. Moreover, among the IL-10 and TNF-α producing cells from elderly donors, we show a significant amount of naïve B cells. Data are expressed as percentage (Mean ± SD). (*P* value < 0.05, obtained by one way ANOVA nonparametric test, is considered significant)

Table 4 IL-10 producing cells among B cell subsets in 10 young (Y) vs 10 old (O) donors after CpG/PMA/IONOMYCIN activation

	Distribution of B populations inside IL-10 producing B cells		
	CpG/PMA/IONOMYCIN		*P*
	Y	O	
Naive	28.6 ± 4.6	37.7 ± 15.4	0.6
Memory unswitched	64.3 ± 9.2	59.3 ± 16	0.8
Memory switched	5.6 ± 4	1.4 ± 0.2	0.3
DN	1.4 ± 0.6	1.6 ± 0.7	0.9

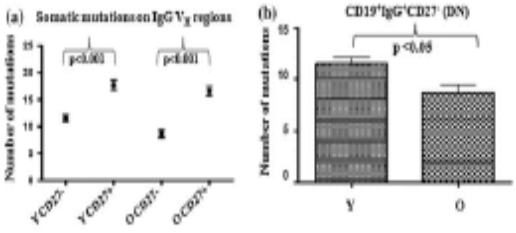
Data are expressed as percentage (Mean ± SEM). (*P* value < 0.05, obtained by one way ANOVA nonparametric test, is considered significant)

of the disease (Wei et al. 2007). Intriguingly we have reported (Colonna-Romano et al. 2010) that DN B cells are not increased in a "genetically advantaged" cohort of centenarian's offspring that also show a higher level of naïve B cells compared to their age-matched controls (70–80 years old).

In the present paper we have further studied memory/naïve B cells in the elderly, evaluating surface Immunoglobulin (sIg), production of pro-and anti-inflammatory cytokines and somatic hypermutation.

Our data confirms a previous report of reduced IgM only ($IgM^+IgD^-CD27^+$) memory B cells in old subjects (Shi et al. 2005), although the compensatory increase in the expression of IgG and IgA on switched memory $CD27^+$ B cells did not reach significance. No significant differences were observed in the expression of the different immunoglobulin classes between old and young donors in DN B cells.

It has been described (Duddy et al. 2004; Duddy et al. 2007; Sanz et al. 2007, 2008; Lund 2008) that



Subjects studied	Number of sequences analyzed	Comparison between p value
Young (Y) = 3	Y CD27$^+$ = 79	Y CD27$^+$ Vs Y CD27$^-$ $p < 0.001$
	Y CD27$^-$ = 125	O CD27$^+$ Vs O CD27$^-$ $p < 0.001$
Old (O) = 3	O CD27$^+$ = 123	Y CD27$^+$ Vs O CD27$^+$ $p > 0.05$
	O CD27$^-$ = 117	Y CD27$^-$ Vs O CD27$^-$ $p < 0.05$

Fig. 3 Evaluation of somatic mutations on IgG V_H regions in 3 young (Y) and 3 old (O) subjects. a Both in young and in the elderly donors, CD19$^+$IgG$^+$IgD$^-$CD27$^-$ (CD27$^-$) B cells have shown significantly fewer mutations than CD19$^+$IgG$^+$IgD$^-$CD27$^+$ (CD27$^+$) B lymphocytes. b Frequency of somatic mutations on CD19$^+$IgG$^+$CD27$^-$IgD$^-$ (DN) B cells evaluated in young and old subjects. The latter show a significantly reduced rate of mutations when compared to the former (P value < 0.05, obtained by one way ANOVA nonparametric test, is considered significant). The number of sequences analyzed are summarized in the bottom of the figure

naïve and memory B cells are able to produce different levels of pro- and anti-inflammatory cytokines (TNF-α and IL-10). Moreover it is known that elderly people show a pro-inflammatory micro-environment that has been related to the increased risk of morbidity and mortality (Licastro et al., 2005; Vasto et al. 2007). With this in mind we have evaluated whether changes in the relative proportions of different B cell populations could affect the cytokine environment. Here we show that in both young and old donors the physiological (α-CD40 and IL4) stimulation of B cells induces a good IL-10 and TNF-α production by unswitched (IgD$^+$CD27$^+$) memory B cells. Interestingly, in the elderly, naïve B cells are also highly activated to produce cytokines under these conditions. Not all studies are in agreement with respect to the identification of B cell subpopulations responsible for IL-10 (a very relevant anti-inflammatory cytokine) production in humans. In fact this feature has been attributed to both memory (CD27$^+$, principally IgD$^+$CD27$^+$) (Anna et al. 2007) and immature transitional (CD38^{high}CD24^{high}) B cell pools (Blair et al. 2010). On the other hand, Bouaziz et al. (2010), have shown that both IgD$^+$CD27$^-$ (classically naïve) and IgD$^+$CD27$^+$ (memory unswitched) B cells participate in IL-10 production by evaluating the differential expression of IgD and CD27, or CD38 and CD24 on IL-10-producing B cells after a "strong" (CpG/PMA/Ionomycin) activation. In our study we confirm that the "strong" activation induces IL-10 production by IgD$^+$CD27$^-$ naïve and IgD$^+$CD27$^+$ unswitched memory B cells in the control (young donors), we also show that the old donors behave similarly in that both naïve and unswitched memory B cells from old subjects are activated to produce IL-10. Taken together our IL-10 production data might suggest that naïve B cells from young donors need an adequate stimulus (e.g. TLR engagement) to be activated "in vitro" but B cells from old subjects have higher "in vitro" basal levels of IL-10 and TNF-α production (not shown) and therefore may already be basally activated, possibly by bacteria (which can be harbored in places such as the urinary tract) or viruses (such as CMV). It has been demonstrated (Lamperopoulou et al. 2008) that TLR2, TLR4 and TLR9 engagement induces IL-10 production by splenic B cells in the mouse. In addition, as proposed by Rieger and Bar-Or (2008), IL10 production by naïve B cells may act as a control mechanism to prevent the exacerbation of inflammation in an autoimmune context, whereas IL10 production by memory B cells might be active in

the resolution of the disease. In the evaluation of these results we have kept in mind that transitional ($CD24^{high}CD38^{high}$) B cells are also present in the IgD^+CD27^- (naïve) gate. These are known to have regulatory properties by production of the anti-inflammatory cytokine IL-10 (Blair et al. 2010), so it could be hypothesized that in the elderly there is an increased number of IL-10-producing transitional B cells with regulatory function. We suggest that the cytokines produced in young donors are active in modulating the size of immune response, whereas in the elderly the higher production of cytokines by naïve B cells may be due to a basal overstimulation of the immune-inflammatory system.

To gain insight into the biological significance of the different naive and memory B cell subsets, we have studied the level of mutation in the V region of IgG and showed that $CD27^+$ B cells have a significant higher number of somatic mutations both in young and old donors compared with the $CD27^-$ B cells. No significant differences in the level of hypermutation in $CD27^+$ cells are observed between the two age groups. In contrast, the rate of mutations in the DN ($IgG^+IgD^-CD27^-$) B cell population is lower in the elderly. It is well known that memory B cells are characterized by the high rate of somatic hypermutation, and that this event occurs in the germinal center and that it involves T-B cell interaction. So our results might be due to different circumstances. As previously published (Bulati et al. 2011), and as shown here, $IgG^+IgD^-CD27^-$ DN B cells show a low frequency of somatic mutation, and this supports the theory that these cells might emerge independently from T cell help, or from CD40-CD154 interaction. Alternatively, the reduced amount of somatic mutation in the old group might be due to reduced co-stimulation of B cells in the elderly (Weisskopf et al. 2009) as a result of intrinsic T cell defects, although this would also affect the rate of mutation in the classical switched memory ($IgG^+IgD^-CD27^+$) B cells which is not seen here. This leads us to the hypothesis that there is a large population of DN cells that are unrelated to classical memory B cells. The increase of the double negative memory B cells in the elderly (Colonna-Romano et al. 2009; Bulati et al. 2011), together with the reduced rate of mutation shown here, 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 et al. 2002; Frasca et al. 2005).From this and our previous papers (Colonna-Romano et al. 2009, 2010) we can conclude that DN B lymphocytes are exhausted cells. In fact they are not activated by anti-CD40/IL4, or by CpG/PMA/Ionomycin and behave differently as CMV-specific effector- memory CD8 lymphocytes. These are T cells usually supposed to be terminally differentiated and unable to proliferate, although they can be activated when appropriately stimulated "in vitro" (Waller et al. 2007). It might be interesting to know whether these cells are a by product of time-enduring stimulation by an unknown, infectious agent. One possible candidate would be CMV, since Chidrawar et al. (2009) have demonstrated that CMV infection in the elderly influences not only T lymphocytes (Pita-Lopez et al. 2009; Pawelec et al. 2009, 2010), but also negatively affects B cells.

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CHAPTER 7

Immune profiling of Alzheimer patients.

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Immune profiling of Alzheimer patients

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ABSTRACT

Alzheimer's disease (AD) is characterized by extracellular senile plaques in the brain, containing amyloid-β peptide (Aβ). We identify immunological differences between AD patients and age-matched controls greater than those related to age itself. The biggest differences were in the CD4+ rather than the CD8+ T cell compartment resulting in lower proportions of naïve cells, more late-differentiated cells and higher percentages of activated CD4+CD25+ T cells without a Treg phenotype in AD patients. Changes to CD4+ cells might be the result of chronic stimulation by Aβ present in the blood. These findings have implications for diagnosis and understanding the aetiology of the disease.

1. Introduction

Alzheimer's disease (AD) is an age-related neurological disorder that leads to progressive dementia. AD is histopathologically characterized by extracellular amyloid plaques, formed by amyloid beta peptide (Aβ), and by intracellular neurofibrillary tangles. Deposits of highly aggregated amyloid beta fibrils trigger an inflammatory process that plays an important role in AD pathogenesis (Griffin and Mrak, 2002; Akiyama et al., 2000; Rojo et al., 2008). It was suggested that the inflammation occurring in the brain of AD patients has systemic parallels, and there are many reports supporting the concept that AD is a systemic inflammatory disease (Britschgi and Wyss-Coray, 2007). A number of reports also show that more T-cells are activated in AD patients than age-matched controls, and that these cells are present both in the periphery and as infiltrates in the brain (Togo et al., 2002; Town et al., 2005; Li et al., 2009). In vitro studies have shown that Aβ induces the production of chemokines such as MIP-1α, RANTES and MCP-1 by PBMCs of AD patients and the expression of CCR5 on brain endothelial cells; this might enhance the migration of peripheral T cells across the blood brain barrier (BBB) (Li et al., 2009; Reale et al., 2008). We have recently demonstrated that PBMC from AD patients produce high levels of RANTES and MIP-1β after in vitro activation with Aβ 42, and that the expression of CCR2 and CCR5 on T cells and of CCR5 on B cells is increased in AD patients after in vitro stimulation of PBMC with Aβ peptide (Pellicanò et al., 2010). Furthermore, evidence is accumulating for an altered distribution of lymphocyte subsets in the peripheral blood of AD patients compared to age-matched controls (Speciale et al., 2007). A general decrease of B and T cell numbers has been reported, while the number of natural killer (NK) cells was not affected (Speciale et al., 2007; Richartz-Salzburger et al., 2007). Within the T cell population, a slight increase of the percentage of CD4+ and a decrease of CD8+ lymphocytes was found (Richartz-Salzburger et al., 2007). We recently reported a significant reduction of the percentage of naïve CD4+ cells (CD45RA+CCR7+), and an increase of effector memory (CD45RA-CCR7-) and TEMRA (CD45RA+CCR7-) cells in a pilot study of Canadian AD patients (Larbi et al., 2009) compared to age-matched controls. A reduction of $CD4+CD25^{high}$ cells, considered as potentially Treg cells, was also found (Larbi et al., 2008). No differences were discernible between AD patients and controls within the CD8 compartment because the effects of age were already so marked in the latter.

Our previous study was limited to a small group of Canadian patients with mild AD. To determine whether the immune signatures seen in these patients are to be expected generally in AD, we undertook a more detailed analysis on a completely different population from Italy. We have studied CD4+ and CD8+ subsets investigating the expression of the isoforms of CD45 (CD45RA and CD45RO), which can be informative for the differentiation stage of T cells (Michie et al., 1992; Sallusto et al., 1999), and the expression of two major positive T cell costimulatory receptors CD27 and CD28 and two negative receptors expressed by late-stage differentiated cells, CD57 and KLRG1 (sometimes referred to as markers of "senescence"). CD57 is expressed on NK cells and late-stage CD8+ T cells with slight expression on CD4+ cells sometimes reported (Tarazona et al., 2000;

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Ilbegbu et al., 2005), whereas KLRG1 is the Killer Lectin receptor G1, expressed on larger proportions of CD4 + T cells as well as CD8 + T cells and NK cells. It was initially suggested that the expression of KLRG-1 marked replicatively-senescent cells (Voehringer et al., 2001; Voehringer et al., 2002). Finally we have analyzed the percentage of the activated CD4 + CD25 + population that is increased in AD patients compared to young and old subjects. The emerging data suggest the existence of characteristic immune profile limited to the CD4 + T cell subset in AD, which might be useful diagnostically as well as in furthering understanding of the etiology of this intractable disease.

2. Material and methods

2.1. Subjects

A total of 40 AD patients (22 women and 18 men; age range: 62–94 years, mean 75 ± 8), 21 healthy old controls (11 women and 10 men; age range: 72–92 years, mean 84 ± 5) and 11 young subjects (4 women and 7 men; age range: 21–28 years, mean 25 ± 2) from Italy have been investigated. None of the controls had a history of neurodegenerative disorders. Diagnosis of probable AD was according to standard clinical procedures and followed the NINCDS/ADRDA and DSM-III-R criteria (McKhann et al., 1984; American Psychiatric Association, 1987). Cognitive performance and alterations were measured according to the mini-mental state evaluation (MMSE) and the global deterioration scale. The MMSE was normal (30/30) for the young and the healthy elderly groups. The patients were mild-to-moderate AD with MMSE scores between 25 and 10, of which 16 had mild AD (MMSE mean 22.8 ± 2) and 24 moderate AD (MMSE mean 14.4 ± 2.9). All AD cases were defined as sporadic because their family history did not mention any first-degree relative with dementia. AD patients included in the study did not present with any major co-morbidity such as cancer, symptomatic (present or previous) cardiovascular diseases, or major inflammatory diseases such as autoimmunity and infections. Control subjects also 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, C-reactive protein, liver function tests, iron, proteins, cholesterol, triglycerides). The controls were collected from the same population as the patient cohort. The University Hospital Ethics Committee approved the study, and informed consent was obtained from all guardians of patients and controls according to Italian law.

All subjects were tested for CMV serostatus by ELISA using CMV-IgG-ELISA PKS assays (Genesis Diagnostics, UK). All the elderly subjects were positive for CMV antibody (both healthy old and AD) while none of the young were infected.

Whole blood was collected by venepuncture in vacutainer tubes containing ethylenediamine tetra acetic acid. Peripheral blood mononuclear cells (PBMC) were separated using a Ficoll/Hypaque gradient and frozen according to standard protocols.

2.2. Flow cytometry

To analyze T lymphocyte subsets, direct immunofluorescence was performed with anti-CD3-AlexaFluor700, CD8-PercP, CD28-PercP-Cy5.5 (Becton Dickinson, Heidelberg, Germany), CD4-Qdot705, CD27-Qdot605 (Invitrogen, Karlsruhe, Germany), CD45RO-eFluor650 (eBioscience, San Diego, California), CD45RA-PacificBlue (Bio-Legend, Biozol, Eching, Germany), and CD57-FITC (Immunotools, Friesoythe, Germany). For indirect immunofluorescence, anti-human KLRG1 (clone 13A2), kindly provided by Prof. H.P. Pircher (University of Freiburg, Germany), was used as primary antibody. The secondary antibody was goat anti-mouse IgG-Qdot565 (Invitrogen). Treg analysis was performed by extracellular labeling with anti-CD3-AlexaFluor700 (Becton Dickinson), anti-CD4-Qdot705 (Invitrogen), anti-CD127-Alexa647 (Bio-Legend) and intracellular staining with anti-FoxP3 with (Bio-Legend).

Cell viability was determined with RedVid (Invitrogen). All staining steps were performed in PFEA buffer (PBS, 2% FCS, 2 mM EDTA and 0.01% Na Azide). Blocking of non-specific 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 re-suspended and measured using an LSR-II flow cytometer and the acquisition software FACSDiva (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star, Portland, OR). For data analysis, dead cells (Red-Vid-positive), were excluded. CD3 + living cells were gated within the SSC/FSC lymphocyte gate. Further analysis was performed using CD3 + CD4 + and CD3 + CD8 + gated populations.

2.3. Statistics

All statistical analyses were performed with Graph-Pad Prism 4.0 using the Mann-Whitney nonparametric *U* test to compare two independent groups. The Bonferroni correction for multiple comparisons was applied. Differences were considered significant with a p value ≤0.005. Significant differences are indicated by * $p \leq 0.005$, ** $p \leq 0.001$, *** $p \leq 0.0005$ as mentioned in the figure legends.

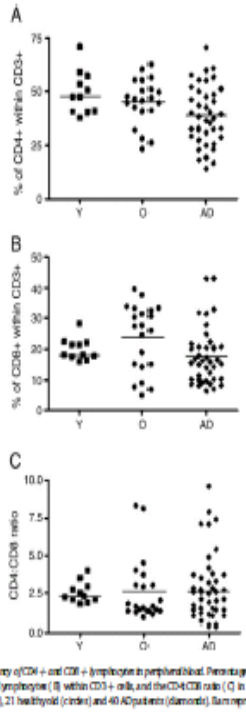

Fig. 1. Frequency of CD4 + and CD8 + lymphocytes in peripheral blood. Percentages of CD4 + (A) and CD8 + T lymphocytes (B) within CD3 + cells, and the CD4:CD8 ratio (C) in 11 young controls (square), 21 healthy old (circles) and 40 AD patients (diamonds). Bars represent medians.

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3. Results

3.1. Frequency of CD4 and CD8 cells in young controls, old controls and AD patients

It has been shown that the CD4:CD8 ratio can be a marker predictive of mortality in an elderly Swedish population. A cluster of this marker and others constitutes the Immune Risk Profile (IRP) predicting mortality in the very elderly on 2, 4 and 6-year follow-up (Pawelec et al., 2002). Because our aim was to investigate the immune status in AD patients, who have a reduced lifespan compared to healthy old individuals, we have analyzed CD4 and CD8 distribution in PBMC of AD patients compared to healthy age-matched old controls and young subjects. As shown in Fig. 1A, there is a slight but non-significant decrease in the median percentage of CD4+ cells within the CD3+ T cell population in AD patients as a group compared to the age-matched controls. The decrease in CD4+ cells was marginally more marked for AD patients compared to young controls, but this difference also failed to reach significance. However, within the broad range of values recorded in this group, individual AD patients showed the lowest percentages of CD4 cells, lower even than the lowest age-matched controls. These tendencies were not observed in the CD8 compartment (Fig. 1B). These slight shifts also failed to affect the CD4:CD8 ratio in the three groups. However, individual patients within the AD group again showed the most extreme differences, with the lowest CD4:8 ratios (Fig. 1C). The CD4:8 ratio cut-off for inclusion in the IRP is < 1; the data summarized in Fig. 1C indicate that, as expected, no young controls fall into this group, whereas 1/21 old controls (5%), but 5/40 AD patients (12.5%) have a CD4:8 ratio of < 1. No significant differences were observed comparing CD4:8 ratios obtained from subjects with mild vs moderate AD (data not shown).

3.2. Costimulatory molecules on CD4+ and CD8+ cells

The distribution of T cells at different stages of differentiation (naïve, memory etc.) is likely to be more important than the mere percentages of CD4 and CD8 cells. We therefore compared T cell differentiation status in AD patients versus old controls, using multiple markers including the CD27 and CD28 costimulatory molecules, which are crucial for T cell activation. It has been reported that expression of these two markers is reduced proportional to the degree of differentiation of the T cell (Romero et al., 2007). Naïve cells are CD28+CD27+ while the latest stage of T cell differentiation is accompanied by the loss of both CD28 and CD27 (Koch et al., 2008). As shown in Fig. 2A (left-hand side) there is a slight but not significant decrease in the percentage of CD28+CD27+ cells within the CD4+ subset in AD patients compared to the elderly controls. The young controls in this population have a highly significantly greater percentage of CD27+CD28+ cells within the CD4 subset than either the old controls or AD patients, albeit more marked in the latter. Reciprocally, significantly increased percentages of late-differentiated CD4+ cells (CD28-CD27-) are seen in both elderly controls and AD patients, with no real difference between the latter two groups (Fig. 2A, right-hand side).

Within the CD8 subset, the anticipated highly significantly lower percentage of CD27+CD28+ and higher percentage of double-negative cells is seen in the old compared to the young, with no difference at all between AD patients and age-matched controls (Fig. 2B).

3.3. Frequencies of naïve and late-differentiated cells within the CD4+ and the CD8+ populations

Although assessing the expression of CD27 and CD28 provides some information on T cell differentiation status and potentially T cell function, a combination of markers is required to distinguish between naïve and memory cells. To this end, we have analyzed the expression of CD45RA and CD45RO together with CD27 and CD28. CD45RA and CD45RO are isoforms of the protein phosphatase CD45, with naïve cells more likely to express CD45RA and memory cells CD45RO. However, re-expression of CD45RA by late-differentiated memory cells makes this marker unsuitable for identifying naïve cells by itself (Hamann et al., 1997). Thus we used four markers to identify naïve and memory subsets, as follows: CD28+CD27+

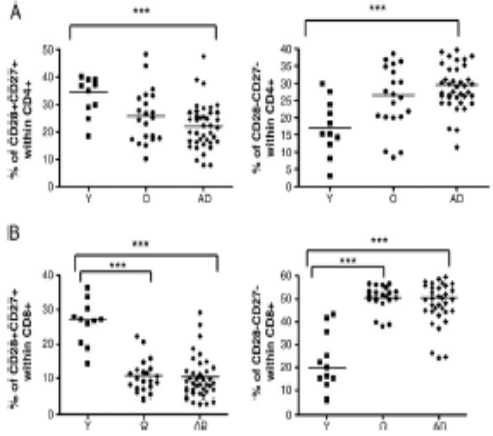

Fig. 2. CD28 and CD27 expression in young controls, old controls and AD patients. PBMC of 11 young controls (squares), 21 healthy old (circles) and 40 AD patients (diamonds) were stained for CD3, CD4, CD8, CD27, CD28 to distinguish early- and late-differentiated cells. The percentage of CD28+CD27+ (early) and CD28-CD27- (late) lymphocytes are shown for CD4+ (A), and CD8+ T cells (B). Bars represent medians. Significant differences are indicated by *** $p \leq 0.0005$ by Mann–Whitney nonparametric U testing with Bonferroni correction.

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CD45RA+CD45RO- presumptive naive, and CD28-CD27-CD45RA+CD45RO+ late differentiated, comparing CD4 and CD8 lymphocytes in the young, old and AD patients. Fig. 3A gives examples of flow cytometry data as dot-plots for CD4+ cells from a young donor, an old control and an AD patient, and Fig 3B shows the summed data from 11 young controls, 21 healthy elderly and the 40 AD patients. Although the percentages of naive CD4 cells in both old controls and AD patients are very low relative to the young, the decrease in AD compared to age-matched controls reaches significance. Similarly, the reciprocal increase in late-differentiated memory cells is also significantly greater in AD patients than in age-matched controls. These differences are not seen in the CD8 subset analyzed in the same manner (Fig. 4).

The same analysis was done comparing patients with mild vs. moderate AD and no significant differences have been observed (data not shown).

3.4. Expression of "senescence" markers on CD4+ and CD8+ T cells

In an effort to refine our definition of naive and late-differentiated T cells in AD, in order to increase our ability to distinguish immune signatures in AD from age-associated changes in age-matched controls, we employed two additional markers. Previous studies have shown that chronic antigenic stimulation of the immune system, such as in the case of cytomegalovirus (CMV) infection, led to increased frequencies of late- or even end-stage differentiated CD8+T cells. These cells highly express KLRG1 and CD57 which are sometimes designed "senescence" markers (Brenchley et al., 2005). Therefore, we tested the possibility that putative chronic stimulation of the immune system by AD-derived end products (Aβ) would influence the expression of these two markers. As shown in Fig. 5, within the CD4 subset, the percentage of cells expressing KLRG-1 was very significantly greater in AD patients compared to age-matched controls (Fig. 5, upper right-hand panel). This was not the case for their CD8+ cells (Fig. 5, lower right-hand panel), and nor was it the case for either CD4 or CD8 cells regarding their expression of CD57 (Fig. 5, left-hand panels). Also in this case no significant differences have been observed comparing data obtained from patients with mild vs. moderate AD (data not shown).

3.5. CD4+CD25+ cells and Tregs

CD25, the IL-2 receptor α chain, is expressed by activated T cells, but is also taken as a marker of regulatory T cells. We have confirmed a significantly higher frequency of CD4+CD25+ T cells in AD patients compared to both groups of controls. Examples of staining for CD4 and CD25 in a young control (left-hand panel), old control

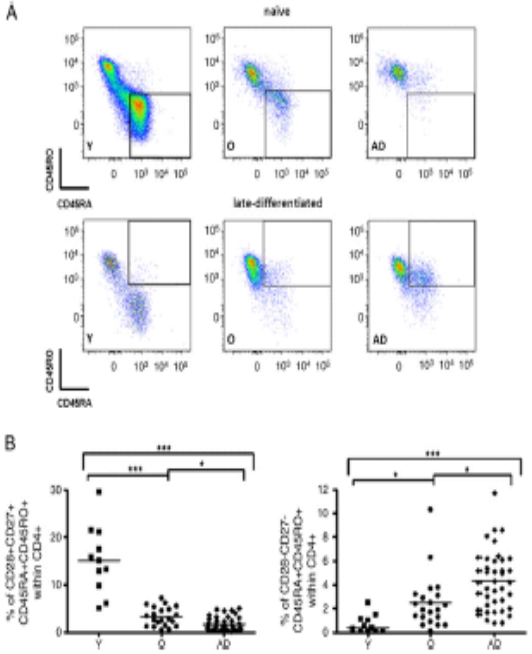

Fig. 3. CD4+ naive and late-differentiated subsets in AD patients and controls. PBMCs were stained with CD3, CD4, CD28, CD27, CD45RA, CD45RO to identify naive and late-differentiated cells. (3A) Representative dot plots of naive (top) and late-differentiated (bottom) CD4+ T cell distribution in a young control (left panels), in an old control (middle panels) and in an AD patient (right panels). (3B) Percentages of CD28+CD27+CD45RA+CD45RO- naive cells (left) and of CD28-CD27-CD45RA+CD45RO+ differentiated cells (right) of 11 young controls (black squares), 21 healthy old (black circles) and 40 AD patients (black diamonds). Bars represent medians. Differences between control subjects and AD have been assessed by Mann–Whitney nonparametric U testing with Bonferroni correction. Significant differences are indicated by * $p \leq 0.005$, *** $p \leq 0.0005$.

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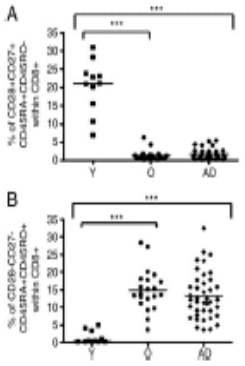

Fig. 4. CD8 + naïve and late-differentiated subsets in AD patients and controls. The percentages of CD8+ naïve CD28+ CD27+ CD45RA+ CD45RO- cells (4A) and late-differentiated CD28-CD27-CD45RA+ CD45RO+ cells (4B) within PBMC of 11 young controls (black squares), 21 healthy old (black circles) and 40 AD patients (black diamonds) are shown. The bar represents the median. Differences between control subjects and AD have been evaluated by Mann–Whitney nonparametric U testing with Bonferroni correction. Significant differences are indicated by *** p ≤ 0.0005.

(middle) and AD patient (right-hand panel) are shown in Fig. 6A and the summed data from 11 young controls, 21 healthy old and 40 AD patients in Fig. 6B, left panel. There is a highly significant difference between the old controls and the AD patients, with the latter possessing a greater percentage of CD4+ CD25+ cells. However, CD25 by itself is not sufficient to identify regulatory T cells; to this end we used FoxP3 nuclear staining and weak CD127 surface staining as markers for these cells. As shown in the right panel of Fig. 6B, the percentage of CD4+ CD25+ FoxP3 + CD127low T cells is higher in both elderly controls as well as AD patients compared to the young, but there is no difference between old controls and AD. This suggests that AD patients have increased levels of CD4+ T cell activation but not Tregs. No significant differences were observed comparing CD24 + CD25 + obtained from subjects with mild vs moderate AD (data not shown).

4. Discussion

Alzheimer's disease is the most common form of dementia and represents one of the main causes of disabilities among elderly people. The diagnosis of AD is made following clinical criteria and only post-mortem autopsy can really confirm the disease (McKhann et al., 1984). Such clinical criteria do not allow early diagnosis of Alzheimer's disease even if the pathological alterations are present years before a certain diagnosis. The availability of reliable minimally-invasive biomarkers for AD progression and especially for incipient AD would be vital for an early diagnosis and a timely start of appropriate treatment to slow disease progression (Schupf et al., 2008; Mocali et al., 2004; Uberti et al., 2010; Padovani et al., 2001).

The accumulation of senile plaques in the CNS formed by Aβ deposits is the main hallmark of the disease. For this reason AD has been always considered as a brain disease (Selkoe, 2001). Recently it was suggested that the inflammation induced by the accumulation of Aβ is not an only a local phenomenon but can induce systemic symptoms or be caused by systemic events (Britschgi and Wyss-Coray, 2007; Richartz-Salzburger et al., 2007; Fiala et al., 2005). Moreover, it is likely that Aβ is not only accumulated in the brains of AD patients, but is also present in the periphery and can be detected in the blood (Britschgi and Wyss-Coray, 2007; Mayeux et al., 2003; Sagare et al., 2011).

In this study we have tested the hypothesis that patients suffering from Alzheimer's disease show systemic changes at the immunological level, consistent with chronic antigenic stress potentially resulting in immune exhaustion. This question has been sporadically

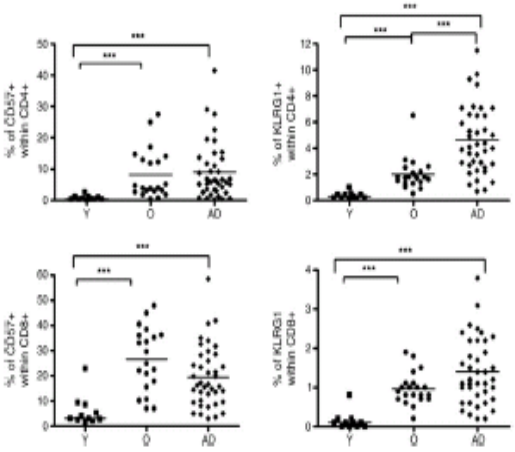

Fig. 5. "Senescence" marker expression on CD4+ and CD8+ cells. PBMC were stained with CD3, CD4, CD8, KLRG1 and CD57. Percentages of CD4 + CD57 + (upper left) and of CD8 + CD57 + (bottom left) cells in 11 young controls (black squares), 21 healthy old (black circles) and 40 AD patients (black diamonds) are shown. Frequency of CD4+ KLRG1 + (upper right) and of CD8+ KLRG1 + (bottom right) cells of young (black squares), healthy old (black circles) and AD patients (black diamonds). Bars represent medians. Differences between control subjects and AD have been evaluated by Mann–Whitney nonparametric U testing with Bonferroni correction. Significant differences are indicated by *** p ≤ 0.0005.

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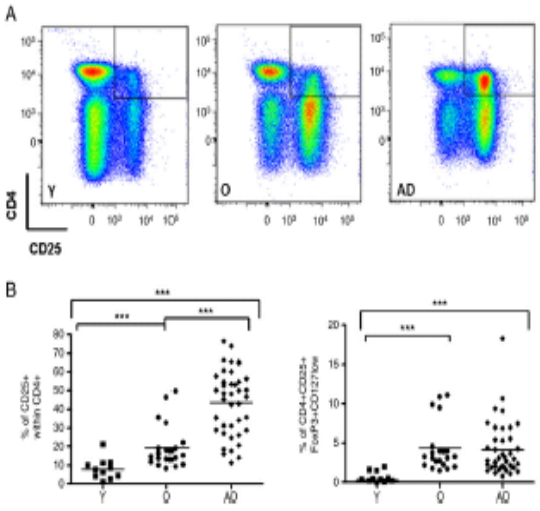

Fig. 6. Percentage of activated and Treg cells within CD4+ cells of AD patients and controls. Representative dot plots of CD4+CD25+ cells in a young control (left panel), an old control (middle panel) and an AD patient (right panel) (6A). Median of CD4+CD25+ cells in PBMC of 11 young controls (black squares), healthy old (black circles) and AD patients (black diamonds) are shown (6B left). Percentage of CD4+CD25+Foxp3+CD127low, putative Tregs, in young (black squares), healthy old (black circles) and AD patients (black diamonds) (6B right). Bars represent medians. Differences between control subjects and AD have been evaluated by Mann–Whitney nonparametric U testing with Bonferroni correction. Significant differences are indicated by *** $p \leq 0.0005$.

investigated by others in the past, with disparate results, which may have been partly the result of technical issues with analytic techniques, among other possibilities, especially regarding AD diagnostic criteria. A significant decrease of B and T cell percentages without changes of natural killer (NK) cells was previously reported (Richartz-Salzburger et al., 2007). These and other investigators also reported a slight increase in the percentage of CD4+ T cells and a decrease of CD8+ cells (Richartz-Salzburger et al., 2007; Lombardi et al., 1999). These data are not in complete agreement with other reports of no differences in the percentage and absolute number of CD8+ T cells, NK cells or CD4+ and CD8+ T cells (Speciale et al., 2007). In our own pilot study we showed that major changes are seen within the CD4+ T cell subset in mild AD patients compared to healthy elderly controls (Larbi et al., 2009) whereas the more marked changes in the CD8 subsets were seen equally in both patients and age-matched controls. To confirm and extend our previous findings in a different population (Italian not Canadian), here we tested a larger group of AD patients (n=40, one group of mild and one group of moderate AD) compared to healthy old (n=21) and young subjects (n=11) using multiparameter flow cytometry, including new markers to better characterize the immunological profile of these patients.

The analysis of costimulatory molecules revealed a slight reduction of CD28+CD27+ (early differentiated cells) and an increased percentage of CD28-CD27- (late differentiated cells) within the CD4+ subset of both mild and moderate AD patients compared to healthy elderly controls. We extended this analysis to include CD45 isoform expression, and found a significantly reduced median percentage of CD28+CD27+CD45RA+CD45RO- naïve CD4+ T cells in AD patients compared to old controls and a reciprocal increase of CD4+ CD28-CD27-CD45RA+CD45RO+ late-differentiated memory cells. Furthermore, we tested the expression of the potential senescence markers CD57 and KLRG1 on these cell subpopulations. The results with KLRG-1 revealed the most marked differences found here between AD and age-matched controls; with highly significantly greater percentages of CD4+ T cells carrying this marker in the former. The CD57 marker was not informative in this respect. Neither were any of these markers informative within the CD8 subset, probably because the age-associated changes already seen in the controls were so marked that any additional alterations in the patient group were not visible. We know that the age-associated changes seen in CD8+ T cells are predominantly caused by persistent infection with, and hence chronic antigenic stimulation by, CMV, because CMV-negative individuals do not manifest these changes at older age (Chidrawar et al., 2009; Derhovanessian et al., 2010). In contrast, the CD4+ cells are only marginally or much less affected by CMV serostatus (Derhovanessian et al., 2010). Thus, the striking findings of differences within the CD4 subset between AD patients and age-matched controls reported here cannot be caused by CMV; also all elderly controls as well as AD patients were CMV-seropositive. Because all young controls were CMV-seronegative, the even more marked differences between young and old groups are likely to be predominantly a result of CMV stratification. It was discussed by Goronzy et al. that there is an effective homeostatic control mechanism that allows the maintenance of an appropriate number of naïve CD4+ T cells in elderly people, in contrast with the CD8+ compartment that is more shifting with a decrease of naïve CD8+ cells already during middle age (Goronzy et al., 2007). Only in some pathological conditions with inflammatory components (e.g. rheumatoid arthritis) it was shown that chronic antigenic stress can be responsible for premature immunosenescence of CD4+ cells (Weyand and Goronzy, 2002). We hypothesize that the differences in CD4+ naïve, memory and late differentiated (KLRG1+) T cell distribution between AD and age-matched controls are causes by chronic antigenic stimulation by Aβ present in the blood but this has not been directly tested and a differential effect of CMV in AD-

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vs-age matched controls cannot be ruled out. Alternatively the shift from naïve to late memory CD4+ cells in AD patients can be the result of the capture of Aβ by local APC in the brain and the migration of these cells towards secondary lymph nodes inducing T cell stimulation, as suggested by Monsonego et al. (Monsonego et al., 2003). The present results are consistent with our previous report of a pilot study of mild AD patients (Larbi et al., 2009), but extend that study beyond the diagnosis of mild AD to include moderate AD. Moreover, the employment of a different set of markers to define naïve and differentiated memory cells in these two studies in patients from two different countries, nonetheless with consistent results, suggests that these findings are likely to be robust. Furthermore, it may be of consequence that the immune profile seen here in moderate AD is already present in the mild AD group. This suggests that immune changes occur early in disease progression and raises the possibility that they may occur even before cognitive symptoms manifest. If this were so, it has implications for early diagnosis of alterations leading to the future development of AD. We are currently testing this possibility in patients with mild cognitive or no impairment.

In our previous paper we also showed a higher percentage of CD4+ CD25+ putative Treg cells in AD compared to healthy controls (Larbi et al., 2009). However, the IL2rα chain (CD25) expressed on Tregs is also expressed on activated cells. To better identify these cells we used several other Treg markers, and found a markedly higher frequency only of CD4+CD25+FoxP3- cells (i.e. activated T cells, not Tregs) in AD patients compared to old controls. These data are in agreement with others reporting a higher frequency of activated cells in the PBMC of AD patients (Pellicanò et al., 2010; Monsonego et al., 2003; Miscia et al., 2009; Cicconcioppo et al., 2008), but seem discordant with a recent report on increased levels of Tregs in AD (Saresella et al., 2010). The presence of activated CD4+ T cells might be the result of Aβ-specific chronic T cell stimulation, creating a pro-inflammatory environment, and enhancing disease progression. Possibly, the level of Treg induction depends on disease progression, because Saresella et al. found greater suppressive activity in mild cognitively impaired patients than in AD patients (Saresella et al., 2010).

We conclude that there is a common peripheral immune profile for Alzheimer's disease which mainly involves CD4+ T cells, changes to which are consistent with chronic antigenic stress leading to immune exhaustion. Whether Aβ is really the driving force is unknown but efforts will be concentrated on this issue in order to define how and why CD4+ T cells undergo changes in AD. It will be crucial to determine whether such changes to CD4 T cells precede the development of AD, whether they are discernible in MCI, or even in people not yet showing any cognitive impairment. To this end, we are going to study the immune profile of different forms of dementia to see whether the differences we observed for CD4+ cells are specific to AD and can be used as biomarkers for early diagnosis. Moreover, focusing intervention efforts on CD4 cells to alleviate their potential dysregulation could be of therapeutic benefit. This could possibly be approached by targeting CCR5.

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CHAPTER 8

Novel human late memory B Cell Population revealed by CD38/CD24 gating strategy: characterization of $CD38^{-}CD24^{-}$ B cells in Centenarian Offspring and elderly people

Manuscript in preparation

Manuscript in preparation

Novel human late memory B Cell Population revealed by CD38/CD24 gating strategy: characterization of CD38$^-$ CD24$^-$ B cells in Centenarian Offspring and elderly people

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Abstract

The development of the B cell branch undergoes significant modifications with age. Elderly people are characterized by the impairment of the B cell response that reflects the inability to effectively respond against viruses and bacteria. The modifications of the immune system with age (immunosenescence) are widely studied in centenarians who are considered the main example of successful aging. In the recent years, attention has shifted to the centenarian offspring (CO), as a model of people genetically advantaged for health aging and longevity. In this study, we show the preliminary characterization of a new population of late memory B cells, CD19$^+$CD38$^-$CD24$^-$, that we describe increased in the elderly and contemporary decreased in CO when compared with healthy

age-matched controls (AM). Here, we demonstrate that human $CD19^{+}CD38^{-}CD24^{-}$ is involved in TNF production. We hypothesize that the expansion of these cells, also negative both for IgD and CD27, might be related to the inflammatory status of elderly people as the result of a time-enduring stimulation of the immune system. In addition, we evaluate the expression of RP105 (CD180), the toll-like receptor (TLR) associate molecule, expressed on B cells. In this paper We describe an age-related increase of RP105-negative B cells in peripheral blood of elderly people and its up-regulation in memory B cell subsets. In this paper we discuss the modified expression of CD180 and its potentially role as marker of immunosenescence.

Introduction

B cells are key mediators of immunity. The humoral immune response, due to the B cell compartment activation, involves a response to pathogens by producing antibodies to neutralize bacteria and viruses, secreting cytokines and interacting with other components of the immune system. The early step of B cell development occurs in the bone marrow from hematopoietic stem cells (HSC). The early progenitors of B lymphocytes develop into pro- , pre- and immature B cells [1,2], after subsequently stages of differentiation that involve light and heavy chain genes rearranging and the expression of cell surface markers. When immature B cells become independent from stromal factors, they leave the bone marrow via the blood stream as transitional B cells, [3-6], up to secondary lymphoid organs. Only a small fraction of immature B cells become mature naïve. Therefore, transitional B cells are considered as a negative selection checkpoint for autoreactive B cells [5,7]. Transitional B cells have been widely studied in mice [8,9]. As in the mouse, human transitional B cells can be found in the bone marrow (BM), peripheral blood (PB) and spleen [7]. The absence of ABCB1 transporter activity has been used to distinguish transitional B cells from mature naïve B cells [10]. In human

peripheral blood, naïve and memory B cells have been described on the basis of the differential expression of IgD and CD27 [11-14]; naïve B cells have been identified as $IgD^{+}CD27^{-}$, whereas the expression of CD27 allowed to distinguish memory B cells subsets: $IgD^{+}CD27^{+}$, indicated as unswitched memory B cells or marginal zone-like B cells [15], $IgD^{-}CD27^{+}$ as classical switched B cells. Afterwards, a population of DN B cells ($IgD^{-}CD27^{-}$) has been identified too [16,17]. The $IgD^{-}CD27^{+}$ population has not been further subdivided. As a consequence, this is a mixed population of IgM^{+} and Ig class-switched memory B cells [12,18,19]. In addition, the complexity of the memory B cell pool has been demonstrated by the evidence of a IgM memory B cell population identified as $IgM^{+}IgD^{+}CD27^{+}$ and of the "IgM only" memory B cells identified as $IgD^{-}IgM^{+}CD27^{+}$. $IgM^{+}CD27^{+}IgD^{+}$ and $IgM^{+}CD27^{+}IgD^{-}$ have not always been distinguished each other. The former, known as "natural effector B cells", have reduced replication history and SHM levels compared with the latter. So, they seem to be a mixed population of GC-derived and splenic marginal zone-derived memory B cells [18]. On the contrary, IgM-only memory B cells appear to be generated from germinal center responses, [16,18,20]. In these last years, a different flow cytometric approach has been used to distinguish naïve from memory B cells [7-9]. The use of two developmentally regulated markers, CD24 and CD38, in association to the B-lineage marker CD19, allowed to identify three different B cell populations: $CD19^{+}CD38^{high}CD24^{high}$, a population of transitional B cells that includes immature B cells, $CD19^{+}CD38^{int}CD24^{int}$ defined as mature B cells, and $CD19^{+}CD38^{-}CD24^{high}$ called "primarily memory B cells". In a recent paper [21], the authors have shown as the stimulation with CD180 (RP105), an homologous of TLR4 that regulates TLR4 signaling, has been able to induce intrinsic B cell proliferation and differentiation from transitional to mature B cells. The expression of RP105 surface marker has been also evaluated on peripheral blood B cells from patients with

different autoimmune diseases. The number of RP105-negative B cells has been shown increased in SLE patients compared with normal subjects [22]. It was hypothesized that RP105 may regulate B cell activation and that RP105-negative B cells might be involved in the production of autoantibodies and take part in pathophysiology of SLE.

The number of total B cells and the composition of the B cell subsets shows age-dependent changes[23]. The frequency of transitional B cells as well as naïve B cells decreases rapidly during the first years of life, while the frequency of switched and unswitched memory B cells increases. After these changes the absolute number of B cells remains stable while the shift from naïve to memory continues [24]. In elderly people $IgD^{+}CD27^{-}$ naïve B cells result significantly reduced [25,26]. On the other hand, DN B cells ($IgD^{-}CD27^{-}$) are significantly increased in the elderly [17,27]. Different studies have shown that the same population is expanded in patients suffering by the chronic stimulation of the immune system, such as SLE [13], HIV [28] and in healthy subjects challenged with RSV [29]. The evaluation of the processes involved in the modifications of the immune system in the elderly (immunosenescence) led to consider the immunological features of those subjects predisposed to longevity. Centenarians have been widely studied in order to identify key factors associated with exceptional longevity in humans. However, centenarian studies posed the challenge of whom to use as control. Unlike their parents, centenarian offspring (CO) have an appropriate control group, i.e. common elderly, who haven't a familiar history of longevity. In these years, we and other groups have focused our attention on the study of centenarian offspring, trying to evaluate any possible correlation between their genetic background, predictor of longevity, and their immunological profile. CO don't show the typical naïve/memory shift observed in elderly. In particular, we have not seen the increase of DN ($IgD^{-}CD27^{-}$) B cells. Also the evaluation of IgM secreted in the serum by CO has shown

that the detected values are within the range of the levels observed in young subjects [27].

Several distinct memory B cells have been identified in humans, although the association between their specific phenotype profile and function remains to be further clarify. In this study, we show the preliminary characterization of a new population of late memory B cells, $CD19^{+}CD38^{-}CD24^{-}$, performing analysis in order to understand its biological role and function. Moreover, as we are interested in the modification of the immune system during aging, we carried out comparative studies using B cells from elderly people. Indeed, data from literature show how in the periphery of elderly people, the extended survival of memory B cells and the clonal expansion contribute to the limited repertoire and the collapse in B cell diversity that is correlated with poor health status and the impairment of antibody response [30-36]. We have extended our analysis to centenarian offspring (CO) that, like their centenarian parents, have genetic and functional advantages that predispose them to healthy aging and longer survival, with the purpose to evaluate the immunological features that may be related with their healthiness. Moreover, we have examined the expression of CD180 on total B cells and CD38/CD24 B cell subsets, in order to evaluate the age-related modulation of this marker.

Material and Methods

Subjects

12 healthy Sicilian Centenarian Offspring (age range: 70.1 ± 8.3) with almost one of their parents centenarian (≥ 100 years) have been investigated. A total of 8 young (age range: 28.5 ± 1.9), 8 Old (age range: 86.4 ± 3.8), and 7 Age-Matched Controls (age range: 69.1 ± 9) were also included in the study. All subjects were in good health according to their clinical history and none of the them had neoplastic, infectious, autoimmune diseases, or received any medications influencing

immune function at the time of the study. The controls were collected from the same population as the patient cohort. The study received approval from local ethic 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 cytometry

1. *Phenotype analysis*

To characterize the phenotype of B lymphocyte subsets, extracellular labeling was performed with anti-CD19-PE, CD24-Pe-Cy7, CD5-V450, IgD-FITC, IgG-APC-H7, IgM-PerCP-Cy5.5 (BD Biosciences), CD27-Qdot605 (Invitrogen), CD80-PerCP (Abcam), CD38-eFluor650 (eBioscience) and intracellular staining with anti-FoxP3-AlexaFluor647 (BD Biosciences).

2. *Stimulation of PBMC with CpG/phorbol myristate acetate (PMA)/ionomycin for B cell IL-10, IL-6, TNF production*

PBMC (10^6 cells/ml) were suspended in EX VIVO medium with or without CpG-B 2006 (3 µg/ml, Tib Molbiol), PMA (50 ng/ml), ionomycin (1 mg/ml) and monensin sodium salt (2 mM, SIGMA ALDRICH) in 24-well flat-bottom plates for 5h, at 37°C (CO_2 5%). Cells were harvested, washed and directly stained with anti-CD19-PerCP-Cy5.5, CD24-Pe-Cy7, IgD-FITC, IgG-APC-H7, CD180-PE (BD Biosciences), CD27-Qdot605 (Invitrogen), CD80-PerCP (Abcam), CD38-eFluor650 (eBioscience). For indirect immunofluorescence, anti-human 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). Citofix&Citoperm (BD Biosciences) was used. Finally cells were stained with anti-IL-10-APC (Miltenyi Biotec), TNF-AlexaFluor700 and IL-6-V450 (BD Biosciences), washed and analyzed.

Cell viability was determined with RedVid (Invitrogen). All staining steps were performed in PFEA buffer (PBS, 2% FCS, 2 mM EDTA and 0.01% Na Azide). Blocking of non-specific 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 re-suspended and measured using LSR-II flow cytometer and 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 SSC/FSC lymphocyte gate.

3. Cytokine Detection

For analysis of cytokine production, serum samples from patients were tested with Cytometric Bead Array (CBA) Human Multiflex System (Becton Dickinson).The CBA was used according to the manufacturers' protocol (Becton Dickinson, Oxford, UK). Two-color flow cytometric analysis was performed using a flow cytometer LSR-II. Analysis was made using CBA dedicated analysis software, "FCAP Array™ software", to generate results in graphical and tabular format.

Statistical Analysis

All statistical analysis 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 < .05, ** P <.01, *** P < .001 and it is also shown in the figures. All values are expressed as mean ± SEM.

RESULTS

Characterization of CD38 +-/CD24+- B cells subsets

Analysis of B lymphocytes from our samples, carried out by using CD38 and CD24 markers, allowed us to discriminate four distinct populations: $CD19^{+}CD38^{hi}CD24^{hi}$ (classical transitional), $CD19^{+}CD38^{int}CD24^{int}$ ("mature naïve"), $CD19^{+}CD38^{-}CD24^{+}$("primarily memory) and $CD19^{+}CD38^{-}CD24^{-}$ (Fig. 1a). Afterwards, we evaluated the expression of IgD and CD27 on the four B cell subpopulations above identified. On the basis of their functional organization into the different naïve/memory B cell subsets, we observed as $CD19^{+}CD38^{hi}CD24^{hi}$ and $CD19^{+}CD38^{int}CD24^{int}$ show a naïve phenotype (IgD+CD27-). The $CD19^{+}CD38^{-}CD24^{+}$ B lymphocytes, just called primarily memory B cells, begin to express CD27 and contemporary loss IgD ($IgD^{+}CD27^{+}$/$IgD^{-}CD27^{+}$). The analysis of the $CD19^{+}CD38^{-}CD24^{-}$ B cell subset indicates that these cells are predominantly IgD- ($IgD^{-}CD27^{+}$/$IgD^{-}CD27^{-}$) (Fig. 1b). In order to deeply characterize these populations we have evaluated the expression of different immunological markers: IgD, IgM, CD27, CD5, CD80 and CD180. Further phenotipical identification revealed as the largest part of $CD19^{+}CD38^{hi}CD24^{hi}$ are IgD^{hi}, IgM^{hi}, $CD27^{-}$, $CD5^{+}$, $CD80^{hi}$, $CD180^{hi}$; $CD19^{+}CD38^{int}CD24^{int}$ are IgD^{hi}, IgM^{int}, $CD27^{-}$, $CD5^{-}$, $CD80^{-}$, $CD180^{hi}$; $CD19^{+}CD38^{-}CD24^{+}$ are IgD^{int}, IgM^{hi}, $CD27^{hi}$, $CD5^{-}$, $CD80^{int}$, $CD180^{hi}$; finally, $CD19^{+}CD38^{-}CD24^{-}$ are classified as IgD^{-}, IgM^{-}, $CD27^{int}$, $CD5^{hi}$, $CD80^{-}$, $CD180^{-}$ (Table 1). The table shows the percentage of positive cells of a representative experiment.

Age-related changes on B cell branch show a different distribution of naïve/memory subsets

To determine whether aging shapes the distribution of peripheral immune cell subsets, we investigated the B cell branch of a cohort of young and elderly people. Moreover, in order to assess if centenarian offspring, who are enriched for longevity, displayed a different immune profile, we compared data with those obtained by the study of age-matched control group. We performed a comparative analysis of these four subpopulations, evaluating their distribution in our different age groups. We did not observe any significant difference for $CD19^{+}CD38^{hi}CD24^{hi}$ and $CD19^{+}CD38^{int}CD24^{int}$ (data not shown). Concerning $CD19^{+}CD38^{-}CD24^{+}$, we observe a trend of age-related increase. Interestingly the amount of these cells in CO is more similar to that observed in young donors than in their age-matched controls (Fig. 2a). Moreover, $CD19^{+}CD38^{-}CD24^{-}$ B cells showed an highly significant age-related increase. So, concerning these lymphocyte subsets, centenarian offspring behave in the middle between young and elderly (Fig.2b).

Age-related changes of $IgM^{+}CD27^{-}$ (naïve mature), $IgD^{+}IgM^{+}CD27^{+}$ (natural effector), and $IgD^{-}IgM^{+}CD27^{+}$ (IgM-only) B cell subsets.

The complexity of the memory B cell pool has been further demonstrated by the evidence of different IgM^{+} B cell subsets. According to a recent gating strategy based on CD38/CD24 expression within $CD19^{+}$ B cell compartment, $CD38^{dim}CD24^{dim}$ were separated into three B cell populations: $IgM^{+}CD27^{-}$ (naïve mature), $IgD^{+}IgM^{+}CD27^{+}$ (natural effector) and $IgD^{-}IgM^{+}CD27^{+}$ (IgM-only) (Fig.3a) [21]. The comparative study between our four groups of subjects revealed a different distribution of B cells identified on the basis of these markers between young and old donors. Besides, centenarian offspring

show a youth immune profile when compared with age-matched controls. Indeed, the percentage of $IgM^{+}CD27^{-}$ (naïve mature) (Fig 3b) is significantly increased in the group of people genetically advantaged for longevity. The analysis of the other two $IgM^{+}CD27^{+}$ memory B cell subsets showed a significantly age-related reduction (fig 3c, d).

CpG + PMA/Ionomycin stimulation induce intracellular cytokine production by human blood B cells in vitro

To study the functional properties of peripheral blood B lymphocytes, we tested their ability to produce cytokines under "in vitro" combined action of CpG/PMA/Ionomycin. We evaluated the production of IL-10, IL-6 and TNF by CD38/CD24 B cell subsets. Our analysis revealed that 5h after this "strong" stimulation, the main IL-10 producing cells resulted $CD19^{+}CD38^{hi}CD24^{hi}$ (Table 2a). $CD19^{+}CD38^{-}CD24^{+}$ and $CD19^{+}CD38^{-}CD24^{-}$ resulted the more responsive B cell populations for TNF production (Table 2b). On the other hand, this kind of stimulation was not able to induce IL-6 production. Indeed, we did not detect a significant increase in production of IL-6 compared to unstimulated PBMC (not shown).

We analyzed the levels of IL-10 produced by $CD19^{+}CD38^{hi}CD24^{hi}$ comparing the different groups. Our analysis revealed a not significant age-related increase (not shown). Afterwards, we compared the two major B cell subpopulations involved in the TNF production. We did not detect any differences comparing the different groups (Table 2b).

Cytokines detection in the serum

We next evaluated any possible correlation between the level of cytokines detected after "in vitro" stimulation and basal levels observed in the serum of patients. For this purpose we performed the high sensitive assay CBA human multiflex

system in order to evaluate IL-10 and TNF production. In the present study, serum levels of IL-10 in the young resulted to be three to four time higher than those of elderly people (Fig. 4). Concerning TNF levels detected in the serum of patients, our preliminary data underlined individual differences that didn't allow us to perform a statistical analysis (data not shown).

RP105-negative B cells and its expression on IgD/CD27 B cell subsets

As the lack on B cells of the TLR4-homologous CD180 has been described in chronically stimulated SLE patients, we analyzed the percentage of $CD19^{+}CD180^{-}$ B lymphocytes obtained by peripheral blood of young, elderly, centenarian offspring (CO) and their age-matched controls (AM). In Fig.5a we report a significant increase of this population in the elderly when compared with young. No differences were obtained by the comparative analysis of CO and their controls although, the percentage of CD180- B cells in CO and AM controls is increased and decreased when compared respectively to that observed in young and old donors . Afterwards, we evaluated the presence of CD180 on cell subsets identified on the basis of the expression of IgD and CD27. Interestingly, the analysis of $IgD^{-}CD27^{-}$ (DN) B cells revealed that these cells are for the most part negative for CD180, whereas the other populations are $CD180^{+}$ (Fig. 5b).

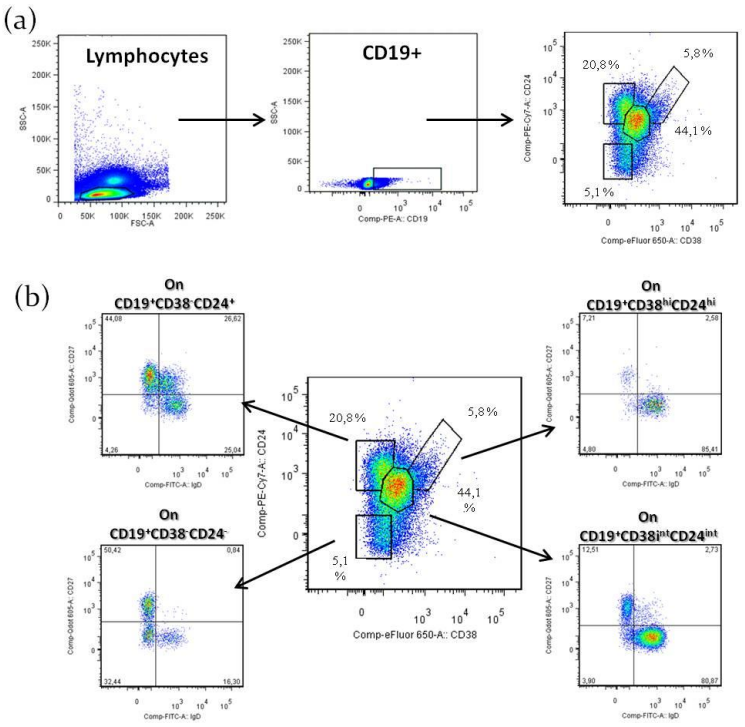

Fig. 1. (a) Gating strategy to identify 4 CD38/CD24 B cell subsets: $CD19^{+}CD38^{hi}CD24^{hi}$ (upper right), $CD19^{+}CD38^{int}CD24^{int}$ (the region in the middle), $CD19^{+}CD38^{-}CD24^{+}$(upper left), and $CD19^{+}CD38^{-}CD24^{-}$ (lower left). (b) Characterization of CD38/CD24 B cell subsets according to IgD/CD27 organization into 4 naïve-memory compartments. This phenotypical identification confirmed that the majority of $CD19^{+}CD38^{hi}CD24^{hi}$ and $CD19^{+}CD38^{int}CD24^{int}$ are naïve ($IgD^{+}CD27^{-}$), while $CD19^{+}CD38^{-}CD24^{+}$ begun to express a memory profile: some of them appear memory unswitched ($IgD^{+}CD27^{+}$), others memory switched ($IgD^{-}CD27^{+}$). Moreover, $CD19^{+}CD38^{-}CD24^{-}$ B cell subset, principally IgD^{-}, show features of late memory B cells ($IgD^{-}CD27$).

	Percentage of Positive cells *(MFI)			
Markers	$CD19^{+}CD38^{hi}CD24^{hi}$	$CD19^{+}CD38^{int}CD24^{int}$	$CD19^{+}CD38^{-}CD24^{+}$	$CD19^{+}CD38^{-}CD24^{-}$
IgD	86.2	82	48.4	12.3
CD27	8.4	14.2	68.8	51
IgM	51.3	30.1	45.8	8.1
CD5	21.3	8.5	6.4	47.2
*CD80	698	272	396	141
CD180	93.3	89.4	94.8	4.1

Table 1. A typical experiment showing the expression of IgD, CD27, IgM, CD5, CD80, CD180 on $CD19^{+}CD38^{hi}CD24^{hi}$, $CD19^{+}CD38^{int}CD24^{int}$, $CD19^{+}CD38^{-}CD24^{+}$, $CD19^{+}CD38^{-}CD24^{-}$ B cell subsets. Results, expressed as percentage of positive cells (CD80 as MFI), are representative of all subjects analyzed.

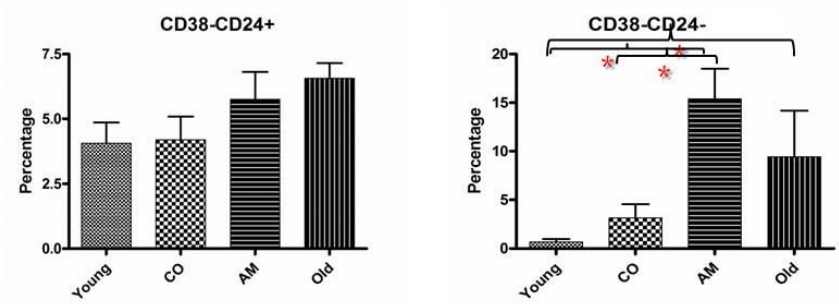

Fig. 2a (Left). Trend of increase age-related of $CD19^{+}CD38^{-}CD24^{+}$ B cell subset. B (Right). Significant increase of $CD19^{+}CD38^{-}CD24^{-}$ B cell subset in elderly people (Old and AM) when compared to young. CO, like young, show a lower percentage of this population when compared to their age-matched controls.

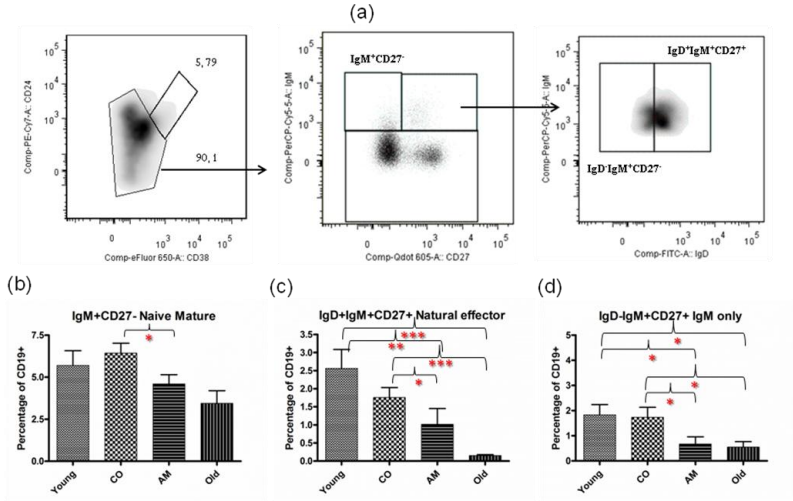

Fig. 3. (a) Diversity in the human B cell compartment and peculiar distribution in young and old, centenarian offspring (CO) and their age-matched (AM) controls. Within the $CD19^{+}$ B cell compartment, B lymphocytes are organized on the basis of CD38 and CD24 expression. According to Berkowska's gating strategy [21], $CD38^{dim}CD24^{dim}$ B lymphocytes were subdivided on the basis of IgM, CD27 and IgD expression, defining $IgM^{+}CD27^{-}$ naïve mature, $IgD^{+}IgM^{+}CD27^{+}$ natural effector and $IgD^{-}IgM^{+}CD27^{+}$ IgM-only. (b) A direct comparison between CO and AM underline as the former show a significantly higher percentage of naïve mature. (c) A substantial and significant age-related decrease was observed in the natural effector subset. (d) Moreover, elderly people display a decrease of IgM-only subset when compared with young such as CO exhibit higher level of the same population respect their AM controls.

(a)

IL-10				
Subsets	Young	Old	CO	AM
$CD38^{hi}CD24^{hi}$	917.4±310.5	2916.5±892.9	2059.3±277.8	2575.2±484.6
$CD38^{int}CD24^{int}$	316.4±37.1	2017.2±947.5	1066.8±427.2	924.2±523.7
$CD38^{-}CD24^{+}$	541.4±136.5	1137.7±106.9	780.7±111	746.5±157.9
$CD38^{-}CD24^{-}$	248.4±37.7	869±401.9	688.2±232.4	479±233.8

(b)

TNF				
Subsets	Young	Old	CO	AM
$CD38^{hi}CD24^{hi}$	194.4±39.4	380.5±67.1	317.5±45.1	313±20.4
$CD38^{int}CD24^{int}$	497.6±129	326.7±30.1	355.3±87.1	142.1±56.4
$CD38^{-}CD24^{+}$	881.6±176.2	453.7±65.1	605.3±122.9	265±112.4
$CD38^{-}CD24^{-}$	700.3±300.9	743.7±226.6	809.7±391	268±122

Table 2. Evaluation of IL-10 and TNF production by CD38/CD24 B cell subsets after CpG/PMA/Ionomycin stimulation. (a) $CD19^{+}CD38^{hi}CD24^{hi}$ is the main B cell subsets involved in IL-10 production. An age-related trend of increase was observed after 5h of stimulation. In the table 2(b) it is shown as the major contribute on the production of TNF is given by $CD38^{-}CD24^{+}$ and $CD38^{-}CD24^{-}$. No significant differences have been observed between the four groups analyzed. All the data are expressed as MFI (Mean ± SEM) and they are representative of 5 young, 4 elderly, 6 centenarian offspring and 4 age-matched controls.

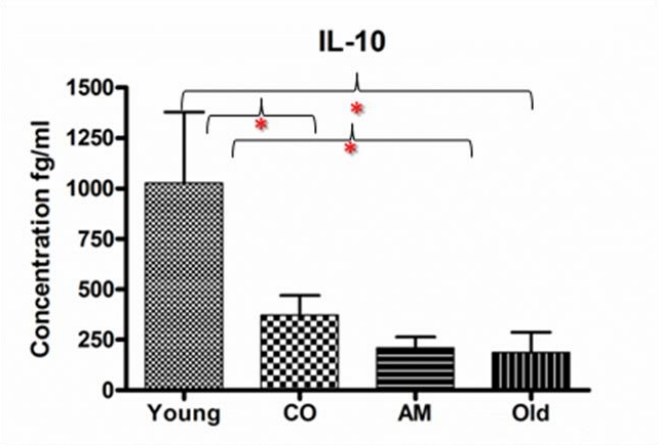

Fig.4. IL-10 detection in the serum of patients with cytometric bead array (CBA) human multiflex system (Becton Dickinson). Our analysis have revealed an higher concentration in young subjects compared with old people. No differences between centenarian offspring (CO) and age-matched (AM) was observed.

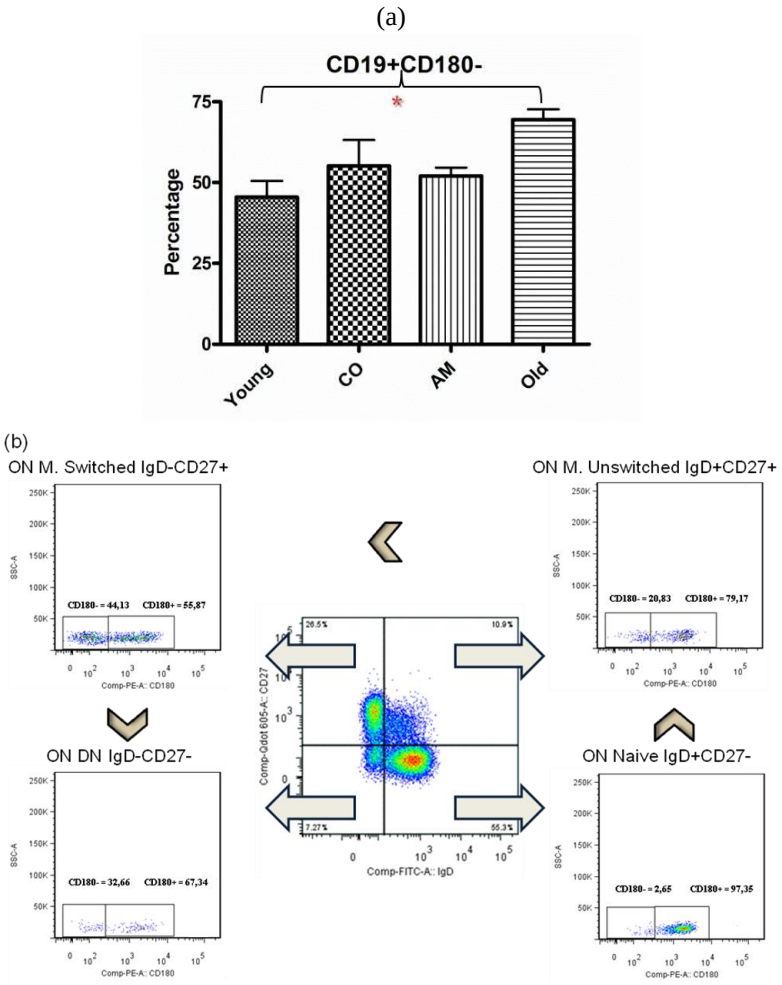

Fig. 5 (a) Evaluation of $CD19^{+}CD180$ (RP105)$^{-}$ B cell population among the different age related groups analyzed. Old people show a significantly increase in percentage of the B cell subset, that lack CD180 expression marker, when compared with young. ($p = 0.01$). Histograms are representative of all subjects included in the study. Bar graphs indicate mean ± SEM. (b) Representative dot plots showing CD180 modulation among naïve/memory B cell compartments identified by the expression of IgD and CD27. A progressive down-regulation of the expression marker from naïve ($IgD^{+}CD27^{-}$) towards DN ($IgD^{-}CD27^{-}$) is demonstrated.

BRIEF DISCUSSION

A typical feature of ageing is a pro-inflammatory status which is related to many age-related diseases (as cardiovascular, Alzheimer disease,). The increased health risk in old age is the result of a combined action of environmental stressors, that accumulate over time, and the individual genetic background. Besides, not all subjects or population groups are equally susceptible to the effects of the time-enduring stimulation of the immune system. It is known that in the elderly, there is an impairment of both innate and adaptive immune response that renders them less able to respond to new infectious agents and to vaccines [30-36]. On the other hand, recently evidences have shown a well preserved immune profile of a group of healthy elderly centenarian offspring (CO) who seem to have genetic and functional advantages associated with the reduced risk of disability with age [17, 38].

The B cell lineage in aging, undergoes dramatic alterations in the cellular composition. In these last years, several new B cell populations have been described although their phenotype has not been always associated with biological function. In the present work, we have characterized age-related changes in distinct peripheral B cell populations.

According to the gating strategy proposed by Carsetti et al., 2004, our results have shown 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 the never described $CD19^{+}CD38^{-}CD24^{-}$. We have confirmed data from literature that affirms that $CD19^{+}CD38^{hi}CD24^{hi}$, transitional B cells, have a regulatory function, as this population produce a large amount of IL-10 (Breg) [37]. Previous study have reported that the frequency of transitional B cells is rapidly reduced during the first years of life, to stabilize after 5 years of age [24]. Here, we observed no related changes with old age of this population. Interestingly, elderly people produce more IL-10 than young people after a

"strong" stimulation with CpG/PMA/Ionomycin. Probably, the increase in the production of this cytokine could be a mechanism of compensation of the typical pro-inflammatory status of elderly people. This observation is corroborated by the increase of the percentage of $CD19^{+}CD38^{hi}CD24^{hi}$ IL-10 producing cells in SLE patients [37]. Alternatively, in elderly people these cells are not the main source of IL-10. In our previous paper, we have described that both in young and elderly donors the physiological (α-CD40 and IL-4) stimulation of B cells induces a good IL-10 production by unswitched ($IgD^{+}CD27^{+}$) memory B cells. This kind of stimulation was also able to stimulate naïve (IgD+CD27-) B cells of elderly people to produce IL-10. Moreover, we observed that the "strong" stimulation (CpG/PMA/Ionomycin) induced both naïve and unswitched B cells of young donors to produce the anti-inflammatory cytokine. All the data suggest that naïve B cells from young donors need an adequate stimulus to be activated "in vitro". On the other hand, we believe that elderly people produce higher level of IL-10, maybe because they are basally activated by bacteria or viruses. So, another hypothesis could be related to the kind of the stimulus. It is also possible that any B cell might have the capacity to produce IL-10 if activated appropriately.

In this work, we have demonstrated for the first time, the existence of a new population of memory B cells with functional capacity. We have shown an age-related increase of the $CD19^{+}CD38^{-}CD24^{-}$ B cells. This B cell subset, as $IgD^{-}CD27^{-}$ (DN) B cells, lack the expression of IgM and CD80. Interestingly, we have observed that the great part of these cells express the marker CD5. We have also observed the ability of this population to produce "in vitro" the pro-inflammatory cytokine, TNF. We have performed the same analysis in CO and their AM controls. Our data show that CO behave as young donors, in fact they have a similar percentage of this B cell subset. We have hypothesized that the age-related increase of this population, as it has been previously demonstrated for IgD-

CD27- B cells [17,27], might be the result of the time-enduring stimulation of the immune system. For this purpose, we have assessed the distribution of the two markers, IgD and CD27 on the four CD38/CD24 B cells population above described. We have confirmed that, $CD19^{+}CD38^{hi}CD24^{hi}$ and $CD19^{+}CD38^{int}CD24^{int}$ have a naïve phenotype (IgD+CD27-); $CD19^{+}CD38^{-}CD24^{+}$ begin to express CD27 and contemporary loss IgD ($IgD^{+}CD27^{+}$/$IgD^{-}CD27^{+}$). Interestingly, the analysis of the $CD19^{+}CD38^{-}CD24^{-}$ B cell subset indicates that these cells are predominantly IgD- ($IgD^{-}CD27^{+}$/$IgD^{-}CD27^{-}$). This last subset could represent a population of late memory B cells. Moreover, all this data suggest a modulation of the expression of CD38 and CD24 from transitional to late memory developmental stage.

A further evaluation of CO B cell branch was performed evaluating the expression of CD27, IgM and IgD. It is in fact known that IgM memory B cells ($IgD^{-}IgM^{+}CD27^{+}$, "IgM-only" and $IgD^{+}IgM^{+}CD27^{+}$, "Natural effector" B cell cell subsets), are reduced in the elderly, so predisposing them to pneumococcal infection [12,19]. Here again, we show that CO has a "younger" B cell profile. In fact, the percentage of these cells are not reduced as their age-matched controls and are more similar to the percentage observed in young people. In addition, also the population of $IgM^{+}CD27^{-}$, called Naïve mature, is significantly increased in CO when compared with their controls.

It is well known that B cells participate in the immune response, not only producing antibodies, but also catching the pathogens by PRR (pattern recognition receptor) as TLRs. It has been recently demonstrated the expression, on murine B cells, of RP105 (CD180), the toll-like receptor- (TLR-)associated molecule. It has been shown that B cells from RP105-deficient mice were hypo-responsive to TLR-4 and TLR-2 stimulation [39,40]. In a recent work, authors have evaluated the expression of RP105 on peripheral blood B cells from patients with different autoimmune diseases. The number of RP105-negative B cells has been shown increased in SLE patients compared

with normal subjects [22]. Here, we have evaluated the percentage of B cells defective for CD180 expression on young, old, centenarian offspring and age-matched controls. Interestingly, we have observed an age-related increase of $CD19^{+}CD180^{-}$ B cell subset. Afterwards, we have checked the expression of CD180 on IgD/CD27 B cell subpopulations, observing a down-modulation of this marker from naïve ($IgD^{+}CD27^{-}$) to DN ($IgD^{-}CD27^{-}$) B cells. So, the reduction in CD180 expression observed in the elderly and the age-related increase of DN B cells, defective for CD180, could be related to increased susceptibility to GRAM- infectious diseases of elderly people. On the other hand, the amount of CD180-negative B cells in the other groups is similar: this result suggest that the loss of CD180 is probably an age-related phenomenon. The age range of CO is, in fact, 70.1 ± 8.3, whereas age range of old is 86.4 ± 3.8. We believe that CD180 may regulate B cell activation and that RP105-negative B cells might be involved in the production of autoantibodies and take part in pathophysiology of different autoimmune diseases. Further investigations could be necessary to better understand the biological role of CD180 that could be used as marker of senescence.

Next, we have evaluated the IL-10 levels detected in the serum of the four groups of subjects: we show that only young subjects have high levels of IL-10, whereas all the other groups (CO, AM and O) show a progressive decrease. This is an interesting data, although we have not significant differences between CO and AM or O, as CO show a slight higher level of this regulatory cytokine.

In the whole, our data suggest that the B cell branch of centenarian offspring behave similar to those observed in young subjects. This hypothesis is corroborated by the reduced number of “late memory” B cells and a higher level of IgM B cell subsets. These observations could suggest a good bone marrow reservoir of centenarian offspring. This apparently immunological advantage, could help them both to fight the

main age-related diseases and to properly respond to vaccinations.

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CHAPTER 9

Summary and General discussion

Summary and general discussion

The progressive and cumulative modifications of the immune system with age, known as “Immunosenescence” (Franceschi C et al., 1995; Pawelec G et al., 2005), have a great impact on immune performance in late life, contributing to the decreased ability of the elderly to respond to new antigens and vaccinations (Grubeck-Loebenstein B et al., 2009). The direct consequence of these modifications is the increased susceptibility of old people to infectious diseases. These changes occur in all leukocytes and accordingly, affect innate and adaptive immune functions. The acquired compartment of immune systems shows significant modifications in the elderly, in fact both T and B cell branches are compromises, resulting in a reduction of the number of virgin antigen-non experienced cells and, simultaneously, filling the immunological space with expanded clones of memory and effector, antigen-experienced cells (Franceschi C et al., 2000; Miller JP, Cancro MP, 2007).

The humoral immune response of elderly people is qualitatively and quantitatively diminished when compared with the immune response of young people. The amount of

the different Ig isotypes changes with age: in fact, although the number of B cells is decreased, it has been described an increase of serum concentration of IgG, IgA and, to a lesser extent, IgE. On the contrary, both IgM and IgD are decreased (Listì F et al., 2006).

A typical feature of aging is a chronic, low-grade pro-inflammatory status observed in the old people. In this context, elderly people are characterized by a general increase in the production of pro-inflammatory cytokines and inflammatory markers (Bruunsgaard H et al., 2003a, b, c; Krabbe KS et al., 2004; Fulop T et al., 2006;Cevenini E et al., 2010). The consequence of this situation is an age-dependent up-regulation of the inflammatory response, "Inflamm-aging", that render elderly prone to frailty (Balistreri CR et al., 2008; Franceschi C et al., 2005; Lio D et al., 2004; Pes et al., 2004).

Moreover, it has been suggested that chronic antigenic stimuli, such as herpes virus infection (e.g. CMV) appears to accelerate immune aging (Pawelec G et al., 2005; Akbar AN and Fletcher JM, 2005). The continuous attrition caused by chronic antigenic load results in a 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), that represents one of the main causes of disabilities among elderly people. The sequelae of central nervous system (CNS) inflammation processes, observed in the brain of AD patients, may be related primarily to waning microglial neuroprotection resulting in aging-related neurodegenerative changes (Streit WJ and Xue Q-S, 2010). Moreover, changes in lymphocytes distribution and in cytokines levels in the plasma of AD patients have been identified too (Richartz-Salzburger E et al., 2007; Larbi A et al., 2009; Pellicanò M et al., 2011).

In this scenario, however, centenarians represent a group of select survivors who have, at least, markedly delayed diseases that normally cause mortality in the general population. They are rightly considered an example of successful aging, a human model of disease-free or people in which the onset of the diseases is delayed (Franceschi C, Bonafè M, 2003). In addition, centenarian offspring (CO) have a genetic advantage that could predispose them for longevity and 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 the changes of the immune system with age observing any possible correlation between the genetic profile, predictor of longevity, and the immunological profile. In this context, we focused our attention principally on the naïve/memory B cell compartment of centenarian offspring. Concerning, instead, the study of the age-related diseases due to the pro-inflammatory microenvironment typical of the elderly, we have analyzed some immune parameters of people with Alzheimer's disease.

In **chapter 2**, we did a short report about the mechanisms of immunosenescence. We analyzed the modifications of the immune system in elderly people and their increased susceptibility to infectious diseases and pathological conditions relating to inflammation or autoreactivity. We discussed about the relationship between chronic CMV infection and adverse health outcomes, and the others immunological parameters define an immunological risk profile (IRP) predicting 2, 4 and 6 years of mortality. We also analyzed the modifications of the B cell branch during aging and the shrinkage of the immunological repertoire.

In **chapter 3,** we performed a comparative study of the B cell compartment of centenarian offspring (CO) and their age-matched controls (AM). In particular, we observed as

CO don’t show the typical naïve/memory shift of the elderly. This trend was confirmed also after the evaluation of immunoglobulins secreted in the serum. The concentration of IgM, a marker of the primary response, has shown higher levels in CO when compared to AM. All these data suggest a good bone marrow cell reservoir, supporting the hypothesis of a “familiar youth” of the immune system that could be a big advantage both to fight the main age-related diseases and to properly respond to vaccinations.

In **chapter 4**, we have analyzed blood lymphocyte subsets and the expression of activation markers on peripheral blood mononuclear cells (PBMC) from AD patients and their age-matched controls (HC) after in vitro activation with human recombinant amyloid beta peptide (rAβ-42). We have reported a basal expression of the activation marker CD69 and of the chemokine receptors CCR2 and CCR5 on T cells, and CCR2 on B lymphocytes after activation. We reported also the increased expression of the scavenger receptor CD36 on monocytes from AD subjects with or without stimulation. PBMC of AD patients stimulated in vitro with rAβ-42 are able to produce different chemokines and cytokines. These data support the involvement of the immune system in Alzheimer’s disease.

In **chapter 5**, we have reviewed recent works on B cell immunosenescence, focusing our attention on memory B cell subsets. We discussed about the origin and function of a specific memory B cells, IgG+IgD-CD27-, that is expanded in elderly people and in patients suffering by the chronic stimulation of the immune system, such as SLE and in healthy subjects challenged with RSV, but reduced in centenarian offspring and not increased in patients affected by Alzheimer's disease. We hypothesized that the expansion of these memory B cells might be the manifestation of an age-related physiologic modification (elderly) or a pathologic deregulation (SLE patients) of the immune system. Alternatively they might develop outside the germinal center (idea supported by the low rate of somatic hypermutation) or they might represent the first wave of memory B cells. Again, they could be represent exhausted/terminally differentiated memory B cells.

In **chapter 6**, we gained insight into the biological significance of the naïve/memory B cell subsets and their different function in young and elderly people, evaluating surface immunoglobulins (Igs), production of pro- and anti-inflammatory cytokines and somatic hypermutation. Our data suggest that naïve B cells (IgD+CD27-) of young subjects need an adequate stimulus to be activated "in vitro",

while the elderly has higher “in vitro” basal levels of IL-10 and TNF-α, maybe because they are basally activated, possibly by bacteria or viruses. Moreover, we evaluated the level of mutation in the V region of IgG genes in the DN (IgG+IgD-CD27-) B cells, observing as the rate of mutations is lower in the elderly. As we know that memory B cells are characterized by the high rate of somatic hypermutations, we hypothesized a different origin for these cells.

In **chapter 7**, we have tested the hypothesis that patients suffering from Alzheimer’s disease show systemic changes at the immunological level, which mainly involve CD4+ T cells, consistent with chronic antigenic stress potentially resulting in immune exhaustion. We observed an increase in percentage of CD4+CD28-CD27- late differentiated cells in AD patients compared to healthy elderly controls and a contemporary reduction in naïve T cells (CD4+CD28+CD27+CD45RA+CD45RO-). We hypothesized that the differences in naïve/memory CD4+ T cell subsets are caused by chronic antigenic stimulation by Aβ present in the blood of AD patients.

In **chapter 8**, 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+CD38hiCD24hi (Breg) is the main IL-10-producing B cell population. We have also observed the higher production levels of TNF by CD19+CD38-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.

Conclusions

"More than 1000 scientific paper on the biology of aging and longevity are published every month" (Hugo Aguilaniu, CNRS, Lyon). A long life in healthy, vigorous, youthful body has always been one of the humanity greatest dreams. The aspiration to delay aging and achieve immortality has

led researchers to study the characteristics of centenarians. Indeed, centenarians may represent the prototypes of successful aging, who have escaped major age-related diseases, reaching the extreme limit of human life in good clinical condition. The interest in centenarians as a model for healthy aging is driven by the desire to identify key factors associated with exceptional longevity in humans. Subsequently, the study of centenarian offspring came from the idea to find markers of successful aging in people genetically advantaged for longevity. Centenarian offspring, like their centenarian parents, have genetic and functional advantages that predispose them to healthy aging and longer survival.

In the present thesis we have focused our attention on the immunological changes that occur in old people and their relationship with the age-related pathologies. We try to explain the distinct roles played by humoral and cellular responses in protective immunity and the memory B and T cell differentiation in long-lived subjects. In contrast to T cells, B lymphocytes have not been extensively studied in the elderly, although B cell compartment is defective too (Cancro MP et al., 2009; Frasca D et al., 2011); indeed, in elderly people, the quality and the size of the antibody response is substantially impaired, the number of circulating

B cells is reduced and a shift from the naïve (CD27-) to memory (CD27+) B cells was also described (Gupta S et al., 2005; Listì F et al., 2006; Colonna-Romano G et al., 2008). Studying B cell populations in the elderly, our attention has been drawn to B lymphocytes negative for both IgD and CD27. We demonstrated that these cells are significantly increased in the elderly (Colonna-Romano G et al., 2009). Others have shown that the same population is expanded in patients suffering by the chronic stimulation of the immune system, such as SLE, HIV and in healthy subjects challenged with RSV (Wei C et al., 2007; Sanz I et al., 2008; Cagigi A et al., 2009). Recently, we have also observed that CD27- memory B cells have a low rate of somatic hypermutation when compared with classical CD27+ memory B cells (Buffa S et al., 2011). All these data support the hypothesis that these cells are senescent memory B cells that have down-modulated the expression of CD27, as specific CD8 T cells versus chronic antigenic stimuli, like infection with herpes viruses (e.g. CMV) (Pawelec G et al., 2005). Another hypothesis is that these cells might develop outside the germinal center (idea supported by the low rate of somatic hypermutation) or, alternatively, they might represent the first wave of memory B cells.

Moreover, it has been known that naïve and memory B cells produce different pro- and anti-inflammatory cytokines (Duddy ME et al., 2004, 2007; Sanz I et al., 2007, 2008; Lund FE, 2008). We have evaluated whether changes in the relative proportions of different B cell populations could affect the cytokine environment. In our recent paper we observed that under physiological stimulation (anti-CD40 and IL-4) both in young and in old people, a population of memory unswitched B cells (IgD+CD27+) is involved in IL-10 and TNF-α production. Interestingly, in the elderly, naïve (IgD+CD27-) B cells are also highly activated to produce cytokines under these conditions. Moreover, both memory unswitched than naïve B cells produce IL-10 with a strong activation (CpG/PMA/Ionomycin). We have observed that old donors behave similarly to young. So, we have hypothesized that naïve B cells from young donors need an adequate stimulus to be activated in vitro, while B cells from old subject have higher in vitro basal levels of IL-10 and TNF-α production, maybe because they may already be basally activated (e.g. by bacteria or viruses). We imagine a scenario where IL-10 production by naïve B cells may act as a control mechanism to prevent the exacerbation of inflammation, while IL-10 production by memory B cells might be active in the resolution of the disease.

On the other hand, we have studied the B cell compartment of centenarian offspring, comparing all the data with those observed in age-matched controls, trying to understand whether any differences exist. CO don't show the typical naïve/memory shift observed in elderly. In particular, we didn't see the increase of DN (IgD-CD27-) B cells. Also 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. In our manuscript in preparation, we have characterized a new population of memory B cells (CD38-CD24-) that result increased in the elderly. Again, comparing data obtained by CO with those observed in their age-matched controls, we have shown as the percentage of this population is significantly reduced in centenarian offspring. We suggest that this B cell population, that is negative for IgD expression, may represent a late stage of differentiation of memory B cells and that its increase could be a typical phenomenon due to the aging of the immune system. They participate to the generation of the inflammatory environment of elderly people by producing pro-inflammatory cytokines ,such as TNF. We have also observed the increase in percentage of CD19+CD180- B cells in the elderly. This marker, recently described as homologous of TLR4 that regulates TLR4 signaling, could

be involved in the recognition of exogenous pathogens and subsequent activation of the immune system. So, the reduction in CD180 expression observed in the elderly could explain their increased susceptibility to GRAM- infectious diseases. On the other hand, the difference of the immune system of CO, could be related with the major ability of bone marrow to generate new B cells and to properly respond to new antigens and vaccinations.

Besides, it has been known that aging is associated with chronic, low-grade pro-inflammatory status that, interacting with the genetic background, is potentially linked to the most-important age-related diseases. Alzheimer's disease (AD) is the most common form of dementia of elderly people. Many studies have reported alterations of the immune system in AD and the involvement of both the innate and acquired branches of the immune system (Vasto S et al., 2008; Britschgi M et al., 2007). In addition, low PBMC phagocytosis of amyloid beta (Avagyan H et al., 2009), and the increased expression of pro-inflammatory molecules (Reale M et al., 2008) confirm this hypothesis. A significant decrease of B and T cell percentage without changes of NK cells was previously reported (Richartz-Salzburger E et al., 2007). On the other hand, others investigators (Speciale L et al., 2007) reported no differences

in the percentage and absolute number of CD3+, CD4+, CD8+ and NK cells. In our previously study, we have reported a good response of T lymphocytes to beta amyloid; in fact, we have shown an over expression of CD69 activation marker and of the chemokine receptors CCR2 and CCR5 on T cells of AD patients when stimulated with rAβ-42. The same stimulus was able to induce production of different chemokines and pro-inflammatory cytokines by PMBC of AD patients. Moreover, some activation markers and chemokines receptors are over-expressed in unstimulated AD cells when compared to controls. We think that this data confirm the existence of the pro-inflammatory status that characterize AD patients. Our recent studies on AD patients revealed that the major changes are seen within the CD4+ T cell subset compared to healthy elderly controls (Pellicanò M et al., 2011), whereas the more marked changes in the CD8 subsets were seen equally in both AD and age-matched controls. The age-associated changes seen in CD8+ T cells are predominantly caused by infection (e.g. CMV), because CMV-negative individuals do not manifest these changes at older age (Chidrawar S et al., 2009). In contrast, the CD4+ cells are only marginally affected by CMV serostatus (Derhovanessian E et al., 2010). So, we hypothesized that the differences in CD4+ naïve, memory

and late differentiated T cell distribution between AD and their age-matched controls are caused by chronic antigenic stimulation by Aβ present in the blood. So, we believe that the increasing knowledge of the immunological aspects of different forms of dementia, could help us to see whether the differences we observed for CD4+ cells are specific to AD and they can be used as biomarkers for early diagnosis.

Moreover, we have studied the B cell branch of AD patients, in order to clarify the modifications that affect the immune system. Our observations about the B cell reduction both in percentage than in absolute number confirmed data discussed by Speciale (2007). In our previously work we observed an increased expression of the chemokine receptor CCR5 on B cells, after stimulation with rAβ-42, suggesting the involvement of B cells in the complex cellular alterations of this pathology. We have also shown a reduced capacity of B cells of AD patients to uptake Aβ compared to young and aged controls. We believe that the ineffective clearance of Aβ in the blood could explain the higher frequency of chronically stimulated CD4+ T cells. Further studies about the B cell branch of AD patients are necessary to understand its implication on the physiopathology of the disease.

In conclusion, in the present thesis I have reported the experimental observations that I obtained from the study of

the immune system during aging. I focused my attention on phenotypic, genetic and functional characteristics of elderly people. Moreover, I have also studied the B cell branch in healthy centenarian offspring trying to find some immunological markers that could explain their genetically advantaged for longevity. On the other hand, the analysis of the immune system of AD patients was important to understand the relevant modifications that could be related with the unsuccessful aging and usefully for an early diagnosis of disease.

CHAPTER 10

Sommario e Discussione generale

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 antigeni e vaccini (Grubeck-Loebenstein B et al., 2009). Il risultato più evidente di queste modificazioni risulta essere l'incrementata suscettibilità degli anziani alle malattie infettive. Questi cambiamenti avvengono in tutti i leucociti e, di conseguenza, riguardano sia le risposte immunitarie innate che quelle adattative. Il compartimento acquisito del sistema immunitario presenta evidenti variazioni negli anziani, infatti risultano compromesse sia la branca B che quella T, con il risultato di avere una riduzione del numero di linfociti vergini che non hanno mai incontrato l'antigene e, nello stesso tempo, 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 ai giovani. La quantità dei diversi isotipi immunoglobulinici cambia con l'età: infatti, sebbene il numero delle cellule B sia ridotto, è stato descritto un incremento della concentrazione sierica di IgG, IgA e, in misura minore anche di IgE. Al contrario, la concentrazione sierica di IgM e IgD è diminuita (Listì F et al., 2006).

Un elemento caratteristico dell'invecchiamento è rappresentato dallo stato di infiammazione cronica basale osservato negli anziani. In questo contesto, osserviamo un incremento generale nella produzione di citochine e marcatori di tipo pro-infiammatorio (Bruunsgaard H et al., 2003a, b, c; Krabbe KS et al., 2004; Fulop T et al., 2006;Cevenini E et al., 2010). Questa condizione determina un'aumentata regolazione della risposta infiammatoria, "Inflamm-aging", che predispone gli anziani alla fragilità (Balistreri CR et al., 2008; Franceschi C et al., 2005; Lio D et al., 2004; Pes et al., 2004).

Inoltre, è stato ipotizzato che stimoli antigenici cronici, come infezioni da parte di virus erpetici (es. CMV), possano accelerare il processo di invecchiamento (Pawelec G et al., 2005; Akbar AN and Fletcher JM, 2005). Il logoramento continuo del sistema immunitario causato dalla stimolazione

antigenica cronica determina la generazione di risposte di tipo infiammatorio che sono implicate nelle malattie età-correlate (Pawelec G et al., 2005; Vasto S et al., 2007).

Una tipica malattia età-correlata è la malattia di Alzheimer (AD), che rappresenta una delle principali cause di invalidità tra gli anziani. I processi di immunosenescenza osservati a livello del sistema nervoso centrale dei pazienti AD, potrebbero essere dovuti, principalmente, alla ridotta neuroprotezione a livello microgliale che determinerebbe la comparsa di eventi neurodegenerativi età-correlati (Streit WJ and Xue Q-S, 2010). Inoltre, nei pazienti con AD è stata descritta una diversa distribuzione linfocitaria e livelli diversi di citochine plasmatiche (Richartz-Salzburger E et al., 2007; Larbi A et al., 2009; Pellicanò M et al., 2011).

In ogni modo, in questo scenario i soggetti centenari rappresentano un gruppo di sopravvissuti selezionati in cui le malattie che normalmente causano mortalità nella popolazione insorgono più tardi. Queste persone sono considerate un esempio di invecchiamento con successo, un modello esente da malattie o dove l'insorgenza delle stesse è ritardato (Franceschi C, Bonafè M, 2003). Inoltre, i figli dei centenari (CO) hanno un background genetico che potrebbe predisporli alla longevità. Questi vantaggi sono evidenti, come la ridotta prevalenza di malattie età-correlate, in

particolare malattie cardiovascolari, ictus, 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 osservando, se esistono, dei collegamenti tra il background genetico, predittore di longevità, e il profilo immunologico. In questo contesto, abbiamo focalizzato la nostra attenzione principalmente, sul compartimento linfocitario B vergine/memoria dei figli di centenari. Invece, riguardo lo studio delle malattie età-correlate dovute al microambiente pro-infiammatorio degli anziani, abbiamo analizzato alcuni parametri immunologici delle persone affette dal morbo di Alzheimer.

Nel **capitolo 2**, abbiamo effettuato una valutazione dei meccanismi dell'immunosenescenza. Abbiamo analizzato i cambiamenti del sistema immunitario degli anziani e la loro maggiore suscettibilità a malattie infettive e ad eventi patologici legati a processi infiammatori e ad episodi di autoreattività. Abbiamo discusso sulla relazione esistente tra infezione cronica da CMV e peggioramento delle condizioni di salute. Abbiamo valutato gli altri parametri immunologici che definiscono il profilo immunologico di rischio (IRP) che predice mortalità a 2, 4 e 6 anni. Infine abbiamo analizzato le modificazioni della branca B cellulare durante

l'invecchiamento e il restringimento del repertorio recettoriale.

Nel **capitolo 3**, abbiamo effettuato uno studio comparativo tra il compartimento B dei figli dei centenari (CO) e i controlli della stessa età (AM). In particolare, abbiamo osservato come i CO non presentano il tipico spostamento vergine/ memoria delle persone anziane. Questo andamento è stato confermato anche a seguito della valutazione dei livelli sierici delle immunoglobuline. La concentrazione dell' IgM, un marcatore della risposta primaria, ha mostrato livelli significativamente più elevati nei CO rispetto agli AM. Questi dati, nel loro insieme, suggeriscono una buona riserva midollare, supportando l'ipotesi della "giovinezza familiare" del sistema immunitario che potrebbe essere un grosso vantaggio, sia per combattere le principali malattie legate all'età, sia per rispondere efficacemente ai vaccini.

Nel **capitolo 4**, abbiamo valutato nei pazienti AD e nei loro controlli età-correlati (HC), le popolazioni linfocitarie a livello ematico e l'espressione dei marcatori di attivazione nelle cellule mononucleate di sangue periferico (PBMC) dopo attivazione in vitro con il peptide ricombinate beta amiloide (rAβ-42). Abbiamo osservato un'espressione basale del marcatore di attivazione CD69, e dei recettori

chemochinici CCR5 sui linfociti T e del CCR2 e CCR5 sui linfociti T e B dopo attivazione. Inoltre, abbiamo visto nei monociti dei soggetti AD, sia con che senza attivazione, un'espressione incrementata del recettore scavenger CD36. Le PBMC dei pazienti affetti dal morbo di Alzheimer sono in grado di produrre diverse citochine e chemochine in seguito a stimolazione in vitro con rAβ-42. Questi dati supportano l'idea che prevede il coinvolgimento del sistema immunitario nella malattia di Alzheimer.

Nel **capitolo 5**, abbiamo esaminato i lavori recenti sull'immunosenescenza dei linfociti B, centrando l'attenzione sulle popolazioni di cellule memoria. Abbiamo discusso sull'origine e funzione di una particolare popolazione di cellule B della memoria, IgG+IgD-CD27-, che è aumentata negli anziani e in pazienti che hanno malattie che stimolano cronicamente il loro sistema immunitario, come il LES, e in soggetti sani sottoposti a challenge con virus respiratorio sinciziale, ma che troviamo ridotta nei figli di centenari e non aumentata nei pazienti con Alzheimer. Abbiamo formulato un'ipotesi, secondo la quale l'aumento di queste cellule B memoria potesse essere dovuto a condizioni fisiologiche legate all'età (negli anziani) o rappresentare una de-regolazione patologica del sistema immunitario (pazienti con LES). Alternativamente, tali

cellule potrebbero svilupparsi al di fuori del centro germinativo (idea sostenuta dal basso numero di mutazioni ipersomatiche), o ancora rappresentare una prima ondata di cellule B memoria. Un'altra possibilità potrebbe essere quella di cellule B memoria esauste e/o terminalmente differenziate.

Nel **capitolo 6**, abbiamo incrementato la nostra conoscenza circa il significato biologico delle sottopopolazioni vergini/memoria dei linfociti B, e la loro differente funzione nei giovani e negli anziani, valutando le immunoglobuline di superficie (Igs), la produzione di citochine pro- e anti-infiammatorie, e le ipermutazioni somatiche. I risultati che abbiamo ottenuto hanno evidenziato la necessità di uno stimolo adeguato per attivare in vitro le cellule vergini B (IgD+CD27-) dei giovani, mentre negli anziani le stesse cellule hanno livelli base più alti di IL-10 e TNF-α, probabilmente perché sono attivate già a livello basale a causa di infezioni da parte di virus e batteri. Inoltre, abbiamo valutato il livello di mutazioni nella regione V dei geni IgG nei linfociti B DN (IgG+IgD-CD27-), osservando come queste fossero ridotte negli anziani. Poiché è risaputo che le cellule B della memoria hanno un alto tasso di ipermutazioni somatiche, abbiamo ipotizzato una diversa origine per queste cellule.

Nel **capitolo 7**, abbiamo valutato l'ipotesi secondo la quale i pazienti che soffrono di Alzheimer presentano cambiamenti sistemici a livello immunologico, principalmente nei linfociti T CD4+, a causa di uno stress antigenico cronico con conseguente esaurimento immunitario. Abbiamo osservato un aumento percentuale delle cellule differenziate tardive, CD4+CD28-CD27-, e una contemporanea riduzione di cellule vergini T (CD4+CD28+CD27+CD45RA+CD45RO-), nei pazienti AD rispetto agli anziani controlli in buona salute. Abbiamo ipotizzato che le differenze delle popolazioni vergini/memoria dei linfociti T CD4+ sono determinate dalla stimolazione antigenica cronica indotta da Aβ presente nel sangue dei pazienti con AD.

Nel **capitolo 8**, abbiamo presentato un nostro lavoro recente sulla caratterizzazione dei linfociti B vergini/memoria basata sull'espressione di due marcatori di sviluppo regolato, 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 e, 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 in letteratura che affermano che la popolazione CD19+CD38hiCD24hi (Breg) è la principale popolazione B responsabile della produzione di IL-10 e, osservando come le popolazioni CD19+CD38-CD24+ e CD19+CD38-CD24- siano le principali 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. Questo potrebbe garantire loro una maggiore protezione contro il rischio di infezioni e i processi infiammatori tipici dei soggetti anziani.

Conclusioni

"Più di 1000 articoli scientifici sulla biologia dell'invecchiamento e sulla longevità sono pubblicati ogni mese" (Hugo Aguilaniu, CNRS, Lione). Il raggiungimento di un'età avanzata in buona salute è sempre stato uno dei più grandi sogni dell'umanità. L'aspirazione di ritardare l'invecchiamento e raggiungere l'immortalità ha indotto i ricercatori a studiare le caratteristiche dei centenari. Questi ultimi, infatti, potrebbero rappresentare il prototipo di invecchiamento con successo, esenti da malattie età-

correlate, raggiungendo i limiti estremi della vita umana in buone condizioni cliniche. L'interesse nello studio dei centenari, come modello di invecchiamento in buona salute, deriva dal desiderio di identificare quei fattori chiave associati con un'eccezionale longevità. Successivamente, sono stati studiati i figli dei centenari per cercare dei marcatori di invecchiamento con successo in persone geneticamente avvantaggiate per la longevità. I figli dei centenari, come i loro genitori, hanno vantaggi genetici e funzionali che li predispongono all'invecchiamento in buona salute e ad una migliore sopravvivenza.

Lo scopo di questa tesi è quello di studiare i cambiamenti immunologici che avvengono nei soggetti anziani e in soggetti che presentano delle malattie età-correlate. Abbiamo provato a spiegare le distinte funzioni delle risposte cellulari e umorali nell'azione protettiva del sistema immunitario e le differenze osservate nelle cellule memoria B e T negli anziani. A differenza dei linfociti T, le cellule B degli anziani non sono state studiate in maniera esaustiva, sebbene il compartimento B di questi soggetti risulti essere interessato (Cancro MP et al., 2009; Frasca D et al., 2011).Infatti, negli anziani, la risposta anticorpale è sostanzialmente compromessa, il numero dei linfociti B circolanti è ridotto e si osserva anche la diminuzione delle

cellule vergini (CD27-) ed un contemporaneo incremento di quelle memoria (CD27+) (Gupta S et al., 2005; Listì F et al., 2006; Colonna-Romano G et al., 2008). Durante questi tre anni, ho studiato il compartimento cellulare B in soggetti anziani, focalizzando l'attenzione su una particolare popolazione di linfociti B che non esprime nè IgD e neppure CD27. Dagli esperimenti effettuati è stato possibile dedurre che queste cellule sono significativamente aumentate negli anziani (Colonna-Romano G et al., 2009). Altri ricercatori hanno dimostrato che la stessa popolazione risultava aumentata in pazienti che soffrivano di una stimolazione cronica del sistema immunitario, come nel LES, HIV e in soggetti sani sottoposti a challenge con virus respiratorio sinciziale (Wei C et al., 2007; Sanz I et al., 2008; Cagigi A et al., 2009). Recentemente abbiamo valutato il numero di mutazioni somatiche nella regione V delle IgG delle sottopopolazioni IgG+IgD-CD27- e IgG+IgD-CD27+. Dall'analisi dei dati ottenuti è emerso che nelle cellule B CD27- vi è un tasso di ipermutazioni somatiche ridotto rispetto alle cellule CD27+ (Buffa S et al., 2011). Questi dati supportano la tesi secondo la quale queste cellule siano una popolazione memoria senescente che ha down-regolato l'espressione del CD27, come succede ai linfociti T CD8+ specifici in seguito a stimolazione cronica, ad esempio nel

caso di infezione da virus erpetici (es. CMV) (Pawelec G et al., 2005). Un'altra ipotesi è che queste cellule possano svilupparsi al di fuori del centro germinativo (idea supportata dal basso numero di mutazioni somatiche) o, ancora, rappresentare una prima "ondata" di cellule B della memoria. Inoltre, è noto che le cellule vergini e memoria producono differenti citochine pro- e anti-infiammatorie (Duddy ME et al., 2004, 2007; Sanz I et al., 2007, 2008; Lund FE, 2008). A tal proposito abbiamo valutato se i cambiamenti nelle proporzioni relative delle differenti popolazioni B potessero influenzare l'ambiente citochinico. In un nostro recente lavoro abbiamo osservato la produzione di IL-10 e TNF-α, da parte di una popolazione di linfociti B memoria unswitched (IgD+CD27+), a seguito di uno stimolo fisiologico (anti-CD40 e IL-4). Negli anziani, però, anche i linfociti B vergini (IgD+CD27-) rispondono allo stimolo producendo citochine. Invece, con uno stimolo forte (CpG/PMA/Ionomycin) sia le cellule B memoria unswitched che le vergini di giovani e anziani producono IL-10. Quindi, abbiamo ipotizzato che i linfociti B vergini dei giovani necessitano di uno stimolo adeguato per poter essere attivati *in vitro*, mentre le cellule B degli anziani producono elevati livelli basali di IL-10 e TNF-α,, probabilmente perché sono già attivati, per esempio a causa di infezioni da

parte di batteri o virus. Pertanto, immaginiamo uno scenario in cui l'IL-10 prodotta dai linfociti B vergini possa agire come un meccanismo di controllo in grado di prevenire l'esacerbazione dei processi infiammatori, mentre l' IL-10 sintetizzata dalle cellule B della memoria possa avere un ruolo nella risoluzione della malattia.

Inoltre, abbiamo studiato il compartimento cellulare B dei figli di centenari (CO), effettuando un'analisi comparativa con un gruppo di soggetti avente la stessa età, al fine di valutarne eventuali differenze. I CO non hanno la caratteristica distribuzione vergine/memoria che invece contraddistingue gli anziani. In particolare, non abbiamo osservato l'incremento della popolazione cellulare B DN (IgD-CD27-). Anche la valutazione dei livelli sierici della IgM nei CO ha mostrato dei livelli simili a quelli osservati nei giovani. Nel nostro lavoro in fase di preparazione, abbiamo caratterizzato una nuova popolazione di linfociti B memoria (CD38-CD24-) che è aumentata negli anziani. Ancora una volta, confrontando i dati ottenuti dall'analisi dei CO con quelli relativi ai loro controlli, abbiamo osservato come la percentuale di questa popolazione risultasse ridotta nei figli di centenari. Quindi, questi linfociti B, che non esprimono IgD, potrebbero costituire una popolazione memoria tardiva il cui incremento sembrerebbe essere

correlato al fisiologico invecchiamento del sistema immune e che contribuire alla generazione del caratteristico ambiente pro-infiammatorio dell'anziano, producendo TNF-α. Oltre alla popolazione descritta precedente, nei soggetti anziani, abbiamo osservato l'incremento di un altro gruppo di cellule B, ossia quelle CD19+CD180-. Il CD180, descritto di recente in letteratura, è un omologo del TLR4 e ne regola il pathway. Si pensa che possa essere coinvolto nel riconoscimento dei patogeni esogeni e nella successiva attivazione del sistema immune. Di conseguenza, la ridotta espressione del CD180 negli anziani potrebbe spiegare la loro incrementata suscettibilità alle infezioni da parte di batteri GRAM-. Inoltre, le differenze del sistema immune degli figli di centenari, potrebbero essere correlate con la maggiore capacità del midollo osseo di questi soggetti di generare nuove cellule B e di rispondere in modo migliore sia ai nuovi antigeni che alle vaccinazioni.

Inoltre, è noto che l'invecchiamento è associato con uno stato pro-infiammatorio che, insieme al background genetico, aumenta il rischio di sviluppare diverse malattie età-correlate. La malattia di Alzheimer (AD) è la più comune forma di demenza negli anziani. Molti studi hanno riportato alterazioni del sistema immune innato e adattativo in pazienti con AD (Vasto S et al., 2008; Britschgi M et al.,

2007). Tale ipotesi è stata confermata dalla valutazione della ridotta fagocitosi della proteina beta amiloide da parte delle PBMC (Avagyan H et al., 2009) e dall'aumentata espressione di molecole di tipo pro-infiammatorio (Reale M et al., 2008). Studi precedenti hanno riportato una riduzione significativa della percentuale di cellule B e T ma non di quelle NK (Richartz-Salzburger E et al., 2007). Altri ricercatori, invece, non hanno osservato differenze né in termine percentuale, né di numero assoluto, delle cellule CD3+, CD4+, CD8+ e NK (Speciale L et al., 2007). In un nostro lavoro precedente, abbiamo riportato una buona risposta dei linfociti T alla stimolazione con la proteina beta amiloide; i pazienti AD, infatti, a seguito della stimolazione con rAβ-42, presentavano l' over-espressione del marcatore di attivazione CD69 e dei recettori chemochinici CCR2 e CCR5 sui linfociti T quando confrontati con i controlli sani. Inoltre, sempre in questi soggetti, lo stesso stimolo induceva l'incremento della produzione di diverse chemochine e citochine pro-infiammatorie da parte delle PBMC. Diversamente da ciò, alcune popolazioni cellulari di soggetti con malattia di Alzheimer non stimolate, presentavano un' over-espressione di alcuni marcatori di attivazione e recettori chemochinici rispetto i controlli. Questa è una chiara evidenza dello stato pro-infiammatorio

che caratterizza la malattia di Alzheimer. In un nostro recente studio, abbiamo osservato come i maggiori cambiamenti nei pazienti AD rispetto ai loro controlli, siano a livello della popolazione CD4+; invece, i cambiamenti del compartimento CD8+ , osservati sia nei soggetti con malattia di Alzheimer che nei loro controlli, sembrano essere causati fondamentalmente da infezioni (es. CMV). Soggetti negativi al CMV non presentano le stesse caratteristiche dei soggetti anziani (Chidrawar S et al., 2009). E' stato dimostrato che i cambiamenti relativi ai linfociti T CD4+ sono condizionati solo marginalmente dalle infezioni da CMV (Derhovanessian E et al., 2010). Abbiamo ipotizzato, pertanto, che le differenze nel compartimento T CD4+ di cellule vergini, memoria e differenziate tra i pazienti Alzheimer e i loro controlli, siano causate dalla stimolazione antigenica cronica della proteina Aβ presente nel sangue. Noi pensiamo che sia importante approfondire lo studio del sistema immunitario nelle diverse manifestazioni di demenza, per vedere se tali differenze osservate nel compartimento CD4+ siano specifiche della malattia di Alzheimer o possano essere utilizzate come dei marcatori biologici per fare una diagnosi precoce.

Inoltre, abbiamo cominciato a studiare la branca B cellulare dei pazienti con malattia di Alzheimer, al fine di

avere un'idea più chiara circa i cambiamenti che affliggono il sistema immunitario di questi persone. Abbiamo confermato la riduzione sia percentuale che di numero assoluto dei linfociti B già discussa da Speciale (2007). In un nostro lavoro precedente, abbiamo osservato l'aumento dell'espressione del recettore chemochinico CCR5 sui linfociti B a seguito della stimolazione con rAβ-42, suggerendo il coinvolgimento della branca B nelle complesse alterazioni che caratterizzano la malattia. Abbiamo inoltre mostrato la ridotta capacità dei linfociti B dei pazienti AD di riconoscere il peptide Aβ rispetto ai giovani e agli anziani controllo. Noi crediamo che l'eliminazione inefficace di Aβ dal sangue possa essere correlata all'aumento dei linfociti T CD4+ cronicamente stimolati.

Studi futuri sul compartimento cellulare B in pazienti con malattia di Alzheimer saranno necessari per comprendere il loro probabile coinvolgimento nella fisiopatologia della malattia.

In conclusione, in questa tesi ho riportato le osservazioni sperimentali che ho ottenuto dallo studio del sistema immune durante l'invecchiamento. In particolar modo, mi sono occupato della caratterizzazione fenotipica, genetica e funzionale del sistema immune dei soggetti anziani. Inoltre,

ho anche studiato il compartimento cellulare B nei figli di centenari col fine di trovare qualche marcatore immunologico di longevità. Lo studio del sistema immunitario dei soggetti con malattia di Alzheimer potrebbe permettere di identificare dei marcatori di invecchiamento senza successo, utili per una diagnosi precoce della malattia.

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Curriculum Vitae

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Silvio Buffa was born on 3th April 1983 in Palermo, Italy. In 2001 he got a Scientific Diploma. In 2007 he graduated in Biological Science, cellular and molecular specialty at the University of Palermo. In 2008 he qualified as a professional biologist. In January 2009, he started his doctorate under the supervision of Prof.ssa G. Colonna Romano purchasing advanced specialized studies in Immunology with particular attention on mechanisms of aging.