Successful immunosenescence and the remodelling of immune responses with ageing

Claudio Franceschi1, Daniela Monti1, Daniela Barbieri1, Stefano Salvioli1, Emanuela Grassilli1, Miriam Capri1, Leonarda Troiano1, Marcello Guido1, Massimiliano Bonafé1, Franco Tropea1, Paolo Salomoni1, Francesca Benatti1, Enrica Bellesia1, Sabrina Macchioni1, Roberta Anderlini1, Paolo Sansoni2, Stefano Mariotti3, Mary Louise Wratten4, Ciro Tetta4 and Andrea Cossarizza1

Abstract. In recent decades, major theoretical and technological advances have been achieved in the field of immunology. These have allowed the scientific community to analyse the immune system in a much more sophisticated manner than was possible even 20 years ago. Moreover, great theoretical changes have also occurred in gerontology—in particular, the hypothesis has been put forward that ageing and diseases are two different phenomena, and that successful ageing, i.e. ageing in good psychophysical conditions, is really possible for most humans and animals. Immunosenece was then carefully investigated, either in selected healthy people of advanced age or in the oldest old people, such as healthy centenarians. The main results showed that most immune parameters are indeed well preserved even at this far advanced age. This paper deals with some of the most important theoretical problems of immunosenescence. An immunological tenet was that the most important phenomenon of immunosenescence is the involution of the thymus. In most textbooks and papers it is taken for granted that the thymus starts its involution immediately after puberty. When people aged 60–65 were considered old, it was not difficult to think that they could live for the rest of their life with a fully involuted thymus. The findings on centenarians challenge this tenet, as they have only a small reduction of T lymphocytes, and a relatively normal number of virgin and memory T cells, together with a functional T cell repertoire. Other observations reported here on centenarians, concerning the activity of B lymphocytes and the cytokine network, as well as those on the well-preserved innate immunity and the cells’ capability of undergoing proliferation after appropriate stimuli, suggest that complex immune changes occur with age, but also indicate that we have to modify our attitude, to grasp the new scenario which is emerging. Immunosenescence can no longer be considered as a unidirectional deterioration, and this complex phenomenon is much better described by terms such as ‘remodelling’, ‘reshaping’ or ‘retuning’.

Key words: ageing; longevity; centenarians; immunosenescence

Ageing and longevity: a theoretical approach

Ageing has been studied extensively. However, longevity, and particularly human longevity, has been neglected [1]. Hundreds of theories are available on ageing, indicating that scientists are still far from understanding the biological and cultural basis of this process. To this long list of theories, we have added a new one, based on the consideration that the maintenance of soma integrity is the consequence of a continuous activity of a limited number of cellular defence mechanisms [2–4]. We have hypothesized that DNA repair, enzymatic and non-enzymatic antioxidants, production of heat shock and stress proteins, and activity of poly(ADP-ribose)polymerase form a network of interconnected cellular defence mechanisms, whose global efficiency has been evolutionarily set at different levels in different species and in different individuals of the same species. We have also speculated that apoptosis is a fundamental biological process which can join the list of cellular defence mechanisms, being an ancestral process used to eliminate damaged, mutated, viral-infected or transformed cells [5,6]. Overall, the above-mentioned cellular defence mechanisms can be considered as the basic molecular and cellular anti-ageing systems.
However, it can be argued that ageing is not simply a cellular mechanism but that it represents the failure of more integrated systems whose purpose is to preserve body integrity. The nervous, the endocrine and the immune systems are all devoted to the maintenance of body homeostasis. Moreover, we and others have argued that these three systems, which co-evolved together, have to be studied as a whole [7], and that the more appropriate approach is to consider immune-neuroendocrine cells and organs as part of a unique system devoted to cope with all kinds of internal and external damaging agents [8,9]. In previous papers we have suggested that the immune-neuroendocrine system relies upon the above-mentioned molecular and cellular defense mechanisms for its continuous activity [3]. This point of view represents a tentative attempt to combine molecular and cellular with systemic theories of ageing. It can be predicted that the optimal functioning of this immune-neuroendocrine system is of major importance for survival, ageing and longevity. Accordingly, as far as human longevity is concerned, we hypothesized that people who survived in good conditions for long periods, close to the maximum lifespan of our species, should be equipped with an optimal immune-neuroendocrine system [3]. For this reason, research started some years ago on the biological basis of human longevity, in a selected group of healthy centenarians. We will report here some of the data collected in the last few years on the immune system of centenarians. These data are the first part of a broader investigation in which immune and neuroendocrine parameters will be analysed in order to understand some of the molecular and cellular bases of human longevity. A genetic search for longevity assurance genes is also in progress.

An old tenet: immunosenescence = immunodeterioration

The ageing of the immune system has been thoroughly studied in a variety of models and, particularly, in rodents and humans.

The result of this intense investigation can be summarized with the equation immunosenescence = immunodeterioration [10]. Increased sensitivity to infectious diseases and cancer, decreased antibody production to non-self antigens and increased levels of autoantibodies, defective natural killer cell (NK) activity and decreased T lymphocyte proliferation have been considered a paradigm of a defective immune responsiveness with age. Moreover, it has also been assumed that most of this age-related immune deterioration has to be ascribed to profound and early changes in the thymus, whose involution starts immediately after puberty [11]. As a consequence, another popular tenet in immunogerontology is that the cellular, or T lymphocyte branch, suffers because of age more than the humoral, or B lymphocyte, branch. This scenario is mainly based on data obtained in rodents. In this review, we will try to demonstrate that in humans the deterioration of the immune system with age is not as dramatic as that reported in experimental animals. Moreover, we will review data suggesting that the time is right to challenge the above-mentioned tenet.

Immunogerontological bias

We and others have been interested in analysing human immunosenescence. Some years ago, Dutch immunologists suggested that strict biochemical and clinical inclusion and exclusion criteria, known as the SENIEUR Protocol, should be adopted in studies of immunosenescence [12,13]. This proposal was aimed at avoiding a classical bias in gerontological studies, i.e. the confusion between ageing and age-related diseases. Indeed, an entirely new scenario emerged when the immune system of carefully selected, healthy, elderly people was investigated. Lessons have been derived from this approach. In humans, physiological ageing of the immune system is probably not as altered as previously thought [14,15]. This hypothesis is a great challenge owing to the importance of the immune system for immunological pathologies but also for other diseases not traditionally included among immune diseases, in which immune responses can play an important or even crucial role (e.g. atherosclerosis, dementia, cancer).

In comparison with the enormous literature on immunosenescence in rodents and in elderly humans, only scanty and anecdotal data are available on people older than 80–85 years. This is strange for two main reasons: firstly because human lifespan is considerably longer, i.e. 110–120 years, and secondly because the number of old people is increasing dramatically in all countries, and particularly in those that are economically (and immunologically!) developed [16,17]. Thus, the last three or four decades of human life have been left unexplored immunologically. Moreover, the fact that most studies simply compare immune parameters from 'young' and 'old' subjects does not allow investigators fully to understand the biological significance of the changes as they occur over time. Human ageing is a slow process, and it is difficult to choose appropriate and reliable criteria for assessing it. Finally, the literature on immunosenescence is confused since, in many reports, data referring to lymphocyte subsets are presented as percentages but not as absolute numbers. These inconsistencies can create the illusion that nothing changes with age (e.g. the percentage of CD3+ cells), whereas upon closer examination, a significant decrease in these cells does occur [18,19].

Centenarians are exceptional individuals

For all these reasons—namely (i) selection and healthy status of the subjects; (ii) necessity to explore the last decades of human life; (iii) importance of successful ageing to appreciate physiological immunosenescence)—we began to investigate the immune system of...
centenarians. We will refer to centenarians as people who are not only older than 100 years, but also in good mental (e.g. practising pharmacist) and physical condition (able to chop firewood!). Generally, centenarians are considered to be a rare curiosity. However, this is not the case. First, their number is increasing dramatically, and, according to recent predictions, those surviving longer than 95 or 100 years will soon represent a consistent group [16,17]. In Italy, preliminary data based on a nationwide investigation co-ordinated by Professor Luciano Motta (University of Catania) indicate that there are now ~6000 centenarians. About one-third of them are in relatively good mental and physical condition, and can be considered 'healthy' centenarians. We think that the SENIEUR Protocol, proposed by Ligthart et al. for elderly people [12,13], should be reviewed for application to people aged over 100 years. We are elaborating such a protocol, to be adapted to healthy centenarians, within the framework of the Concerted Action Programme on Molecular Gerontology of the EU BioMed Research Programme.

Secondly, healthy centenarians are the best example of successful ageing, namely people who have escaped major age-related diseases and reached the extreme limit of human life in good clinical conditions [20]. In most cases, the histories of these exceptional individuals reveal them to be free of cancer, dementia, diabetes, cardiovascular diseases and cataract. Moreover, as discussed above, in order to reach such an advanced age, centenarians should be equipped with well preserved and efficient immune and defence mechanisms, and have optimal combinations of appropriate lifestyle and genetic background [21,22]. Thus, the study of centenarians, and particularly that of healthy centenarians, is not only of broad biological and medical interest, but can help in identifying genes that prevent the above-mentioned age-related diseases.

The study of humoral immunity and the first paradox of successful immunosenescence: increase in immunoglobulin, decrease in B cells, lack of organ-specific autoantibodies

As far as humoral immunity is concerned, the tenet that ageing equals deterioration is based on the observation that there is an increased frequency with age of pathological processes involving B cells and antibody production, such as B chronic lymphocytic leukemia (B-CLL), presence of autoantibodies or monoclonal gammapathies, and amyloidogenesis. Moreover, the decreased antibody response may also result in a propensity for infectious diseases, particularly pneumonia, or recurrent infections, as well as poor responses to vaccinations against the causative agents, resulting on the whole in an increased morbidity and mortality in elderly subjects. Most of the data on this topic have been collected before the 'revolution' in immunosenescence caused by the use of SENIEUR Protocol in immunogerontological studies. For this reason, we begun the analysis of humoral immunity in healthy elderly people, including centenarians. First of all, we faced an unpredicted paradox concerning humoral immunity with age, i.e. an increase of serum immunoglobulin (Ig) and a concomitant decrease in peripheral blood lymphocytes [19,23,24].

Most studies in the past two decades have addressed the assay of Ig classes and subclasses in sera from aged human subjects, in attempts to establish the normal range for laboratory purposes, as well as to investigate the physiology of this classical parameter of humoral immunity with ageing. By analysing 87 sera of healthy subjects belonging to several age groups, carefully selected according to the established criteria of the SENIEUR Protocol, and including a group of healthy centenarians, we found that both serum IgG and IgA significantly increase with age, whereas IgM remains unchanged [23]. Moreover, among IgG subclasses, we observed that IgG1, IgG2 and IgG3 showed a significant increase, whereas IgG4 did not [23]. An increased in vitro Ig production by B cells from aged people has been previously reported [25]. It is interesting to remember that IgG1 and IgG3 are mainly involved in the humoral responses to viral and bacterial antigens; IgG2—together with IgM—are responsible for responses to polysaccharides (mainly outer wall antigens of capsulated bacteria); and IgG4, with IgE, are increased in response to parasite antigens, as well as being the 'memory' isotype in conditions of chronic high dose exposure [26]. This increase of IgG and IgA antibodies may afford greater protection against viral and bacterial infections in healthy aged people and centenarians. It is also noteworthy that very few IgG subclass defects were found in elderly subjects, with the exception of IgG4 deficiency [23,27].

Decreased numbers of lymphocytes were found in selected healthy elderly subjects [19,25]. Despite small changes in percentages of lymphocyte subsets, all were significantly decreased as absolute numbers [19]. B lymphocytes in centenarians were reported to be increased, although not significantly. In contrast, we observed a striking decrease of CD19+ B cells both in the 70–83 year age group and in centenarians [23,24]. The percentage of these cells also decreased, from 13.5% in young controls, to 9.4% and 3.2% in the two groups of aged people. Further, we found that the CD19+ cells co-expressing the CD5 molecule also decreased with age [23,24]. These cells represent a distinct subset of B lymphocytes able to produce polyreactive autoantibodies, and can originate B-CLL. This finding confirmed that we did not select subjects with unrecognized B-CLL, which is common in elderly subjects.

The scenario regarding the changes in humoral immunity that occur in elderly subjects with age is even more complex if we consider the problem of autoimmunity and, in particular, that of autoantibodies. It has been reported that the frequency of subjects with detectable serum organ-specific or non-organ-specific autoantibodies increases with age. This tenet was challenged by our observation that organ-specific
autoantibodies are practically absent in the plasma of healthy centenarians [24]. We subsequently showed that this is not a peculiar characteristic of centenarians, as the absence of autoantibodies also occurs in healthy old people [24]. In contrast, unselected, elderly people presented an age-related increase in these autoantibodies [24,28]. Non-organ-specific autoantibodies follow a different trend, increasing also in healthy aged donors, including centenarians (manuscript in preparation).

These data on the humoral immunity of elderly subjects raise several questions, and different possibilities can be envisaged to explain these age-related changes. In particular, it is possible to hypothesize that increased number of B lymphocytes and plasma cells occurs in organs other than peripheral blood, or that the lifespan of B lymphocytes and plasma cells in germinal centers is increased in aged people. Finally, an increased production of Ig per cell has to be ruled out. According to the first and second hypotheses, alterations with age of B cell homing and propensity to apoptosis can be predicted. Indeed, recent data from our laboratory suggest that the membrane expression of certain molecules involved in homing processes and of certain cell adhesion molecules changes with age (manuscript in preparation). A different propensity to apoptosis has been observed in peripheral blood lymphocytes from centenarians after exposure to damaging viral or chemical agents (manuscript in preparation).

The study of cellular immunity and the second paradox of successful immunosenescence: presence of a well-preserved number of T cells in elderly subjects and centenarians, despite a thymus involuting since puberty

Unexpectedly, age-related changes in the T cell compartment were much less dramatic than we would have predicted according to the data in rodents and in unselected aged subjects. Moreover, it is noteworthy that some parameters were found to decrease, while others increased.

In particular, we found that the absolute number of CD3^hi, CD4^hi and CD8^hi T cells decreased with age [19,29], while activated peripheral T cells (HLA-DR^hi) were markedly augmented. In both cases centenarians did not escape this destiny.

Another interesting trend has been recorded as far as percentage and absolute number of ‘virgin/unprimed’ (CD45RA^hi) and ‘memory/activated’ T cells (CD45RO^hi) was concerned: these parameters did not change significantly after the fourth decade of life [30]. Indeed, a dramatic decrease in CD45RA^hi T cells occurred from birth to the age of 30 years, with a concomitant increase in CD45RO^hi lymphocytes. Old people and centenarians still showed a consistent number of either CD4^hi or CD8^hi virgin T cells [30].

Thus, the following questions arise: where do these T lymphocytes come from? Why are sharp changes in expression of CD45 isoforms mainly observed in the first two or three decades of life? These modifications seem to mirror the involutive changes of the thymus, which take place immediately after puberty [31], i.e. at least 80 years ago. Assuming that a lifespan of several decades is highly improbable for most memory T cells, we need to understand the origin and continuous renewal of virgin and memory T cell compartments when the thymus has probably undergone a profound involution, as in centenarians. In any case, it is difficult to explain why a consistent number of T cells shows a virgin phenotype, waiting for possible new antigens, even in far advanced age. Notwithstanding the fact that reversions from one isoform to the other have been described [32–35], and that the presence of antigens is required for ‘memory’ cells to survive [36–38], the possibility exists that the thymus becomes progressively less important as the T lymphocyte producer, and that its role is taken over by other ‘peripheral’ lymphoid organ(s). We must assume, therefore, that negative and positive selection are occurring successfully in these organ(s), as demonstrated by a lack of autoimmune responses in healthy centenarians. Thus, thymic remnants or substituting organ(s) are probably able to produce and select high numbers of T cells every day, until the extreme limit of human life.

The Cell division in centenarians: slightly delayed but longer lasting

Ageing is characterized by a variety of alterations which occur in most organs and cell types. Loss of proliferative vigour is considered a marker of the ageing process and is related to the Hayflick phenomenon, i.e. the limited number of replications that normal cells can undergo. It has been demonstrated that there is a correlation between proliferative capability and maximum lifespan in different species, and an inverse correlation between proliferative capability and donor age. Thus, a decreased capability to proliferate may be considered a characteristic of cellular senescence.

In a recent paper we argued that an intriguing relationship exists between cellular senescence, tumour growth and longevity [6]. All these phenomena are deeply related to programmed cell death or apoptosis [39]. A possible scenario is the following: cells may be equipped with genes which actively promote cellular senescence thus controlling cell death in order and escaping transformation [5,40]. This situation is balanced by other genes responsible for survival and viability. Circumstantial evidence suggests that cellular senescence may be considered a peculiar type of cell differentiation whose biological function is to counteract uncontrolled cell proliferation. From this point of view, cellular senescence can be considered one of the most important mechanisms in avoiding the continuous onset of tumours. The most effective evolutive way for a cell to control neoplastic growth is probably to set up genes which promote apoptotic cell death [41–49].
Moreover, we were able to show that there is an intriguing relationship at the molecular level between cell proliferation and apoptosis [50-55]. Interestingly, and unexpectedly, we have recently found that lymphocytes become progressively resistant to apoptosis with increasing age of the donor, and centenarians follow this trend (manuscript in preparation). At present, it is difficult to reconcile this findings with the above-mentioned hypothesis based on the consideration that apoptosis is the main mechanism for getting rid of mutated and potentially transformed cells. In any case, resistance to apoptosis could contribute to cellular longevity, and, possibly, to organismic longevity.

It has been suggested that a crucial change in the immune system is the reduced capability of its component cells to proliferate, a problem related to clonal expansion after exposure to antigenic stimuli [56]. We found that T lymphocytes from healthy centenarians were fully capable of proliferating, and that the only difference vis-à-vis young people (20-30 years old) is a delay in peak responsiveness [21]. Recent data on the proliferative capability of fibroblasts from centenarians are in full agreement with the notion that they experience no major change in proliferative capability (manuscript in preparation). These data cast some doubt on the proposed relationship between ageing and loss of proliferative vigour [57].

**Soluble regulatory mechanisms: the unbalanced cytokine network**

In recent years it became clear that the immune orchestra depends on a subtle and well-tuned network of humoral mediators, collectively called cytokines, that are responsible for differentiation, proliferation and survival of lymphoid cells. They include interleukins, colony-stimulating factors, interferons, and others, such as tumour necrosis factors (TNFs). These molecules, most of which have been characterized and cloned, constitute a complex network, and act by the interaction with, and binding to, specific membrane receptors, which must be considered as an integral part of the cytokine network. There are cytokines, such as interleukin-2 (IL-2), which have a particular importance for the proliferation and differentiation of T, B and NK cells. IL-2 and IL-10 lead to increased production of IgM, IgG and IgA, whereas IL-4 and IL-13 induce IgE and IgG4 synthesis [58,59].

Other cytokines, such as IL-1, IL-6 and TNF-α are considered pro-inflammatory agents, and play an important role not only in the immune responses but also in inflammation. IL-6 also amplifies Ig synthesis by committed B cells.

It has been reported that the production and utilization of one of the most important cytokines, IL-2, declines with age [60]. However, we have shown that the altered production and utilization of this cytokine by cells from aged donors were rescued by the exposure of cells to low-frequency pulsed electromagnetic fields, suggesting that the above-mentioned alterations are not irreversible, and can be positively modulated [61].

What about other cytokines, whose production and utilization has not been critically analysed during human immunosenescence? We reported that the capability of mononuclear cells from healthy, aged subjects [62], as well as from centenarians (manuscript in preparation), to produce pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α increases with age. These data suggest that the cytokine network undergoes profound but complex changes with age. Indeed, the production and/or utilization of some cytokines decreases with age, while the production of other cytokines increases. This field is far from being clear, as the data on changes of other cytokines are lacking, especially in humans. Moreover, no data are available on possible changes in cytokine receptor on target plasma membrane (number per cell, affinity, etc.), soluble cytokine receptors, as well as receptor antagonists. These data are urgently needed, as cytokines and their receptors are heavily involved in the mechanisms responsible for many age-associated pathologies (atherosclerosis, dementia, autoimmune diseases, etc.).

**Innate immunity: the first to come, the last to go**

T and B cells are classical examples of adaptive immunity, being clonally distributed and capable of specifically reacting with single epitopes of a given antigen. However, for millions of years lower creatures had to survive in environments full of pathogens, such as viruses and bacteria, without an immune system functioning at a clonal level of recognition. Mechanisms have been used, and evolved, in order to overcome such a problem: they are collectively called 'innate immunity', and include responses such as chemotaxis, phagocytosis and natural cytotoxicity, among others. Evolutive studies from different laboratories, including ours, suggest that these mechanisms are fully capable of preserving body integrity even in the absence of adaptive immunity [7-9,63-73]. However, for the sake of economy, they did not disappear with the onset of adaptive immunity. Indeed, they are still present and effective in more evolved animals such as mammals, where they are intermixed with, and collaborate with, clonally distributed T and B cells, and still represent a first line of defence against a variety of pathogens.

On the basis of these considerations, it can be predicted that the most sophisticated immune responses are also the most fragile, and prone to age-related alterations. Conversely, ancestral innate immune responses should be more resistant to age-related changes, being 'simpler', more economical and conserved throughout evolution.

Accordingly, innate natural immunity was studied in healthy elderly subjects and centenarians. In particular, we focused on NK cell activity and chemotaxis. NK cells and activity during ageing have been studied extensively by several groups. Different results (decrease, increase, no change) have been reported,
probably because of a poor selection and insufficient inclusion and exclusion criteria [15,19]. In large groups of healthy centenarians, middle-aged (40–50 years old) and young (20–30 years old) subjects a detailed cytofluorimetric analysis allowed us to demonstrate an age-related increase in cells with high NK activity (CD16⁺,CD57⁻) [19]. However, cells with intermediate (CD16⁺,CD57⁻) or low (CD57⁺,CD16⁻) NK activity showed only minor modifications. In centenarians, an increase in this high-activity NK subset is mirrored by well-preserved cytotoxicity, as measured by both NK and redirected killing assays. In Down’s syndrome, an example of precocious ageing in humans [31,74–85], an expansion of NK cells occurs, suggesting that this is peculiar to immunosenescence [83,86]. However, in this syndrome, NK cells were, functionally, highly inefficient [86]. Recent data suggest that a persistently low NK activity is a predictor of impending morbidity [87].

Conversely, it can be speculated that well-preserved NK activity can help in becoming a centenarian. The age-related increase of cells bearing NK markers, and of non-MHC-restricted T lymphocytes [19], could be interpreted as a compensatory mechanism to cope with decreases in T cells.

Recent preliminary data on chemotaxis suggest that the capability of peripheral blood mononuclear cells to respond to chemotactic stimuli is well preserved in centenarians (manuscript in preparation).

Overall, the above-mentioned data indicate that, as predicted, innate immunity does not undergo a significant deterioration with age. This is probably one of the reasons why healthy aged subjects such as healthy centenarians are apparently fully capable of coping with infectious agents, and have no increased frequency of infectious diseases. Elderly subjects who show an increased susceptibility to infections (influenza, tuberculosis, etc.) are probably those in whom pathological changes in the immune system have occurred. The hypothesis can be put forward that physiological ageing per se does not represent a major risk factor for most infectious pathologies.

Taking into account the complex (positive and negative) changes which occur in the immune system with age, in comparison with young subjects, we prefer to use the words ‘reshaping’ and ‘retuning’, instead of ‘alteration’, ‘deterioration’, ‘decline’, to describe the complexity of immunosenescence [88–91]. It is our opinion that these pejorative descriptions do not grasp its substance, and that terms such as ‘continuous remodelling’ are clearly more appropriate to describe a situation where some immune parameters increase, others decrease, whereas still others remain unchanged.

In other words, we think that the body undergoes continuous adaptation as a consequence of the continuous exposure to low levels of internal and external damaging agents, such as oxygen free radicals, glucose and other reducing sugars, radiation, and so on. This is a dynamic point of view that considers centenarians as the end-product of very effective cellular defence mechanisms selected throughout phylogenesis and ontogenesis.

Investigations on centenarians, the best example of successful ageing, can clarify the trend and direction of the immunosenescence, and go far beyond immunology sensu strictu, since the most important age-related pathologies (e.g. atherosclerosis, dementia, cancer) do have an immunological component.

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