[Frontiers in Bioscience 7, d1056-1183, May 1, 2002]

T CELLS AND AGING, JANUARY 2002 UPDATE

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1. ABSTRACT

Age-related changes in the immune system may contribute to morbidity and mortality due to decreased resistance to infection and, possibly, certain cancers in the aged. Many studies mostly performed in mice, rats and man but also including monkeys and dogs have established that age-associated immune decline is characterized

bydecreases in both humoral and cellular responses. The former may be largely a result of the latter, because observed changes both in the B cell germline-encoded repertoire and the age-associated decrease in somatic hypermutation of the B cell antigen receptors are now known to be critically affected by helper T cell aging. As antigen presenting cell (APC) function appears to be well-maintained in the elderly, this review will focus on the T cell. Factors contributing to T cell immunosenescence may include a) altered production of T cell progenitors (stem cell defects, stromal cell defects), b) decreased levels of newly-generated mature T cells (thymic involution), c) aging of resting immune cells, d) disrupted activation pathways in immune cells (stimulation via the T cell receptor for antigen, costimulation, apoptosis control), e) replicative senescence of clonally expanding cells. This review aims to consider the current state of knowledge on the scientific basis for and potential clinical relevance of those factors in immunosenescence in humans. Experiments in other species will be touched upon with the proviso that there are clearly differences between them, especially between humans and rodents, but exactly what those differences are is not completely clear. Given its potential importance and the increasing proportion of elderly people the world over, coupled with the realisation that whereas mortality is decreasing, morbidity may not be decreasing in parallel (1), a better understanding of the causes and impact of immunosenescence may offer the possibility of identifying where prevention or delay of onset, as well as therapeutic intervention, might be beneficial. Amelioration of the effects of dysregulated immune responses in the elderly by replacement therapy, supplementation therapy or other approaches may result in an enhancement of their quality of life, and significant reductions in the cost of medical care in old age.

2. INTRODUCTION: HOW DO WE ESTABLISH WHETHER IMMUNOSENESCENCE EXISTS AND IF SO WHETHER IT IS RELEVANT TO IMMUNOLOGICAL DEFENCE MECHANISMS IN AGING?

T cell function is altered *In vivo* and *In vitro* in elderly compared to young individuals. This is found in both long and short-lived species as a function of their age relative to life-expectancy rather than chronological time (2). Why should similar changes which occur over 100 years in humans take place more rapidly during 3 years in mice, were these changes not in some way pre-programmed? On the other hand, the relative lack of antioxidant defences in short-lived mice compared to long-lived humans, whereby mouse cells are much more susceptible to oxidative damage and malignant transformation, might suggest that deleterious changes are simply occurring faster in the former. Another difference between these species is that mouse cells (at least those from commonly-used laboratory strains) possess exceptionally long stretches of telomeric repeats at the end of their chromosomes, whereas those of humans are much shorter. This should give mouse cells the advantage in terms of number of cell divisions that telomerase-negative cells can undergo prior to growth arrest caused by critically-short telomeres. However, mouse cells either stop growing for other reasons or undergo transformation ("immortalisation") in culture. In humans, loss of telomeric repeats triggers a completely different set of growth arrest/apoptosis processes. These considerations alone make comparisons between humans and mice difficult; certainly there are species-related differences which have not yet been elucidated. The current update of this review will build on the previous version (February 1999) to cover the most recent work on immunosenescence in man, but reference to studies in mice and other species are inevitable; the latter must be viewed in the light of the above considerations.

The changes of immune status in the elderly are generally perceived as reflecting a deterioration of immune function (hence the common term "immunosenescence"), but it remains controversial whether they themselves cause or are caused by underlying disease in humans. Despite strenuous efforts to circumvent this problem by separating "disease" from "aging", as exemplified by the application of the "SENIEUR" protocol and its modifications (3,4), this problem is not solved. Although extremely healthy "successfully" aged people may still behave immunologically differently from the young, this may not necessarily impact on clinically important health parameters (5,6). The whole question of proband selection for immunogerontological studies is the subject of much discussion, for example, see the recent series of commentary articles in Mech Aging Dev (7-10).

Many years ago, it was established that several tests of T cell function give different results in the elderly than in the young, and this was interpreted as indicating depressed responses in elderly individuals (11). Even such very strong reactions as the rejection of allogeneic skin transplants in mice (12) and rats (13) and alloantigen recognition *In vitro* (14) may be depressed. Particularly the ability of T cells to undergo clonal expansion when stimulated *In vitro* is repeatedly identified as an age-associated decrease in immune function. Various mechanisms have been proposed to account for such suboptimal proliferation but they probably still offer only an incomplete explanation of this phenomenon. Nonetheless, several early studies already suggested a positive association between T cell function as it could be measured *In vitro*, and individual longevity (15-17), and between absolute lymphocyte counts and longevity (18). In these and very many other studies, the "chicken and egg" question has to be asked: ie. do people live longer because of "good" T cell function, or do they possess good T cell function because other factors have enabled them to survive longer? This question remains difficult to answer even today, and even in mice, which are of course easier to study than humans. One problem is that immunogerontology relies on two main types of study design, cross-sectional and longitudinal. The former is easier and faster to perform but may result in comparing heterogeneous populations (genetically and environmentally). For this reason, most information that we have on human immunosenescence is derived from cross-sectional studies. Thus, one cannot be sure that any age-associated alterations identified are not in

fact due to selection for other traits in the population. Centenarians as a model of successful aging are an extreme example of this; clearly they constitute an exceptional group and changes in their immune systems may not apply to the majority of the normal (less long-lived) population. Even in populations with the greatest longevity, they form by definition an outlying group at the extreme of the normal distribution (eg. currently ca 1 in 7,000 in Sardinia (19)). That a high proportion of centenarians has relatively well-preserved immune functions compared to the less elderly (20,21), may reflect their general lower biological age (under the implicit assumption that aging takes place at different rates even in members of the same species).

The other main type of study design is much more expensive and time-consuming but may yield more valuable data because it monitors the same population over time (longitudinal study). Data accumulated in this way may offer the power of prediction, the "gold-standard" for scientific inquiry. However, very few such studies exist, and where they do, immunological parameters are rarely measured. Little has been published so far, but immunological components could be "bolted on" to several such ongoing studies. It is once again the Scandinavians who have led the way in this type of study approach. A series of recent publications has reported on the monitoring of a longitudinal study of the very old at two year intervals. Relatively simple immunological factors assayed indicated that decreased longevity in this already very old population was associated with an immune risk profile (IRP) including poor T cell proliferative responses to mitogen, high CD8+ T cell counts, and low CD4 and CD19 cell counts. In a remarkable series of follow-up studies over 8 years (thus far), it was found that no single parameter was predictive for survival, but that a cluster of the above parameters was indeed predictive (22-25). These data are the strongest so far to suggest that well-preserved immune function is associated with extended longevity in a very small fraction of the population. However, the study is far from complete: a careful analysis of cause of death (anything to do with immunity?) was lacking and numerous other environmental factors (psychosocial, physiological etc) still need to be taken into account. Closer analysis revealed that nutritional disorders, medication, chronic inflammation or other diseases were not associated with the IRP. On the other hand, an intriguing correlation between the IRP and changes in T cell homeostasis associated with persistent cytomegalovirus infection was observed. The association involved higher numbers of CD8 cells being CD28-negative, CD56+, and CD57+ indicating subset alterations with increased numbers of CD8 cells in activated states in the very old. Fortunately, many additional data are available from the main part of the study (which was also not originally conceived as an immunological study – this is a brilliant example of a successful "bolt-on"). More studies of this type, especially those monitoring individuals only middle-aged at initiation, are desperately required.

In humans, it can be difficult to distinguish the effects of aging from alterations in immunity caused by underlying pathology. For this reason, when reviewing the available published data, it is important to be clear about the health status of the subjects. For this purpose, the SENIEUR protocol, as mentioned above, is a strict donor selection procedure based on laboratory and clinical parameters, whereby only a small fraction of the elderly are classified as perfectly healthy (4). It has been established, for example, that the reduced proliferative responses stimulated by phytohaemagglutinin (PHA) are still observed even in perfectly healthy SENIEUR donors, although it has been reported that the response to immobilized CD3 mAb may not be reduced (26). In SENIEUR-selected elderly women with good nutritional status, the only immune parameter found to be depressed was the lymphoproliferative response to another mitogen, Con A (27). The reason for these discrepancies is unclear. This example illustrates the fact that for a critical review of the data both the experimental system and the status of the donors must be taken into account. Conceivably, a whole range of factors has not yet been taken fully into account in any of these studies. For example, it is clear that nutritional status influences the level of responses, as well as caloric intake and exercise levels. Some studies are beginning to address these variables. For example, a randomized controlled trial comparing exercise with and without micronutrient-enriched food supplementation in frail elderly concluded that exercise but not supplementation was beneficial for cellular immunity (28). An intriguing recent discovery concerns the consequences of parity on T cell proliferative responses, where it was found that elderly nulliparous women had decreased responses compared to young nulliparous women, but that elderly multiparous women did not show reduced responses (29). This may be related to the length of lactation time, which also affects thymic involution (30), and which was shown by the same group to correlate with immune parameters in mice (31). Age-related changes in myelopoiesis and B lymphopoiesis in mice have also been reported to be influenced by previous parity (32). Hormone replacement therapy may also be a factor affecting immune status in post-menopausal women. Little is known of this, but one report indicates that the slight reduction in numbers of peripheral lymphocytes in elderly women is prevented by HRT (33). Moreover, some functional parameters, such as T cell proliferation and TNFα production, may be normalised by HRT (34). Therefore, a large number of parameters need to be taken into account when discussing human immunosenescence, some of which are reviewed below.

3. FACTORS CONTRIBUTING TO IMMUNOSENESCENCE

3.1. Hematopoiesis

Basal hematopoiesis is maintained through old age. However, there is a decreased capacity to cope with hematopoietic stress (eg. severe bleeding) (1). This points to a possible decline in the function of hematopoietic stem cells. This decline may be intrinsic to the stem cells themselves and/or to microenvironmental factors (eg. stromal cells, cytokines, hormones). Taken together, this decreased hematopoietic potential could contribute to altered immune function in the aged. Age-related changes in hematopoiesis could contribute to immunosenescence at three levels: 1) a decreased capacity for self-renewal of the whole stem cell pool; 2) a reduced capacity to generate

blood cells as a whole; and 3) a reduced commitment to lymphopoiesis. In principle, all three types of mechanism may operate. Bone marrow cellularity decreases in late life, but only from 80 – 100 yr.; however, proliferative activity in BM peaks in middle age and then gradually decreases, associated with increased percentages of apoptotic cells (2). Hematopoiesis in man may be compromised because of a severely reduced capacity to produce colony stimulating factors (3) and increased production of pro-inflammatory factors such as IL 6 (4). However, plasma from old people and centenarians contains more stem cell factor (SCF) than in the young, possibly contributing to a compensatory mechanism to a certain extent (5). Hematopoiesis may also be affected by the lower numbers of progenitor cells present in elderly BM (6) and because of an age-related decline in proliferative potential of putative hematopoietic stem cells (HSC) (7). Thus, there may be fewer cells carrying the CD34 stem cell marker in the peripheral blood of elderly compared to young healthy donors (8). However, those present seem to retain function as assessed *In vitro* by response to growth factors and colony formation (5). A survey of 500 BM autotransplant patients (9) concluded that aging was associated with reduced numbers of committed hematopoietic progenitor cells, as measured both by surface phenotype ($CD34^+$, $Thy1^+$, $CD38^{lo}$) and function (long-term culture initiation). Additionally, the bone marrow stroma may be less supportive of lymphopoiesis (10). On the other hand, the number of CD34+ cells which can be collected from donors >60 years old, and the time to engraftment after transplantation, has been reported not to differ from these values in younger patients (11). In mice, the repopulating potential of murine fetal liver-derived HSC is higher than that of adult BM-derived stem cells (12), showing age-related changes in the stem cell compartment.

Normal cells with shorter telomeres may possess less remaining replicative capacity than those with longer telomeres (see section 5.2). Accordingly, HSC from adult BM were found to have shorter telomeres than fetal liver-derived or umbilical cord-derived stem cells, consistent with aging of HSC in humans as well (13). Telomere lengths decreased on culture despite low level expression of telomerase in these cells (14,15), although the rate of base-pair loss per population doubling of cells in culture was lower during the first two weeks, when telomerase was upregulated, than in the next two, when it was downregulated (16). The telomerase expressed is therefore functionally active but may not be able to completely maintain telomere length in aging HSC cells. True embryonic stem cells (ESC), on the other hand, which may really be immortal, do retain high levels of telomerase activity (17). ESC from telomerase-knockout mice show gradual telomere shortening over many population doublings (PD) resulting in slowing and eventual cessation of growth (18). Importantly, a survey of young recipients of allogeneic family BMT documented that telomere lengths in the patients 4 - 82 months post-transplant were significantly less than in their donor´s cells (19). It was estimated that assuming the rate of telomere shortening was linear, the deficit on the part of the recipients was equivalent to 15 years on average, with some patients having a much greater deficit (up to 40 years). This observation has been causally related to the enforced extensive replication of the transplanted stem cells to reconstitute the recipient´s stem cell pool. It was suggested that this might contribute to increased frequency and earlier onset of clonal disorders of hematopoiesis in later life. A previous study had provided concordant results, in that telomere lengths were found to be shorter in recipients than their BM donors, and also indicated that the degree of reduction in the recipients correlated with the reciprocal of the number of HSC originally infused (20). Another study along the same lines produced data remarkably consistent with Wynn´s above, and concluded that the telomere shortening in BMT recipients was equivalent to a mean 41 years of "donor aging" (21). Moreover, cells from transplant patients receiving HSC from older donors had shorter telomeres than those receiving HSC from younger donors, suggesting potential exacerbation of this “transplant stress” effect when using older donors (22). However, a recent study examined very long-term survivors of BMT (up to 30 years) and although telomere shortening was confirmed, it was not greater than that seen in the above studies earlier after transplantation, ie. according to these data, telomere shortening is not linear with time, and mostly occurs fairly shortly after BMT (23). Perhaps it may be limited only to the first year, ie. reflects the proliferative stress of reconstitution (24). Nonetheless, these findings are all consistent with the conclusion that reducing the amount of proliferative stress on HSC results in better retention of telomere length, despite the presence of telomerase and provide evidence for replicative aging of haematopoietic stem cells. They also suggest that ways of manipulating telomere length (see section 5.2) may be applicable therapeutically in this and other contexts (25,26). Serial transplantation of HSC in mice also showed decreased telomere lengths and increased numbers of cycling HSC (27), suggesting that the mouse may be suitable as a model for such studies.

The finding that $CD34^+$ cells mobilise less effectively in cytokine-treated elderly compared to young donors is also of direct clinical significance (28), although this has not been confirmed in all studies (11). Moreover, in mice, the capacity of progenitor T cells from old BM to develop in the young thymus may also be compromised (29). However, this has also not been found in all models (30) where young and old BM was identical in reconstitution ability but the age of the thymic stroma was found to be critical for the development of autoimmunity. The reasons for these differences are presently unclear, but may be related to the earlier finding that although BM from old mice can reconstitute young mice, a delayed deterioration of the T cell compartment is observed later - a kind of premature T cell aging in these mice (31). This type of experiment performed *In vitro* yielded similar results, viz. BM cells from mice seeded into fetal thymic organ cultures (FTOC) showed an age-related decline in their ability to generate T cells (32,33). The thymus of aged mice contains increased proportions of CD4,8-double negative (DN) and double-positive (DP) cells. Among the DN phenotypes there is a high level of CD44+ cells. *In vitro* studies based on coculture of thymocyte progenitors with

FTOC suggested a decline in transition to DP cells at the stage of downregulation of CD44 (34). This does suggest an intrinsic change in the stem cells with age Similar results were obtained thereafter in humans, where the ability of CD34+ cells from the BM to generate T cells *In vitro* (using a mouse thymic environment) showed an age-associated decrease (35).

Other studies in mice have found that hematopoietic stem cells are more frequent in old individuals and more likely to be in cycle, although less efficient at homing to and engrafting bone marrow of irradiated recipients (36). Being in cycle seems to provide some compensation for the reduced frequency of functional stem cells by enabling an immediate increase of the stem cell pool (37,38). Some of the previously published inconsistencies in the data may be resolved by the study of de Haan *et al*. (39), who showed that aging significantly alters the primitive hematopoietic compartments of mice in several ways. First, the proliferative activity of the primitive cells is greatly reduced over the first year of life. Second, there is a (compensatory) increase in relative and absolute stem cell number with age. Third, the changes are strain-dependent and related both to the longevity of the strain as well as to the age of the individual mouse. A strong inverse correlation was observed between mouse lifespan and the number of autonomously cycling progenitors in 8 different strains of mice; a gene controlling this frequency was mapped to mouse chromosome 18 (syntenic to human chromosome 5, involved in various haematological malignancies) (40). These investigators more recently re-examined this question in allophenic mice made between relatively long-lived (C57BL/6) and short-lived (DBA/2) strains, in which they had previously demonstrated that DBA/2 stem cells senesced at around the average lifespan of the mice and all remaining hematopoiesis was carried out by C57/BL cells (41). They have now mapped genetic loci associated with the variation of both the traits of stem cell cycling activity and longevity. Two loci were identified, mapping to chromosomes 7 and 11, the latter in an area containing the IL 3, 4, 5, 13, GM-CSF cytokine gene cluster (42). One of the effects of this genetic background might directly relate to cell cycle kinetics and dynamics of stem cell development; thus, in short-lived DBA mice the frequency of cobblestone area forming cells (= "stem" cells) cells is higher in fetal liver than in long-lived B6 mice, and remained high until 12 - 20 months of age, when it suddenly dropped (43). This may reflect faster "using up" of stem cells and their loss correlating with the lifespan of that mouse strain. Others have implicated a gene on chromosome 12 in controlling differences in HSC senescence seen in different strains of mice (44). A recent study in C57BL/6 mice confirmed the increased number of HSC but suggested that these showed less ability to differentiate into lymphoid cells; the impact of this effect on the immune status of the animals was not assessed (45). In outbred long-lived species such as human, this type of variation would make analysis difficult. Therefore, more work needs to be done to definitively answer the question of whether any alterations in hematopoiesis in the elderly may contribute to immunosenescence. However, taken together, the results so far suggest compromised ability to generate progenitor cells from BM in the elderly. Such results seem consistent with the large literature on sequential transplantation of bone marrow cells in animals, where a limited number of retransplantations is possible before cells lose the ability to reconstitute. Most recently, similar results were obtained using mobilised peripheral stem cells retransplanted up to five times, but with progressively decreasing ability to repopulate the irradiated recipients (46). Some studies which argue for an apparent lack of exhaustion of stem cells during serial transplantation have measured expansion factors which seem at first glance to be large, but still exhibit a limited capacity for sequential replications, showing that stem cells are unable to divide indefinitely (47). Taken together these studies show a variety of mechanisms at the stem cell level that can contribute to a decreased level of generation of T cells in aging. One may expect that these are just part of the possible multi-factorial mechanisms to be considered. In addition, these studies show individual variability in manifestations of the various causes, which would need to be taken into account in any attempts at corrective intervention.

3.2. Thymus

One of the reasons that T cell differentiation is compromised with age is thymic involution, so that thymic output of T cells declines with age. This has also been deduced from pathological situations in humans (48). However, there is probably a great deal of inter-individual variation with more or less residual thymic function being maintained into later life by some people. A recent study of thymic samples from donors from one week to 50 years old showed an early decrease of cellularity but with two early peaks at 9 months and 10 years of age. Moreover, the adult thymus still contained thymocytes with similar surface phenotypes to those seen in young donors (49) and retained TCR rearrangement activity (at least up to 56 years of age in this study) generating functional thymocytes (50). This suggests that the thymus can remain active at least up to middle age. However, the functional activities of the thymus output could not be studied in this investigation. Data have also been available for many years to document that the replacement of thymic parenchyma with adipose tissue is a discontinuous process, reaching a maximum at around 50 years of age in humans and thereafter not progressing further (51). Moreover, the amount of non-fatty material in the thymus may not decrease further after the age of about 30 years (51). Secretion of the important immunoactive hormone thymulin continues throughout life, although blood thymulin levels do decrease with age (52). A more recent study concurred but reported that thymulin levels remain constant from around 40 up to 80 years of age (53). There is evidence here, however, that lower levels of thyroid hormones and insulin, rather than thymus dysfunction, are responsible for lower thymulin levels (52). There is also evidence that lower levels of available zinc, commonly seen in the elderly, may result in decreased thymulin production (54). These findings, together with the genetic heterogeneity of outbred populations probably influencing the occurrence and rate of thymic involution, make it difficult to assess the contribution of such involution to changes in T cell function in individual

humans. There is evidence to suggest that even in the very old, sufficient thymic function may be retained to allow for naive T cell differentiation (55). It should be noted that these studies relate the findings of peripheral T lymphocyte levels to thymic function. Yet, this extrapolation is not necessarily correct. There is a possibility that T lymphocytes may be generated at extra-thymic sites or expanded in the peripheral lymphoid tissues. Thymic telomerase positivity has also been observed in a minority of the elderly, possibly implying retention of thymic function in some individuals (56). It has been estimated that complete thymic atrophy in humans would not occur until the age of about 120 years (57). An important more recent study in transgenic mice has shown that even aged animals may retain a significant ability to generate mature T cells; this was demonstrated by reconstituting young or aged recipients with T cell-depleted bone marrow from mice transgenic for a TCR not recognising antigen in the recipients. In this way, any peripheral expansion of residual T cells was excluded and it could be shown that aged mice could still generate about half the amount of mature T cells as the young mice. However, functional studies were not carried out to ascertain whether these T cells really behaved normally (58). In mice, T cell export to the periphery from the thymus peaks in the young animal (ca. 4 weeks old) but by mature adulthood (6 months) this has strikingly decreased (by >95%). A recent simple experiment correlated this event with responsiveness by measuring reactivity of female mice to male H-Y antigen. Unprimed thymectomized mice could mount a response to H-Y, but this decreased with time after thymectomy and was lost by 6 months. Mice primed before thymectomy, on the other hand, retained responses for more than a year afterwards (59). This suggests that the lifespan of naive cells in mice is only half that of memory cells. In humans thymectomized at least 8 years before the study because of myasthenia gravis (MG), mild T cell lymphopenia and TCR-Vß family expansions were seen, but functional studies were not done (60). Long term thymectomized patients displayed an expansion of some Vß families among circulating CD4+ and CD8+ cells and a polyclonal increase in serum IgM and IgG associated with high plasma levels of organ and non-organ-specific autoantibodies (60). There is one other report on the effects of thymectomy on human peripheral blood T cell pools (61). They found that although MG itself was associated with lower levels of probable recent thymic emigrants in peripheral T cells (defined by TREC, see following paragraph), thymectomy did not further decrease these. However, a prospective study indicated that in those patients where thymopoiesis could be shown to be active, thymectomy did reduce levels of TREC but did not affect the number of memory-phenotype cells (61). Again, no functional studies were reported. Although thymectomy for myasthenia gravis is relatively common, and the question of thymic function of central importance for immunology and aging, and that it was reported a long time ago that adult thymectomy reduced life span in mice (62), it seems that thus far, no-one has taken this opportunity to investigate retention of T cell function after adult thymectomy.

Until recently, it has also been practically impossible to assess thymic output in man, but new non-invasive techniques are now being developed to estimate the presence of recent thymic emigrants (RTEs), or, at least, newly-generated T cells. It has been proposed that episomal DNA circles generated during excisional rearrangements of TCR genes may provide stable markers for RTEs (63). These TCR-rearrangement excision circles (TRECs) are not duplicated during division and because they are stable they are diluted out as cells divide (64). In the case of TCR2 cells, TRECs generated during the deletion of TCR-δ locus are identical in about 70% of the T cells (65,66). Therefore, this molecular marker allows the fate in terms of cell divisions of the majority of T cells to be followed. *In vitro* stimulation of human CD3 cells resulted in decrease in TREC in parallel to increasing cell numbers (67). These investigators then surveyed TRECs in CD4 and CD8 cells from donors of different ages and found a 1 - 1.5 log decrease in their numbers from 0 through to 80 years of age (whereas the number of T cells with a "naive" phenotype decreased only fourfold). However, they also found high levels of TRECs in the thymocytes of elderly individuals, showing that old thymi can still generate functional T cells with actively rearranged TCR genes (67). As Rodewald pointed out (68), the relative numbers of TRECs found by Douek *et al*. at different ages agree well with quantitative data on remaining lymphoid mass at different ages. Therefore, with thymic involution, the number of RTEs decreases radically but residual functional integrity is maintained, correlating with the anatomical measurements of lymphoid mass. It must be borne in mind, however, that T cells in the elderly with large numbers of TRECs may also have arisen by extrathymic differentiation (68), or possibly, that certain TREC+ cells have remained quiescent in the body for an extended period. Measurement of TREC should therefore be carried out following adult thymectomy, as mentioned above. Nonetheless, measurement of TRECs in T cell subsets of the elderly will enable the contribution of freshly generated and/or antigen-inexperienced (thymic or extrathymic) T cells-versus-antigen-experienced T cells to immune responses to be assessed for the first time in the aged. More recently, Steffens *et al*. measured TRECs in T cell subsets of children and adults (69). They found that the number of TRECs correlated with the number of “naive” subsets identified by the phenotypes CD45RA+RO-negative, CD45RA+ CD62L+ and CD45RO-negative, CD27+ CD95low in children but only with the CD45RA+CD45RO-negative phenotype in adults. Their data suggested that during the first 5 years of life thymic output decreased to a small extent, but between 23 and 58 there was a steady age-associated decrease (69). A decreased but still detectable number of TREC+ cells is present in the peripheral blood of centenarians (C. Franceschi *et al*., unpublished results).

There is probably a significant genetic contribution influencing thymic involution. In an extreme example, rats of the Buffalo strain do not experience thymic involution at all and in parallel do not manifest decreased T cell function with age (70). However, these rats are an extreme example in that they develop a benign thymoma. In sheep, thymic involution does occur, but gram for gram, the remaining thymic tissue retains its function; it

was estimated that one in 50 peripheral T cells of old sheep had been exported very recently (during the past 24 h) (71). Careful experiments in the rat have shown that thymic involution cannot be viewed simply as a progressive shrinkage, but as complex remodeling dependent on unknown factors (72,73), and therefore susceptible to manipulation when these factors are properly identified. One of these factors may be the status of the T cells themselves in the individual; for example, thymic involution is reported not to occur in TCR-transgenic mice, leading to the conclusion that successfully matured T cells can maintain thymic integrity (74,75). Intriguingly, on the other hand, mice lacking the transcription factor NF-AT, generally considered necessary for T cell activation, have been reported to undergo retarded thymic involution (76). This was not associated with inhibited T cell responses but rather with an inability properly to terminate responses in the animals, leading to an accumulation of activated T cells. This paradox has not yet been resolved. Other findings are hard to fit in as well, eg. those of Lau & Spain, who found that thymic involution was also retarded by disrupting both MHC class I and II genes in mice, or by disrupting CD4, as well as by generating TCR transgenics (77). Since CD4 and MHC disruption results in dysregulated thymocyte development this is hard to integrate with the idea that properly functioning T cells prevent thymic involution. The state of health of the individual also influences thymic involution in as yet not clearly determined ways. In tumor-bearing animals, arrest of thymocyte proliferation at an early stage of development results in accelerated thymic atrophy, consistent with the transgenic T cell data above (78). The cytokine environment also markedly influences thymic atrophy, with IL 7 and IL 4 maintaining the thymus as mentioned below, and LIF, OM, and IL 6, levels of which increase with age, accelerating involution (79). Interestingly, OM may also stimulate extrathymic T cell generation (80,81).

Reconstitution experiments indicate that the observed accelerated maturation of T cells to an “activated” or memory phenotype in old mice is largely due to the aged environment and involves interactions via the TCR which are, however, not antigen-specific (82,83). These findings are related to experiments in which naive TCR transgenic T cells transferred to T cell-depleted syngeneic hosts proliferate in the absence of antigen, and acquire a CD44-strongly positive memory phenotype, and so may not be limited to old animals (84). This proliferation seems to represent a kind of homeostatic control, and takes place only when the total number of T cells in the animal is reduced (85). Further analyses (86) showed that lymphopenic mice reconstitute the T cell compartment by a process requiring cell division (but apparently not requiring IL 2 or costimulation), resulting in acquisition of memory phenotype and function (response to lower antigen doses than naive cells, more rapid development of cytotoxicity, faster interferon production). Others, however, have reported that once T cell reconstitution is accomplished, the cells revert to a naive phenotype (87). Nonetheless, this process would still be associated with increased previous proliferative stress and probably therefore with decreased remaining replicative lifespan of such cells. This may explain previous data on reconstitution of mice with thymus and bone marrow grafts, which, in middle age, failed to extend lifespan (88). At the same time, there may be a developmental block which results in an increase in the frequency of $CD3^+$ CD4/8-DN thymocytes (89). In humans, this may result in higher proportions of apparently immature T cells being present in old individuals (90-92). However, the markers used to discriminate immature T cells in these latter studies do not seem to have allowed for distinguishing between $CD2^+$ T cells and $CD2^+$ NK cells, so that the increase in immature T cells might actually represent an increase in NK cells. This would resolve the paradox of age-associated accelerated maturation of T cells to memory phenotype cells at the same time as apparently immature T cells also increase in numbers. In fact, one group specifically tested this and concluded that such cells were indeed functionally active NK cells (93). Other important changes related to altered thymic function may include changed restriction repertoires of the T cells generated, such that even TCR2 (TCR-$\alpha\beta$) cells acquire responsiveness to antigen presented by non-self MHC in man (94) and mouse (95). Evidence has also been presented for increased levels of extra-thymically-differentiated T cells (defined as CD3+ CD57+) in elderly humans, as well as increased NK-phenotype (but not NK-functional) cells (96). The increase in extra-thymically-differentiated T cells may also represent some sort of compensatory mechanism for decreased thymic integrity.

T cells are not only affected by thymic involution; they themselves also influence the thymus via a feedback effect and provide survival signals for the medullary microenvironment (75). The influence of T cells in the thymus on thymocyte development was shown by Fridkis-Hareli *et al*. (97) and also pointed to different regulatory effects of T cells from old and young mice. An important survival signal produced by T cells in the thymus may be IL 4 (M. Ritter, cited in (98)), considered to be a Th2-type cytokine. Perhaps even more importantly, IL 7 could play a critical role (99) although this factor is not produced by T cells. IL 7 is an anti-apoptotic survival factor for naive T cells, which by itself can induce telomerase activity (see 5.2) (100). IL 7 receptor expression did not decline with age in mouse thymocytes and IL 7 but not stem cell factor *In vivo* could reverse the decline of thympoiesis and increased thymocyte apoptosis otherwise observed in aged mice (101). CD4 T cells appear to be the most effective at maintaining thymic function and a decreased collaboration between thymocyte progenitors and mature CD4+ T cells from aged mice could also result in a defective feedback of aged CD4+ cells on thymocyte development and differentiation (1,102). Signals controlling thymic status may also be derived from the nervous system, either directly from sympathetic innervation or indirectly via the hypothalamic-pituitary axis (103). There are increased numbers of noradrenergic sympathetic nerves and 15-fold increases in concentration of norepinefrine in the thymi of 24 month-old mice (104). Beta-adrenoreceptor blockade partially restores the pattern of thymopoiesis in old mice to that of young (105). There is also an age-associated increase in acetylcholinesterase-positive structures in the human thymus (106). In rats, the

development and involution of the thymus may be under the control of the balance of growth hormone-releasing hormone (GHRH) and GH-release inhibitory hormone, produced by the hypothalamus (107,108). It is interesting to note that GHRH is one of the increasing number of "non-immunological" factors found to be expressed by cells of the immune system; indeed, the level of GHRH expressed by lymphocytes in the elderly is decreased compared to the young (109).

The decrease in thymic size and alterations in thymic architecture and functionality for T cell differentiation which do occur up to middle age are the results of a controlled process independent of stress and lack of repair mechanisms. Thus, infection, pregnancy, stress, drug or hibernation-induced thymic involution are all reversible in younger individuals, conforming with the suggestion that thymic atrophy is an energy-saving process according to the disposable soma theory of aging (110). Few studies have addressed themselves to energy requirements during immune responses, but one study of (T cell-dependent) antibody production concluded that young and old mice used equivalent amounts of "extra" oxygen to mount an immune response (111). One intriguing recent study (in bumblebees) demonstrated that in starved individuals triggering an immune response could exact a high price in use of energy reserves, and itself result in significantly decreased survival (112). However, according to the "energy-saving" view of thymic involution, the evolutionary pressures on maintaining thymic function for constant full T cell repertoire generation were secondary to the generation early in life of a memory cell repertoire for a mostly tribally-limited pathogen presence. Thymic function did not need to be maintained beyond reproductive maturity because the number of new infections experienced by early humans in later life in the wild was too limited to make thymic maintenance worthwhile. This presupposes that early humans did not come into contact with very many new pathogens, suggesting a sedentary existence. However, early humans were nomadic, only recently becoming sedentary, so it is unclear whether this does apply. George & Ritter suggested (110) correlating thymic involution rates and function in animals and birds which migrate long distances, the hypothesis being that the more varied the environment, the more evolutionary pressure there would be to maintain the thymus. Another possibility to explain early thymic involution may relate to avoidance of undesired tolerization of newly generated T cells to pathogens which in later life have entered the thymus (113). Some intriguing recent preliminary results suggest a further possibility: in primates, it was tested whether the normal peripheral leukocyte count correlated with group size, population density, exposure to soil pathogens or mating promiscuity (114). It was reported that differences in leukocyte count did correlate with the latter, implying differences in the immune system driven by the requirement to combat sexually-transmitted disease: one could hypothesize that thymic involution would be delayed in the most promiscuous. This remains to be examined.

Studies on depletion of $CD4^+$ cells by CD4 mAb indicate that recovery of this population, which is dependent upon the presence of the thymus, is much slower in aged mice than young mice (115). In humans, CD4-depletion by mAb treatment in rheumatoid arthritis (RA) results in a very prolonged effect. T cell reconstitution is slow, there is a predominance of T cells with memory phenotypes, and there is limited TCR diversity (116). The ability to generate new T lymphocytes after chemotherapy (117) or T cell-depleted BMT (118) is inversely related to the patients´ age, probably an indirect indication of thymic involution. During the first year of recovery after chemotherapy, the CD4 cells in adults also mostly carry memory markers, but in children they carry markers of naive T cells (119). Recovery of CD4 cells was inversely related to the age of the donor and was enhanced in patients with thymic enlargement after chemotherapy (120). Interestingly, the faster recovery of CD45RO+ cells shortly after chemotherapy (3 - 6 months) was followed by another decrease in these cells, at 9-12 months; this was due to increased susceptibility to on the part of these cells (121). These data are reminiscent of events noted in tissue culture, where CD4 clones become more susceptible to apoptosis as they age (122). However, CD8 cell recovery was much more rapid and was not associated with age or thymic enlargement. The CD8 cells were mostly CD57+ CD28-negative. However, depletion of both CD4 and CD8 naive cells has been observed in Hodgkin´s disease patients, and this persisted up to 30 years after T cell-depleting treatment (123). All these data can be interpreted to imply that the prime source of reconstituting cells in adults is from peripheral expansion of pre-existing CD4 T cell subsets which survived conditioning, and not by thymus-dependent generation of new T cells. CD8 cell generation is thought to be extrathymic here (120). A similar phenomenon may be observed in HIV infection, where antiviral therapy results in an increase of "naive" CD4 cells only if some were still present before initiation of therapy (124), and where recovery of such "naive" T cells with a CD4+ CD28+ phenotype is age-associated, being greater in the young (125). Transfer of genetically-marked CD4 cells also indicates that the peripheral T cell pool in adults is maintained mostly by division of pre-existing mature T cells rather than by thymic production of new T cells (126).

It is noteworthy that in BMT patients, even as long as 5 years after transplantation, CD4 cell counts are still depressed and cells with a naive phenotype are also rare. Cells with a memory phenotype ($CD45RA^{lo}$, $CD29^{hi}$, $CD11a^{hi}$) were abundant in these patients and many of these were CD28-negative (see section 4.3.3.1). Moreover, there was a negative correlation between patient age and the ability to produce naive T cells after BMT (127), independently of the presence of graft-versus-host disease. These findings seem to apply only to naive T cells, as might be expected; thus, Koehne *et al*. reported that in human peripheral blood stem cell transplantation, only recovery of the $CD4^+CD45RA^+$ population, but not the $CD45RO^+$ population, was thymus-dependent (128). A bone marrow-transplanted young thymectomized patient mimicked this phenotype (129). The patient showed preferential recovery of CD45RO+ cells in the CD4 subset, although in CD8 cells, CD45RA+ cells were generated as well as in age-matched euthymic patients. It was therefore concluded that a functional thymus was essential for the

generation of naive CD4 cells, although extrathymic pathways for naive CD8 cell generation appeared functional (129). T cells with a CD8α/α-positive, CD28-negative phenotype, thought to represent extra-thymic T cells, increase shortly after BMT even in children, whereas CD4+ CD45RA+ cells appear much later (130). Following T cell-depleted BMT, loss of TCR diversity in slowly reconstituting cells is also seen, again consistent with peripheral expansion of a very limited number of T cells transferred with the graft (131).

Many studies mostly performed in mice, rats and man but also including monkeys and dogs (132) have established that age-associated immune decline is characterized by decreases in both humoral and cellular responses. The former may be largely a result of the latter, because observed changes both in the B cell germline encoded repertoire and the age-associated decrease in somatic hypermutation of the B cell antigen receptors (BCR) are now known to be critically affected by helper T cell aging (133). An explanation for this may be that a T cell product induces recombination-activating gene-1 (RAG-1) in athymic mice, which usually lack this in the bone marrow (BM) and therefore cannot rearrange BCR (134). Hence, the thymus is also necessary for B cell development, via its production of T cells and T cell-derived factors. These factors, produced predominantly by CD8 rather than CD4 T cells, have now been identified as Interleukin (IL) 16 (135). This is a fascinating and unexpected finding, considering that IL 16 was up to that point known as a CD4 ligand inhibiting T cell activation (136), and as a chemoattractant factor for CD4 cells, produced by mast cells and fibroblasts (137,138). Additionally, in normal mice, there is an age-associated decrease in RAG-1 levels in BM, and this can be rescued by treatment with IL 7 (139). This cytokine is not a T cell product, so that RAG-1 expression is not exclusively T cell-dependent; a likely source of IL 7 in situ would be the BM stromal cells. It has been established that stromal cells from aged mice secrete less IL 7 than the young (Andrew & Aspinall, in press). Moreover, there is decreased expression of RAG 1 and 2 in mouse aged thymus, causally related to stromal cell, not stem cell, status (140).

4 POST-THYMIC AGING

Once produced, mature T cells are of course also subject to aging processes, either of the type affecting post-mitotic cells (when quiescent) or of the "replicative senescence" type (during the repeated intermittent clonal expansion and contraction required for effective immune responses, or caused by the proliferative stress resulting from chronic stimulation). This will be discussed at length in the following sections.

4.1. T cell subsets

In humans, young and aged individuals commonly differ regarding the proportion of T cell subsets they possess, in particular in terms of an increased proportion of memory cells in the aged. There may be an overall decrease in mature $CD3^+$ T cells with age (1-4). However, this is not necessarily a continuous process; according to one report, the number of T cells decreases until the third decade, then stays constant until the 7th, and then decreases again (5). Reciprocally, increased numbers of apparently activated T cells ($HLA\text{-}DR^+$, $CD25^+$, although possibly not CD69+ (6)), as well as increased numbers of natural killer (NK) cells (7), also activated (8) are seen. There is a decrease in the percentage of T cells carrying the alternative TCR1 (gamma/delta) receptor, and these also appear to be in an activated state (9). There is a decrease in the number of CD7+ cells within the CD3+ population, and the density of expression of the CD7 molecule is also decreased on the positive cells (10). As CD7 is possibly involved in regulating lymphocyte adhesion and activation (11), and CD7-negative cells accumulate under conditions of chronic antigenic stimulation (12), this may be an important finding. The percentage of CD3+ cells bearing another potential stimulatory molecule, CD2, is not reduced; in this case, the density of CD2 molecules on the surface of T cells from the elderly has been reported to be increased (10). These data in human apply only to peripheral cells; the situation in the lymphoid organs is mostly unexplored, but could be different. For example, in rats, the effects of aging on numbers and types of cells in spleen and the periphery are different (13). Preliminary work in humans has begun to examine IL 2 and IL 6 production by spleen cells, but thus far only 6 donors have been studied (14). It is known that activation of T cells from human secondary lymphoid organs compared to peripheral blood has different costimulatory requirements (15). Moreover, in the largest compartment of the immune system, and one of extreme importance in host defence against infection, ie. the mucosal compartment, there may be evidence for more rapid age-associated changes than in the systemic compartment in mice (16), although it has been reported that mucosal immune memory is at least as long-lived as peripheral (17). In non-human primates, aging may compromise mucosal immunity to a similar extent as systemic immunity (18), and alterations consistent with decreased responses have also been observed in both humans and animals (19-21). The latter study reported that human lamina propria lymphocytes, but not intraepithelial lymphocytes of the gut, like peripheral cells, produced less IL 2 and proliferated to a lesser degree to a variety of stimuli in the elderly (21).

Similarly to the possible slight reduction in numbers of CD3+ cells with age, there may also be a reduction in the density of TCR expressed. Not many studies have examined the intensity of TCR expression as opposed to percentage of positive cells, but an early study indicated that the density of CD3 on CD4 but not CD8 cells of the healthy elderly was slightly but significantly decreased compared to the young (3). A more recent study also found slight but significant decreases in the density of expression of CD3 on human T cells ex vivo, which was somewhat more pronounced in CD45RO+ cells than in the general CD3+ population (10,22). The differences reported in these studies were, however, not terribly great and their physiological relevance was not investigated. One other study has recently identified a population of CD4+ cells, increased in the elderly, with a lower than usual density of

CD4 coupled with a lower density of CD3 (23). These cells also had lower CD28 and CD95, and in addition expressed CD25 and HLA-DR. No functional studies were reported. Apart from these reports, there seems to be little evidence for decreased density of TCR expression on old cells, either ex vivo or *In vitro*, in mouse or man. Other studies have failed to find any differences in the level of expression of CD3 in mouse and man (24,25). In long-term cultured human CD4+ CD45RO+ T cell clones, there is also no age-associated decrease in density of CD3 expression (26). However, other subtle differences may exist in terms of the dynamic of the expression levels of TCR. Thus, stimulation results in internalization and degradation of the receptor. For continued responses, re-expression is required. This seems to have been little studied in the context of aging (27).

The "homing environment" in murine spleen seems to deteriorate with age (28). In mice, both secondary lymphoid organs and blood lymphocyte subsets have been studied in parallel. Thus, Poynter *et al*. reported that the proportion of T cells bearing the NK marker NK-1 increases with age in mice in blood and secondary lymphoid organs and that these cells rapidly produced large amounts of IL 4 on stimulation (29). Others have confirmed an age-dependent accumulation of NK-1+ cells in the liver, and also showed a preponderance of IL 4 production by these cells *In vivo* (30). Moreover, the level of IL 4 produced by these cells increased with increasing age (31). There are strong arguments for the extrathymic nature of such NK-1+ T cells (32), which may therefore increase in compensation for decreased thymic output of conventional T cells. On the other hand, the fraction of CD8+ T cells in mice which are thought to be extra-thymically derived and which also increases with age, produce large amounts of IFN-γ and little IL 4, perhaps as a counter-balancing compensatory mechanism (33). Although murine CD8 cells staining brightly for CD44 are commonly referred to as "memory" cells, many of them may also in fact be naive extrathymic T cells (34). Whether similar cells exist in huamns is, however, controversial.

The composition of the T cell compartment changes during aging as a result of antigen exposure, clonal expansion and contraction, regulatory T cell interactions and memory cell formation, and changed thymic output, as discussed above. Thus, many studies have addressed the question of whether the numbers and proportions of T cells and other lymphocytes are altered during aging. In general, alterations in numbers of T cells are relatively small (35) and may be influenced by underlying disease (36), although not all studies agree on this (37). It is probably true to say that all in all, the consensus is that age-associated alterations in T cell numbers are not striking (38). However, age-associated changes in the proportions of T cell subsets have been repeatedly documented in rodents and humans. Mice seem to show a relative loss of CD4 cells in the blood with age compared to CD8 cells (39), and the same may be true in humans, although the reduction in CD4 cells is also associated with poor nutritional status (40).

There are clearly more $CD4^+$ $CD45RO^+$ "memory-phenotype" cells and less $CD45RA^+$ "naive-phenotype" cells in PBMC from elderly individuals. In a large study on more than 200 healthy subjects from newborns to people older than 110 years a rapid increase of CD45RO+ cells was observed in the first 2-3 decades of life, this phenomenon being more pronounced on CD4+ cells (5,41,42). In humans, a decrease in the number of CD62L+ T cells has been reported, consistent with a decreased number of naive cells, but those cells which do bear this "naive" marker express it, and CD49d, another adhesion molecule, at higher densities (43). Both CD45RA+ and RO+ cells also express the adhesion molecule ICAM-3 (CD50) at higher densities in the elderly, whereas the percentage of CD50+ cells was lower in the RO+ but not RA+ cells in the elderly (but not the young) (44). Clearly, it is important to consider both percentage of cells positive for a particular marker, as well as the density of expression of that molecule. How far these measured changes are reflected in functional alterations is not yet clear. They may not even underlie the altered cytokine secretion patterns observed in aging, because it has been reported that elderly donors who retain a "young" naive/memory cell ratio may nonetheless show the same pattern as old donors with a changed ratio (45). Interpretation is also complicated by the fact that exact definitions of memory and naive are problematic, as most studies relied solely on CD45 isoform determination and did not consider other markers such as CD62L. Because "antigen-experienced" cells may revert to a CD45RA phenotype, eg. the CD8 memory compartment for responses to the predominant CMV epitope pp65 was found to contain 6-10-fold more cells of CD45RA than CD45RO phenotype (46), this complicates interpretation of these data. Combinations of other markers may be useful to define "true" memory" cells, eg. decreased CD62L+ cells as mentioned above and increased CD60 on memory cells (47), or indeed the simply expedient of assessing CD95, its lack on a T cell indicating antigen-inexperience (48). A mathematical model based on these data predicts that the decrease of CD8+CD95- antigen-inexperienced cells is a reliable biomarker of longevity in humans (49). According to the latter criterion, the decline in naive cells with age may be even more marked than previously believed, especially among CD8 cells (but also among CD4 cells), where centenarians possessed essentially lacked naive CD8 cells (48). Thus, decreased proliferative responses in humans have been attributed to decreased numbers of "true" naive cells with the phenotype CD62L-high, CD45RO-negative (50) and probably CD95-negative. Increases in the proportion of CD45RO+ cells are also observed in strictly selected elderly populations and seem to occur independently of health and nutritional status (40). True naive cells (at least the CD8+ population) in the sense of those which have certainly not yet divided, as assessed by TREC analysis, have recently been characterized as having a CD45R0-negative, CD103+, CD11a-dim, CD95-dim, CD27-bright and CD62L-bright staining phenotype (51). Awareness of the complexity of the "memory" cell compartment and a more sophisticated analysis of changes in aging may still be to come from examining the distribution of chemokine receptors on T cells. Thus, it has been proposed that true naive T cells are CCR7+, but that this receptor is lost on differentiation to effector cells.

Memory cells with a limited lifespan are also CCR7-negative (these are the ones also losing CD28) but there is evidence for another population of memory cells, designated "central memory cells" which retain self-renewal (stem cell-like) capacity and remain CCR7+ although they do express CD45RO (52). In this way, immunological memory could be retained beyond the limited number of cell divisions that effector memory cells could manage, by calling when necessary on the central memory cells, which would provide a pool for generating effectors on rechallenge with antigen (see Chapter 5.3). These populations have not yet been studied in the context of aging.

If the $CD45RO^+$ cells generally represent at least the majority of memory cells, and if exportation of naive $CD45RA^+$ cells from the thymus decreases with age, then an accumulation of $CD45RO^+$ memory cells would be expected in elderly donors. This would be coupled with a predicted reduced ability to respond to new antigens, and a retained ability to respond to recall antigens, as long as the memory cells remained present and functional. This is observed in mice (53). Splenectomy may also have a similar effect, ie. decrease in CD4+ CD45RA+ naive-phenotype cells (54), presumably because the spleen represents a reserve of naive T cells or their precursors. However, not only does the proportion of memory-phenotype cells increase with aging, but in mice at least the memory cells themselves may function less well in old than in young donors (55-57). Moreover, in the oldest old, decreases in memory cell phenotype RO^+ cells have also been recorded (58) and in whole blood analyses, a relative decrease of $CD45RO^+$ cells may also be seen in the CD8 but not in the CD4 population (59). Nonetheless, in exceptional individuals (healthy centenarians) the decrease of RA^+ cells, especially in the CD8 subset, may be markedly less than in the ordinary old population (60). The meaning of this is unclear, because functional tests were not performed, and it is known from other studies that the $CD4^+$ cells responsible for the increase of RO^+ elements express lower levels of CD45RO than do young $CD45RO^+$ cells, but whether this is related to their impaired function is not yet known (61). Furthermore, evidence has accumulated that at least some CD45RA+ cells are indeed antigen-experienced, which would require a different interpretation of previous data considering RA+ cells to be exclusively naive. For example, there is subpopulation of CD8+ CD45RA+ cells which lacks the CD27 marker (62). CD27 seems to be a signal-transducing molecule involved primarily in activating naive but not memory cells and no longer expressed by the latter; its absence therefore implies that these cells were previously stimulated. (63). Hamann *et al*. found that CD8+ CD45RA+ CD27-negative cells had shorter telomeres than CD8+CD45RA+CD27+ cells, as well as oligoclonal TCR-Vß expansions, strongly suggesting that they were indeed antigen-experienced, despite expression of CD45RA (62). Others have confirmed that it is the oligoclonally-expanded cells within the CD8+ CD28-negative population which has shorter telomeres (64). These data are consistent with earlier studies in rat and man (65,66). Virus-driven oligoclonal expansions shown by TCR sequencing in CD8 but not CD4 cells can still be detected a year after resolution of disease (in the case of AIM) and these TCR can be shown in both CD45RO and CD45RA subsets (67). The number of dominant clones decreases with time, so that after 2 years the greatly expanded clones (still up to 10% of all peripheral CD8+ cells) may consist of different dominant clonotypes, ie. the originally expanded clones have been lost and replaced by others (68). Such monoclonal expansions are not observed in B cell populations (69).

More subtle analyses may continue to reveal further differences in surface phenotype and function, which remain to be collated and understood. In mice, for example, aging leads to an increase in the proportion of splenic cells expressing high activity P-glycoprotein and therefore able to extrude rhodamine 123. P-glycoprotein (P-gp), a member of the ATP-binding cassette (ABC) transporter protein, was originally identified by its ability to confer multidrug resistance to a variety of tumor cells by extruding a wide range of structurally-related amphiphilic hydrophobic drugs (70). In mammals, a small family of closely-related genes encodes P-gp with two members of the family in humans (*MDR1* and *MDR3*) and three members in rodents (*mdr1a, mdr1b,* and *mdr2*). *MDR1, mdr1a,* and *mdr1b* confer multidrug resistance, whereas closely related *MDR1 and mdr2* do not. P-gp is expressed on a subset of both CD4+ and CD8+ T cells (CD8+>CD4+) and was suggested to play a role in cytokine secretion and T cell-mediated cytotoxicity (71-75). This was based on *In vitro* inhibition of these functions by high concentrations of anti-P-gp antibodies. However, in more recent studies Eisenbraun and Miller (76) and Gollapudi *et al*. (77) have observed no difference in the secretion of IL-2, IL-4, IL-10, and IFN-γ in *mdr1a* single KO and *mdr1ab* double KO mice compared to wild-type mice, suggesting that P-gp is not essential for the secretion of these cytokines. Furthermore, Gollapudi *et al*. (77) demonstrated that human Jurkat T cells that lack P-gp produce more IL-2 than activated peripheral blood T cells, and that wild-type Jurkat T cells produced similar amounts of IL-2 as mdr-1-transfected and P-gp-expressing Jurkat Cells. Moreover, they also demonstrated that purified $P\text{-}gp^+$ $CD4^+$ and $P\text{-}gp^+CD8^+$ peripheral blood T cells secreted similar amounts of IL-2 as $P\text{-}gp^-CD4^+$ and $P\text{-}gp^-CD8^+$ T cells. Intracellular IL-2 was also similar in $P\text{-}gp^+$ and $P\text{-}gp^-$ T cells. These data would argue against a requirement of P-gp for IL-2 secretion in human T cells. That would not be surprising, because these cytokines have signal sequences that are required for their transmembrane transport and therefore may not require additional mechanism for their secretion. P-gp, however, may be required for the secretion of certain cytokines such as IL-1β, which lack signal sequences (78). In humans, the P-gp molecule has an extracellular domain against which monoclonal antibodies have been raised and used as a tool to identify and isolate $P\text{-}gp^+$ and $P\text{-}gp^-$ negative cells in order to study their functions as well as to specifically block the function of P-gp. However; some of these antibodies are now known to bind to some additional intracellular molecules. In mice, P-gp lacks the extracellular domain; therefore, $P\text{-}gp^+$ and $P\text{-}gp^-$ cells cannot be purified and isolated to study their functions. Thus, functional studies of P-gp in mice are based upon an

indirect method of intracellular accumulation and efflux of Rhodamine 123 dye (Rh123) in the presence or absence of pharmacological inhibitors of P-gp. As with any other pharmacological inhibitors, none of these inhibitors is entirely specific for P-gp and many of them (especially calcium channel blockers) may themselves modify T cell functions. Furthermore, it must also be pointed out that Rh123 is a mitochondrial dye and commonly used to study mitochondrial membrane potential. Therefore, its accumulation could be influenced by the size of mitochondria as well as the charge of the mitochondrial membrane; depolarization decreases and hyperpolarization increases the accumulation of Rh123. Witkowski and Miller (79) reported increased numbers and function of P-gp^{high} T cells in aged mice. However, the same group later reported evidence of dysfunction of P-gp in aged mice (see below). In humans, several studies have demonstrated age-dependent increases in P-gp expression in T cells both at the protein (73,80-83) and mRNA levels (73). Furthermore, increased P-gp expression with age in humans was observed in both CD4+ and CD8+ T cells and their naïve (CD45RA+) subsets (73). No difference was observed in P-gp expression between CD45RO+ memory T cells in either CD4+ or CD8+ T cells in humans. However, the data in mice are conflicting. Bomhardt *et al*. (84) have reported that P-gp^{high} naïve CD4+ T cells exhibit increased functional responsiveness to activation that is consistent with human data of increased P-gp expression in naïve CD4+ and CD8+ T cells. This would also be consistent with the observation that prolonged activation of T cells leads to downregulation of P-gp (85) and therefore, memory T cells are more likely to express less P-gp. In contrast, Witkowski *et al.* (57) reported that P-gp^{high} memory CD4+ T cells from young mice are significantly impaired in their ability to proliferate and release cytokines. The reasons for conflicting data in mice are presently unclear. Witkowski and Miller (86) also reported defective calcium signaling in T cells with high P-gp from aged mice. These authors concluded that P-gp in aged mice is dysfunctional. However, P-gp has no role in calcium signaling. Jurkat T cells lacks P-gp but have better calcium signaling than P-gp^{+} T cells and *MDR-1*-transfected Jurkat cells. Calcium signaling defects were reported earlier in aged humans (87). P-gp and TAP1 protein (that plays a role in transport of antigen from the Golgi to the cell surface in the context of MHC class I-restricted antigen presentation) both belong to the superfamily of ABC transporter proteins and share some structure homology. Witkowski *et al*. (88) reported age-associated increased MHC I expression on P-gp^{high} CD4+ memory cells and decreased levels of TAP-1. It was suggested that P-gp may be taking over the function of TAP1 in aging. However, P-gp appears to have no role in antigen processing because *mdr1a* and *mdr1ab* knock out mice have no defect in antigen-induced proliferation. This is again not surprising because the localization of P-gp and TAP1 molecules is completely different and therefore conceptually P-gp should not have a role in antigen processing. Therefore, P-gp^{high} in aging T cells may be a marker for cells with impaired signaling function. Interestingly, Russ *et al*. (89) reported data in humans to suggest that P-gp is unable to substitute for TAP as a peptide transporter and fails to enhance MHC I expression in T cells.

The question of whether antigen-independent functional changes in naive T cells can occur has also been addressed. As discussed above, differentiation of T cells to memory cells coupled with age-related changes in memory cell characteristics may be responsible for much of the altered functional phenotype of the aged individual. Linton *et al*. looked at TCR-transgenic mice with T cell specificity for pigeon cytochrome C antigen, which lacks cross-reactivity with environmental antigens. They found that in aged animals, the TCR- transgenic CD4+ cells were decreased in number and in antigen responsiveness but that they maintained a naive cell phenotype. They concluded that the defects observed were therefore due to aging of the naive cells *per se* and not to environmental stimulatory influences (90). Proliferative functions of these CD4 naive cells can be restored with IL 2, 4, 7, and 15 but effector function is solely responsive to IL 2 (91). Such findings are clearly consistent with several studies showing different patterns of cytokine production by young and old cells despite possession of the same "naive" phenotype (92). The importance of costimulation is emphasized by the finding that the depressed responses of naive T cells to influenza antigens in old mice can be partially reconstituted by upregulation of costimulation (53).

In human, most T cells are CD7+, but the frequency of CD7-negative cells increases with age (22,93) and although isolated T cell clones retain stable expression of their CD7-positive or negative phenotype (94), repeated stimulation and propagation of uncloned lines results in accumulation of CD7-negative cells in the CD4 but not CD8 subset (95). It is these CD7-negative cells, with a CD45RO+ RA-negative “memory” phenotype, which show increased spontaneous apoptosis, another difference between CD4 and CD8 cells. These CD4+ CD7-negative cells can be rescued from apoptosis by soluble IL 15 and membrane-bound IL 15 on fibroblasts (96). Increased proportions of CD7-negative cells are also found in situations of chronic antigenic stimulation *In vivo*, eg. in rheumatoid arthritis (97) and in kidney transplant recipients (98). Such CD7-negative cells show low proliferative responses to CD3-stimulation, low IL 2 secretion but high IL 4 and IL 10 secretion (99). These results suggest that loss of CD7 expression may be age-associated, but the fact that long-term cultured T cell clones retain high CD7 levels imply that factors other than merely the number of PD undergone are critical for CD7 expression.

Thus far, CD28 is perhaps the closest to a biomarker of aging found for human lymphocytes. Both *In vivo* and *In vitro*, the proportion of CD28^{+} cells decreases with age. In monoclonal populations, the density of expression of CD28 decreases with age (100). Effros *et al*. observed a decreasing percentage of CD8 cells carrying CD28 in the elderly, paralleling their observations in CD8 cell lines in tissue culture (101). Others have confirmed that particularly the CD8 subset shows progressively decreasing CD28 expression with age CD28 expression with age and that in healthy aged people including centenarians a concomitant marked expansion of CD8+CD28- cells characterized by high cytotoxic activity is present (48,102) .CD4+ T cells, almost all of which are CD28+ in young adults, also show an increasing CD28-negative fraction in the elderly (103). The fraction of CD8+ CD28-negative cells in centenarians is somewhat higher than in the elderly

(70-90 year-old) population (103). Moreover, telomere lengths in the CD28-negative cells were less than in the $CD28^+$ cells from the same donors, implying that the former had undergone more rounds of cell division than the latter. This type of proliferative senescence may therefore be responsible for the commonly observed accumulation of CD28-negative oligoclonal populations in elderly people (104). Thought to be caused by responses to common viruses, according to the immunological history of the individual, they may also be detected in the young (105). Although originally described only in CD8 cells, the number of individuals with such clonal expansions in both CD4 and CD8 cells was found to be very similar when sensitive spectratyping methods were used to examine complementarity-determining region 3 (CDR3) of the TCR (ca. 70% of individuals over 65); moreover, these expansions were stable over a two-year observation period (106). Similar observations on clonal expansions in CD4 populations have also now been made in mouse (38) and monkey (107). It may well be that the origin of at least some of these clonal expansions resides in anti-viral immunity (108). In mice, such CD8 expansions may indeed by monoclonal, and their onset is accelerated by decreased thymic output (109). Although described as CD28-negative, it is possible that such clonal expansions do retain some CD28+ cells; Chamberlain *et al*. reported that CD8 clonal expansions in healthy elderly people contained mostly CD28-negative cells proliferating poorly in culture, but that small CD28+ populations were almost always present too (110). The CD28-negative cells contained larger amounts of perforin than the CD28+, suggesting that the former were end-stage differentiated CTL. One interpretation is that CD8 clonal expansion, say, against viral antigen, represents a stem-cell system in which most progeny undergo terminal differentiation but some must retain stem cell characteristics for further cell division.

In diseases with chronic antigenic stimulation, further circumstantial evidence in favor of the hypothesis of proliferative senescence indicated by downregulated CD28 expression can be garnered. To give some examples: the percentage of $CD28^+$ cells decreases during Chagasic progression (111); both CD4 and CD8 cells show decreased CD28 expression in chronic B lymphocytic leukemia (112) and in hairy cell leukemia (113); in Crohn´s Disease, the ability of CD28 to mediate costimulation of CD4 cells is compromised (114). It may also be interesting to note that in long-term allogeneic kidney graft transplant recipients, decreased CD28 expression correlated with graft survival over extended periods of time (115) and this was accompanied by reduced proliferation *In vitro* by graft recipient lymphocytes stimulated by donor cells (116). Others have also found that the percentage of CD28+ cells goes down and the percentage of CD57+ cells goes up on both CD4 and CD8 cells from long-term kidney graft recipients (117). In rheumatoid arthritis, the percentage of CD4 cells carrying CD28 is reduced (118) and in both RA patients and normal controls the $CD4^+$ CD28-negative cells show TCRVB oligoclonality (119). However, the loss of TCR diversity in CD4 cells was not limited to memory phenotype cells, but was also seen in naive phenotype cells, suggesting that they may not have arisen as a consequence of repeated cell division T (120). Alternatively, the naive phenotype cells may have been CD45RO to RA "revertants" (121) especially since they did show shortened telomeres consistent with an extensive replicative history (120). Such CD4+ CD28-negative cells appear also to be CD7-negative (119). This phenomenon is influenced by HLA type, however, with RA-associated HLA-DRB1*0401+ donors having higher proportions of CD4+CD28-negative cells (122), and it is not yet clear whether this is due to selective presentation of autoantigen by these particular HLA-DR-alleles. It is however clear that T cell clones derived from these cells are in fact autoreactive (119,122) and functional in that although they are CD40-ligand-negative, they expressed perforin and were cytotoxic (123). These cells differ from *In vitro* expanded CD4+CD28-negative cells in that they are more not less resistant to apoptosis (124). In systemic lupus erythematosus (SLE), T cells also show decreased levels of CD3-zeta chain (125), increased levels of bcl-2 (126), and increased levels of CD28-negative cells expressing increased levels of CD152, as in aging (see 4.2.1) (127). Mostly they have short telomeres and cannot undergo as many population doublings as controls in culture (128). Chronic antigenic stimulation in helminth-infected humans is also associated with an anergic-type effect together with increased CD152 expression (129). The relationship of these CD28-negative cells to aging as opposed to genetically-influenced autoimmune disease is therefore unclear at present. However, it is clearly not generally the case that CD28-negative cells ex vivo are apoptosis-resistant; for example, in B-CLL and hairy cell leukemia, the CD28-negative cells while retaining normal functions such as cytotoxicity and cytokine release, show decreased clonal expansion capacity and increased susceptibility to apoptosis (113). The difference therefore may be related to clearly distinct pathological process in autoimmune disease and other situations of chronic antigen stimulation. In the former, eg. in RA and SLE, T cells are pathogenic partly at least because of their resistance to apoptosis, whereas in chronic infections and cancer, "normal" replicative senescence occurs. These examples suffice to illustrate the range of situations in which T cell proliferative senescence may play a role in modulating immune responses independently of the age of the host. The effects of this kind of "clonal exhaustion" in the elderly may simply be more noticeable than in the young because of thymic involution reducing effective generation of naive T cells and because T cells present in the old may already have undergone many rounds of division.

It is conceivable that alterations in other surface molecules might compromise T cell function, but this has not been studied extensively. Human T cell clones do not show obvious alterations of integrins and other adhesion molecules during long-term culture (130), although this has been observed in mice ex vivo, at least for CD11a, CD49e, CD54 and CD62L in tuberculosis (131). There may also be changes in levels of expression of other markers, such as MHC antigens, although the implications of these findings are unknown (132). Increases in CD4,8-double positive T cells may also occur *In vivo* (133). A recent intriguing study reported that there is an age-associated increase in

level of expression of the cellular prion protein on T cells, particularly CD8+ cells; the meaning of this finding is unclear (134).

4.2. T cell repertoire

Accumulating evidence suggests that even where the overall numbers of T cells are not obviously changed, and even within a particular T cell subset as characterized above, there may be further age-associated alterations in terms of the specificities of the TCR expressed. For example, whereas the TCR2 ($\alpha\beta$) repertoire of CD4 cells in mice was initially reported not to be obviously changed compared to young cells, the CD8 repertoire was found to be markedly altered in early reports, suggesting expansion of a small number of CD8 cells during aging (135). In human, early reports also indicated that it was the CD8 cells rather than the CD4 cells which were primarily affected in this way. Thus, in $CD8^+$ but not $CD4^+$ T cells, up to 30% of the entire population may consist of oligo- or even monoclonal cells expressing the same TCR-Vß markers (104). Surveys have shown that childhood illnesses or vaccination histories do not explain the oligoclonal expansions seen in later life, and whether they are antigen-driven still remains obscure (136). On the other hand, because naive cells contribute >95% of the diversity of the T cell repertoire, whereas memory cells, even though they may represent one-third of the T cell population, only contribute 1% of the diversity (137), it is likely that such phenomena are the result of previous immune responses. Within the $CD8^+$ cells, these oligo- or monoclonal populations are prevalent in the CD28-negative subset (104) and the CD57-positive subset, which essentially overlaps with the CD28-negatives (138). It is interesting to note that it is this CD28-negative, CD8-positive subpopulation which was identified many years ago as containing so-called "suppressor" cells (139), still of interest in transplantation immunology (140). These data may explain the observation that alterations in proportions of different T cell subsets may also be more marked in $CD8^+$ than in $CD4^+$ cells of aged humans (141). However, this phenomenon seems not be absolutely limited to CD8 cells. Although "forbidden" CD4 clones are not present in 24 month-old mice (usually the uppermost limit in mouse aging studies), they do appear in those few mice reaching 30 months of age (142). It was suggested that these potentially self-reactive CD4 cells were derived extra-thymically because thymectomy increased rather than decreased their numbers. If this is generally the case, then the possibility of enhancing extra-thymic development with factors such as oncostatin-M may offer the opportunity for manipulation of this pathway (143,144). Moreover, the realization that mature thymus-derived T cells can re-acquire sensitivity to positive and negative selection outside the thymus, in germinal centers (145), indicates that in theory the generation and selection of T cells may take place even in the absence of a functional thymus. Other data suggest that the thymus may promote TCR expression but that T cell specificity can be selected elsewhere, eg. in the BM (146). The generation of functional mature T cells with diverse TCR2 repertoires from CD34+ human stem cells in the absence of thymic influence *In vitro* indicates a potential approach to enhance T cell generation despite compromised thymic function (147).

More sensitive methods of TCR analysis (CDR3 spectratyping) recently showed that oligoclonal expansions are not commonly limited to CD8 cells in human or mouse. One study found that every old mouse tested presented a skewed spectratype for at least one of the 24 Vß families examined, some even 50% (148). Moreover, this was clearly the case to the same extent for the CD4 as well as CD8 cells (148). Each individual mouse presented a different variant spectratype, although they were genetically identical and shared the same environment. The meaning of these findings for the immune response status of the mice remained to be determined in that study (148), but even ´though quantitative differences in $CD4^+$ cells of old mice are not always found, qualitative changes in function can be dramatic eg. a striking decline in the ability of CD4+ cells to cause rejection of allogeneic skin grafts (149). In human, although not observed by all investigators (150), it may be the rarer CD4 expansions which are observed at increasing frequency in the aged (151). CD4 expansions may be easier to find in the CD45RO memory-phenotype population in the oldest old (152). Using a sensitive PCR-heteroduplex analysis and sorted CD45RA+ and CD45R0+ populations it has been reported that CD4+ expanded clones are rare and accumulate predominantly in the CD45RO+ compartment of exceptionally old donors (centenarians). In contrast, the CD8+ cells contain expanded clones which are already detectable in young adults and become very frequent in 70- to 75-years-old donors in both CD45RA+ and CD45RO+ compartments (152). These results indicate that the age-dependent accumulation of expanded clones starts earlier and is more pronounced in CD8+ than in CD4+ cells, reinforcing the concept that clonal expansion in the two major T cell subsets is controlled by substantially different mechanisms. More recently, using CDR3 spectratype and peripheral blood lymphocytes from 35 centenarians, it was found that TCR Vß1, Vß8 and Vß20 families are expanded in centenarians (153). The spectratype of TCR Vß families in T cells from centenarians displayed a non-gaussian like peak distribution, indicating clonal expansion of particular subfamilies. In contrast, in some disease states, eg. rheumatoid arthritis, CD4 cells may show striking oligoclonal expansions (154). Whilst not seen in normal donors, it is interesting to note that such CD4 expansions were also seen in unaffected siblings of rheumatoid arthritis patients, suggesting that they are a risk factor for rather than a consequence of rheumatoid arthritis (154). As with the the CD8+ cells, in normal donors, the CD4 cell expansions were found in the CD28-negative population (155); moreover, the same CDR3 spectratype was identified in a subset of CD4+CD8+ cells (but not single-positive CD8 cells) which are rare in young donors but also increased in the elderly in these donors. This led the authors to suggest that the double-positive cells (expressing CD4 and CD8 alpha/alpha homodimer) originated from the CD4 single-positive CD28-negative cells (155). That these cells indeed represented late-differentiation stage terminal cells was supported by their lack of expression of CD7, and their possession of the unusual phenotype CD45RA/RO

double-positive. As discussed above, many such changes may be influenced or even caused by past infection history, especially clonal expansions caused by common viruses such as CMV (156,157) or EBV (158,159). This may not be surprising considering the extreme effects that such viruses have on the immune system at the acute phase of disease; thus, in AIM patients, it has been estimated that up to 60% of all CD8 cells are EBV-specific (160). Such exaggerated alterations in the repertoire may well leave a lasting impression through the life of the host.

4.3. T cell function

Decreased T cell responses in the elderly may be due to decreased T cell function, decreased accessory cell function, or both. First, accessory cells.

4.3.1 Accessory cells

There is some evidence for age-associated changes at the level of the accessory cell (161). For example, in human, early data suggested that the decreased cloning efficiency observed for T cells from elderly individuals was caused not only at the T cell level, but also by a defect in accessory function of old PBMC (162). In mice, the precursor frequency of memory cytotoxic T cells which respond to influenza was reported to be entirely dependent upon the age of the antigen presenting cell donor. These studies demonstrated that memory T cells from influenza-primed old mice showed a significantly higher response in limiting dilution cultures on stimulation with influenza-infected splenocytes from young compared to old mice (163). Additional studies on age-associated decreased proteasome function (essential for generation of antigenic peptides in APC) may yield information on the molecular basis of altered antigen processing / presentation (164,165). At least for epidermal cells, proteasome function decreases with age *In vivo*, showing that this may be a general finding not limited to *In vitro* conditions (166). However, proteasome function in fibroblast cultures from healthy centenarians is reported to be more similar to that from the young whereas it is decreased in the elderly (167). Proteins damaged by oxidative processes can no longer by efficiently removed by proteasomes and accumulate in the cell. Indeed, there is a “vicious circle” whereby lipofuscin itself blocks proteasome function and contributes to this accumulation (168). One of the protective mechanisms of the dipeptide carnosine may reside in preventing the age-associated glycation of proteins (169,170), thereby also contributing to maintaining proteasome function. There is some evidence that carnosine may be active *In vivo* by the same mechanism (171).

In other respects also, APC may function suboptimally in the aged. For example, the number of monocytes bearing CD11a/CD18 decreases with age, although the density of expression on those cells which are positive increases (172). The number of monocytes expressing CD14 at high density, however, decreases with age, in parallel to an increase in low density CD14+ cells, which show signs of activation and produce higher levels of IL 6 and IL 10 than young donors´ monocytes (173). In contrast, they may secrete less IL 1, and show decreased cytotoxicity and protein kinase translocation (174). Lipopolysaccharide (LPS)-stimulated monocytes from the elderly produced less G-CSF, GM-CSF, IL 8, TNF-α, and MIP-1-α as well as less IL 1ß compared to those from young donors (175), although they may secrete equivalent amounts of the critical Th cytokine IL 12 (176). In some clinically-relevant animal models, it is the accessory cells which seem to contribute critically to age-associated suboptimal responses, eg. in the response of mice to trypanosome antigens (177). Another example where T and B cell function appears to be normal, but accessory cell function is compromised in aged mice comes from a vaccination model using pneumococcal preparations (178). Using purified T cells in the absence of accessory cells can show deficiencies clearly dependent on the T cells themselves. Using mitogenic CD2 mAb and soluble costimulatory factors (cytokines, phorbol esters, mAb), Beckman *et al*. (179) have shown that in $CD45RO^+$ CD4 cells, the only pathway not comparable between young and old donors was for stimulation by CD2 in combination with IL 7. Perhaps this is related to the finding that unlike most potential costimulatory receptors, neither the percentage of CD2+ CD3 T cells, nor the density of their CD2 expression, is reduced in the elderly (10). Thus, signaling may be intact in old memory cells, except for IL 7-dependent pathways. In contrast, $CD45RA^+$ cells from old donors responded less well than young naive cells to CD2 + IL 2, IL 6, IL 7, IL 1 or phorbol ester, suggesting multiple deficiencies in the naive cells but not the memory cells of old donors. Few studies have addressed age-associated changes in APC activity in different anatomical locations, but there is at least one report that human alveolar macrophages have impaired function the elderly (180). Given the importance of pulmonary infection, clinical and subclinical, and lung function in the aged (see 7.1), this may be an important finding.

On the other hand, dendritic cells (DC) obtained from elderly persons are reported to be able to present antigen at least as well, if not better, than DC from young donors after differentiation with cytokines *In vitro* (181-183). Although peripheral blood DC may express lower levels of HLA-DR in the elderly, other surface markers analyzed were similar (184). Steger *et al*. also reported that DC from the elderly were able to inhibit apoptosis and stimulate proliferation in pre-senescent cultured T cells (185). Thus, Steger *et al*. (181,185) reported that DC comparable in terms of surface phenotype, morphology and tetanus toxoid antigen presenting function could be generated by culture of adherent PBMC from the elderly and the young in GM-CSF and IL 4. This is consistent with previous reports on a similar ability of monocytes from the young and the elderly to present tetanus toxoid (186). Steger *et al*. were able to generate larger numbers of DC from the elderly than from the young, and it was suggested that this might indicate that DC from the elderly may fail to cross tissue barriers properly and therefore be retained in the peripheral blood. However, Pietschmann *et al*. found transendothelial migration unaffected in old DC (184). These results suggest that at least a subset of APC in the elderly retain good or even optimal function. On the other hand, it must be borne in mind that these results were obtained using DC generated *In vitro* using IL 4 and GM-

CSF. Since the production of GM-CSF in the elderly is decreased (see section 3.1) there may not be so many functional DC available in old donors. Thus, less physiological activation of DC in situ might take place in the elderly, but not be easily observed *In vitro* in the presence of exogenous GM-CSF and other factors. Possibly related to these findings are results in the mouse where defects in the transportation of antigens by DC to germinal centers of lymph nodes may also contribute to decreases in immune responsiveness (187,188).

4.3.2. T cell receptor signal transduction

If APC function reasonably well in the elderly, what is happening at the T cell level? To be stimulated, the antigen-specific T cell receptor must be ligated, and must remain capable of signal transduction (signal 1). In addition, non-polymorphic costimulationn receptors must be ligated and signal (collectively, signal 2). That early events in T cell activation are compromised in the elderly is reflected in findings that calcium influx is reduced (87,189) and the earliest cell surface alterations associated with activation are decreased, eg. CD69 and CD71 (190). Given properly functional APC, incomplete T cell activation may be caused in the first instance by disturbed signal transduction. In T cells, compromised function might be sought at the level of expression and re-expression of TCR, signal transduction through either or all of the TCR components, or costimulatory receptors, or growth factor receptors. There is evidence for age-associated alterations at all three of the latter levels. In terms of the expression levels of TCR, stimulation results in internalization and degradation of the receptor. For continued responses, re-expression is required. Surprisingly, this has been little studied in the context of aging. However, it has emerged that a novel action of CD28-signaling is to enhance surface replenishment of TCR, again contributing to an explanation of the consequences of age-associated CD28 downregulation (191).

Antibody against the signal-transducing TCR-associated CD3 zeta chain precipitates a series of tyrosine-phosphorylated proteins in activated T cells. Although the levels of these, and of zeta chain itself (192), as well as the association of ZAP-70 with the zeta chain (193) are retained, their degree of phosphorylation after T cell activation declines with age in mouse and human (192,194). Similarly, the level of expression of ZAP-70 also remains the same, but its activity is decreased in the elderly (195). On explanation may be the increased levels of the zeta/Fc-epsilonR structure in old T cells, a stable change still seen in T cell clones isolated from old mice according to this report (25). In mice, initial biochemical events following TCR triggering are compromised. Although total phosphatidylinositol turnover is not diminished in old T cells (196), there is decreased formation of second messengers such as IP3 and DAG, despite conservation of the activity of PLC (which is responsible for IP3 and DAG generation) (197). However, the actual amount of PLC present in freshly isolated cells may be decreased with aging (198), and there may be differences in the isoform composition with differential expression of the ß2 isoform in the elderly (199).

In human T cells, selective reduction of one isoform of protein kinase C (PKC) (200) might contribute to decreased T cell proliferation. This applies to both resting and activated human T cells (201), and can be partially prevented by maintaining aerobic fitness (202), presumably reflecting a general effect of health status. In mice, the clustering of PKC-theta seen at the contact points of APC and T cell membranes is reduced in aging (203). This may be important for T cell activation, because PKC-theta is an essential component of the CD3/CD28 signaling pathway for T cell activation, via NF-kappaB and calcineurin activation (204). PKC requires phospholipase D for its activation; this is inhibited by ceramide, which accumulates in old cells, at least in fibroblasts (205). Moreover, addition of ceramide to young cells causes changes identical to those seen in senescence; therefore, interfering with the elevation of sphingomyelinase in old cells, which generates excess ceramide, might help to prevent senescence (205). Ceramide is also involved in induction of apoptosis, which may be enhanced in old CD4+ T cells.

Kinases are commonly counter-regulated by phosphatases, and even if kinase decrease were not to occur, increase in phosphatase activity might have the same result. In T cells, signaling through the TCR, CD4, CD8 or the IL 2R resulted in lowered protein tyrosine kinase activity in cells from old compared to young donors, although direct activation of protein tyrosine kinases (PTK) by pervanadate (an inhibitor of phosphatases) was normal in the old (206). It is therefore not yet clear whether the age-related decreased tyrosine phosphorylation observed in CD3-stimulated human T cells is related to changes in PTKs or phosphatases (PTP). However, data from Whisler *et al*. indicate that CD45-PTP activity in old cells after CD3-stimulation is not increased compared to young cells (207). They further found that TCR-associated p59fyn enzymatic activity (which is essential for signaling via CD2, (208)) but not p56lck activity was reduced in a high proportion of T cells from the elderly compared to the young, although protein levels were the same. They concluded that decreased p59fyn activation but not increased PTPase activity may contribute to lowered responses in the elderly (207). Similarly, in old mouse CD4+ cells, activation of fyn and ZAP-70, and turnover of IP3, was impaired although protein levels were not reduced (25). Nonetheless, some deficiency in the p56lck pathway could also contribute to decreased activation, because the usual association between CD4 and p56lck may be compromised in T cells from old people (209). A different study did in fact conclude that both the amount and degree of phosphorylation of p56lck were decreased in T cells from the elderly (210). This was associated with defects in IL 2 but not IFN-γ production (210). Others have also reported that there is an age-related impairment of p56lck activity, as well as ZAP-70 activity, but not the levels of either protein, in CD3-stimulated T cells from the elderly (24). Exactly in contrast to Whisler *et al*., they found no reduction in p59fyn; the explanation for these reciprocal findings is unclear.

Among five tyrosine phosphorylated proteins found in activated T cells from young and elderly donors, just one was found to be consistently less phosphorylated in

the old; this was identified as the ZAP-70 structure, associated with the TCR, and critical for transducing activating signals (211). Taken together, these results in mouse and man suggest that the very earliest signal-transduction pathways required for T cell activation are compromised in T cells from old individuals. Moreover, the reduction in PTK activity is unlikely to be due to an increase in PTP activity. Accordingly, downstream signaling pathways mediated by the family of mitogen-activated protein kinases (MAPK), which are considered essential for normal cell growth and function, are also compromised. In rat, MAPK/ras(21) activities are decreased in old T cells (212), and in man, CD3-stimulated T cells from 50% of old subjects were found to show reductions in MAPK activation (213). Stimulation with phorbol ester in combination with calcium ionophore resulted in greater MAPK activation in old cells, but still not to the same extent as young cells (213), suggesting signaling deficits between the TCR and the inducers of MAPK. Similar findings (ie. age-associated decline in induction of MAK) have been reported in mouse using CD3/CD4-mAb-stimulation of T cells (214). Other important signaling pathways may also be affected by aging. Thus, Liu *et al*. reported that the ERK and JNK kinases were diminished in CD3/PMA-stimulated T cells from elderly humans, accompanied by decreased Raf-1 kinase activation (215). ERK2 activation correlated with the ability to produce IL 2 in these studies, and may represent the rate-limiting step for IL 2 production by old T cells (215). Similar findings apply also to rat T cells (216) and mouse cells (217,218). A recently-discovered human phosphatase, VHR, blocks both TCR- and CD28-mediated Erl/Jnk activation (219), and needs to be examined in the context of aging.

Other negative regulators of T cell receptor signaling may also play a part in reduced responses of old T cells, but have not yet been investigated in this context. One example is the so-called SLAP, which associates with CD3-zeta, ZAP-70, Vav etc in T cells, blocks IL 2 transcription via NF-AT, AP-1 blockade (220).

4.3.3. Costimulatory pathways

4.3.3.1. CD28 family coreceptors and CD80 family ligands

Resting T cells require stimulation via the antigen-specific TCR for activation. However, in addition they also require stimulation via non-polymorphic antigen-nonspecific costimulatory receptors by molecules expressed on APC. Furthermore, activated T cells restimulated through the TCR alone may become growth arrested and apoptotic (221). Altered expression of these costimulatory molecules and/or their receptors would also lead to altered T cell responsiveness, as repeatedly illustrated in the literature. One striking example in a model system shows that peptides usually acting as weak agonists inducing anergy can be converted to good agonists fully activating T cells by increasing the level of CD80-mediated costimulation (222). Thus the TCR and its signal transduction is not critical in determining outcome of T cell stimulation; it is the balance of stimulation together with CD80/CD28-mediated costimulation (and probably other costimulation) which determines outcome. CD28 is an important costimulatory receptor, the expression of which is decreased in aging in man (101,102,141) and monkeys (223). Although most prominent in CD8+ cells, normal healthy individuals (but not SENIEUR-characterized) also show decreased CD28-positivity in their CD4 cells (224). Therefore, it can be hypothesized that CD28-mediated costimulation, together with the contributions of other costimulatory pathways are critical for determining the outcome for the T cell each time it is exposed to antigen. Although it is commonly assumed that it is primarily naive T cells which require costimulation, our and others´ data (225) provide many examples of costimulation-dependent stimulation of antigen-experienced T cells. Such costimulatory pathways may deliver positive or negative signals allowing the T cell to respond to the antigen presentation environment in a "highly tuned" fashion. Clearly, any age-associated alterations in these components would have a big impact on the resulting immune response. Accumulating data are consistent with this hypothesis. For example, the ability of centenarians´ T cells to respond by medium-term proliferation to alloactivation and mitogen-activation correlates with the percentage of CD28+ cells in their PBMC (103), and that the level of proliferation correlates with CD28 rather than, say, CD2 or other structures (226). However, it was also reported that the proliferation of T cells from centenarians stimulated by anti-CD3 or PMA and costimulated by CD28 was similar to that of young and middle aged subjects (227). Paralleling ex vivo findings, *In vitro* longitudinal models using long-term cultured CD8 cells, the percentage positive for CD28 decreases with time in culture (101). During culture, it is the CD28+ cells which first show a CD28-dim phenotype and then become CD28-negative, rather than selective expansion of cells originally CD28-negative (228). Concomitantly, the cytokine secretion pattern of the cells was altered (228). A second study showed that the loss of CD28 from CD8 cells in culture could be prevented by IL 4 (229). Therefore, the balance of costimulation both influences and is influenced by the cytokine environment.

In human monoclonal populations of CD4 cells, an age-associated decrease in density of expression of CD28 on the surface correlated with autocrine proliferative capacity (230) and altered cytokine secretion patterns (26). Therefore, investigations on the molecular control of CD28 expression and on the reason for an age-associated reduction in CD28 expression, would be very valuable for the study of immunosenescence. Recent work by Goronzy and colleagues has begun to address the important issue of the genetic regulation of CD28 expression, suggesting loss of binding activities to at least two regulatory motifs of the CD28 promoter in T cells from the aged; this is more prevalent in CD8 than in CD4 cells (224). Mutations in sites corresponding to DNA/protein interaction sites in either of these resulted in loss of nuclear factor binding activities and abrogation of promoter activity (224). In replicative senescence, however, only one of these two factors is downregulated (231). Therefore, the functional phenotype of CD28 loss is the same in *In vivo* chronically antigenically stimulated T cells and in *In vitro* replicative senescence; however, the mechanism is different. Possibly

this helps to explain the potential discrepancy in findings suggesting that CD4+ CD28-negative cells ex-vivo (from rheumatoid arthritis patients) are resistant to apoptosis, including activation-induced cell death (AICD) (124), whereas *In vitro* cultured CD4 cells become more susceptible to AICD (232).

In mice, Dobber *et al*. (233) reported that aged mouse $CD4^+$ cells stimulated with Con A or anti-CD3 plus anti-CD28 mAb showed decreased IL 2 production compared with young cells. This suggests that aged CD4+ (and CD8+) cells show diminished responsiveness to CD28 costimulation despite equivalent expression of CD28 on cells from young and old mice (234). However, CD28-signaling, at least as far as the induction of Raf-1, appears not to be blocked in CD4+ cells of aged mice, although activity levels are reduced compared to the young (235). There may be dysregulation of the MAP kinase phosphatase MKP6, but this has not been tested in aged T cells yet (236). Engwerda *et al*. have also more recently shown that AICD is increased in T cells from old mice, as a direct consequence of their decreased levels of CD28-mediated costimulation, which otherwise may protect stimulated cells from apoptosis (237). One of the mechanisms whereby CD28 signaling protects against apoptosis is by preventing CD95L upregulation and by increasing the expression of the anti-apoptotic proteins c-FLIPshort and Bcl-x(L) (238,239). There may also be additional mechanisms of protection against apoptosis, some of which do not involve CD28 (240) but which may no longer function in CD28-negative cells. Thus, Borthwick *et al* have reported that costimulation of CD8 cells via CD11a and CD18 reduces AICD in CD8+ CD28+ cells, this is not the case for CD28-negative cells which remain more susceptible to apoptosis (241). Conversely under certain circumstances CD28 costimulation may actually enhance apoptosis by upregulating apoptotic mediators (242) so the end result will depend on the particular circumstances of stimulation and costmulation and the cytokine milieu.. However, CD28 costimulation most often protects against apoptosis, even that caused by ligation of CD95 (fas) on the T cell surface (243), and CD28-negative cells may be more susceptible to apoptosis (244). One reason for this, and for the increasing loss of CD28+ cells with age, may be related to the increase of CD95 expression on CD28-negative cells (245). Emphasizing its importance, this "biomarker" of aging, CD28 downregulation, is accompanied by the presence of shorter telomeres in CD28-negative cells (101,246,247) (see section 5.2). Despite their antigen-experienced, short telomere phenotype, the CD8+ CD28-negative T cells which are increased in aged humans may nonetheless bear the CD45RA "naive" cell marker (248). This is consistent with previous reports on age-associated increased levels of CD45RA+ CD8+ cells (249) and the documented reversion of CD45RO to CD45RA isoforms in rats (121) and the potential re-acquisition of RA by RO+ cells in humans, albeit with retention of RO expression (250). Such cells secreted little IL 2, but very large amounts of IL 4 and IL 10, as well as IFN-γ (248), a combination of cytokines which may have immunosuppressive effects (251). In the case of IL 4, this may be a critical factor protecting CD28-negative cells against apoptosis, as well as acting as a growth factor (252).

Decreased signaling via CD28 might therefore be expected to contribute to the lack of telomerase upregulation as well as the deficit of IL 2 production observed in old cells *In vivo* and *In vitro*. This would result in an anergic state, as well as arrested proliferation and increased apoptosis. In addition, these cells, like certain other anergic (young) cells (253) may be able actively to suppress other cells in a mixed population, cells which otherwise would be capable of proliferation (254). In more than one experimental model, a type of anergy induced in chronically-stimulated CD4+ cells is associated with their production of IL 10, and associated suppressive activity (255,256). Anergic cells may even be able to interact with APC such that their function is also compromised (257). In this way, even though *In vitro* assays suggest retained APC capacity in the elderly (181), old anergic T cells might downregulate APC activity. In addition to CD28, expression of the related reciprocal coreceptor, CD152, thought to be involved primarily in downregulating T cell responses, may be upregulated in aged T cells. This molecule had been thought to deliver "off" signals to the T cell when ligated by the same structures as CD28 (CD80, CD86) (258). However, this concept might be an oversimplification of the situation and other 'competition' models have been proposed (259). The increased amounts of CD152 expressed by old T cells may make them harder to turn on even if CD28 functions normally (27). On the other hand, however, CD152 signaling may also result in blockade of certain inhibitory events, such as AICD (260). This increase in CD152 is another property shared with young anergic T cells (A. Engel & G. Pawelec, unpublished results) and T cells in some disease states, such as RA (261). Moreover, T cells with a CD4+ CD152+ phenotype, which express the IL 2R, have been identified in mice and more recently in humans (262,263) and designated “regulatory” suppressor cells; these cells have many of the characteristics of anergic and/or aged cells (including secretion of little IL 2 but higher levels of IL 10 and IL 4, increased susceptibility to apoptosis, decreased clonal proliferation).

Age-associated changes in levels of expression of the natural ligands for the positive and negative costimulatory receptors CD28 and CD152 would, of course, also contribute to altered function. CD86, by nature of its constitutive expression, affects early responses with CD28, and models investigating CD28 and CD152 interplay suggest that CD80 preferentially binds CD152 (259). However, there is very little data thus far on age-associated changes of costimulatory ligand expression on APC. One study in human failed to find any decreases in expression of CD86 on either resting or IFN-stimulated monocytes from the elderly compared to the young (226). In contrast, DC in germinal centers of aged mice may lack expression of CD86 (264) which would encourage the induction of anergy or apoptosis in the antigen-specific T cells with which they interacted. This is clearly an area where more data are needed. Moreover, in addition to the CD28/CD152 receptors with CD80/CD86 ligands, there is

a third CD28-family member designated ICOS, expressed on activated T cells which, when ligated, results in costimulation producing little IL 2 but large amounts IL 10 (265). It is identical to the previously described T cell activation molecule H4 (266). Therefore costimulation via this receptor might result in immune response suppression, or diversion to IL 10-directed immune responses. Consistent with this idea, blockade of ICOS ligation has been reported to attenuate lung mucosal inflammation induced by Th2 but not Th1 effector populations (267). We have some preliminary evidence for higher levels of ICOS expression on old TCC cells compared to young cells from the same clone. Few other studies have compared the behavior of T cells from young and old donors at the clonal level (268). The function of ICOS is not yet fully elucidated however: ICOS knockout mice have severely compromised immune responses, with deficient B cell responses, consistent with IL 10 involvement. However, ICOS-KO T cells are able to secrete IFN-γ but little IL 4 (IL 10 was not tested in that study) (269). Such mice display increased susceptibility to experimental autoimmune encephalomyelitis, indicating a protective role for ICOS in this context (270). Germinal center generation and antibody class switching is deficient in ICOS-KO mice, and this can be reconstituted by stimulation via the CD40/CD154 pathway, showing that the effects of ICOS on humoral immunity are CD40-dependent (271). ICOS can be upregulated on CD28-negative cells and is completely independent of CD28 and CD28 ligands; like CD28, ICOS ligation can protect stimulated cells from apoptosis (272). This latter effect may even be at least partly caused by its superinduction of IL 10, which may protect against T cell apoptosis due to IL 2 withdrawal (273).

An ICOS ligand has now been identified as a third member of the CD80/CD86 family, designated B7-H1, has also now been identified in human (274). Although the authors concluded that this molecule was not an ICOS ligand, costimulation via B7-H1 did result in IL 10 but not IL 2 production, and, as pointed out by others, might nonetheless be the ICOS ligand (275). This would be consistent with reports in the mouse system, also appearing at the end of 1999, which reported expression of this third ligand by resting B cells and its induction on fibroblasts by TNF-α (276). The ICOS ligand, designated LICOS by some, also binds to CD28 and CD152, but only at non-physiologically low temperatures (277). Others have designated the ligand B7-H2 and shown that it is expressed on immature dendritic cells (278).

Furthermore, our knowledge of the B7 family of coreceptor ligands and receptors may still be incomplete, as witnessed by the discovery of another immunoinhibitory receptor designated PD-1. Engagement of this receptor results in inhibition of TCR-mediated proliferation and cytokine secretion, and can at least partly overcome the positive effects of CD28 ligation. It appears that the PD-1 ligand is also a member of the B7 family (279). Age-associated alterations of PD-1 expression and function need to be investigated.

Thus, CD28 costimulation increases the sensitivity of IL 4 responsiveness and may bias towards a Th2 phenotype (280) especially when the CD28 ligands CD80 or CD86 rather than an anti-CD28 mAb are employed (281). CD152 signaling, on the other hand, may preferentially block Th2 rather than Th1 development, and, if not present at generally inhibitory levels, thereby facilitate Th1 development (282).

4.3.3.2. Other costimulators

There is a large range of potential coreceptors which have not been extensively or not at all examined from the aging point of view but which may show important age-associated alterations in expression or function. Thus, not only CD28 costimulatory mechanisms but other important accessory/adhesion pathways may be compromised in aging. Jackola *et al*. (283) reported defects in cell-cell binding amongst healthy elderly donors, which was associated with altered activation capacity of the integrin LFA-1. Indeed, LFA-1 itself may act as a costimulatory molecule in synergy with CD28, so alterations in levels of expression may impact greatly on T cell stimulation (284). Moreover, other surface receptors implicated in costimulation may be downregulated with aging. Preliminary evidence is beginning to show that the density of expression of the CD40 ligand CD154 is decreased on activated T cells aged *In vivo* (176,285,286). However, the soluble form of CD154 has been reported to retain biological activity (287). Surveying a number of CD4+ TCC derived from several different donors and quantifying levels of expression of surface molecules at different times in culture revealed certain other age-associated alterations in putative costimulatory structures (130). Consistent with the *In vivo* observations, the level of expression of CD154 (CD40-ligand) was also reduced on TCC (130). In addition, not only was the level of expression of CD28 decreased with age, but also of CD134 (OX-40), which is expressed on activated T cells and can costimulate them together with anti-TCR signaling (288,289). This is particularly important because it implies that if OX-40 as well as CD28 were reduced, then OX-40 could not substitute for CD28-costimulation, even though it is known that it can costimulate independently of CD28 (290) and even break tolerance in CD4+ cells (291). Expressed by cells which do not carry CD28 ligands, OX-40 ligand can costimulate T cell proliferation and IL 2 secretion (289). In OX-40-KO mice, initial T cell activation and proliferation is normal, but clonal expansion cannot be sustained in the CD4 cells (292). Alterations in levels of costimulatory ligands and receptors can thus modify the outcome of TCR-mediated signaling, so that T cell function is changed. Particularly the pattern of cytokine sproduced may be altered, eg. decreased levels of CD40/CD154 and CD28/CD80-86 signaling result in upregulated IL 10 production (293), also seen in aging (section 4.4) and in TCC (130).

Other candidates with potentially important costimulatory effects on T cells, which should be examined in the context of aging, include the 4-1BB (CD137) and 4-1BB-ligand system. The receptor, CD137, is expressed on CD4 and CD8 cells and can provide a CD28-independent activation signal when TCR signaling is strong (294). Under certain circumstances, other molecules could

possibly take over the function of CD28; thus, in CD28 knockout mice, ICAM-1 substitutes for CD28 signaling for CD3-TCR-mediated stimulation (295).

Furthermore, the so-called "killer inhibitory receptors", originally described on NK cells, and in fact consisting of different families of both activatory and inhibitory receptors, may also be expressed on T cells, especially CD8+ T cells. Whether they are relevant to the aging process is not yet clear, but in other contexts of chronic antigenic stimulation, they may play very important roles. This is illustrated by a study on CD8+ tumor-infiltrating lymphocytes (TIL) in renal carcinoma, where CD8 cells, in addition to being CD28-negative, also expressed the CD158b inhibitory receptor (296). It has also been shown that melanoma TIL with a CD28-negative phenotype can express the CD94/NKG2 inhibitory receptor (297). Indeed, some of the disappointingly low success rates of treating cancer patients with TIL by adoptive immunotherapy may relate to the fact that the TIL which are able to grow in culture are for the most part not those which have been specifically stimulated by tumor (298); the former may not be expandable because they are senescent (299,300). In mice, CD8+ memory cytotoxic cells expressing such receptors (eg. Ly-49) steadily increase in numbers with age; moreover, these receptors are functional in inhibiting TCR-mediated activation of the CD8 cells bearing them (301). Therefore, these chronically-stimulated cells may have a functionally senescent phenotype, contributing to their decreased ability to reject tumor. On the other hand, cells with the CD4+ CD28-negative phenotype seen at increased levels in RA, which also express CD158 etc, may possess activating variants thereof which substitute for costimulation via the missing CD28 (302). Although CD94-negative, they may express CD161 and even the CD8α/α homodimers characteristic of NK cells (303). However, the relationship of these RA-derived cells to the CD28-negative cells in the elderly remains unclear.

4.4. Cytokine production and response

4.4.1. Regulation of gene transcription

Once stimulated, T cells must transcribe T cell growth factor (TCGF) genes, secrete growth factors, upregulate TCGF receptors and respond to the cytokines. Most commonly, the TCGF is IL 2, but other TCGF certainly play a part. In this way, autocrine and/or paracrine clonal expansion, a prerequisite for successful immune responses, is effected. A set of transcription factors involving complexes of the various c-jun and c-fos proteins is involved in regulating transcription of many genes, including IL 2, and activation of AP-1 is detected a few hours after T cell stimulation (304,305). Specific defects in AP-1 activation have been reported in young T cell clones rendered anergic *In vitro* (306). The anergic phenotype is in some ways similar to the senescent phenotype (ie. cells can be stimulated via the TCR to secrete cytokines, be cytotoxic, but they cannot expand clonally via autocrine IL 2 production). This phenotype of lack of clonal expansion capacity but retained cytotoxicity is also seen *In vivo* in antigen, eg. viral antigen-specific T cells (307). AP-1 activation may be impaired in *In vivo*-aged human or murine T cells as well (308,309). However, c-fos, c-jun and AP-1 were found to be still fully inducible in fibroblasts from centenarians (310) that maintain a well preserved responsiveness to a variety of proliferative stimuli and growth factors (311) Using SENIEUR donors´ T cells, it was found that the PHA-stimulated activation of AP-1 was commonly impaired in the elderly. In many donors, addition of phorbol ester partially compensated for this defect, but a minority remained refractory. The defect appeared to be in the amount of AP-1 activity produced, since the AP-1 protein that was produced by cells from old donors behaved in the same way as that from young donors and also contained c-fos and c-jun (309). Thereafter, the same group reported that both AP-1 and NF-AT were reduced in elderly donors´ stimulated T cells (312). However, whether these changes were associated with alterations in T cell subset composition was not reported. These data are consistent with those of Song *et al*. (313) demonstrating decreased c-jun mRNA but normal c-fos mRNA responses to PHA in T cells from elderly donors. Moreover, fewer lymphocytes from elderly donors exposed to influenza virus *In vitro* expressed fos and jun compared to cells of younger donors, possibly as a reflection of compromised activation of anti-viral responses (314). In Fischer rats, the age-associated decrease in IL 2 mRNA and protein correlates with a decreasing ability of nuclear extracts of freshly isolated T cells to bind an oligonucleotide representing the transcription factor NF-AT (315), suggesting differences in transcriptional regulation in young and old cells. NF-AT forms an important family of at least four transcription factors; NF-AT DNA binding activity has been found in nuclear extracts of stimulated T cells (316) and is thought to be important for IL 2 gene transcription (317) as well as for IL 2R (CD25) transcription (318). Transactivation of of one of these NF-AT family members, NF-ATc, is mediated by calcineurin, the activity of which also declines with age in rats (319). Additionally, there is evidence in old mice for differential expression of the negative transcriptional regulator for IL 2, Nil-2a (320).

Amongst other transcription factors of known importance for IL 2 production, CD3-stimulated induction of NF-κB was also found to be decreased in old mice (321) and humans (322). One reason for insufficient NF-κB activation may be that the natural inhibitor I-κB is not adequately degraded because of compromised proteasome function (323). Even such immunologically-oriented phenomena as endocytosis of IL 2 may be dependent upon proteasome function, so that age-associated alterations in the latter could have far-reaching effects (324). Age-associated inactivation of proteasome function has been independently reported and attributed to the effects oxidative damage in different tissues ex vivo in the rat, (325), and in replicative senescence in cultured human fibroblasts (326). Such effects may be partly preventable by hsp90 (164), but hsp90 levels are themselves decreased with age in PHA-stimulated T cells (327). The importance of hsp90 for T cell stimulation is emphasized by the finding that a specific hsp90 inhibitor, geldanamycin, blocks T cell activation (328). Whisler *et al*. (312) also found reduced NF-kκB in some elderly human donors´ stimulated T cells,

but they did not find a correlation with depressed IL 2 production (unlike their findings with NF-AT, see above). Interpretations may be complicated, however, by the unexpected finding that NF-AT may exert negative regulatory, not stimulatory, effects on the immune response (329). Finally, in rats, Pahlavani *et al*. reported that the induction of AP-1, NF-κB and Oct-1 DNA binding activity in nuclear extracts of spleen cells from old animals was significantly lower than that of young animals, and the decrease of AP-1 was due to reduction of c-fos mRNA, whereas c-jun remained the same in young and old cells (330). On the other hand, constitutive NF-κB activation in lymphoid tissue of old animals (which can be corrected by dietary anti-oxidants) may contribute to dysregulated cytokine synthesis (331). Moreover, this oxidative stress-induced constitutive NF-kappa B expression can be decreased by activating peroxisome proliferator-activated receptors (PPAR) by agents such as dehydroepiandrosterone sulfate (DHEAS) and WY-14,643. This results in restoration of the cellular redox balance and reduction of spontaneous inflammatory cytokine production (332) in the mucosal as well as systemic immune systems (333). Independently of its anti-oxidant effects, Vitamin C may specifically block NFκB by preventing the degradation of its inhibitor I-κB via an effect on p38 MAPK (334). Vitamin E supplementation may have the same sort of effect as activators of PPAR on high inflammatory cytokine production and nitric oxide secretion in old animals (335). Aging in mice is accompanied by decreased levels of PPAR transcripts, but this decrease can also be reversed by DHEAS administration (332). Moreover, increased ROS generation is associated with increased cyclo-oxygenase (COX-2) production, prostaglandin synthesis and enhancement of NFκB binding activity (336). During the aging process, this is associated with decreased I-κB activity (337).

4.4.2. Cytokine secretion

As seen above, age-associated alterations in T cell function may be related at least partially to changed molecular regulation of cytokine production due to differences in signal transduction pathways in old cells (section 4.3.2). It has been long believed that a major dysfunction in T cells from elderly donors is a selectively decreased ability to secrete T cell growth factors since the first report of this phenomenon 20 years ago (338). A more recent manifestation of this idea is that there are age-associated changes in immune responsiveness characterized by a type 2 cytokine phenotype in neonates, developing towards a predominant type 1 phenotype in adults and reverting to a type 2 phenotype in the elderly. This hypothesis was primarily based on studies in the mouse (339), but is certainly an oversimplification in humans, where neither neonates (340) nor the elderly necessarily show this trend and where the clear distinctions between type 1 and 2 seen in the mouse are anyway not so obvious. However, in carefully-controlled colonies of rhesus monkeys, there is some evidence for a decrease in PHA-stimulated production of the hallmark Th1-type cytokine IFN-γ and an increase of LPS-stimulated IL 10 production by PBMC, whereas there was little or no change in IL 1ß, IL 6 or TNF-α (341). The cytokine response is of course highly dependent on the circumstances under which it is measured, of which aging is just one parameter. There will probably be no general rule, but different outcomes depending on the conditions, technical or otherwise. Thus, the strongest data for an age-associated shift from Th1-dominated responses in adulthood to Th2 in old age do not always apply even in mice. In two different systems eliciting Th2 responses in young mice, it has been found that in aged mice it is the Th2 responses which are depressed and Th1 which are increased (342). In addition, two Th2-like patterns of cytokine secretion may be discernible, characterized either by IL 4 production, or IL 5 and IL 13 production (343). Therefore, it should come as no surprise that in highly heterogeneous human populations, it is hard to find a consistent pattern in the disparate results in the literature at present, as can be seen from what follows.

Many studies agree that T cells from aged humans can show defects in secretion of a major TCGF, IL 2, expression of its receptor IL 2R and subsequent DNA synthesis following stimulation with mitogens like PHA. However, not all data agree even with this, despite large numbers of studies, and where they do, it has been argued that one reason for this lies in altered relative proportions of different T cell subsets at different ages (see 4.1). In fact, several investigators reported that the decrease in IL 2 production in old mice, at least, was solely a result of different subset composition. Engwerda *et al*. (344) reported that purified CD4 or CD8 cells from aged mice, stimulated with CD3-epsilon mAb and CD28 mAb, produced the same amounts of IL 2 and IL 4 as young cells of the same $CD44^{hi}$ or $CD44^{lo}$ phenotype (although they produced increased amounts of IFN-γ). Kirman *et al*. (345) reported that the age-associated increase in IL 4 secretion by mouse spleen cells was not caused by an increase in the numbers of IL 4-secreting cells. On the other hand, the decrease of IL 2 secretion was indeed associated with a decreased number of secretor cells. This could be prevented by exposing the animals constantly to high levels of IL 2 *In vivo*, which could therefore correct the age-associated cytokine imbalance in these mice (see 7.5). However, Kurashima *et al*. (346) found that naive cells produced mainly IL 2 and memory cells mainly IL 4 in young mice, although the reciprocal was observed in old mice: ie. naive cells produced more IL 4 than memory cells and memory cells produced more IL 2 than naive cells, although overall levels were reduced in old compared to young mice. Thus, it seems more likely that in mice age-associated alterations in cytokine production are not determined solely by the subset changes, but by alterations within each of those subsets.

However, data on cytokine secretion in human are inconsistent, and have been so for a long time. The situation is still not clarified. There are many reasons for this state of affairs; in practical terms, one is that there has never been a concerted coordinated study at the multi-center level to control for different possible variables in cytokine status measurement. Such approaches have been extremely valuable in elucidating complex biological phenomena in other areas, eg. histocompatibility (HLA

Workshops), leukocyte cell surface molecule definition (CD Workshops), genome studies (HUGO). There are many reasons why the data on the effects of age on cytokine status in human are so inconsistent. For one thing, early studies employed apparently healthy elderly donors without rigorously excluding underlying illness, or assessing nutritional or psychological status. Studies frequently included only very small numbers of subjects, stimulation of cytokine secretion was different in the different studies, and different techniques were employed to quantitate the cytokines. These points have been considered in an excellent recent review by Bernstein & Murasko (347), which has recently been updated (Biogerontology, in press). In addition, more subtle factors may also influence cytokine status, such as exercise, as reported by Shinkai *et al*. (348), or possibly even the time of day of blood collection because of circadian rythm in cytokine secretion (349). And, of course, the techniques employed for assessing cytokine levels play an important part, and have been notoriously variable (350).

It was nonetheless anticipated that when data became available using donors selected according to the strict standard criteria of the SENIEUR protocol (351)), results on cytokine secretion and other immunogerontological parameters would become more reproducible. Studies with SENIEUR donors have indeed confirmed age-associated alterations in cytokine production, and a few examples are given here. However, the desired level of agreement and consistency in the data has still not been achieved. Nijhuis *et al*. found that IL 2 production in old Dutch SENIEUR donors (compared to young donors also selected with the SENIEUR protocol = JUNIEURs) was not decreased, but they did find increased IL 4 production (352). They also reported that this increase in IL 4 production in elderly donors did not correlate simply with the larger fraction of memory-phenotype cells in the elderly, although this was confirmed to be the case for young donors (352). This suggests that in young donors, different levels of IL 4 production are determined solely by antigen exposure and amount of memory cells, but that in aged donors, other regulatory mechanisms are operating. Unchanged IL 2 and IFN-γ production, but significantly decreased IFN-α and soluble IL 2 receptor secretion has been reported in German SENIEUR donors (353). Equivalent levels of IL 2 secretion by cells from SENIEUR and JUNIEUR donors has also been reported by others (40,354). However, this has not been the case with all studies, despite the use of the SENIEUR selection protocol. Thus, Candore *et al*. reported age-associated decreased IL 2 and IFN-γ production but unaltered IL 4 and IL 6 secretion after PHA stimulation in Sicilian donors (355). Both IL 2 and IL 4 secretion have been reported to be reduced in other Italian SENIEUR donors (356). The frequency of T cells responding to PHA by secreting IL 2 and therefore overall level of IL 2 decreased with age in American SENIEUR donors (357). Other changes in cytokine secretion patterns have also been established. Increased IFN-γ production (7), and, as also shown in mouse, enhanced IL 10 production have been reported (59,358). Taiwanese SENIEUR donors´ CD4 and CD8 cells produced larger amounts of IFN-γ than JUNIEUR cells, but here IL 4 was produced predominantly by SENIEUR CD8 cells (359). Increased production of IL 5 from PBMC of the elderly *In vitro* has also been reported (360). Discrepancies between different reports may also arise as a result of previous antigenic exposure of the populations studied; thus, for example, increased IFN-γ production may be noted in populations which had experienced greater viral exposure, because it is mostly produced by the CD8+ CD28-negative CD57+ subset (361) which contains memory cells for viruses like EBV and CMV (156). On the other hand, influenza–vaccinated elderly donors´ cells produced less IFN-γ after *In vitro* stimulation with the ´flu vaccine than young donors, and this was true both for those selected according to the SENIUER protocol and for those classified as frail (362).

Data on other cytokines and also chemokines are also gradually accumulating. For example, production of IL 8 by monocytes has been studied; constitutive IL 8 secreted *In vitro* was reported to be lower in the elderly than in the young, but on stimulation with LPS more IL 8 was produced in elderly males than in young (363). A different study showed that LPS-stimulated monocytes from healthy elderly donors produced smaller amounts of IL 8 (as well as G-CSF, GM-CSF, IL 1ß, TNF-α and MIP-1α) than young donors (175), as well as less IL 3 (364), while other studies suggested increased production of IL 6, IL 8 and TNF-α by the elderly (353,365). While most studies agree on age-associated increases of plasma IL 6 and IL 6 production *In vitro*, there are conflicting data even for this widely studied biomarker (see below). It has indeed been argued that the reason for this resides with inadequate exclusion of underlying disease from the study population (366), with IL 6 being a very sensitive marker of inflammation. Production of several chemokines has been reported to increase in the elderly, namely, MIP-1α, RANTES, MCP-1 and also IL 8 (367). Here again, therefore, disparate data on production in the elderly have been presented. One reason for this may be differences in production of IL 8 by different cell types; according to Mariani *et al*., T cells from nonagenarians when stimulated with CD3 mAb show a greater increment of IL 8 production than those from young donors, whereas IL 2 stimulation of IL 8 production from NK cells is lower in the old (368).

These examples suffice to illustrate the current difficulties still prevalent in the interpretation of data even when derived from extremely strictly selected populations. A detailed assessment of all published studies up to a couple of years ago led Bernstein & Murasko (347), contrary to received wisdom, to conclude that even the parameter of a decrease in IL 2 production could not be taken as a definitive age-linked change in humans. They were led to conclude that the one single most important reason for this was indeed heterogeneity within the elderly population tested. They also concluded that for the same reasons data on other cytokines, less well studied than IL 2, were even more unreliable. The reader is referred to their review for a good summary of the details of each study and possible explanations for some of the differences in results. We suggest here that approaches to obviate these problems

include performance of longitudinal rather than cross-sectional studies, use of standardized techniques at all stages of the assays, and inclusion of sufficient numbers of individuals for statistically rigorous comparisons. The latter point may be particularly important given the well-known extreme heterogeneity within the human population.

Models of immunosenescence may also be useful in this regard, where monoclonal T cell populations can be studied longitudinally throughout their proliferative lifespan in culture. Many of the parameters observed In vivo or ex vivo are reproduced in such cultures, particularly alterations in cell surface markers (see eg. Section 4.1 above) and in cytokine secretion patterns. In long-term expanded uncloned cells the ability to secrete IL 2 and undergo autocrine proliferation was reported to be lost at around 27 PD, long before the end of the proliferative lifespan of the lines was reached at a maximum of ca. 40 PD (369). This is comparable to our results with monoclonal populations, which show greatly reduced IL 2 secretion coupled with increased levels of antigen-specific IL 10 secretion and maintained IFN-γ (130). Very few studies have compared the behavior of T cells from young and old donors at the clonal level. Paganelli et al. (370) reported on T cell clones (TCC) obtained from two centenarians compared to those obtained from three young donors. CD4+ TCC made up 38% of TCC obtained from the young, but 53% of those from the old. Cytokine production from the CD8-TCC was the same in young and old-derived clones, but the CD4-TCC were different. Most TCC derived from young donors produced IFN-γ but not IL 4, whereas those from the centenarians produced both. This was interpreted to indicate a shift in the CD4 population from predominantly Th1 to Th0 phenotype in the centenarians. They did not record clonal longevity In vitro. We have derived TCC from an elderly SENIEUR donor under serum-free conditions and compared cytokine secretion patterns with those of clones derived from a young donor under identical conditions (268). The data suggested that the frequency of TCC which secreted IL 2 did not differ whether they were derived from the old or the young donor, but the amount of IL 2 released was greater in the TCC from the young donor. Twice as many clones from the old donor secreted IL 10, and the amount per clone was much greater in the old than young. These results fit with expectations from ex vivo studies. However, whereas only 17% of TCC from the old donor secreted IL 6, this figure was 75% for TCC from the young donor, although levels per clone were fairly similar. This might be explained by a previous report suggesting that IL 10 can suppress secretion of IL 6 by T cells In vitro (371). Production of other cytokines (IL 4, IFN-γ, GM-CSF) remained similar, both in terms of frequency of clones secreting the cytokine, and the amount secreted per clone. These results suggests that differences observed between the young and old in mixed populations of PBMC directly ex vivo (eg. decreased IL 2 production, increased IL 10 production) may be a stable property of the cells within those populations, even after cloning and propagation for > 30 PD.

4.4.3. Confounding factors affecting cytokine secretion results

Even strict selection criteria, such as the SENIEUR protocol, may fail to take psychological and nutritional factors sufficiently into account. That age-associated decreased immunological function may be linked to psychological factors (356) and nutritional factors (40) has rarely been considered in published studies not specifically directed towards evaluating these parameters as the study objective. Where nutritional factors have been rigorously considered, reports have appeared indicating lack of effects of aging on production of IL 2, IL 1ß or IL 6 in PHA-stimulated whole blood cultures from the healthy elderly (354). However, effects of stress may be important: recent studies are beginning to suggest clinically relevant alterations, eg. in the decreased antibody responses to influenza vaccination of chronically stressed elderly carers of dementia patients (372). This report confirmed an earlier one where the humoral response to influenza vaccination was altered by the chronic stress associated with caring for a demented spouse, resulting in significantly lower antibody titers, as well as lower IL 1 and IL 2 production in the elderly caregivers (373). Acute stress may also alter response patterns in a way often associated with an age-induced change, for example, medical student exam stress was reported to induce a Th1 to Th2 cytokine shift (374). In mice, restraint stress causes thymic involution, which can be reversed by vitamin E administration in young but not in old mice (375). Such factors may need to be taken more into account in studies of immunosenescence (376) A large meta-analysis suggested impaired proliferation to mitogens, lower NK activity, altered lymphocyte subsets and numbers in clinical depression, with older patients showing more extreme effects than younger (377). On the other hand, another study failed to reveal any differences in levels of IL 1ß, IL 6 or TNF-α in the plasma of elderly patients with major depressive disorder compared with age-matched healthy controls (378). However, this study also unexpectedly failed to show any differences between old and young donors, and included very few patients; its general applicability may therefore be doubtful. A more recent study concluded that there was a correlation between increased plasma levels of TNF-α and depression in the elderly (379). Some data are beginning to emerge concerning possible mechanisms for such interactions, eg. on the regulatory effects of neuropeptides on cytokine secretion in young and elderly donors (380). The expression of dopamine D3 receptors on human lymphocytes is decreased precipitously between 40 - 50 years of age (381). Conversely, increasing levels of expression of cellular amyloid precursor protein by human lymphocytes are significantly associated with age (382). Although the significance of these findings is unclear, studies of this type may begin to help shed some light on the mechanisms responsible for neuroimmunological communication and age-associated alterations. That this could be a two-way communication is illustrated by the ability of IFN-γ and other cytokines to modulate the production of melatonin (383). Other factors perhaps not sufficiently taken into account in previous studies of cytokine release may be not only differences between sexes but differences between females dependent on their reproductive history which also affect cytokine production (384). Since leptin, a hormone known to affect the Th1/Th2 balance (385) apparently is induced by estrogens (386) and suppressed by testosterone (387) and since both sexual hormones decline with age, changes in leptin levels may be another factor to consider.

It may even be that unsuspected population genetic influences could be playing a role, since the distribution of MHC alleles differs even within different groups of the European population, and levels of immune responses and cytokine secretion as well as possibly longevity are known to be associated with MHC type (388). Particularly the MHC class II alleles, which are mostly responsible for presenting foreign antigen to CD4+ helper T cells, may influence longevity (389) However, it should also be considered that technical differences in experimentation, particularly the measurement of cytokines by immunoassays, could be contributing significantly to the discrepancies in the data obtained by different groups (390). The relatively new technique of intracytoplasmic cytokine staining may help resolve some technical difficulties, because it allows identification of the type of cell secreting the cytokine, at the single cell level. Using this technique, age-associated increases in TNF-α and IL 6 secretion by CD3+ cells have been confirmed (391).

Another point to consider is not only the kinetic aspect of the release of cytokines *In vitro*, but also *In vivo*. Thus, the IL 2 secretion defect reflected in many but not all studies may in fact be transient, with T cells from old donors re-acquiring this ability after a period in culture (392). Different donor states might then explain discrepancies found in cytokine secretion patterns even amongst SENIEUR donors, as noted above. Huang *et al*. found that old donors with apparent IL 2 secretion defects *In vitro*, in fact had high serum IL 2 concentrations *In vivo*. Moreover, vaccination of young donors mimicked this effect and resulted in their T cells becoming refractory for IL 2 production shortly thereafter *In vitro*. These investigators therefore suggested that apparent defects in IL 2 secretion in elderly donors are a result of *In vivo* activation of their T cells by unknown mechanisms and reflect a normal event also seen in young donors after *In vivo* T cell activation by immunization (392). These findings are also consistent with those made in SLE patients and lupus mouse models, where IL-2 serum levels tend to be increased but IL-2 secretion is clearly suppressed (393). Both in the elderly and in SLE patients increased TNF levels may eventually play a role in effecting such phenotype. If these results are confirmed, a reassessment of the meaning of depressed IL 2 secretion by old T cells *In vitro* will be required. The immune "defect" observed here will then actually represent a normal consequence of activation, possibly a kind of "exhaustion", which in animal models can even result in extra-thymic clonal deletion of the activated cells (394). In this paradigm, altered immune responses in elderly persons are more likely to be associated with dysregulation of the response in terms of lack of suppression of responses to self, consistent with the perceived association of aging and autoimmunity. On the other hand, "memory" cells accumulate in elderly donors, and may be in active division required for their maintenance of memory (66,395). Not only might these activated cells explain the data of Huang *et al* (392), but since they might eventually arrive at a post-mitotic state, this would also explain their eventual loss from the system altogether. Moreover, in mice, such memory cells seem to be maintained in proliferation by IL 15 rather than IL 2, and may even be actively deleted from the system by the latter (396). IL 2 may increase clonal expansion during a primary immune response, thus increasing the size of the cell pool from which memory cells are derived, but as shown in IL 2-KO mice, it may thereafter reduce the size of the maintained memory pool (397). The large clonal expansions of CD8+ cells are also maintained by IL 15 and inhibited by IL 2 in old mice (398). Because CD8 clonal expansion is also regulated by perforin and clonal contraction by IFN-γ (as shown in KO mice) (399), any decrease in IFN production with age might contribute to CD8 persistence in old individuals.

It has to be remembered that the presence of one cytokine may also affect the production of others, or in many cases, almost certainly does so. Therefore, in studies measuring only one single cytokine, confounding factors for the production of the target cytokine may be differences between donors in their production of other cytokines. IL 6, for example, may influence the responsiveness of T cells to other factors such as PDGF, and hence alter their cytokine production profiles (400). Decreased anti-viral CD8+ CTL activity in aged mice has also been attributed to deficient IFN-γ production caused by IL 12 deficiency, and reconstituted by IL 12 *In vitro* (401). Different proportions not only of cell subsets but different proportions of various cell types in the population may also need to be taken into account. So, again, one sees a great deal of difficult-to-interpret variability in the data. In whole blood cultures, LPS stimulation was also reported to result in lower TNF-α and IL 1ß production in the elderly, with unchanged IL 6 (402). Using PHA-stimulated whole blood cultures, others have found no change in IL 1ß or IL 6 production in well-nourished SENIEUR females (354) . On the other hand, the dose of LPS required to stimulate IL 6 and TNF production may be greater for monocytes of elderly compared to young donors (403). Others have found increased production of both IL 10 and IL 12 from SEB-stimulated PBMC of the elderly (404), whereas IL 12 has also been found to remain unchanged (176) or decreased after CD3 stimulation; in the latter case this could be reversed by adding IL 2 to the medium (405). Thus, production of certain cytokines *In vitro* may increase rather than decrease with age (406), although serum titers in the healthy elderly may not (407). Increases in TNF-α may be directly relevant for decreased T cell responses, because TNF-α can inhibit proliferation of some human TCC (408) and can attenuate TCR-signaling *In vivo* in mice (409).

4.4.4. Levels of cytokines in plasma

In vivo studies of plasma levels of factors such as IL 6 and TNF-α also generally reveal age-associated increases; in fact, it has been proposed that IL 6 levels may be a good overall biomarker of health in aging because plasma levels are correlated with functional status and prospectively with mortality (410-412). This may in turn be related to subclinical disease status, however. IL 1 and TNF-α, as well as IL 6, together with IL 3 and IL 4, the levels of which are increased in aged mice and humans (365,413), are known to control isotype switch and immunoglobulin production during B cell differentiation. In particular, along with B cell differentiation, IL 6

stimulates proliferation of thymic and peripheral T cells and in co-operation with IL 1 induces T cell differentiation to cytolytic-T cells and activates NK cells. Moreover, IL 6 induces increased expression of the heat shock protein hsp90 in human lymphocytes *In vitro* and in mice *In vivo*; in the latter, this results in the generation of anti-hsp90 autoantibodies (414). These observations emphasize the importance of IL 6 in both non-specific and specific immune responses, as well as in a wide variety of other systems and may also be relevant to several aspects of age-associated pathological events including atherosclerosis, osteoporosis, fibrosis and dementia. The postulated concept of "inflammaging" (415), whereby many age-associated pathological processes are due to the inflammatory activities of the immune system (416), represents a modern restatement of the original "Immunologic Theory of Aging" (417). Here, the inflammatory process is made responsible not just for autoimmuity but a whole range of other afflictions, from Alzheimer´s to atherosclerosis. For example, increased levels of IL 6 (and also TNF-α and CRP) are associated with senile osteoporosis in both females and males (418), although in healthy old donors, the increase in IL 6 may be limited to males (419). Those elderly with the highest levels of TNF-α are also reported to suffer more from atherosclerosis (420). The same group also observed that the higher TNF-α levels in the elderly were correlated with higher sIL 2R levels and lower PHA-stimulated IL 2 production (421). Recently, the IL-6 promoter genetic variability at -174 C7G locus and its effect on IL-6 serum levels was studied in a total of 700 people from 60 to 110 years of age, including 323 centenarians. The proportion of homozygotes for the G allele at -174 locus decreases in centenarian males, but not in centenarian females, and only among males, homzygotes for the G allele have higher IL-6 serum levels in comparison with carriers of the C allele. These data suggest that those individuals who are genetically predisposed to produce high levels of IL-6 during aging, i.e. -174 GG homozygous men, are disadvantaged for longevity (422). Conversely, subjects homozygous for GG at 1082 position of IL-10 locus, a polymorphism associated with high plasma levels of IL-10, are significantly more frequent than expected among centenarians, in comparison with younger control subjects (423). In these studies, IL 6 was not assayed, but there may well be a correlation between TNF-α and IL 6 levels. Strikingly, it has been reported that the levels of TNF-α in nursing home residents can predict mortality (424). On the other hand, TNF-α levels are not always reported to be higher in the healthy elderly (407) and in elderly patients with pneumonia are lower than in the young (175). Others have observed increased levels of IL 6 in elderly females also, enhanced by stress, which may even outweigh the age effect (425). This may offer a possibility for pharmacological intervention by IL 6R blockade (426). Even for IL 6, however, not all investigators have found elevated serum levels in perfectly healthy elderly humans in the past (407,427). This may have been technical, because more recent studies agree on the correlation between elevated IL 6, elevated CRP and mortality, including longitudinal, prospective, studies (411). The possibility remains that genetically different populations behave differently, because the distribution of polymorphisms in the promoters of genes such as the IL 10 gene is known to vary (428), although serum levels of IL 2 have been reported to be the same in young and old donors in the one available study (407). Therefore, despite the presence of some of the same difficulties as when measuring cytokine secretion profiles, the rather more recent data summarized above may be more rigorous and allow more definitive interpretation. Nonetheless, here again care must be exercised as the numbers of subjects were often quite small and thus far there are few data from different studies to compare.

Direct measurements of IL 2 levels in the serum of SENIEUR donors have also failed to detect age-associated decreases (429), although a second study suggested that serum IL 2 levels were reduced in the very old (58). However, both studies agreed that the level of soluble IL 2R in the blood was increased in the elderly, which could contribute to decrease of IL 2 function. Decreased soluble IL 2R secretion has been noted before in non-SENIEUR donors and may be of greater significance than possible IL 2 secretion defects (430). Interestingly, the presence of several types of autoantibodies was positively correlated with the presence of increased soluble plasma IL 2R, but neither were associated with a particular HLA type, even though there was a significant increase in HLA-DR7 in the elderly (431).

Total serum IL 12 levels in the elderly have been found to be increased compared to the young (432). Regarding chemokines, serum MCP-1 levels are higher in the elderly than in the young while RANTES has been detected in serum of centenarians only (433)

4.4.5. Cytokine antagonists

As well as altered cytokine levels in aging, altered levels of cytokine antagonists might also influence cytokine networks, as mentioned for IL 2R above. These possibilities are now beginning to be explored. Thus, Catania *et al*. (434) reported a study of 122 healthy aged compared to 39 unhealthy (urinary tract infections) and 100 young controls regarding plasma levels of IL 1R-antagonist and sTNF-R. These were higher in the healthy old than in young controls, and were even higher in the infected subjects. Production of IL 6 and IL 1Ra but not IL 1ß or TNF-α by elderly PBMC was reported (435). A marker of activated macrophages, neopterin, is elevated in the plasma of apparently healthy aged human donors, in correlation with higher titers of cytokine antagonists like soluble TNF-α-receptor and IL 1Rα. These findings were more extreme in patients with infections. It was therefore suggested that subclinical infections may have been responsible for this even in apparently healthy elderly donors (434). Another study reported that serum levels of sIL 6R and IL 1Ra were not significantly different in healthy young, middle-aged or old donors. However, sTNF-R levels (and M-CSF) were increased in the elderly (436). Both types of TNF-R, RI and RII, are increased in the elderly, and even more so in centenarians (433).

The balance of altered cytokine levels (agonist) and altered soluble cytokine receptor levels (antagonist)

will always need to be considered together when discussing age-related changes, as there are probably differences in both, also for IL 6 (437). While the production of factors such as IL 6 is clearly influenced by health status, even in "near-SENIEUR" donors (438), their IL 6R (antagonist) status needs to be determined too (439). The same also applies to TNF-α (440).

4.4.6. Cytokine receptor expression

Other than the IL 2R few cytokine receptors have been studied, and even for the IL 2R most but not all studies were limited to measuring expression of the alpha chain only (CD25). Nonetheless, there is a fair degree of consistently amongst the published studies, saying that levels of IL 2R expression are decreased in the elderly (347). Measuring CD25 does not say much about the function of the receptor, which consists of two other chains in addition to CD25. However, defects in upregulation of the high affinity receptor for IL 2 were reported early on (441). Interestingly, even that lower proportion of cells expressing the high affinity receptor and retaining their ability to internalize the IL 2 still fail to respond properly (442). Thus, even under conditions where IL 2 secretion is apparently normal, and where IL 2R expression is also apparently normal in terms of receptor affinity and number (443), aged T cells may still proliferate less vigorously than young cells, even in the presence of exogenous IL 2 (443), although this has not been seen in all studies (444).

4.4.7. Cytokine gene polymorphisms

Cytokine and cytokine receptor genes are generally highly conserved in terms of exon sequences. In fact, many of the reported polymorphisms within cytokine genes are silent mutations that do not affect amino acid sequence. However, they occur within known or putative regulatory regions, thus influencing protein expression by altering mRNA splicing, mRNA stability and levels of gene transcription. In fact, polymorphisms located within promoter regions in several cytokine genes have been recently described, mostly single nucleotide polymorphisms (SNP), or microsatellites, which affect gene transcription, causing interindividual variations in cytokine production (445). These polymorphisms might confer flexibility on the immune response by allowing differential production of cytokines. The presence of certain alleles may influence the outcome of certain diseases and therefore have been selected within specific populations (446). Accordingly, several studies are attempting to identify immunogenetic cytokine markers for a given disease. Like studies performed with HLA antigens, association is sought between specific cytokine gene polymorphisms and clinical outcome by direct comparison of individual cytokine genotypes and the susceptibility, duration and severity of the disease under study. Thus, many studies have identified statistically significant associations between cytokine alleles and diseases (445,447).

Several studies have been performed on the association between cytokine polymorphisms and age-related inflammatory diseases, responsible for "unsuccessful" aging, such as Alzheimer's disease (AD) and atherosclerosis and, on the other hand, more recently, with longevity. Here we will briefly discuss available data on proinflammatory cytokines IL-1, TNF-α, IL-6, IFN-γ and anti-inflammatory cytokine IL-10. AD is a complex disease involving several genetic and environmental components. Genetic studies have yet to identify all the genes involved in the pathogenesis of AD. Established genetic causes of familial AD involve genes for β-amyloid precursor protein, presenilin-1, and presenilin-2. For the more common sporadic forms of AD, increased risk has been associated with a number of genes, the most important of which is the ε allele 4 of apolipoprotein E (ApoE). Recent studies now show increased risk for AD associated with certain polymorphisms in the genes encoding the proinflammatory cytokines, since local inflammatory processes can exert direct neurotoxicity, interfere with β-amyloid expression and metabolism and maintain chronic intracerebral acute phase protein secretion, in turn favouring formation of β-amyloid fibrils (448,449). In studies in which a positive association was found, the effect of inflammatory cytokine gene polymorphisms on the risk of developing AD may be evident in ApoE ε 4 allele non-carrier patients, suggesting a complex interaction between these genetic risk factors (449). Atherosclerosis is an inflammatory disease. Because high plasma concentrations of cholesterol, in particular those of low-density lipoprotein cholesterol, are one of the principal risk factors for atherosclerosis, the process of atherogenesis has been considered by many to consist largely of the accumulation of lipids within the artery wall; however, it is much more than that. In fact, the lesions of atherosclerosis represent a series of highly specific cellular and molecular responses that can best be described, in aggregate, as an inflammatory disease. Thus, atherosclerosis is clearly an inflammatory disease and does not result simply from the accumulation of lipids (450). In fact, epidemiologic studies clearly indicate that chronic low-grade inflammatory activity in aging promotes an atherogenic profile and is related to enhanced mortality risk (7). Accordingly, recent studies now show increased risk for atherosclerosis-related diseases associated with certain polymorphisms in the genes encoding cytokines involved in the control of inflammation.

The polypeptide proinflammatory cytokine IL-1 family represents a group of proteins that have contrasting and synergistic biologic responses. IL-1α and IL-1β and their precursor forms are heavily involved in the enhancement of inflammation and host defence. Within this family of gene products, there is also a naturally occurring receptor antagonist, IL-1ra, as well as a family of receptor proteins that have differential signaling functions and activities (451). IL-1 levels are elevated in AD patients´ brains, and overexpression of IL-1 is associated with β-amyloid plaque progression. IL-1 interacts with the gene products of several other known or suspected genetic risk factors for AD, mostly the ApoE ε allele 4. IL-1 overexpression is also associated with environmental risk factors for AD, including normal aging and head trauma. These observations suggest an important pathogenic role for IL-1, and for IL-1-driven cascades, in the pathogenesis of AD (452). A large number of studies clearly demonstrates an involvement of IL-1α -889 SNP (a C to T

transition, also designated as allele 2) (453-456). In particular, Grimaldi *et al*. (454) found a strong association between the IL-1α T/T genotype and AD onset before 65 years of age, with carriers of this genotype showing an onset of disease 9 years earlier than IL-1α C/C carriers. A weaker association with the age at onset was also shown for the IL-1β and IL-1RA genes. Another group found the IL-1α T,T genotype significantly increased in a group of neuropathologically confirmed AD patients from four centers in the United Kingdom and United States. Homozygosity for both alleles T of IL-1α and allele T of IL-1β conferred even greater risk (455). The IL-1α polymorphism was analyzed in a population of 247 AD patients and 187 control individuals. In support of the published findings, the IL-1α T/T genotype was associated with an increased risk of early onset AD. Clinically, the IL-1α T/T genotype was associated with an earlier age of onset, but not with a change in the rate of progression of AD (456). Plasma levels of IL-1ß were found to be increased in patients with AD and the high levels of the cytokine were linked to the IL-1β TT genotype. This genotype in the presence of a particular α1-ACT genotype increased the risk of AD and decreased the age at onset of the disease (457). Finally, in a recent paper, surprisingly, in a sample of 114 patients followed for an average of 3.8 years, individuals homozygous for the SNP C allele declined significantly more rapidly on the basis of the decrease of the score of cognitive function than the others (458). However, on the whole these results clearly demonstrate that IL-1 polymorphisms play a significant role in AD. No study has been yet published on the association between IL-1 polymorphisms and both longevity and atherosclerosis.

TNF genes might also play a role in inflammatory age-related diseases, because TNF influences the strength, effectiveness, and duration of local and systemic inflammatory reactions, as well as repair and recovery from infectious and toxic agents. The TNF cluster genes, located in the HLA region on chromosome 6, encode three inflammation-related proteins, TNF-α, TNF-β and lymphotoxin-β. All three are important mediators of the immune response with multiple biologic activities. Several polymorphic areas have been documented within the TNF gene cluster. Notably, some two-allele polymorphisms for the TNF promoter region and several microsatellite polymorphic sites have been described. Polymorphisms in the TNF promoter region have been observed to result in differences in the rate of gene transcription and in the rate of protein production. Many associations with immune-mediated diseases have been described, both with TNF gene promoter polymorphisms, and TNF region microsatellites (459,460). TNF may be involved in the pathogenesis of AD based on observations that senile plaques have been found to upregulate proinflammatory cytokines (448). However, discrepant results have been obtained. In fact, results of one study indicate that increased intrathecal production of TNF-α in AD is preferentially controlled by environmental stimuli rather than genetic makeup (461). On the other hand, a collaborative genome-wide scan for AD genes in 266 late-onset families implicated a 20 centimorgan region at chromosome 6p21.3 that includes the TNF gene (462). Three TNF polymorphisms, a -308 TNF promoter polymorphism, whose TNF2 allele is associated with autoimmune inflammatory diseases and strong transcriptional activity, the -238 TNF promoter polymorphism, and the microsatellite TNFa, whose 2 allele is associated with a high TNF secretion, were typed in 145 families consisting of 562 affected and unaffected siblings. These polymorphisms formed a haplotype that was significantly associated with AD using the sibling disequilibrium test. Singly, the TNFa2 allele was also significantly associated with AD in these 145 families. A further polymorphism in the regulatory region of the TNF-α gene was analysed in a case-control study. The polymorphism (C850T) was typed in 242 patients with sporadic AD, 81 patients with vascular dementia, 61 stroke patients without dementia, and 235 normal controls. The distribution of TNF-α genotypes in the vascular dementia group differed significantly from that in the stroke and normal control groups, giving an odds ratio of 2.51 for the development of vascular dementia for individuals with a CT or TT genotype. Logistic regression analysis indicated that the possession of the T allele significantly increased the risk of AD associated with carriage of the ApoE ε4 allele for those with this allele but no TNF-α T versus those with ApoE ε4 allele and TNF-α T. Possession of the TNF-α T allele significantly increases the risk of vascular dementia, and increases the risk of AD associated with ApoE (463). Ample evidence supports a role of TNF-α in the development of cardiovascular disease. TNF-α is expressed in atherosclerotic plaques but not in healthy vessels. In atherosclerotic plaques, TNF-α may contribute to foam cell formation, to T-lymphocyte activation and to the expression of matrix metalloproteinases that may destabilise the plaque by degrading the extra-cellular matrix. A significant positive association has been demonstrated between the TNF-α G-308 A polymorphism and levels of extracellular superoxide dismutase and homocysteine which are consistent with TNF-α acting as an oxidative stress relevant to atherogenesis. However, the TNF-α polymorphism was not associated directly with the occurrence or severity of atherosclerosis documented angiographically (464). Another study, retrospective, on an autopsy series that comprised 700 Caucasian Finnish men, aged 33-70 years (465) measured coronary stenosis and surface area of atherosclerotic changes and the presence of myocardial infarction and coronary thrombosis was recorded. Two biallelic polymorphisms within TNF gene locus-TNFA at the position -308 in the promoter region of the TNF gene and TNFB in the first intron of the lymphotoxin were studied. A weak association with the TNFA and TNFB polymorphisms and atherosclerotic morphometric changes in coronary arteries was found, but there were no differences in coronary stenosis and in the frequency of old or recent myocardial infarction or coronary thrombosis between men with different genotype status in either locus. Thus TNFA and TNFB polymorphisms are unlikely to contribute to progression of atherosclerosis in a way clinically important. No study has been yet published on the association between TNF polymorphisms and longevity.

IL-6 is a pleiotropic cytokine capable of regulating proliferation, differentiation and activity of a variety of cell types, and plays a major role in bone remodelling, neuro-endocrine homeostasis, hemopoiesis, immune system regulation. In particular, IL-6 plays a pivotal role in acute phase response and in the balancing of the pro-inflammatory/anti-inflammatory pathways (466). Recently, the magnitude of IL-6 plasma levels has been associated with the degree of functional disability and mortality (410,411,467). In fact, in a large study of 675 healthy elderly patients, high IL-6 levels were associated with a two-fold increase in risk of death, comparing subjects in the highest quartile with the lowest quartile (467). Despite the high sensitivity of IL-6 plasma levels to acute and chronic infections, as well as to other environmental conditions, there is a strong genetic control of IL-6 plasma level that suggest different reactivity in aging people. In fact, a C/G polymorphism at 5'-upstream of IL-6 has been identified (468). This SNP influences the rate of IL-6 gene transcription and is associated with different IL-6 plasma levels. As far as the functional implications of this SNP, carriers of the G allele at –174 locus appear to be prone to develop lipid abnormalities and have a worse glucose handling capacity, a higher blood glycosylated haemoglobin, a higher fasting insulin levels, and an higher insulin sensitivity (469,470). Accordingly, recent data suggest that C+ men may be protected from cardiovascular diseases. In particular, an under-representation of homozygous for C allele at –174 locus has been found in a group of Swedish men having a myocardial infarction under age 40 (471). Moreover, in middle-aged men, homozygous for the G allele at –174 C/G locus have an increased artery intima-media thickness in the carotid bifurcation, a predilection site for atherosclerosis. Concerning IL-6 in AD patients, Bhojak *et al*. performed a genetic screening of sporadic, late-onset AD cases and age-matched controls to evaluate the role of this IL-6 polymorphisms in AD. Data indicate no significant association between this SNP and the risk of AD (472). These data suggest that the IL-6 polymorphisms do not significantly alter the risk of AD in case-control cohort. Recent studies also showed that another IL-6 polymorphism, i.e. a variable number of tandem repeat polymorphism (VNTR) in the 3' flanking region of IL-6 gene, plays a complex role in AD. In fact, it was demonstrated that the allele C of this polymorphism was associated with a reduced risk of sporadic AD and delayed initial onset. In a further study, they examined the role of G/C polymorphism at position -174 in 102 AD patients and two control groups of 191 healthy subjects and 160 depressed patients. There was no evidence for an allelic association between this IL-6 polymorphism and earlier age of onset or risk of AD. However, an interaction, with possible additive effects, between IL-6 VNTR and IL-6 SNP could modify AD risk (473). Recently, in a study performed on centenarians (422), it has been reported that those individuals who are genetically predisposed to produce high levels of IL-6 during aging, i.e. C– men at IL-6 –174 C/G locus, have a reduced capacity to reach the extreme limits of human life span. On the other hand, low level IL-6 production throughout the life-span (C+ individuals) appears to be beneficial for longevity, at least in men. From a demographic point of view, the decreased proportion of C– men in centenarians could therefore be attributed to a higher mortality of C– men for cardiovascular diseases, which are the most prominent causes of mortality among the elderly in Western countries (see above). Taken together, these data suggest that in an aging population, those people who have the tendency to produce elevated IL-6 quantities are less likely to attain maximum longevity, and this fact is likely due to their increased likelihood of developing age-related inflammatory diseases.

The representative type 1 cytokine IFN-γ plays a pivotal role in the induction of immune mediated inflammatory responses. Recently, it has been reported that the 12 CA repeats microsatellite allele at the first intron of IFN-γ gene is associated with a higher level of cytokine production *In vitro*. More recently the same group reported an absolute correlation between the 12 CA repeat allele and the presence of the T allele at a SNP located at the +874 position (+874T→A) from the translation start site coinciding with a putative NF-kB binding site that may be important in the induction of constitutively high IFN-γ production (474). No study has been performed on the association between age-related inflammatory diseases and IFN-γ polymorphisms. However, both in experimental animals and in human beings IFN-γ is involved in the development of atherosclerotic plaques (475). Also, in AD patients, increased spontaneous and IL-2-induced release of IFN-γ and TNF-α from NK cells were found compared to healthy subjects and furthermore, significant negative correlations between the spontaneous release of IFN-γ and TNF-α from NK cells and the decrease of the score of cognitive function were found in patients (476). Thus, it is conceivable that IFN-γ polymorphisms can be involved in these diseases. On the other hand, Lio *et al*. have studied the distribution of +874T→A IFN-γ polymorphism in a large number of Italian centenarians to evaluate if the two alleles might be differently represented in people selected for longevity (477). The +874T allele was found less frequently in female centenarians than in controls, whereas allele frequencies in centenarian men were not found significantly different from controls. Possession of the +874T allele, known to be associated with low IFN-γ production, significantly increases longevity.

Conversely, it can be hypothesized that anti-inflammatory cytokines might also be involved in successful aging and longevity. The cytokine IL-10 seems to be an appropriate candidate. IL-10, a cytokine produced by macrophages, T cells and B cells, is a major immunoregulatory cytokine, usually considered to mediate potent downregulation of the inflammatory responses (478). IL-10 production, independent of interaction with other cytokine gene products (459), is genetically controlled by polymorphisms in the IL-10 promoter sequence (479,480). In fact, the presence of multiple SNPs in the human IL-10 5' flanking region has been demonstrated, and some of these (i.e. - 592, - 819 and – 1082) combine with microsatellite alleles to form haplotypes associated with differential IL-10 production. A

recent extensive study suggests that IL-10 polymorphisms are not associated with an increased risk of myocardial infarction (479) although serum levels of IL-10 are decreased in patients with unstable angina, in keeping with previous data from animal model studies that suggest that IL-10 has a protective role in atherosclerosis (481). Concerning AD, in a recent study whole blood samples from AD and controls were stimulated ex vivo with endotoxin under standard conditions and cytokine levels assessed. Patients with AD had seven- to ten-fold higher IL-1β production relative to the amount of IL-10 (482). The data suggest that an absolute (or relative) reduced production of IL-10 may contribute to the development of AD, but no study has been performed on the association between AD and IL-10 polymorphisms. A recent study has instead demonstrated a role of IL-10 polymorphisms in successful aging. The 1082G homozygous genotype was increased in centenarian men but not in centenarian women. No difference was found between centenarians and control subjects regarding the other two polymorphisms. The presence of the –1082GG genotype, suggested to be associated with high IL-10 production, significantly increases the possibility of reaching the extreme limit of human lifespan in men (423).

Taken together, these data strengthen the suggestion that inflammatory status may be detrimental for successful aging since polymorphic alleles of inflammatory cytokines, involved in high cytokine production, play an important role in age-related inflammatory diseases, i.e. in "unsuccessful" aging. Reciprocally, they suggest that controlling inflammatory status may allow more "successful" aging. Indeed, the major findings reported in recent papers on cytokine polymorphisms and longevity (422,423,477)suggest that those individuals who are genetically predisposed to produce low levels of inflammatory cytokines or high levels of anti-inflammatory cytokines have an increased probability of achieving extreme longevity.

5. CLONAL EXPANSION AFTER T CELL ACTIVATION

5.1. Culture models for immunosenescence: does the Hayflick Limit apply to normal T cells and if not, why not?

Once naive T cells have been successfully stimulated, costimulated and cytokine availability and utility assured, the T cell response requires waves of clonal expansion followed by contraction when antigen is no longer present and re-expansion on contact with antigen again. The number of cells re-entering proliferation and the degree of proliferation required is tightly regulated (1). Limits to the proliferative capacity of T cell clones might impact deleteriously on the overall response and disturb this delicate homeostasis. Cells that have previously proliferated *In vivo* may have a decreased remaining proliferative potential. T cell clones *In vitro* provide a good model for investigating age-associated changes in a longitudinal manner. Do they undergo replicative senescence? Human T cells can be maintained for extended periods in tissue culture, and in most cases they are recognized to have finite lifespans, eg. see refs. (2-10). A small number of studies has approached the question of whether T cells from old donors have shorter proliferative lifespans than those from young donors. Early reports showed a correlation between advanced donor age and decreased proliferative capacity (11). However, in these experiments, T cells were stimulated only once with mitogen at the initiation of culture and subsequently provided with IL 2 but no restimulation via the TCR. These data are nonetheless valuable in showing a decreased capacity for clonal expansion on the part of old T cells after a single stimulation. Replicative capacity under appropriate culture conditions for T cells, ie. intermittent restimulation via the TCR, is greater than the value found in the above experiments. Thus, by restimulating T cells every two weeks, McCarron *et al*. (2) achieved far greater T cell expansion ranging from 62 - 172 PD (as calculated from their figures of cell expansion). In that study, there were no differences in longevity of T cell cultures derived from neonatal, young adult or old adult donors. This is in agreement with recent work done on human fibroblasts, where the long-established view that fibroblast longevity in culture was dependent on donor age was shown not to be the case when donor health status and biopsy conditions were rigorously controlled (12,13). However, in bulk cultures, be they T cells or fibroblasts, the final longevity of the cultures will be determined by the longest-lived clones. Therefore, cloning experiments should reveal a truer picture of average lifespans of all the cells in a population and not just the longest-lived. The elderly might well possess rare clones as long-lived as those in the young, but nonetheless still have a majority of clones much shorter lived. Such experiments in bulk cultures would not reveal this. A decreased proliferative capacity been reported for some T cell clones established from aged healthy donors´ cells, particularly for $CD8^+$ cells (3). Apart from this and own work, the McCarron study is unique in that it also examined longevity of (a small number of) T cell clones (2). Here, the findings were rather different. T cell clones derived from neonates averaged 52 PD, while those from young adults (20-30 yr) managed 40 PD, but those from the elderly (70-90 yr) only 32 PD. In our own experiments, using CD3+ cells from young adult donors, average cloning efficiencies of around 50% were obtained. About half of these clones achieved a population size of one million (equivalent to 20 PD). TCC were continuously lost during culture, so that there were fewer survivors reaching 30 and still fewer accomplishing 40 PD. The average longevity of the established clones in these experiments works out at around 33 PD, whereas the average lifespan of all cells in the starting population is estimated at approximately 17 PD. This contrasts markedly with the lifespan of the longest-lived clone in these experiments, which in our hands is thus far about 170 PD. Clearly, rare long-lived clones are the exception rather than the rule, but all else being equal, these would remain *In vivo* when other specific clones had already died out. At the end of their lifespan *In vitro*, the TCC die by apoptosis and thus would be deleted from the repertoire *In vivo*. Cloning T cells from old (>80 yr) SENIEUR donors using the same culture conditions (RPMI 1640 + 10% human serum and IL 2) did not result in the generation of any clones at all, suggesting

that aging may have occurred *In vivo* such that the replicative lifespan of the clones *In vivo* was close to exhaustion. However, this has recently been shown not to be the case, since cloning and culture in a new serum-free medium supported equally high cloning efficiencies in the old as in the young. Thus far, the proportion of clones achieving 20 PD has also been shown to be similar; experiments taking all derived clones further are ongoing, but some of the clones have already reached 39 PD. These results suggest that the replicative capacity of clonable T cells derived from very old perfectly healthy donors is probably not less than that of T cells from the young and implies that senescent cells have in fact not been accumulating in the clonable T cells of these (highly selected) donors (14). In none of these experiments were immortal T cell clones obtained.

There may nonetheless be rare exceptions to the rule that T cells have finite lifespans. Certain human TCC which have been cultured for many years must be presumed to have exceeded the Hayflick limit, although this has not been formally measured by any of the investigators working with such clones. How can these potential discrepancies be resolved? Human T cells infected with HTLV-1 or Herpes saimiri virus can become immortalized (15). In the latter case, the cells retain a normal functional phenotype, ie. they remain dependent upon exogenous growth factor for their continued proliferation, and they still respond specifically to stimulation via their antigen receptor and non-specifically via the alternative activation pathway (CD2/CD58-dependent). Therefore, inadvertent infection with H. saimiri would result in retention of apparently normal immunological attributes coupled with indefinite lifespan. However, the chances of inadvertent infection with this non-human virus are presumably very low, and can be excluded by screening for known viruses. Nonetheless, there always remains the possibility that rare events featuring transformation with unknown pathogens might account for some examples of apparent immortality of TCC. For example, one putatively immortal line which developed from a culture whose sister cultures all senesced was found to be infected with mycoplasma (16). SV40 large T antigen, with well-known transforming properties, rarely immortalizes human T cells, but it has been reported that this may occur (17). In this case, however, the resulting cell line expressed only cytoplasmic, not surface, TCR but did express surface CD1a, not representative of normal mature T cells (17).

It may still be questioned whether suboptimal culture conditions for T cells are responsible for the short lifespans of the majority of TCC. Since the rare long-lived TCC are cultured under apparently very similar conditions to the normal, short-lived, ones, this may seem *a priori* unlikely. However, some simple manipulations of tissue culture conditions may be sufficient to affect longevity, and for some reason certain rare T cells might more successfully adapt than others to particular culture conditions. For example, it has become apparent that simply reducing the oxygen content of the culture environment from the supraphysiological tension commonly employed (air) to a more physiological level can result in considerable lifespan extension of human fibroblasts (18-21). Moreover, young cells are more resistant to increased oxygen tension than old cells (19) and lifespan extension is 2-3-fold greater in mouse cells than human cells, consistent with lower ROS defences of mice compared to humans (22). Conversely, increasing the oxygen content from 20% to 40% rapidly rendered young fibroblasts senescent and gene expression changed accordingly (23). One interesting effect of hypoxic culture is the induction of telomerase, at least in vascular smooth muscle cells (24). Whether T cells behave similarly and whether they are more sensitive to oxidative damage, has not yet been reported, with the exception of one study on primary proliferation, cytokine secretion and Ig production *In vitro* where benefits of lower oxygen tension were observed (25). What is known is that oxidative stress does suppress transcription factor activities involved in proliferation (AP-1, NF-AT) in quiescent human T cells, which would be expected to compromise their proliferative capacity (26). Mortality and metabolic changes caused by oxidative stress are also more severe in lymphocytes from elderly humans compared to young, although this is not associated with decreased levels of antioxidant enzymes in old lymphocytes (27). Other possible manipulations which have been reported to extend the lifespan of fibroblasts, but which have not been tested on T cells, include using hydrocortisone, carnosine (but see below) anti-sense oligonucleotides for p53 and Rb, high albumin concentrations, additional growth factors and other hormones (reviewed in ref. (28)). Optimisation of culture conditions using capillary bed culture cartridges may also better mimic the *In vivo* environment and lead to extended lifespans, but this has also not yet been demonstrated (29). Two TCC, one from a young donor and one from an old SENIEUR donor (30), grown in serum-free medium, displayed age-associated increases in oxidative damage to DNA as assessed in modified Comet assays (31). Carnosine added to the culture medium did not decrease this, although for one clone (from the young donor) it did result in an extension of lifespan from 60 to 80 PD (31). These preliminary results therefore suggest that if carnosine can extend lifespan of human TCC in culture, it is unlikely that this is associated with its antioxidant effects.

Most human TCC are generated from cells obtained from peripheral blood, but such recirculating cells may not be truly representative of the T cell pool. The major lymphoid organs from which T cells can be obtained are skin and gut. T cells infiltrating the skin in various disease states can be cultured *In vitro* using the same techniques as employed for peripheral cells, and these have also been found to have limited lifespans (eg. ref. (32)). However, skin-infiltrating cells cultured in the presence of IL 4 in addition to IL 2 but in the absence of antigen presenting cells (which were, unusually, found to have a negative effect on cell growth in this system) have been reported to grow apparently indefinitely (33). These cells were found not to harbor HTLV-1 (although it cannot be formally excluded that some other, thus far unidentified, virus is involved). These long-lived T cells manifested various chromosomal abnormalities at different frequencies

(34). Generation of these lines was reproducible in different donors with different diseases, suggesting that isolation of apparently immortal cells under these conditions was not a rare event. While some of the donors were cancer patients, perhaps displaying generalized genetic instability and chromosomal fragility, the majority were atopic dermatitis patients (not known to fall into this category). Moreover, one T cell line was established from a skin nickel patch test which retained a normal karyotype up to 300 PD (way beyond the Hayflick limit) and acquired an abnormal karyotype thereafter (K. Kaltoft, unpublished). Even such T cells showed decreasing levels of CD28 expression with increasing age, but they retained telomerase expression (35). There is independent evidence that T cells in the skin are markedly different in their behavior to blood-borne cells, including lack of ability to respond to standard activating stimuli (36). Therefore, depending on the source of cells and the culture conditions, normal T cells may be able to proliferate indefinitely. Why this should not be the case for the majority of the peripheral T cells cultured in many different laboratories under similar conditions is not clear presently.

5.2. Does telomere attrition contribute to the replicative senescence of normal T cells?

Loss of telomeric DNA, and gradual shortening of telomeres, has been proposed to result, after a certain number of cell divisions, in the inability of cells to divide again in culture, ie. replicative senescence (37). Such findings seem to be applicable in human, where telomeres are quite short, but not in (laboratory) rodents, where telomeres are very long. This may reflect a major difference between long-lived animals, which maintain their cells for long periods in a good state of repair, and short-lived animals which do not have so many defences. Thus, rodent cells may stop proliferating with long telomeres because of other reasons, whereas human cells stop replicating because of short telomeres, as argued repeatedly by Shay & Wright (38). This explains older data that embryonic mouse cells may proliferate for a very extended time if provided with appropriate culture conditions (39), because their telomeres are so long. Recently, two instances of very extended rat cell culture have been recorded, one oligodendrocytes and one Schwann cells. However, "indefinite" growth is always hard to pin down: in these two reports, despite the title "Lack of replicative senescence...", the Schwann cells were taken to 75 PD (well within the normal range even of some human cells) whereas for the oligodendrocytes, no PD data were given (40,41). Nonetheless, these publications are being cited as evidence for the ability of normal somatic cells to divide indefinitely in culture without experiencing senescence (42). This latter paper also even cites a publication by Romanov *et al*. (43) entitled “Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes”. However, this interesting paper emphatically does not support the idea of indefinite growth under certain conditions in culture; quite the opposite. It describes a crisis point in the cell lines associated with telomere shortening and genetic instability, resulting in a total proliferative potential of 30 – 60 PD and “no immortalized variants have yet been detected”. It therefore remains the case that human cell lines in culture, and even untransformed rodent cell lines as well, possess finite proliferative potential *In vitro* – there is still no convincing evidence against this.

As illustrated above (43), in human, critically short telomeres may cause eventual chromosome instability and formation of dicentric chromosomes (44), possibly caused by essential components of the DNA repair machinery being compromised (45). DNA damage repair programs may also be triggered, for example via p53 and usually but not always (43) resulting in growth arrest and/or apoptosis (46). Other, more subtle effects on the regulation of gene expression depending on telomere lengths have now also been demonstrated in human cells (47). Loss of telomeric repeats is not only an *In vitro* phenomenon as it is also observed in human cells *In vivo* (48) and may have pathological relevance, for example, in impaired wound healing (49) and in aging of cells such as those in the kidney (50). In human monoclonal fibroblast cultures, telomere length was found to be reduced with culture age and was directly proportional to the remaining replicative capacity of the clone (51). Telomere shortening might therefore act as a mechanism counting the number of cell divisions that a cell population has experienced. However, recent studies have shown that telomere lengths in fibroblasts ex vivo are not decreased in the elderly, up to centenarian age, although the same fibroblasts did show telomere length decreases upon *In vitro* culture (52). This is in agreement with more recent studies reassessing the association between longevity of fibroblast cultures *In vitro* and age of the donor (12,13,53,54). There is also no association between telomere lengths and longevity in different strains of laboratory mice (generally with very long telomeres) and wild mice (generally without such extended telomeres) (55). Nonetheless, in humans, telomere length in blood cells ex vivo was shown to be related to donor age (56), even in the same individuals mentioned above, whose fibroblasts failed to show a decrease, although there is a great deal of inter-individual variation (52). The association between this shortening of telomeric repeats and age of the donor is not confounded by differences in white blood cell count (57) and also appears to apply to several different tissue types (58). Telomere attrition occurs more rapidly in premature aging syndromes, eg. Hutchinson-Gilford progeria (59) or trisomy-21 (60). The overall rate of telomere shortening in leukocytes is not necessarily linear with age (61-63), and the same seems to apply to non-lymphoid cells as well (64). This may partly reflect differential activation-induced upregulation of telomerase activity (65), but not always, ie. not in children (63), or genetically regulated differences between donors in telomerase activity which may further complicate cross-sectional comparisons between donors of different ages (66). Most telomere shortening occurs early in life in humans; thus, according to one report, during the first year loss is very rapid, then stabilizes over 8 decades at a 30-fold lower rate, but with lymphocytes showing a more rapid loss than granulocytes (67). It was suggested that granulocyte telomere loss reflects stem cell aging, whereas the additional attrition in lymphocytes represents their clonal expansion, which was greater in the “memory”

subset, especially the CD4 subset early (ie. from birth to 4 yr) in life (67).

In mice at least, a genetic locus controlling differences in telomere lengths has been identified (68). Possibly for the reasons mentioned above, some studies have not found an association between donor age and lymphocyte average telomere length (69). Interestingly, however, that study did show telomere attrition in the white cells of breast cancer patients, where chronic lymphocyte stimulation by tumor may have been taking place. In addition, as suggested by Bohr and colleagues, processes other than cell division, such as DNA repair, which decreases with age, may contribute to telomere shortening (70). This may also be compromised in cancer patients. Moreover, an accumulation of single-stranded breaks caused by oxidative stress is another major cause of telomere shortening, independent of the number of cell divisions undergone by the cells (71). Overexpression of the regulatory protein TRF2 under circumstances not yet defined, can also accelerate telomere shortening (72). There is also a number of other telomerase-interacting proteins, the roles of which are not yet worked out (73). One such is a ribonucleoprotein component, La, which physically associates with telomerase RNA and causes gradual telomere shortening in proliferating cells (74). There are clearly negative regulators of telomerase expression (75), some of which may correspond to previously reported "tumor suppressors". This may contribute to providing an explanation of the variability in lifespans of cell lines under different culture conditions, eg. the decrease in lifespan caused by severe reduction in total cell population size, which may be due to critical shortening of only a small number of telomeres (76), possibly only chromosomes 2 and 11 in mice (77). Intriguingly, even homologous chromosomes may have different telomere lengths, with these differences being stable *In vivo* for some time, but disappearing on T cell culture but not fibroblast culture (78), suggesting that telomerase activation obscures such differences. Therefore, it must also be borne in mind that the different human chromosomes may not all experience telomere shortening at the same rate and that averaging all chromosomes´ telomeres (as usually reported in the literature thus far) may not provide a true picture of critical chromosomal damage (79). Nonetheless, this group did report that fibroblast replicative senescence seems to correlate with the average mean telomere length, rather than with critical short lengths of one or a few particular chromosomes (80). In culture, lymphocytes from normal donors show an estimated telomeric loss rate of 120 bp/cell doubling, comparable to that seen in other somatic cells (60). Weng *et al*. (81) reported that $CD4^+$ memory-phenotype cells showed consistently shorter telomeres than naive-phenotype cells. Interestingly, this difference in telomere length between naive- and memory-phenotype cells was the same whether the cells were isolated from young or old donors. This must mean either that it is the T cell precursor rather than the mature T cell which has "aged", as defined by decrease of telomere length, or that naive and memory cells both divide at the same rate *In vivo*. Weng *et al*. also showed that telomere lengths decreased during autocrine expansion of both naive- and memory-phenotype cells, and that the latter completed less PD than the former. The authors concluded from this that the replicative potential of memory cells was less than that of naive cells and that this might be related to telomere shortening. However, what they actually measured in their experiments was autocrine proliferative capacity, not replicative potential. Autocrine proliferative capacity relies upon the stimulation of growth factor secretion, upregulation of the growth factor receptor and correct signal transduction. As we have shown for culture aged T cell clones (82), exogenous factor-dependent growth of the cells (ie. replicative potential) is retained for a period far longer than the capacity to secrete interleukin 2 (autocrine proliferative potential). It is therefore unlikely that the cessation of growth noted by Weng *et al*., which was only 10 PD for memory cells and 20 PD for naive cells, reflects shortened telomere-triggered blockade of replicative potential. Whether shortened telomeres have anything to do with the blockade of autocrine proliferation which Weng *et al*. actually measured is currently unknown.

Early data showed that telomere lengths in sperm DNA do not decrease with increasing age of the donor, suggesting that a mechanism for maintaining telomere length may be active in germ cells but not somatic cells (59,83). Indeed, activity of telomerase, an enzyme responsible for maintaining telomere length in unicellular eukaryotes, had, in fact, been previously demonstrated in immortalized human cell lines and tumor cells, but not in normal somatic cells with the possible exception of cells of animals which do not reach a developmentally controlled maximum body size, eg. some fish (84). Thus, certain animals which do not downregulate telomerase apparently manifest a permanent growth phase and little or no senescence (84,85). Also, using more sensitive assays, it soon became clear that a strict dichotomy between tumor and somatic cells was not as straightforward as had been previously thought (86). For example, Hiyama *et al*. (65) showed that telomerase activity is detectable at very low levels in normal human T and B cells and that it increases greatly after mitogenic stimulation. Even cells like myocytes may show telomerase activity using appropriate assays (87), and fibroblasts, thought not to express telomerase under any circumstances, may do so after bleomycin injury (at least in rats) (88). The level of telomerase activity in freshly isolated CD4+ cells from normal donors is said not to change with age (89). However, optimal telomerase induction requires optimal stimulation via CD3 and CD28 (90), so age-associated defects in CD28 may contribute to suboptimal telomerase induction. Telomerase activity is regulated in the G1 phase of the cell cycle in normal human T cells, as indicated by the finding that rapamycin (which blocks TCR-signal transduction and cdk2 activation) but not hydroxyurea (an S-phase inhibitor) prevents telomerase induction (91). Human telomerase consists of two essential components, the reverse transcriptase (hTERT) and RNA template (hTER) components, regulated at the transcriptional and post-transcriptional levels (92). The oncogene c-myc can activate h-TERT transcription (93,94), in cooperation with Sp1 (95) and counterbalanced by Mad (96,97). Telomerase is upregulated by T cells within 24 h., increases up to 72 h.

and then decreases again after 96 h if the cells are not restimulated (98). Telomere lengths do not decrease during this period, but possibly because telomerase is downregulated again, decreases in telomere length are not prevented during long-term culture, although they may be prevented initially (99). Moreover, some clonal populations of CD4 cells may survive this decline and show stabilisation of telomere length over a long period (100). The rare occurrence of such cells might explain sporadic reports of the emergence of very long-lived human T cell lines (35,101). Telomerase positivity may also in some way contribute to prevention of apoptosis of these cells (102,103); should those results also apply to T cells, since it clearly has functions other than mere telomere elongation (104). In CD8 cells, Monteiro *et al*. (105) reported that telomere lengths in the CD28-negative population were shorter than in the CD28+ population, and that *In vitro* clonal expansion of CD8 cells is associated with telomere shortening. Pan *et al*. reported that telomerase was upregulated in normal PBMC after their stimulation with PHA (106). Continued culture of these cells with IL 2 resulted in eventual cessation of growth after ca. 23 PD, when telomerase activity was no longer found. Confusingly, the authors interpreted this as demonstrating downregulation of telomerase at senescence. However, they presented no evidence that their T cells were in fact senescent; they had ceased proliferation because they were not restimulated via the TCR. Thus, they merely demonstrated that telomerase expression correlates with proliferation of the T cells. Consistent with this interpretation, they also mentioned a T cell clone which they did maintain by intermittent restimulation, and here they showed the presence of telomerase in stimulated cells and its absence in "resting" cells (106). Thus, the increase in telomerase activity after stimulation is transient but may be upregulated in certain clones at least upon restimulation. In uncloned CD4 cells intermittently stimulated with CD3 and CD28 mAb, the initial strong telomerase upregulation was found to be weaker at each restimulation until it was no longer upregulated at all; at this point, telomere lengths of the cultured cells decreased (89). Similarly, in CD8+ cells, it has been found that the degree to which telomerase activity is upregulated after repeated stimulation of antigen-specific T cells was inversely related to the length of time that they had been in culture (107). Whether the situation is comparable *In vivo* is open to question. In acute infectious (AIM) it was found that CD8 cells upregulate telomerase and maintain or even increase telomere lengths until resolution of infection (108), so other mechanisms may be involved here, which are lacking *In vitro* (similar increases in telomere length, associated with enhanced telomerase expression, have also been observed in germinal center B cells (109), and these fail to develop in telomerase-knockout mice (110)). However, CD4+ memory cells specific for common recall antigens in humans have been shown indeed to possess shorter telomeres even that the general population of CD45RO+ cells (111). This may also be the case even for CD8 cells in AIM, because examination of the antigen-specific CD8 cells at a later time point also showed that despite earlier telomere length maintenance, short telomeres were found in these patients a year or more later (112). Thus, the conditions for up and downregulation of telomerase and the maintenance of an effective level of telomerase in T cells are not clearly defined so far, but may be critical to the fate of the responding T cells and thence to the T cell-mediated immune response itself. In certain cases of apparent immortalization of abnormal (but non-tumorigenic) human T cells, telomerase was found not to be downregulated; its high level of expression, as found in the majority of tumors, may be responsible for the extended longevity of these T cells (35). In some way, these cultures perhaps parallel the *In vivo* situation in AIM more closely.

Telomerase may not be the only factor determining telomere length and cell survival, although specifically inhibiting telomerase can cause growth arrest of immortalized cells (113). Strahl & Blackburn reported (114) that inhibitors of retroviral reverse transcriptase (telomerase itself is a specialized cellular reverse transcriptase) could cause progressive telomere shortening of immortalized human lymphoid cell lines *In vitro*. Telomerase activity was present in these lines and its activity was blocked by the agents tested. Telomeres in the blocked lines eventually stabilized and remained short. It was, however, suggested that telomere lengths in lymphoid cells lines (which were unstable even in the absence of inhibitors) are determined both by telomerase and telomerase-independent mechanisms, as also suggested by others (115), and now being dissected in detail (116). Telomerase-negative lines may maintain telomere lengths by alternative mechanisms (117), one of which possibly involves the NBS1 (Nijmegen Breakage Syndrome) protein, which is involved in DNA repair (118). Moreover, although such lines may not in fact be immortal (but very long-lived), they do maintain stable short telomere length, in the absence of telomerase activity (119). On the other hand, certain tissues appear to undergo senescence correlated to decreased telomerase activity despite their ability to retain telomere lengths (120) and other cells show telomere shortening despite telomerase expression (121). This may have relevance to pathology, eg. in Fanconi anemia, severity of disease correlates with progressively shortening telomeres, despite the presence of high levels of telomerase in these cells (122). Strikingly, HIV patients treated with reverse transcriptase inhibitors clearly showed suppression of telomerase activity, but nonetheless this did not result in accelerated telomere attrition *In vivo* (123).

However, that both telomerase activity and telomere length can be absolutely critical in determining longevity of cells in culture was unequivocally demonstrated some years ago by two independent laboratories (124,125) and indirectly confirmed by a third (126). Since then, many others have duplicated these results with various different cell types. Using telomerase-negative cell types (fibroblasts and keratinocytes), it was found that transfection of the gene for the catalytic component of telomerase extended life-span way beyond the point at which untransfected lines senesced. This life-span extension was not accompanied by acquisition of abnormal karyotype or surface markers, but was associated with telomere length extension and inhibited expression of the senescence-associated ß-galactosidase marker. Such lines

were not tumorigenic and still responded to ionising radiation with p53 and p21 unpregulation, ie. the G(1) cell cycle checkpoint was intact (127). Other studies demonstrating similar effects of hTERT transfection into endothelial cells have also been reported (128). This was the first evidence for a causal relationship between telomere shortening and cellular senescence *In vitro*. Although these experiments were initially criticized on the basis that a proper negative vector control replacing the telomerase-encoding portion with an irrelevant gene should have been used (129), the large number of confirmatory experiments since then has removed any possible doubt. Possible explanations for previous similar results in experiments not using telomerase (129) may be that these involved c-myc over-expression, which is itself able to induce telomerase (93,94), and which is counterbalanced by Mad (96). Additionally, it is now clear that the expression of catalytically active telomerase is not of itself necessarily sufficient for immortalization, as shown for example by the senescence-inducing effect of Ha-Ras transfected into telomerase-immortalised human lung fibroblasts (130), or possibly in cells expressing p16*ink* (131). Nonetheless, the presence of p16 in T cells may not absolutely preclude continued proliferation of telomerase+ cells, provided that other mechanisms which over-ride p16 activity are present. These may involve over-expression of the targets for negative mitotic regulatory activity of p16, eg. cyclin D2, cyclin E, cdk4 and c-myc (101) or a human homologue of the mouse gene bmi-1 which binds and inhibits p16 (132). One recent report of hTERT immortalisation of two human CD8 T cells clones (133) but another indicating lack of immortalisation despite telomerase expression and telomere length maintenance also in human CD8 cells (134) may possibly be explained by different p16 status. Other failures to immortalize human cells with hTERT may also be explicable in this way (134,135). Another recent study transfecting naive CD8 cells also indicates that hTERT expression can markedly extend the lifespan of at least some CD8 clones (108 PD compared to > 170 PD) (136). Thus, some T cells of both CD4 and CD8 subsets respond to hTERT with marked longevity prolongation, but the reason why others do not remains to be clarified. The same kind of so-far unexplained heterogeneous immortalization effects are also seen even in the same fibroblast cell line (137).

5.3. Longevity of naive and memory cells

As discussed above (section 5.1), memory-phenotype cells are generally not immortal *In vitro*, showing a variable but finite lifespan. An important question raised therefore concerns the longevity of naive and memory cells *In vivo*. There is some evidence that memory cells are not quiescent long-lived cells, but represent T cell clones in a constant state of activation. This may need to maintained *In vivo* by the TCGF IL 15, at least for murine CD8+ memory cells, whereas production of IL 2 inhibits them (138). Indeed, IL 15 may also be required at the initiation of T cell responses (where IL 2 was again found to be inhibitory), as well as for the maintenance of memory, whereas IL 2 is required only for clonal expansion (139). Other gamma-chain cytokines such as IL 7 have also been implicated in maintaining the CD8 memory pool, but not in antigen-driven clonal expansion (140). In the absence of IL 2-deletion, memory cells may be very stable in the animal; thus, when the thymus fails to produce any fresh naive cells, the animal retains memory and naive cells, but when the thymus produces new cells, it is the resident naive cells which are deleted to “make room”, whereas the memory cells remain present (141). However, when a new infection stimulates the T cells, and presumably causes IL 2 release, it may be the memory cells which are deleted (by the IL 2) to “make room” (142). It seems increasingly necessary to bear in mind what appear more and more to be fundamental differences between CD4 and CD8 lineage cells when considering development and especially longevity of immunological memory. It is obviously also important to bear in mind that what is seen in the periphery as a CD45RO+ memory cell may represent only the CCR7-negative “effector” memory cell and not the stem cell-like CD45RO+, CCR7+ “central” memory cells, capable of self-renewal and contribution of new effector cells to the memory pool (143). Because the central memory cells home to secondary lymphoid tissues, whereas the effectors home to inflamed, peripheral tissues (144), monitoring peripheral blood would be expected to pick up only the latter.

A method for measuring intermitotic time to investigate longevity of CD45RA+ and RO+ cells in cancer patients following radiotherapy relies on radiation-induced dicentric chromosomal lesions which can be visualized cytogenetically. Small numbers of $CD45RA^+$ cells with these lesions were found up to 10 years after irradiation, consistent with the belief that naive T cells can be very long lived. However, CD45RO+ cells with such lesions had all disappeared by one year, suggesting that they had all attempted unsuccessfully to divide, consistent with memory cells being in cycle (145). A later study extended these data to conclude that proliferation rates of naive cells were 8x lower than proliferation rates of memory cells. They estimated that, on average, naive cells divided once every 3.5 years, whereas memory cells divided every 22 weeks (146). However, as these authors themselves pointed out, there are some problems with these data, viz. they were dealing with cancer patients, whose T cells and immune status were not normal; irradiation can have indirect effects like induction of lymphopenia which further disrupts the system; possibly some cells with dicentric lesions can nonetheless divide; and the proportion of cells dying without attempting to divide is unknown. All these factors could lead to underestimates of memory cell longevity (147). Another approach to measuring longevity of CD4+ CD45RA-positive or negative cells also relied on therapeutic radiation-induced changes, in this case mutations in the TCR leading to loss of CD3 expression. Here, it was concluded that the half life of RA+ or RA-negative cells was the same, ie. 2 -3 years (148). These data on naive cells therefore agree quite well with those of Michie *et al*. The reason for the difference in longevity of memory cells is not clear, except of course that loss of TCR would render the cells insensitive to (re)activation, and without such signals they might die more slowly (by being insensitive to activation-induced cell death).

A direct approach to whether memory cells continue to cycle *In vivo* has been taken using transgenic mice (149). Naive cells deliberately activated with specific antigen were transferred into athymic hosts in the absence of antigen and found to continue to cycle slowly for extended periods. Naive cells, on the other hand, did not begin to proliferate when transferred into the these recipients. The mechanism for the maintenance of slow cycling of the memory cells is unknown. It may be that unlike naive cells, memory cells do not require antigen to survive and proliferate, but only the self MHC molecule (ie. have a lower functional activation threshold) (150). Others have reported that the presence of class I molecules may not be necessary for continued CD8 memory cell turnover, as this occurs even in class I-deficient mice (151). Other newly developed methods for estimating T cell longevity, using, eg. a non-radioactive endogenous labeling technique (ie. the incorporation of ^{2}H-glucose via de novo nucleotide synthesis into newly-synthesized DNA of dividing cells), are being applied. Estimates from these methods vary widely, eg. the latter concluded that CD4 and CD8 cells had half-lives of only 87 and 77 days respectively (152). A consensus is thus difficult to reach. However, it is clear from these experiments that proliferative senescence could play a role in the eventual loss of the memory cells. The above data in man and mouse have to be reconciled with earlier results in the mouse showing that after thymectomy it is the naive population which disappears rapidly, whereas the memory cells are long-lived (153). The reason for this apparent discrepancy is unknown.

According to our *In vitro* data on T cell longevity, where TCC survive on average 35 PD and maximally 80 PD, this would put the life expectancy of a memory cell at 35 x 22 = 770 wk = 15 years average; 80 x 22 = 176 wk = 34 years maximum (without taking the primary immune response into account, for which the number of cell divisions required can only be guessed at but where the initial upregulation of telomerase might offset the effects of the first 10 or so divisions (154)). What might be the source of stimulation of the memory cells, which need to persist in the absence of antigen? Several groups have argued that the solution lies in the hypothesis that antigen does persist somehow. This is certainly true for many viruses, but it is unclear for which other antigens it may apply. For viral infection, there is clear evidence that memory T cells are indeed maintained in a constant activated state even for very long periods. Thus, Rehermann *et al.* studied patients up to 23 years after clinical and serologic resolution of HBV infection and still found evidence for recently activated HBV-specific CTL (155). Similarly, although antibody was undetectable 18-20 years after HCV infection, the same group could still find IFN-γ-producing helper cells (156). This might well eventually allow enough time for "clonal exhaustion" of the originally responding T cell clones, in the absence of the generation of de novo responses (see calculation above). Data from other chronic infections such as HIV may be consistent with the scenario of clonal expansion leading to clonal exhaustion and lack of replacement by new thymic emigrants eventually resulting in diminution of the repertoire and loss of anti-HIV responses (157,158). CD8 cells of the same CD28-negative phenotype as seen in HIV-uninfected senescent cultures and with similarly short telomeres and inability to proliferate have also been described in young persons with AIDS, leading to the suggestion that replicative senescence of virus-specific T cell clones *In vivo* might contribute to disease progression (159,160). These cells are truly HIV-specific as revealed by staining with MHC/peptide tetramers (161). Evidence suggests that the CD28-negative cells are indeed derived from originally CD28+ cells (162). A marked decrease in telomere length has also been recorded in CD4, CD8 and B cells from HIV-infected patients with advanced immunodeficiency, supporting the notion of a high turnover of these cells and suggesting that replicative senescence may be involved in the final immunosuppression of these patients (163). This has been confirmed by others, showing shortest telomere lengths in patients with a CD4 cell count < 200 (164). There is also an increase in $CD4^{+}$ CD28-negative cells in HIV infection, albeit not so marked as in the CD8 subset (165). Indeed, disease progression in AIDS is reported to be marked by an accumulation of CD28-negative cells unable to secrete IL 2, whereas long-term non-progressor patients maintain CD28 levels and IL 2 secretion capacity (166). The rate of destruction of HIV-infected cells in young and old patients seems similar, leading to the suggestion that the elderly cannot replace CD4 cells as rapidly as the young (167). It has been found that T cell responses in HIV patients are characterized by severe TCRVB biases and clonal expansions in CD4 cells, and that such responses are exaggerated with disease progression (168). Despite this, others have found no evidence for increased CD4 turnover in HIV infection on the basis of lack of truncation of telomere length in CD4 cells during progressive disease, although this was clearly confirmed in CD8 cells (169). However, virally infected cells may conceivably express dysregulated telomerase. Others have measured increased rates of loss on follow-up of up to 14 years, depending on progressor status, ie. 140 bp/yr in CD8 cells and 100 bp/yr in CD4 cells in slow progressors, compared with 240 and 160 bp/yr in CD4 and CD8 respectively in fast-progressors (170). Independent evidence for enhanced cycling of CD4 cells in HIV derives from measurements of mutations in the hprt locus, which showed that mutation rates in the CD8 and CD4 cells were similarly high (171), consistent with an increased division rate in both subsets. The cytokine secretion profile of mutant CD4 clones (from healthy or HIV patients) was predominantly Th2-like, whereas the CD8 mutants had the same pattern as wild-type.

Such findings may not be limited to the extreme and rare cases of infection with HIV. Alterations of immune parameters associated with infection by a common Herpes virus, CMV, share some of these senescent characteristics, particularly in the increased numbers of CD8+ CD28-negative cells. Moreover, the increase in CD4+ CD28-negative cells was found in one study to be determined solely by CMV seropositive or negative status, independently of age, ie. only seropositive donors possessed significant numbers of these cells, albeit twice as many in the seropositive old as in the seropositive young

(172). This same group also examined RA patients and found that CD4+ CD28-negative CD57+ cells were only expanded in the 28 patients who were seropositive for CMV but not in the 17 seronegative patients, who were otherwise comparable (173). The most recent point of the OCTO longitudinal study also found an association between CMV-positivity and increased CD8+ CD57+ CD28-negative T cells, a CD4/8 ratio inversion, and decreased longevity (174). On the other hand, another report documented that in multivariate analysis, the age-associated decrease of CD28+ CD57-negative CD8 cells was dependent only on age and not on CMV carrier status, although the expansion of CD28-negative CD57+ CD8 cells was associated with both age and CMV (175). Such clonal expansions may be a general consequence of inflammatory processes of various kinds (176), and accumulate due to a decreased susceptibility to apoptosis *In vivo* (177). These cells may have an "anergic" phenotype and persist without effector function, thus contributing to compromised immune responsiveness (178). Such results are consistent with earlier data showing that prevention of this apoptosis defect restored immune responses (179), and findings that low-dose cyclophosphamide induced apoptosis of thymocytes in aged mice, concomitant with enhancement of immune capacity (180).

For non-viral antigens, and thymus-independent regeneration of T cells in general, it is probable that antigen-driven peripheral expansion commonly occurs (181), implying that a finite proliferative lifespan of the T cells would be of critical import for the functional integrity of the immune system. Experimental data have implicated a type of TCR cross-reactivity responsible for maintaining homeostasis of T cells, eg. in the case of CD4 cells via class II molecules regardless of their peptide loading (182). For CD8 cells, IL 15 produced by a variety of (non-immunological) tissues may be the critical maintenance cytokine (138). Therefore, this may represent a general mechanism for increasing T cell senescence with age even in the absence of pathogen stimulation.

5.4. Activation-induced cell death and aging

Differentation of human $CD4^+$ T cells *In vivo* and *In vivo* can be followed by monitoring changes in the expression of different isoforms of CD45 as noted above (see 3.2, 4.1, 5.3) and by using the unique mAb PD7/26 specific for the CD45RB isoform. Salmon *et al* (183) reported that whereas CD45RA expression is lost rapidly after activation of naive cells *In vitro*, loss of RB expression is gradual, occurring over many cell cycles, and reciprocating the increase in RO expression. The progressive shift from RB^{hi+} to RB^{lo+} is paralleled by gradual loss of bcl-2 (which protects against apoptosis) and acquisition of fas (which can mediate apoptosis), as well as gradual loss of ability to secrete enough IL 2 to maintain autocrine proliferation (whereas IL 4 secretion remains intact). This eventually results in their becoming dependent on exogenous IL 2 not only for growth but also for their survival, because without enough IL 2 they undergo apoptosis. Longevity extension for T cell clones might therefore be achievable by upregulation of bcl-2 or bcl-2 family-member bcl-x(L). It may be bcl-x(L) which is critical here, because apoptosis prevention in resting T cells cultured on fibroblast monolayers is associated with maintenance of bcl-x(L) expression but not of bcl-2 expression (184). On the other hand, accumulation of bcl-2 has also been noted in senescent cultures of CD8 cells (185), which are resistant to apoptosis and show reduced levels of caspase 3 (186). However, the down-side of this could be that suppression of apoptosis by bcl-2 or bcl-x(L) would result in enhanced tumorigenesis, because radiation-induced mutagenesis is increased under these circumstances, consistent with the original isolation of bcl-2 as an oncogene (187). There are also experiments suggesting that under certain circumstances, CD3/CD28-stimulated CD8 cells may be susceptible to apoptosis (not resistant in the way that senescent CD8 cells have been reported to be), despite their expression of bcl-x(L) (188). However, the central role of bcl-x(L) and bcl-2 in regulating all apoptotic pathways has been questioned (189). Two independent pathways have been described; one of which involves caspase 9 and is sensitive to regulation by bcl-2. The other pathway is activated through CD95 ligation and requires caspase 8 generation and is able to bypass the regulatory effects of the bcl-2 family. It is this latter pathway which is activated in peripheral T cells following binding of CD95-L (190). Therefore, the effect of age on DISC generation of the caspases may well help elucidate age-related changes in apoptotic sensitivity.

5.4.1. Apoptosis of T cells in aging

Apoptosis or programmed cell death plays an important role in embryogenesis, metamorphosis, and cellular homeostasis. In the immune system, apoptosis appears to play a crucial role in selection of the T cell repertoire, deletion of self-reactive lymphocytes, deletion of peripheral effector T cells following termination of an immune response, and in the cytotoxicity mediated by CTL and NK cells (191,192). There are two major signaling pathways of apoptosis, the death receptor pathway and the mitochondrial pathway (193,194). The mitochondrial pathway of apoptosis in aging has not yet been explored. In the death receptor signaling pathway, the signal is provided by the interaction between the ligand and death receptor. The death receptors belong to the large tumor necrosis factor receptor superfamily and the ligands to the tumor necrosis factor/nerve growth factor family. Following ligation of death receptor by ligand, the death receptor oligomerizes and recruits adapter molecules and initiator caspases to a complex, the death-inducing signaling complex (DISC), which results in the activation of initiator caspase. Initiator caspase then cleaves effector caspases (caspase-3,-6, and –7), which in turn cleave a number of target substrates resulting in morphologic and biochemical characteristics of apoptosis. The process of apoptosis is tightly regulated by a number of gene products that promote or block apoptosis at different stages. The most extensively studied and perhaps most important are Bcl-2 family proteins (195). Bcl-2 and Bcl-XL are anti-apoptotic, whereas Bax is proapoptotic. These molecules either homodimerize or heterodimerize with molecules of opposing function (i.e. Bax can heterodimerize with Bcl-2 or Bcl-xL). The net influence on apoptosis appears to be dependent upon the relative concentrations of these

molecules in the form of heterodimers. Athough the regulatory role of Bcl-2 family proteins in the mitochondrial pathway of apoptosis is well-established (194), their role in the regulation of death receptor signaling is controversial (189) and may be cell type specific.

Once a cellular immune response has terminated, most of the cells are removed by a process termed activation-induced cell death (AICD). AICD appears to be primarily mediated by Fas (CD95)-Fas ligand (CD95L) interactions (192). However, other molecules may also be involved in this process; thus, the TNF/TNFR system appears to play a role preferentially in the apoptosis of CD8+ T cells (196), although more recent data suggest that TNF-TNFR is involved in apoptosis of both CD4+ and CD8+ T cells. On the practical side, there are basic differences for *In vitro* induction of apoptosis during AICD, CD95-CD95L, and TNF-TNFR signaling. In AICD, pre-activated T cells are re-activated with the same stimulus whereas in the CD95-CD95L system activated cells are re-activated with anti-CD95 monoclonal antibodies or soluble CD95L, and in the TNF-TNFR system, activated T cells are exposed to TNF. In the AICD system, resistance or sensitivity depends upon proper engagement of TCRs by specific antigen bound to MHC molecule, antigen concentration, and co-stimulatory signals (197). It has been suggested that AICD could be inhibited in memory T cells activated *In vivo* by a foreign antigen, but may become operative once antigen has been cleared. These observations are particularly important in the context of apoptosis in aging and perhaps explain some apparently contradictory observations. Differences between *In vitro* senescence and *In vivo* senescence also need to be kept in mind. *In vitro* senescence during repeated stimulation in culture may select an apoptosis-resistant population. Additionally, apoptosis control might be different among various lymphoid compartments (i.e. peripheral blood vs spleen, vs lymph nodes). Finally, other factors including nutritional, socioeconomic status of the aged and the use of medications and nutritional and vitamin supplements also have to be considered.

5.4.1.1. Apoptosis of T cells in aged Mice

There is some controversy regarding the proportions and numbers of CD4+ and/or CD8+ T cells in aged mice, especially with regard to CD4+ T cells. A decreased proportion of CD4+ T cells in lymph nodes or peripheral blood from aged mice (198,199), no change with age in CD4+ T cells in spleen or lymph nodes (200,201) and increase with age (202). Similarly, CD8+ T cells in aged mice are increased, decreased or unchanged (203,204). Hsu *et al*. observed increased CD8+ and decreased CD4+ T cells resulting in reversal of CD4+/CD8+ T cell ratio (205). The reason for such diverse observations is unclear. There appears to be an agreement, however, that memory CD4+ and CD8+ T cell subsets are increased in aged mice.

In mice, apoptosis has been examined predominantly for AICD and no studies of TNF-TNFR apoptosis has been published. Zhou *et al*. generated *fas* transgenic mice and compared immunological status of young and old transgenic mice with wild-type littermates (179). They found that Fas expression and Fas-induced apoptosis was decreased in old wild type mice, but not in old transgenic mice. In old wild type mice, there was an increase in CD44+ Fas- cells, decreased IL-2 production and increased IL-4 and IL-10 production. In the transgenic mice these changes were not observed. Furthermore, age-related thymus involution was prevented in the Fas-transgenic mice. However, the life-span of the transgenic mice was not increased. Spaulding *et al*. reported that T cell apoptosis induced by irradiation, heat shock or CD3-stimulation was reduced in old compared to young mice (206). Polyak *et al*. also reported higher levels of *In vivo* and *In vitro* lymphocyte apoptosis after irradiation in young compared to old mice (207). More recently, Hsu *et al*. have reported decreased apoptosis in activated CD8+ cells as compared to CD4+ T cells from aged mice; however this difference was observed only at 96 hours of activation and no such difference was observed at 48 hours (205). In contrast, Chrest *et al*., using cells from lymph nodes from Balb/C mice, have shown an increased T cell apoptosis upon anti-CD3 engagement (208). Pahlavani and Vargas also reported increased activation-induced apoptosis in old Fischer 344 rats (209). Ex vivo T cells from very old mice have also been reported to be more not less susceptible to TCR-mediated AICD than those from young or old mice and expression of CD95 by CD4 cells of old mice infected with M. tuberculosis is not decreased (210,211). Telford and Miller also observed increased AICD in aged mice and not unexpectedly decreased apoptosis in CD8+ T cells after withdrawal of agonist (212). The reason for contradictory results could be any or a combination of several factors as outlined above.

5.4.1.2. T cell apoptosis in aged humans

In vivo, $CD4^+$ cells in old donors showed significantly decreased CD45RB expression (213). Salmon *et al* (183) studied the effect of *In vitro* replicative senescence on the expression of CD45RB isoforms. They reported that CD45RA (marker for naïve cells) expression was lost rapidly after activation of naive cells *In vitro*, loss of RB expression was gradual and occurred over many cell cycles, and there was a reciprocal increase in CD45RO (marker for memory cells) expression. The progressive shift from RB^{hi+} to RB^{lo+} was paralleled by gradual loss of bcl-2 and acquisition of Fas, as well as gradual loss of ability to secrete enough IL 2 to maintain autocrine proliferation (whereas IL 4 secretion remained intact). This eventually resulted in the cells becoming dependent on exogenous IL 2 not only for growth but also for their survival, because without enough IL 2 they undergo apoptosis. Longevity extension for T cell clones might therefore be achieved by upregulation of bcl-2 or bcl-2 family-member bcl-xL. It may be bcl-xL which is critical here, because protection from apoptosis in resting T cells cultured on fibroblast monolayers is associated with maintenance of bcl-xL expression and not of bcl-2 expression (184). It should be emphasized however, that any of these molecules serve an anti-apoptotic function. Aggarwal and Gupta (214) reported increased Bcl-2 and Bax but no increase in Bcl-xL in lymphocytes from aged humans. Effros (215) also observed increased bcl-2 *In vitro*

replicative senescent cultures of CD8 cells, which are resistant to apoptosis and show reduced levels of caspase 3 (186). There are also experiments suggesting that under certain circumstances, CD3/CD28-stimulated CD8 cells may be susceptible to apoptosis (not resistant in the way that senescent CD8 cells have been reported to be), despite their expression of bcl-xL (188). However, the central role of bcl-xL and bcl-2 in regulating death receptor apoptotic pathways has been questioned (189).

Studies of AICD and CD95-mediated cell death in aging humans have been performed either *In vivo* or *In vitro* replicative senescence. The results of these two types of studies are contrasting. *In vivo* studies show increased apoptosis in human T cell subsets, whereas *In vitro* replicative senescence studies show decreased apoptosis, especially in CD8+ T cells.

The proportion of CD95+ CD4+ and CD95+ CD8+ T cells are increased with age *In vivo* (214,216-219). Aggarwal and Gupta (214) reported increased expression of CD95 in both CD45RA+ naïve and CD45RO+ memory CD4+ and CD8+ T cells. Sinohara *et al* (216) also observed increased CD95+ memory CD4+ and CD8+ T cells in aged humans; however, they did not analyze the expression of CD95 in naïve T cell subsets. Aggarwal and Gupta (214) demonstrated that CD95 overexpression was at both protein and mRNA level. In contrast, a decreased proportion of CD95+ T cell subsets have been reported in very advanced (>90 years of age) aging (220-222). This discrepancy cannot be explained on the basis of different anti-CD95 used in different studies because Aggarwal and Gupta demonstrated upregulation at the gene level as well. The amount of soluble Fas in the blood of elderly donors is significantly increased as compared to young donors (223). Aggarwal *et al*. (224) have also demonstrated that neonatal T cell subsets express less CD95 as compared to young subjects. Therefore, it is likely that there is an age-dependent increase in the expression of CD95 and very advanced aged humans (<90 years or centenarians) are immunological privileged due to down regulation of CD95 (and perhaps CD95L and less apoptosis). Then one can argue whether the study of centenarians is a true reflection of effect of aging on the immune system and for that matter on other systems as well. It is also worthwhile to mention that Fas-mediated apoptosis correlates better with the expression of CD95L rather than CD95. Aggarwal and Gupta (214) reported increased expression of CD95L in both CD4+ and CD8+ T cells from aged humans. A number of investigators have observed increased AICD and anti-CD95-induced apoptosis in aging (214,219,225,226). Aggarwal and Gupta (214) reported increased anti-CD95-induced apoptosis in both CD4+ and CD8+ T cells and their naïve and memory subsets. However, significantly more apoptosis was observed in CD45RO+ memory subsets as compared to naïve CD45RA+ T cells in both young and aging subjects. Increased apoptosis was associated with increased expression and more and early activation of both caspase-8 and caspase-3 in aged subjects as compared to young subjects (227). Furthermore, Bcl-2 (anti-apoptotic) expression was decreased whereas Bax expression (pro-apoptotic) was increased in aging subjects. Herndon *et al* (228) reported increased apoptosis in CD3+CD45RO- (naïve) T cells in aging as compared to young following *In vitro* culture of lymphocytes stimulated with PHA and cultured with IL 2 for up to 6 days. Phelouzat *et al* (218) also reported increased AICD in lymphocytes from aged subjects, using suprapharmacologic concentration of PMA and ionomycin. In contrast, an age-related decrease in CD95-negative cells and a reciprocal increase in CD28-negative cells have been reported (229). Spaulding *et al* (186) observed resistance to apoptosis in human CD8+ T cells that reached replicative senescence after multiple rounds of antigen-specific proliferation. This is associated with upregulation of Bcl-2. It is also suggested that CD8+CD28- T cells that in aged subjects are expanded are resistant to AICD. In TNF-induced apoptosis, CD8+CD28- CD4+ and CD8+ T cells appear to be more susceptible to apoptosis than CD8+CD28- T ells (Gupta, unpublished data). It is likely that in replicative senescence apoptosis-sensitive cell have already been deleted. Furthermore, there might be a differential sensitivity of this subset to different apoptotic stimuli.

Gupta's group has studied TNF-α-induced apoptosis in human aging (191,230). They showed that CD4 and CD8 cells from the elderly were also more susceptible to TNF-α-induced apoptosis. Furthermore, they demonstrated that increased TNF-α-induced apoptosis was both in CD45RA+ and CD45RO+ T cells, suggesting that increased apoptosis in aging was not merely a consequence of increased accumulation of memory type cells. Increased apoptosis in aging lymphocytes was associated with increased expression of TNF-RI and decreased expression of TNF-RII in both CD4+ and CD8+ T cell subsets and increased expression of TRADD, FADD, and Bax in lymphocytes both at the level of mRNA and protein. A decreased expression of Bcl-2, TRAF-2 and TNF-RII and early and increased activation of caspase 8 and 3 were observed in aged humans. No changes were observed in Bcl-x_L expression (214). Furthermore, we have observed increased sensitivity of T cell subsets from aged humans to TNF-α-induced apoptosis that is associated with increased and early activation of caspases. The sensitivity of T cells to TNF-α-induced apoptosis appears to be age-dependent; cord blood lymphocytes are least sensitive whereas aged T cells are most sensitive to TNF-α-induced apoptosis (230). The relative sensitivity of T cells to TNF-α-induced apoptosis is aged>young adults > cord blood.

The amount of soluble fas in the blood of elderly donors was also reported to be significantly increased compared to young donors (223). Moreover, several studies have shown increased CD3- or PHA-mediated AICD and hence decreased proliferation in the elderly (219,225,231). This was found not to be due to IL 2 deprivation, nor was it associated with decreased bcl-2 expression (231). There is a possibility that increased AICD might be associated with decreased levels of IL 6 because IL 6 has been reported to protect neonatal T cells from AICD (232) as well as adult T cells in an IL 2-independent, fas/fas-ligand-dependent manner (233). However, IL 6 production seems not to be decreased in older individuals, as discussed above. Consistent with the findings of increased susceptibility to

AICD ex vivo, and unlike the situation with cultured CD8 cells, CD4+ T cell clones aged in culture also become increasingly susceptible to AICD (234); moreover, although T cell lines derived from old donors upregulated CD95 more slowly than those derived from young donors, their loss of ability to downregulate CD95 occurred faster, resulting in more rapidly increased susceptibility to fas-mediated apoptosis (225). The difference between CD4 and CD8 cells is not completely clear but recent results further implicate a fundamental difference between the two major T cell cell types in their response to fas ligand, which they express in addition to fas. This may be related to the finding that loss of the p75 variant of the TNF-α receptor conferred resistance to AICD on CD8 but not CD4 cells (235). Although on stimulation naive cells of both CD4 and CD8 type rapidly upregulate fas ligand and can be positively costimulated via this receptor (236), later in the immune response, CD4 cells become susceptible to fas ligand-mediated apoptosis, but CD8 cells, or at least some CD8 cells, may remain resistant (237). This rather than the response to fas may therefore be the critical difference. In mice *In vivo*, evidence for age-associated increased apoptotic death of superantigen-stimulated T cells has been forthcoming (238), and in old rats, stimulated T cells are also more susceptible to apoptosis (209). In humans, similar phenomena may be reflected in pathological states such as chronic phase HIV infection, where the fraction of apoptotic cells is greatly increased, especially amongst those with an activated (CD45RO+, DR+, Fas+, CD38+) phenotype, suggesting that chronic stimulation leads to clonal exhaustion by increased susceptibility to AICD (239). Consistent with this is the finding that *In vitro* anti-oxidant treatment, which can inhibit AICD, can to some extent restore the proliferative defect of HIV-infected CD4 cells (240). Also consistent with the idea of clonal exhaustion, monitoring HIV-infected individuals for strength and breadth of proliferative responses to HIV peptides revealed that patients with weaker responses progressed more slowly than those with higher responses (241). This could be interpreted to mean that stronger proliferative responses, while neuroprotective (241), result in more rapid clonal exhaustion and therefore disease progression. Non-HIV models of the effects of chronic antigen stimulation also exist, for example in mice experimentally chronically stimulated with human serum proteins (242) and lymphocytic choriomeningitis virus (243).

However, the idea that increased levels of AICD are detrimental to functioning of the immune system must be reconciled with data from several sources suggesting an age-associated increase in resistance to apoptosis on the part of cells from various tissues including lymphocytes. Thus, Zhou *et al*. (179) generated fas transgenic mice and compared immunological status of young and old transgenics with wild-type littermates. They found that fas expression (like the expression of some other receptors in the same family, eg. TNF-R) and fas-induced apoptosis was decreased in old wt mice, but not old transgenics. In old wt mice, there was an increase in CD44+ fas-negative cells, decrease in autocrine proliferation, decreased IL 2 production and increased IL 4 and IL 10 production. In the transgenics these changes were not found. Even age-related thymic involution was prevented in the fas-transgenics. It was therefore suggested that some of the manifestations of aging on the immune system were related to downregulated apoptosis (179). However, the lifespans of the transgenics were not increased and this seemed to be associated with enhanced production of IL 6 and other factors in these mice (244) including increased acute-phase responses and amyloid A deposition in the kidney (245). How might the transgenic fas expression exert these effects? Perhaps by removing defective cells by apoptosis and making room for fresh cells? More likely may be the alternative function of fas, ie. that of lymphocyte stimulation rather than killing. Thus, low to intermediate fas expression (and ligation) results in apoptosis, whereas high level expression can protect against cell death (246), and thereby result in enhanced responsiveness. Spaulding *et al*. (206) provided evidence partly consistent with that of Zhou *et al*. in normal mice, where they demonstrated that T cell apoptosis induced by irradiation, heat shock or CD3-stimulation was reduced in old compared to young mice, unless the former had been maintained on a calorically restricted diet. Polyak *et al*. (207) also reported higher levels of *In vivo* and *In vitro* lymphocyte apoptosis after irradiation in young compared to old mice. Consistent with the above data in mice, human CD8+ T cell cultures that reach replicative senescence become markedly resistant to apoptosis and show increased bcl-2 expression (214) and PBMC from centenarians are much more resistant than PBMC from younger subjects to oxidative stress-induced apoptosis (247). These data are reminiscent of fibroblast cultures, which show similar senescence-associated changes (248). Some further supporting data for the concept of decreased apoptosis in aged cells may be found in the report of Lechner *et al*. (225) who found decreased inducibility of CD95 after CD3-stimulation of old persons´ T cells compared to young. However, as already discussed above, susceptibility to AICD of T cell lines established from old donors was greater than those from young donors (225) and culture-aged CD4+ T cell clones show enhanced AICD compared to young cells from the same clone, although they do not express higher levels of CD95 (234). In this respect, therefore, human CD4 and CD8 cells may behave markedly differently, partly explaining the altered CD4:8 ratio seen in the elderly (see section 4.1). Ex vivo T cells from very old mice have also been reported to be more susceptible to TCR-mediated AICD than those from young or old mice (210), and expression of CD95 by CD4 cells of old mice infected with M. tuberculosis is not decreased (211). Some of the conflicting data may be explained by the findings of Telford & Miller that in mouse aging leads to an increase in susceptibility to apoptosis induced by repeated TCR stimulation, but a decrease (in CD8 cells) to apoptosis induced by agonist withdrawal (212). Additionally, some differences may also be caused by the effects of other cytokines on apoptosis, eg. type I interferons protect T cells from cytokine withdrawal-induced apoptosis (249). Some differences may also be caused by comparison of ex vivo and *In vitro* data (eg. increased susceptibility of CD8 cells to apoptosis with aging, in Aggrawal & Gupta´s studies cited above, compared with resistance of culture aged cells in Effros *et*

*al´*s studies cited above). Other tissues may also show increasing susceptibility to apoptosis with age, as is the case with hepatocytes (250). Here, caloric restriction also reverses the age-associated effects and reduced apoptosis, the opposite of its effect on lymphocytes. Finally, different apoptosis-inducing agents may have different effects, as in eg. enhanced PBMC apoptosis of the elderly caused by 2-deoxy-D-ribose (251,252).

6. CLINICAL RELEVANCE OF IMMUNOSENESCENCE

6.1. Infectious disease and cancer

The incidence and severity of infectious disease is increased in the elderly, as shown for pneumonia, meningitis, sepsis, urinary tract infections, RSV, HIV, influenza, etc. (1-6), recently summarized by Falsey (7). The relative mortality rates of many infectious diseases in the elderly are more than twice those of the young; in the case of tuberculosis and urinary tract infection this rises to a factor of ten (8). The conventional wisdom that HIV necessarily progresses more rapidly in the elderly is now being challenged, however (9). Autopsy data on the very old suggest that the rates of the accepted prime age-associated causes of death in the "younger old" (ie. cardiovascular, cancer) do not necessarily continue to accelerate with increased age in the very old. Studies from Leiden, Geneva and Tokyo have found the prime cause of death to be infectious disease in the over 80´s (however, whether these were really opportunistic infections is still not clear). Some studies do indeed suggest an age-associated shift towards infection with more opportunistic microbes accompanied by morbidity (10). Differences can be very significant, as illustrated eg. by the finding that mortality associated with Staphylococcus bacteremia is twice as high in the old as in the young (11). An extensive study on major causes of death (of women) in Japan between 1951 and 1990 suggests that unlike those causes showing deceleration or neutrality with advancing age, those showing acceleration in old age (ie. pneumonia, influenza, gastroenteritis, bronchitis) mostly involve infectious agents (12). A large-scale single center autopsy study clearly shows the marked skewing of causes of death in the very old towards infectious disease (13). Another study of men and women in the USA and Czech Republic confirmed decreasing cancer mortality after 80 years of age, and high death rates from infection, but also found maintained frequencies of cardiac deaths (14). An Italian study showed far fewer cancer deaths in the very elderly, and indicated that the prime cause of death was pneumonia (15). The occurrence of certain cancers increases in the elderly, but a contribution of immunosenescence to this progression is difficult to ascertain; studies that have sought to demonstrate increased cancer rates in elderly individuals with poor immune function compared to those with good immune function have not shown such an association, even over a 10-year follow-up period (16).

However, despite the findings discussed above, it remains the case that the precise clinical relevance of T cell immunosenescence is hard to define. There are studies suggesting that the NK status of subjects may also be important or even more important than T cells. Thus, Ogata *et al*. reported that not the numbers but the functional activity of NK cells was the only parameter correlating with death (due to infection) in the follow-up period for 44 elderly subjects (17). However, they did not test T cell function, only numbers, and therefore a contribution of T cells cannot be excluded, particularly since there is a reported correlation in the elderly between high NK activity and high T cell proliferative responses (18). Moreover, inclusion of T cell functional parameters has been shown to predict mortality in a Swedish prospective study (19-21). Nonetheless, a more recent study by Ogata *et al*. followed up on their earlier study and also prospectively examined rates of infection and death due to infectious disease in a larger nursing home population. Significantly, this population was screened according to the SENIEUR protocol, and should therefore have been in good immunological health. Their data showed a correlation between low NK activity and infection, and with shorter survival due to infection (22). In this study, some limited data on T cells were presented, looking at numbers of CD8+ cells (but these include some NK cells as well) and numbers of CD3+ CD56+ cells, in relation to survival after infection. Here, a high CD8 count in addition to low NK activity correlated significantly with survival, but the CD56+ CD3 count did not. Therefore, it is clear that NK activity is an important parameter, but a combination of T cell assay and NK may be critical, and so far untested. Interestingly, a critical association of NK cells with longevity has also been reported in rhesus monkeys; NK status and two non-immune parameters, but not other immunological biomarkers, were predictive of longevity (23). These data are in agreement with the well preserved NK cell activity found in centenarians (24).

In mice, there are several models where age-associated alterations in immune responsiveness correlate with a decreased ability to cope with infection, eg. by trypanosomes (25), and this may also be at least partly a result of defective macrophage function (26). On the other hand, in a mouse model of fungal infection, where clear T cell deficiencies can be demonstrated *In vitro* in old mice, greater susceptibility to the infectious agent *In vivo* was not observed; however, in this model, the explanation may be that T cell responses are in fact detrimental (27). In human, Varicella zoster reactivation is commonly invoked in support of the relevance of immunosenescence, and specific abnormalities in anti-viral immunity have been distinguished in the elderly in some studies (28). However, other examples may be found in the reactivation of other latent viruses, such as EBV, and possibly even mycobacteria (29,30). For example, the well-known age-related increased incidence of shingles in the elderly is associated with a decrease in the frequency of VSV-specific T cells which produce IFN-γ and decreased amounts secreted compared to young immune donors, while the production of IL 4 in the same donors was unchanged (31). Correspondingly, antibody levels to VSV are maintained in the elderly, but this is clearly not always enough to prevent reactivation of infection.

6.2. Vaccination

Decreased T cell function in the elderly is shown most clearly *In vivo* by DTH tests to recall antigens (32) as

well as to clinically relevant immunization procedures where T cell-dependent antibody production is depressed, eg. see refs. (33,34). However, the confounding effects of underlying disease make studies of age-related changes in the response to vaccination even more fraught with difficulty than for the cytokine secretion data (see section 4.4). Thus, although antibody responses following primary immunizations in the elderly are often reported to be decreased, immune responsiveness to the primary antigen Helix pomatia haemocyanin was retained in healthy elderly donors who fitted the SENIEUR protocol (35). By contrast, elderly subjects not fulfilling the SENIEUR criteria had a decreased immune responsiveness to this antigen. Nutritional status of the elderly is also very important in determining the success of vaccination, as indicated, for example, in studies showing up to 40% non-responders to influenza vaccine in undernourished subjects (36). Even when initially successful, vaccination may have a less long-lasting effect in older donors (37). Responses to secondary antigens may normalize after boosting in elderly donors, but the improved response may not be sustained for the same duration as in the young (38). Consistent with this, the majority (62%) of elderly individuals vaccinated with tetanus toxoid had antibodies to tetanus up to 10 years after vaccination, but this halved (to 33%) for vaccination more than 10 years previously. In contrast, almost all young donors retained tetanus antibodies even > 10 years after vaccination (39). Confirmation of weaker immunity to tetanus in the elderly, in terms of decreased frequency and level of cellular immune reactivity, has also been provided in a later study by Schatz *et al*. (40), and also when measured *In vivo* by DTH (41). With the increasing likelihood of therapeutic vaccination against cancer becoming a reality, the aged may have a disadvantage in immune responses to cancer vaccines too. In a mouse model, old animals did worse than young; moreover, grafting old recipients with young thymi or adoptively transferring young T cells did not help (42). The presence of a tumor may itself enhance thymic involution; this has been described in a mouse lung cancer model. Middle-aged animals and old animals also differed in other ways by which the tumor could suppress immune responsiveness (43). These findings suggest that it may be even more difficult to develop successful active immunotherapy of cancer in the elderly than in the young.

There may also be more subtle differences between the responses of young and old individuals, such as a selective impairment of particular classes of antibody production, for example, of IgG1 responses to inactivated influenza virus vaccine in the elderly (44,45). This possibly reflects less efficient or altered T cell help. This shift in immunoglobulin(sub)class distribution may be a reflection of altered cytokine patterns. The response to influenza vaccination may also be highly dependent on the flu strain involved, as several authors have shown (46-50). They found that while the majority of elderly people responded to strains of the influenza A H3N2 subtype, only few responded to strains of the influenza A H1N1 subtype (46-50). These findings are probably due to pre-exposure of the elderly and young to different strains of flu viruses (51). PBMC from the healthy elderly may produce lower levels of IFN-γ both pre- and post-vaccination compared to the young, although levels do go up after vaccination in both young and old (52). Moreover, underlining the importance of IFN-γ, these investigators found that although elderly individuals often responded only with either a cellular or a humoral response to vaccination, production of IFN-γ was correlated with the presence of both (53). The production of a potentially inhibitory cytokine, IL 10, was also found to be greater in the young, whereas IL 6 production was similar (52). Others have found increased IL 10 production in the elderly (54); the reason for this apparent discrepancy is not clear, but may be related to these latter investigators´ findings that responses to different strains of flu virus differ markedly (54). The same group had previously shown that the elderly may also have lower IL-2 production following *In vitro* stimulation with flu vaccine (55), although this is also partly dependent on the strain used (54). Accordingly, subcutaneous low-dose IL 2 treatment prior to vaccination has been reported to enhance protection of the elderly to ´flu (56). Similar findings concerning antibody titer (but not lymphoproliferative responses) have been reported for tetanus toxoid vaccination (57). Another study has examined IgG responses of the elderly to pneumococcal vaccination and found no decrease in the old 4 - 6 weeks after vaccination with Streptococcus pneumoniae (58). However, it could also be that the 4 - 6 week period examined in the latter study after immunization is not long enough to be informative. For example, old mice did not show a decline in antibody responses after an immunization with Streptococcus pneumoniae, but nonetheless, after a second immunization, the old mice showed a marked decrease in antibody production compared to the young. This was traced to a defective function of $CD4^+$ T cells (59). Other studies of the efficacy of ´flu vaccinations in the elderly found, however, that annually repeated vaccination resulted in an improvement of humoral responses to several virus strains, rather than a decrease that might be predicted from clonal senescence (60,61). Thus, it may also be the case that in those studies where poorer responses of the elderly to vaccination were observed, this may reflect their state of health and consumption of medication more than anything else. This was illustrated in a recent study on ´flu vaccination, where response was correlated with the well-being of the vaccinees as assessed according to the "activities of daily living" (ADL) scale (62) and in a study on responses to hepatitis B vaccination (63).

Efforts to increase the efficacy of vaccination in the elderly are currently intense but mostly empirical as the underlying mechanisms of suboptimal responsiveness are not yet properly understood. In this regard, there is interest in using immunopotentiating drugs; thus far a randomized double-blind placebo-controlled clinical trial using aspirin has been carried out on 281 donors >65 yr. immunized with influenza vaccine. Aspirin did not affect post-immunization levels of IL 2 secretion or blastogenesis *In vitro* after vaccine-stimulation, but there was a 4-fold increase in specific antibody titer in the aspirin compared to placebo group. Moreover, this increase in the aspirin group was even more marked in individuals >75 yr. old (64).

Other approaches to enhance responses recently explored may be to use "naked" DNA vaccines, as illustrated by studies in old mice (65,66), or to use technical variations on the theme of viral inactivation-versus-attenuation in elderly humans (67). Further approaches currently being assessed include the use of ISCOM preparations in an effort to enhance immunogenicity of the vaccine (68) or the use of highly active dendritic cells as "professional antigen presenters" to provide a stronger stimulus to the T cells (69). Experimentally, stress also reduces the efficacy of influenza vaccination, theoretically also offering the possibility of enhancing vaccination efficacy by stress reduction approaches (70).

6.3. Benefits of immunosenescence?

Although usually viewed as being deleterious to the individual, immunosenescence may on occasion contribute to decreased pathology in elderly individuals, as in the lesser acute rejection seen in clinical corneal and kidney transplantation (71,72) and in murine models of systemic lupus erythematosus (73,74) as well as possibly in asthma in rats (75). The same may be true for acute rejection in human liver transplantation (76) as well as heart transplantation, where the incidence of rejection episodes in recipients > 65 years old was very significantly reduced ($p = 0.017$) (77). However, another study of acute kidney rejection in humans did not find significantly less rejection in elderly recipients, but there was a trend in this direction (78). A practical consequence of age-associated changes may be that the elderly would require less immunosuppression, welcome in frail patients (79). Furthermore, in some tumor models where immunostimulation contributes to tumor growth, immunosenescence may have what at first glance seems a paradoxical effect, ie. it may result in a reduction of tumor growth (80), whereas usually tumor rejection in young animals changes to tumor progression in old animals (81). Recently it was argued that immunosenescence might impact on levelling off of cancer incidence in the oldest old (82). In particular it was suggested that the expansion with age of NK cells and of T cells which progressively acquire phenotypes intermediate between T lymphocytes and NK cells, together with "inflammaging", i.e. the age-related changes in the production of inflammatory/anti-inflammatory cytokines, might create an environment unfavorable for neoplastic growth.

The elderly may also have less problems with alleregic reactions, as with asthma in rats, mentioned above. Thus, antigen-specific IgE production declines with age *In vivo* and *In vitro* (83,84).

6.4. Autoimmunity

Apparently paradoxically, despite declining immune function, aging is also associated with an increase in some autoimmune phenomena. However, immunological tolerance to foreign antigen established in young mice is retained in old mice, even at an age when de novo tolerance can no longer be induced (85). It may also be that some measured autoreactivity is in fact protective, as seen in a mouse model of adjuvant arthritis, where hsp60-specific self-reactive T cells actually suppress disease on adoptive transfer (86). This type of finding could help resolve the naive paradox of increased rather than decreased autoimmunity with age.

There are increased levels of autoantibodies in the aged; because some such antibodies can penetrate living cells and even activate them (87,88), this might have functional consequences and even help to explain some aspects of dysregulated T cell function in the elderly. Moreover, IgM antibodies specifically directed against T cells may begin to appear in mice at one year of age and are widespread at 2 years, possibly interfering with normal T cell function and/or thymic selection (89). These findings may partly explain why the administration of certain TCR Vß peptides to aged mice reverses some of the signs of immunosenescence (ie. the reduced IL 2 secretion and proliferation, increased IL 6 production etc). The mechanism for this effect might be the absorption of TCR autoantibodies otherwise blocking function (90).

In discordance with the higher prevalence of ANA and other autoantibodies, the typical autoantibody disease SLE becomes milder in the elderly (91). Similarly, in a mouse model of the disease, activity and cytokine production were lower in older mice (92). The same may partly be true for Primary Sjoegren's Syndrome , which is likewise associated with ANA and ENA and where a reduced prevalence of RF and Anti-Ro antibodies as well as less hypergammaglobulinemia have been described by some authors (93,94) Instead, in centenarians, the increased levels of autoantibodies found do not include disease-associated antibodies such as anti-ENA and anti-dsDNA antibodies (95), or the thyroid autoantibodies which cause problems in the less elderly (96) but this could be because of selection pressures in the extremely old. Furthermore, the latter group have recently reported that, at least for thyroid autoantibodies, in healthy free-living persons, titers are low, but increase in those in poorer health (97). A similar finding has been described with regard to ANA and RF, where healthy old persons did not show an increased prevalence (98).This suggests that such autoimmune phenomena may be a result of underlying disease rather than aging itself. In mice (99), and, as recently also shown, in humans (100), old individuals have a quantitatively but not qualitatively altered autoantibody pattern and, because not only autoantibodies in general, but also clearly pathogenic autoantibodies, are routinely generated during normal immune responses to foreign antigen in the healthy young, the requirement for peripheral regulatory control of potentially damaging autoreactivity is paramount (101). It is this which could possibly be dysregulated in aging. Thus, immunodeficiency on the one hand could be reconciled with increased autoimmunity on the other by postulating a compromised cellular regulatory activity with age. Data related to this point are mostly old and controversial, but some are consistent with decreased cellular suppressive activity with age (102-104) or with increased resistance to suppressive influences in the aged (105). Decreased specific suppressor cell activity in aged mice is associated with the appearance of MHC unrestricted T helper cells (106). The appearance of the same kind of MHC unrestricted helper activity has been observed in elderly

humans (107), suggesting that altered suppression in aging may also occur in man. This has not been systematically investigated. The finding of increased autoantibodies in thymectomized MG patients suggest that the thymus may exert a suppressive effect on autoimmunity (108) More recently, further studies also including an examination of splenocyte function have begun to investigate mechanisms underlying altered regulatory status in aging. Thus, Crisi *et al*. showed that the type of regulatory CD8+ cells active in young donors were not present in old donors; this was attributed partly but not exclusively to the decreased capacity of old donors´ CD8 cells to secrete an immunosuppressive cytokine, TGF-ß (109). In another experimental model, of oral tolerance induction, it was found that treatment that suppressed responses in young mice not only failed to inhibit but actually enhanced them in old mice (110). Although many widespread autoimmune diseases commonly occur in the young, there is a set which show late onset (111). In addition, some – probably more Th1-mediated – autoimmune diseases may take a more severe course in the elderly, such as psoriatic arthritis (112) and even rheumatoid arthritis (113). Thus, the possible contribution of age-associated dysregulated immune function to these autoimmune diseases remains to be explored.

6.5. Predictors of mortality and longevity

According to some data, a major predictor of mortality in the elderly is lung function (114), perhaps fitting with the above results of Bordin *et al*. (15). Immunosenescence may also play an important role here, since the defense of this most common pathogen entry portal is critical. In support of this concept, Meyer *et al*. (115) have provided evidence for immune dysregulation in the aging human lung. This was associated with the presence of higher numbers of neutrophils and more IL 8 in the elderly compared to young, suggesting that low-grade inflammation is present in many apparently clinically normal lungs in the elderly (116). Further work by this group showed that lymphocytes obtained from lung by bronchoalveolar lavage (BAL) in apparently healthy elderly volunteers differed significantly from those from young donors. The elderly BAL cells had a higher CD4/CD8 ratio due to higher numbers of CD4 cells, and these had higher expression of activation markers HLA-DR and CD69 (117), consistent with their studies cited above. The higher CD4:CD8 ratio in BAL from the elderly has been confirmed by others, but only in women, not men (118). In the latter study, no change in the CD4:8 ratio in peripheral blood could be found.

In studies done in mice, it was found that the inflammatory response to teflon fumes in the lungs of old mice was greater, particularly in terms of TNF-α induction, than in young mice (119). On the other hand, allergic reactions may decrease with age for the same reason, as with the well-known "growing out" of asthma. In a rat model of asthma, it was found that the level of specific IgE antibody and eosinophils in bronchoalveolar lavage was markedly higher in young rats. This correlated with increased interferon-γ production and decreased IL 5 in old rats T cells (120). The earliest observations on immunosenescence showed that cytotoxic T cells were compromised in old mice (121); in humans it has recently been found that this is a result both of an age-related decrease in the proportion of cells expressing perforin and the amount of perforin per cell (122). Compromised cytotoxic effectors might be expected to contribute to decreased efficacy of immunity.

In most aging studies in human, individuals > 60 years are commonly considered "old". Longitudinal studies are required to establish the critical changes within the immune system and what may be associated with "healthy aging". There may be surprises, as in the recent report that in the over-85´s, high cholesterol levels were associated with greater survival over a ten-year follow-up (123). Cross-sectional studies concur but conclude that age-associated decreased cholesterol levels are a marker of poor health (124). Along similar lines, lipoprotein A is also paradoxically increased in the elderly (125). Cardiovascular death rates were similar in high and low cholesterol groups, but the high cholesterol group had lower mortality from infectious disease - perhaps implicating an immunological mechanism (as diets high in unsaturated fatty acids may be immunosuppressive). Another recent analysis revealed that the elderly with low cholesterol levels experienced more emergency room visits, longer hospital stays and more re-admissions etc (126). The basis of this may be that sufficient cholesterol is required for proper formation of the "immunological synapses" or "lipid rafts" that are required for optimal signal transduction in lymphocytes (127). This may be due the increased viscosity of cholesterol-rich membrane, which, however, may itself lead to decreased responses if too extreme (128). These older data are also consistent with recent findings that cholesterol depletion disrupts lipid rafts (129). The use of methyl-ß-cyclodextrin to deplete cholesterol from the membranes of T cells from the elderly, however, failed to enhance their proliferative capacity (130).

Life span is a multifactorial quantitative trait, which is affected by genetic and environmental factors. It also contains a stochastic component resulting from the interaction between the individual chances of surviving and unpredictable events that occur throughout the course of life. The genome is a prime determinant of differences in maximum life span between species and the variation in life span within a species is also expected to be influenced by genetic variation (131-133). A recent study in Iceland has clearly shown that there is a strong familial component to longevity (134). However, there remains considerable uncertainty about the extent of the genetic versus the nongenetic contribution and about the importance of gene-environment interactions (133,134). In resolving the problem of shared environment, twin studies are the most reliable indicator of a genetic contribution to human longevity, although even these are not entirely without difficulty (133). The relative importance of genetic influence on longevity was so studied on data from the population-based Swedish and Danish Twin Registries (135,136). Intraclass correlation coefficients obtained in Swedish twins suggested that the genetic effect on longevity was small and, for males, perhaps absent.

Examining the whole age range, a maximum of around one third of the variance of "longevity" was attributable to genetic factors. Accordingly, Danish data indicate that the inheritance of longevity was 0.26 for men and 0.23 for women. However, in both Swedish and Danish studies, the twin cohorts taken into account included subjects whose most advanced age did not exceed 80-90 years. Thus, it can be argued that the authors evaluated the inheritance of mortality due to common age-related diseases, rather than the inheritance of longevity *per se*. On the whole, it appears that the real contribution of the genetic component to human longevity, as well as the number of genes playing a major role in reaching a far advanced age, has not yet been properly and/or sufficiently addressed. Therefore, the role of genetics in longevity might be much higher than predicted on the basis of available data on "younger" subjects (137).

As discussed by Schachter *et al*. (138), two strategies can be exploited to identify genes that influence human life-span (these are the same as those used for seeking disease-related genes), i.e. sibling pair analysis and “case-control” studies. A sib pair analysis is designed to detect loci that segregate with the trait (longevity) by relying on non-random segregation at a marker locus, i.e. it consists of analysing the distribution of shared alleles at a polymorphic marker locus in sibships. Classical approaches to the study of human genetics, which are aimed at detecting co-segregation of genetic markers in pedigrees, are not easy to implement. In fact, this requires the sampling of pedigrees that include two or more very old individuals, possible in more than one generation. Besides, the continuing trend of changing of environmental conditions renders direct comparisons between the age of death of parents and offspring virtually meaningless. Nevertheless, a genome-wide scan for longevity-predisposing loci was recently conducted by using 308 individuals belonging to 137 sibships demonstrating exceptional longevity. By using both non-parametric and parametric analysis, significant evidence for linkage was noted for chromosome 4 at D4S1564 (139). However, for the reasons above discussed, gene-longevity association studies of unrelated individuals, which search for non-random associations between polymorphisms at candidate loci and longevity, have been the most popular type up to now, and the literature is growing quickly. In a case-control study, allele and genotype frequencies at polymorphic marker loci are compared between probands (a long lived group in this case) and a control group of randomly selected adults. Case-control studies might be subjected to a number of possible confounding factors, the total number of persons (the oldest old in these studies) and controls and the homogeneity of the population in term of geographical origin, among others (131,138). However, a consistent association with longevity for some genes, including mitochondrial DNA emerges (131,132,140). These associations may be gender-related, suggesting as a working hypothesis that men and women may follow different strategies to reach longevity (141). In particular, APOE is the locus that can most consistently and reliably affects longevity. The common APO ε4 allele is a well-established risk factor for AD and cardiovascular diseases. The biological mechanism underlying the association with AD is not known. Hypotheses include a differential effect of APOE alleles on amyloid deposition, tangle formation, neuronal plasticity and cholinergic function. The association with cardiovascular diseases might be the consequence of higher LDL cholesterol and triglyceride levels in plasma of carriers of this variant. The role of the e4 allele in mortality has been the subject of many studies and we can conclude that APO ε4 allele negatively affects longevity (131,132,142).

Data of Puca *et al*. (139) would fit well with the hypothesis that longevity is an evolutionarily-selected trait. This is based on the idea that pre-literate humans would require a repository of tribal wisdom to assist them survive in times of natural and possibly other disasters repeating outside of the-time frame of the average lifespan. Resources to maintain all tribal members in old age would be not be available, but could be spared for only a small fraction of people whose function would be to remember these earlier events and advise on survival tactics. There is some evidence for this view from studies of modern primitive peoples. However, in human societies, women live longer than men (141) and the survival advantage in women has deep biological roots (143). In fact, it has been demonstrated that in primates, the gender responsible for parental cares lives longer than the other gender, suggesting that it came about mainly through the enhancement of genes favoring caretaker survival through natural selection (143). Thus, these genes should be survival genes.

Several genetic studies have relied on an immunogenetic approach. In fact, as discussed above, there is a positive association between good immune function and individual longevity, suggesting that a global well-preserved immune function is associated with extended longevity. Some of the potential survival genes present in the population may therefore relate to immunity and immunosenescence. Thus, it is not unlikely that one of the genetic determinants of longevity resides in those polymorphisms for the immune system genes that regulate immune responses, such as the MHC. Studies of the very old (i.e. the survivors) may be informative in this regard. Centenarians may be considered to represent such a very small, highly selected, proportion of successfully aged individuals. An examination of healthy centenarians would show whether immunological aging is divorced from overall physiological aging. Were the immune system to be senescent despite health in these individuals, this would indicate the lack of importance of immunity for healthy aging. Healthy centenarians are indeed found to have well-preserved immune functions, much more similar to the "young" immune system than average for less extremely old donors. Thus, as summarized by Franceschi *et al*. some time ago (144), T cell proliferative responses are well-maintained (albeit taking place more slowly), the T cell repertoire still contains all Vß families, hematopoiesis is maintained, and interestingly, there is a high level of lymphocyte genomic stability i.e. low spontaneous breaks etc., which otherwise are thought to increase with age in average, non-centenarian, donors (145). There were some data suggesting that healthy centenarians represented a

group with the best retention of thymic structure and function and that these individuals were also characterized by lower DNA damage (146). The latter ties in with the results on anti-oxidant (147) and DNA repair capacity, and resistance to stress (148). In fact, when lymphocytes from 8 mammalian species were screened for their resistance to oxidative and other stressors, a correlation was found between species lifespan and such resistance (149).

Accordingly to previous suggestions, the lifespan of allophenic mice produced from a long-lived and a short-lived strain (150) is positively correlated with the proportion of lymphocytes derived from the long-lived strain, showing the importance of the immune system to overall lifespan (151), perhaps via the effect of mature T cells on maintenance of the thymic environment alluded to above (see section 3.2). The association may be by way of susceptibility to lymphomas: it has been shown that mice which have higher levels of memory cells, lower levels of naive cells and lower proliferative responses at 6 months of age retain these patterns in later life, and that in genetically heterogeneous populations, mice with this phenotype have significantly shorter life spans caused by increase incidence of lymphomas (152). The CD4 memory correlation holds regardless of whether the mice died of lymphoma, fibrosarcoma, mammary carcinoma or other terminal disease (153). Further genetic analysis of longevity in mice revealed five markers on different chromosomes associated with longevity in males, and two others in females, out of 82 loci genotyped (154). Although the products of these loci are unknown, they may all have something to do with cancer susceptibility, because the old mice in this study all died of various different types of cancer. Another approach to investigating the contribution of genetics to longevity in inbred mouse strains showed that the vigor of T cell responses in old mice is influenced by MHC type, with those mice possessing "low responder" phenotypes succumbing at an earlier age than those with "high responder" phenotypes, mostly due to their increased susceptibility to lymphomas (155). Increased levels of IL-6 in old mice may play a role in the increased occurrence of lymphoma (156). Therefore, the mechanism of the genetic association of MHC with lifespan in mice may be a reflection of decreased immunosurveillance against lymphoma and other tumors, as a result of immunosenescence. Mice genetically selected for high antibody responses were found to have longer life spans, and this was also associated with lower incidence of lymphoma (157). On the other hand, mice selected for high or low T cell responses to PHA also have lower and higher lymphoma incidences respectively, but do not differ in longevity; whereas mice selected for resistance to chemical carcinogenesis show altered tumor incidence and longevity without corresponding alterations in immunity (157). MHC type and longevity associations may also be mediated by lymphocyte subset differences, e.g. numbers and functions of T, NK and NKT cells (158). A recent large longitudinal study examined a broad range of behavioral, physiological, anti-oxidative and immune function biomarkers in genetically heterogeneous (not inbred) mice, and concluded that independent mortality predictors could be found in certain behavioral parameters, but also in natural killer cell activity and T cell proliferative responses to the mitogen concanavalin A (159). Another study sought to predict longevity of middle-aged genetically heterogeneous mice on the basis of subset of distribution of T cells; cluster analysis revealed that relatively low levels of CD4 and CD8 memory cells, but high levels of naive cells, predicted individual longevity (160). What these results may mean in longer-lived, less tumor-prone species like man is unclear. Thus, it is not surprisingly that studies performed on mice have suggested that MHC is associated with the life span of the strains (161). Other mechanisms accounting for a possible MHC-longevity association in mice might be a relationship between MHC alleles and rate of thymic involution (50) as well as related to the differences in DNA repair capacity in lymphocytes observed between mice of different MHC types (162).

The HLA complex (the human MHC genes) contains over 200 genes divided into 3 classes. Most HLA genes involved in the immune response fall into classes I and II, which encode highly polymorphic heterodimeric glycoproteins. These molecules take up antigenic peptides intracellularly and emerge on the cell surface where processed peptides are presented to T cells, regulating T cell responses against specific antigens. The response depends on the ability of HLA antigens to bind some peptides and not others. These are the bases for the antigen-specific control of the immune response. Furthermore, the HLA-B8,DR3 haplotype, that is part of the 8.1 ancestral haplotype (AH) HLA-A1, Cw7, B8, TNFAB*a2b3, TNFN*S, C2*C, Bf*s, C4A*Q0, C4B*1, DRB1*0301, DRB3*0101, DQA1*0501, DQB1*0201, is unique in its association with a large number of debilitating immunopathological diseases and is associated in healthy subjects with changes in the response to mitogens and antigens, strongly suggesting that the haplotype controls the immune response in an antigen non-specific fashion (163). Host MHC phenotype largely determines the type of immune response that develops following infection. Thus, in humans, differences in responses to vaccines, allografts and infectious agents are thought to be a direct result of allelic variations in HLA genes in the population and many studies provide compelling evidence for a direct influence of HLA alleles on survival. In the last 25 years, many cross-sectional studies searched for a role of HLA genes in human longevity by comparing HLA antigen frequencies between groups of young and elderly persons. However, conflicting findings have been obtained. In fact, the same HLA antigens are increased in some studies, decreased in others, unchanged in yet other. On the whole, this leads to the hypothesis that any observed age-related differences in the frequency of HLA antigens are more likely due to bias. This may well be true for many of these studies, owing to major methodological problems, such as the employment of various different serological and molecular typing methods, insufficient sample sizes, different inclusion criteria and age cut-off, inappropriate mixing of data referring to people from 58 to over 100 years of age, inappropriate control matching and neglected consideration of sex-related effects as well as different genetic make-up of studied populations (137,164). However, some studies that are not flawed by these biases have indeed shown an association between

longevity and certain HLA-DR alleles or the HLA-B8,DR3 haplotype. In studies performed in Okinawa, a high frequency of HLA-DR1 was found in centenarians (165,166). It is noteworthy that in the Japanese population this antigen is associated with a strong response to mitogens and some infectious antigens like Candida (166). Interestingly, in Caucasoids it is associated with quantitatively higher levels of T lymphocytes in the periphery (167). In the Netherlands, a decrease in HLA-B40 and an increase in HLA-DR11 characterized women over 85 years (168). The same laboratory later followed this up using a 'birth-place-restricted comparison' in which the origin of all subjects was ascertained and were able to confirm that aging in women was negatively associated with HLA-B40 and positively associated with HLA-DR5 (169). Independent studies performed in France confirm the association of HLA-DR11 with longevity in its familial form, in aged populations (170,171). This increase is consistent with the protective effects of this allele in viral diseases, because the frequency of HLA-DR5, or its subtype HLA-DR11, is decreased in patients with some viral diseases (137,164). However, this allele also increases susceptibility to a variety of autoimmune diseases, although it is not clear whether this impacts on survival statistics (137,164). Finally, an association between longevity and the 8.1 ancestral haplotype (or part of this haplotype) seems to be present. An excess of this haplotype in aged men has been reported in two different studies performed in French and in Northern Irish populations (172,173) This association may be gender-specific, because a Greek study showed a significant decrease of this haplotype in aged women (174). The increased TNF-α production, characteristic of this haplotype (175), might help us to better understand the role played by this haplotype in longevity, because the elderly tend to show a "pro-inflammatory" phenotype with increased plasma levels of inflammatory cytokines including TNF-α, hypothesized to represent an adaptive response to stress (176). On the whole, it can be argued that immune dysfunction characteristic of the 8.1 AH appear to contribute to early morbidity and mortality in elderly women, who are more susceptible to autoimmune diseases than men, but, on the other hand, to longevity in elderly men. Thus, an intriguing association between longevity and 8.1 AH apparently emerges, although further studies are necessary to validate this suggestion. In some studies, the differences in antigen frequencies between young and older people were statistically significant, but not impressive. However, even if HLA genes are associated with longevity, they are not the only genes influencing longevity. Thus, HLA studies in man may be interpreted to support suggestions derived from the studies on congenic mice on MHC effects on longevity. On the other hand, as discussed above, in mice the association may be by way of susceptibility to lymphomas, whereas, in human beings the effect on longevity is more likely to be via infectious disease susceptibility. Longevity is associated with positive or negative selection of alleles (or haplotypes) that respectively confer resistance or susceptibility to disease(s), via peptide presentation or via antigen non-specific control of immune response (see above). This association may be gender-related, but, as previously stated, it is not unexpected on the basis of available data on the genetics of longevity, showing that on several occasions, the association of longevity with particular alleles has been found only in one gender (141).

HFE, the most telomeric HLA class I gene, codes for a class I α chain, which seemingly no longer participates in immunity, because it has lost its ability to bind peptides due to a definitive closure of the antigen binding cleft that prevents peptide binding and presentation (177). The HFE protein, expressed in crypt enterocytes of the duodenum, regulates the iron uptake by intestinal cells because it has acquired the ability to form complex with the receptor for iron-binding transferrin. Thus, it indirectly regulates immune responses too, because iron availability plays a role in specific and non-specific immune responses. The C282Y mutation (a cysteine to tyrosine mutation at amino acid 282) in this gene has been identified as the main genetic basis of hereditary haemochromatosis. It destroys its ability to make up a heterodimer with β2-microglobulin. The defective protein fails to associate with the transferrin receptor and the complex cannot be transported to the surface of the duodenal crypt cells. As a consequence, in homozygous people two to three times the normal amount of iron is absorbed from food by the intestine. Regarding the biological significance of HFE heterozygous status, the mean serum iron concentrations, ferritin levels and transferrin-saturation values were higher in heterozygous subjects than in normal ones, as were mean haemoglobin levels and mean corpuscular volume (177,178). Recently, the distribution of HFE polymorphisms in Sicilian centenarians and nonagenarians was studied to evaluate whether HFE alleles might be differently represented in people selected for longevity. It was found that possession of the C282Y allele, known to be associated with an increase of iron uptake, significantly increases longevity in women (178).

There are certainly also other genetic influences on immune responsiveness, upon which the effects of aging may be superimposed, that must be taken into account. In fact, complex immune traits related to lifelong immune responsiveness and immunosenescence such as the level of circulating immunoglobulins, the peripheral blood T lymphocyte levels, and the telomere length appear to be under strict genetic control (179-181). Finally, as previously discussed, a great deal of data indicate that aging is characterized by an imbalance of inflammatory status and inflammatory markers are associated with age-related disease, predicting disability and mortality in the elderly. Thus, it can be hypothesized that genetic variations in pro- or anti-inflammatory cytokines might influence successful aging and longevity (see previous paragraphs on cytokines). Recently, the distribution of +874T→A IFN-γ, –1082G→A IL-10, -174C→G IL-6 polymorphisms in a large number of Italian centenarians has been studied to evaluate if the alleles of the three cytokines might be differently represented in people selected for longevity (182-184). The +874T allele, involved in high production of pro-inflammatory cytokine IFN-γ, was found less frequently in centenarian women than in control women (182). In contrast, the allele frequency of -1082G, involved

in high production of the anti-inflammatory cytokine IL-10, was significantly increased in centenarian men (182). In addition, the proportion of persons homozygous for the G allele at –174 IL-6 locus (characterized by high IL-6 serum levels) was decreased in centenarian men (184). Aging is characterized by a pro-inflammatory status, which, if not counterbalanced, might contribute to the onset of major age-related diseases such as cardiovascular diseases, diabetes, AD, osteoarthritis and osteoporosis (176). Thus, taken together, these results show that the control of the inflammatory state may be beneficial for longevity. Furthermore, these data add another piece of evidence to suggestions that men and women follow different trajectories in order to attain longevity (141).

7. POSSIBLE APPROACHES TO REMEDIATION

Immunogerontological parameters may be affected by many outside influences rather than aging *per se*, particularly if donors are not selected for perfect health using strict clinical criteria. Some of these may be subject to manipulation. For example, much attention has been paid recently to the effects of exercise on immune function in the elderly, although details of the mechanisms involved in any observed improvement in immune function are completely unknown (1) and not seen for all parameters in all studies (2). In fact, exercise might even be considered an immune-restorative intervention, due to its beneficial effects on cytokine secretion, T cell function and NK activity (3). In mice, moderate exercise in old but not young individuals was associated with increased antigen-specific IL 2 and IFN-γ, but not IL 4 and IL 10 secretion on rechallenge with HSV-1 (4). This is in contrast to the effects of acute fatiguing exercise, which had a non-specific short-lived inhibitory effect on both Th1 and Th2 cytokine production (5).

It is difficult to dissect the effects of the many different interacting and confounding factors, including health status, nutritional status, psychological status etc., which overlay a background genetic influence. There is even one report suggesting that the link between major depression and reduced immunity is in fact indirectly caused by lower levels of physical activity in depressed patients (6). Such factors as exercise and diet are easily manipulated and in the longstanding tradition of empirical medicine have encouraged interventionist approaches simply because these are feasible and not dangerous, even though a firm scientific basis for their efficacy may be lacking. The effects of different fatty acids in the diet may also be influential on immune function, and is amenable to manipulation with relative ease. Thus, for example, lipid peroxidation, considered to be a symptom of normal aging in the rat, can be alleviated by administration of DL-alpha-lipoic acid (7). In humans, plasma levels of certain carotenoids (lutein and beta-cryptoxanthin) correlate inversely with indices of DNA damage and lipid peroxidation (8). It was reported many years ago that there is a direct correlation between the ratio of cholesterol to phospholipids in serum and the microviscosity of lymphocyte membranes, and that both increase with aging in humans (9,10). It was suggested that increased microviscosity was at least partly responsible for decreased lymphocyte proliferation. Limited biochemical studies employing SENIEUR donors have indicated that membrane lipid alterations in the elderly may be important for altered immunological function (11). Rather than the membrane lipid constitution per se that was different between young and old, it was the changes observed upon blastic transformation of stimulated lymphocytes which correlated with decreased proliferative function. Reports are beginning to appear of extensive clinical trials using complex mixtures of anti-oxidants, minerals and semi-defined plant extracts and measuring the effects of consumption of these on indices of oxidative stress *In vivo* (12). However, whether these indices have anything to do with aging or the development of age-associated disease is still not clear.

7.1. Vitamins and minerals; anti-oxidants; "nutriceuticals" – more than just proper nutrition?

Many studies indicate an association between diet and longevity. Some results are striking. One example: a 5-year longitudinal study of risk of death in the elderly indicated significant positive associations with longevity for citrus fruit, milk and joghurt consumption with high intake of vitamin C, riboflavin and linoleic acid, and low consumption of meat. These factors contributed to a relative risk of death over the study period differing between groups by greater than 50% (13). But does a mechanism involving the immune system contribute to such beneficial effects of diet? Several reports suggest that even in carefully selected donors, nutritional status may play a significant role in exacerbating immunological differences (14-17). However, studies have also concluded that an age-increment influencing immunological status exists independently of nutritional effects (15). Nonetheless, nutritional status does seem to contribute significantly to immune status and may be relatively amenable to correction by dietary supplementation. Even common nutrients such as glycine may influence immune responsiveness in aging, for example by acting in an anti-inflammatory manner (18). Simple supplementation with arginine has also been reported to reverse some age-associated changes in immune responsiveness in old mice (19), and thymectomized mice (Tx) (20) as well as in old people and cancer patients (21). Thymus grafts from old arginine-treated but not untreated mice in Tx recipients restore peripheral immunity, at least natural killer activity (20). These findings in Tx and old mice as well as in the elderly reinforce the notion of the beneficial effect of arginine upon the immune system which may occur perhaps by its effects in preventing the accumulation of advance glycation end products (AGEs) (22). Alternatively, various other mechanisms of action of arginine upon the immune system may also be proposed, for example, via the L-ariginine:nitric oxide (NO) pathway. *In vitro* experiments on young thymic explants have shown that inhibitors of NO, such as N^G-nitro-L-arginine-methyl ester (L-NAME) obstruct thymic endocrine activity, which can be restored by arginine (23).

Surveys show that while the intake of many micronutrients is satisfactory in the elderly (in developed

countries at least), there may be measurable deficiencies of certain factors, such as magnesium, copper and vitamin D (21). Surveys show that while the intake of many micronutrients is satisfactory in the elderly (in developed countries at least), there may be measurable deficiencies of certain factors, such as magnesium, copper and vitamin D (24). A European survey concluded that 47%, 23%, 3% and 1% of the elderly showed deficient levels of Vitamins D, B-6, B-12 and E respectively (25). These deficiencies may be even more marked in hospitalized subjects and include zinc, selenium and vitamin C deficits (26); in some studies, eg. a German survey, deficiencies are only seen in geriatric patients, not other elderly (27). Further work by the French group went ahead and supplemented such persons with zinc, selenium and vitamins to compensate; this resulted in increased influenza antibody titers after vaccination and reduced morbidity from respiratory tract infections (28). However, the decreased responses seen to ´flu vaccination in the healthy elderly in one recent study were found not to correlate with plasma ß-carotene, or retinol, Vitamin E or zinc levels, in comparison to the young (29). Nonetheless, a supplementation study in mice showed a benefit of long-term Vitamin E consumption, but not with various other anti-oxidants, on influenza infection, associated with decreased levels of IL 6 and TNF-α in the lung (30). Nonetheless, studies indicate that vitamin E supplementation does not extend lifespan in mice (31). Earlier, nutritional "correction" of immunological parameters coupled with a beneficial effect on resistance to infectious disease had been reported in some studies (32) but not others (33). However, there are many possible explanations for these different results, and more data are needed. There is extensive data in on vitamin C supplementation, which enhances the mitogenic responses of lymphocytes from elderly people (34) and has even been reported to slow down the rate of telomere attrition in dividing cells (35). This may be related to its anti-oxidant properties, since oxidative stress can result in telomere shortening independent of cell division (36,37), even without telomerase inactivation (38), leading to the suggestion that the common GGG sequence exists in order to monitor oxidative damage (39). The same sequence may be involved in general damage recognition, as it is also implicated in UVA-mediated telomere shortening, which however, does not seem to proceed via triggering of oxidative damage (40). On the other hand, oxidative stress can also induce a senescent phenotype dependent on p21 but not p16, which is independent of telomere shortening (41). High glucose-oxidative stress may have the same type of effect *In vivo* also, ie. premature senescence of cells and their death by apoptosis (42). However, even with such a "harmless" supplement as Vitamin C, it may be necessary to take care to protect against over-dosing resulting in pro-oxidant activities (43). There is also extensive data on Vitamin E supplementation which enhances lymphocyte proliferative responses and IL 2 production *In vitro* and DTH *In vivo* in elderly people (44). This correlated with a decline in prostaglandin E_2 (PGE_2) synthesis, which is known to increase with aging. Vitamin E accomplishes this blockade of PGE synthesis via its inhibitory effects post-translationally on COX expression (45). The immunosuppressive effects of PGE_2 are predominantly mediated by increasing cAMP levels; therefore agents which decrease cAMP levels might also be expected to enhance lymphocyte responses. These may include insulin and chromium (46). Unlike a number of other proposed factors, the benefits of vitamin E supplementation have been subjected to fairly rigorous scientific testing. A recent substantial study concluded that vitamin E supplementation for 4 months improved a number of clinically-relevant indices of cell-mediated immunity in the healthy elderly, including DTH and antibody responses to hepatitis B and tetanus vaccines but without increasing autoantibody titers (47). Parameters of immune function *In vivo*, such as DTH responses, may also normalize in the nutritionally-deficient elderly following appropriate dietary vitamin and mineral supplementation for shorter periods (48). Another study looked at 6 month supplementation and found increased DTH and IL 2 production, but notably, decreased IFN-γ and increased IL 4; effects were most noticeable in that subset of the elderly who had started with lower baseline DTH and were less physically active (49). However, as noted above, studies actually recording clinical infection in these trials are few and far between.

In mice, some data indicate that certain senescence-associated biochemical changes which can be measured on T cells are prevented by *In vivo* treatment of mice with the anti-oxidant vitamin E (50). Thus, vitamin E supplementation prevented the observed age-related decline of anion transport by lymphocytes in mice and inhibited the generation of the "senescent cell antigen" (SCA) from the anion transporter "band 3". Prevention or delaying of band 3 aging and subsequent generation of SCA has the consequence that the lymphocytes are not eliminated from the system via SCA-mediated interactions with the reticuloendothelial system. By analogy with the mouse, vitamin E supplementation might be expected to have a greater impact on the old than the young; thus, in a mouse influenza model, high-dose vitamin E significantly enhanced lung virus clearance in old mice, with little effect on young mice (51). However, it is not known whether these effects of vitamin E are attributable to its anti-oxidant or some other function. For example, later studies in this mouse model indicated that Vitamin E increased Th1 cytokine production in old mice, probably by decreasing PGE_2 production and thereby contributed to protection against influenza (52). Vitamin E may block PGE synthesis by inhibiting COX activity (45). In addition to vitamins, several other anti-oxidant substances are being screened for anti-aging effects, and some of these are found to have beneficial effects on the immune system (53,54).

A poor vitamin D status is also frequently encountered in the elderly (55). The significant association of NK cell number and activity with vitamin D stores is of great concern and is consistent with the observations *In vitro* that vitamin D deficiency in humans and animals is associated with reduced innate immunity. Supplementation of $1,25(OH_2)D3$ in elderly subjects significantly increases circulating levels of type I IFN, cytokines involved in modulating NK activity (56) and maintaining T cell viability (57) without increasing levels of bcl-2 (but with divergent data on bcl-(xL) expression (58)). In addition,

vitamin D enhances the differentiation and proliferation of cells that possess the corresponding receptor. These activities may be responsible for antineoplastic effects, because vitamin D, together with IL 12 and retinoids, is a potent inhibitor of angiogenesis induced by tumor cells (59). Therefore, it is possible that maintained vitamin D3 levels and NK activity help to protect individuals from cancer in old age. Additionally, IL 12 itself may be able to reconstitute NK activity in old mice (60). Indeed, the "recommended dietary allowance" (RDA) for vitamin D (and calcium) has been just been increased by the USDA specifically for the elderly (61).

Another supplement commonly believed to enhance immunity is often taken along with other factors, namely ß-carotene. Indeed, in a Danish study, ß-carotene levels (without supplementation) were found to be negatively associated with mortality over a 6-year study period (62). However, results of two careful recent studies do not support immunoenhancing effects of either short (3 weeks) or long-term (up to 12 years) ß-carotene supplementation in randomized double-blind placebo-controlled longitudinal comparisons. There were no pre- to post-intervention changes measured in DTH, lymphocyte proliferation, IL 2 production, PGE2-production or lymphocyte subset composition (63). The same group, however, reported enhancement of NK activity without alterations in cytokine production by ß-carotene in the elderly, however (64). Supplementation with "natural" dietary products may be preferable to single factor supplementation and studies have examined various possibilities so far. Tomato juice is a rich source of ß-carotene and lycopene, and supplementation with it does result in increased plasma levels of these in the elderly, however, with few noticeable immunological effects (65). Similar findings have been reported for ß-carotene and lycopene supplementation, albeit at low doses in well-nourished healthy old individuals (66). Dietary supplementation with lactic acid bacteria as an isolated "natural" food component is also under investigation, and has been shown, for example, to enhance killing of the NK target K562 by PBMC, with a greater enhancement in the elderly (67,68).

Not only vitamin- but also trace element-deficiencies in the elderly may contribute to immunodeficiency. For example, levels of selenium decrease in rat lymphoid tissues with increasing age (69) and selenium supplementation has been reported to reverse low levels of proliferation and CTL generation in aged mice (70). In elderly people, selenium supplementation was reported to enhance lymphocyte proliferative responses to pokeweed mitogen (PWM) (71). Selenium levels, as well as zinc, are decreased in the oldest old (>90 yr), even in studies which do not reveal decreases in extremely healthy (SENIEUR) elderly (72). Moreover, it is well established that the availability of certain essential micronutrients decreases with age; for example, low copper levels result in decreased T cell proliferative response (73), possibly because it compromises the anti-oxidant defences of the cells (74). Another very important mineral factor may be zinc (75-78). Zinc is necessary for the function of many hormones and enzymes, including those known to affect immune responses (eg. testosterone (79), melatonin, thyroid hormones, growth hormone and prolactin (80,81)) The elderly have lower zinc levels and the disabled elderly, lower still, but these can be corrected by supplementation. Moreover, the copper to zinc ratio may be more informative than zinc alone, as this ratio may tell us something about the systemic redox balance of the individual (82). This has practical implications, because even if absorption is compromised in the elderly, sufficient supplementation of zinc might overcome the problem (83,84). However, caution is required because an accumulation of zinc might induce a derangement of brain functions and neuronal toxicity. This occurs because zinc easily enters neurones via Ca++ -A/K channels with subsequent neuronal cell-death or necrosis (85). Therefore, it may be necessary to administer physiological doses of zinc (2-3 times the RDA/day), for short periods (1-2 months), using periodical cycles (86). In rats and mice, there is also a decrease in serum zinc with advancing age, and the levels found in the thymus are lower compared to young animals as well (86,87). In particular, free zinc ion bioavailability (measured by the total thymulin/active thymulin ratio) is compromised in central and peripheral immune efficiency (80).This is a more relevant measure than total zinc content in plasma or tissue. Indeed, old mice display low free zinc ion bioavailability despite plasma zinc levels within the normal range (84). These observations on zinc levels, or better on free zinc ion bioavailability, are not be limited to rodents. Also in humans (the elderly and Down's syndrome as a model of accelerated aging) low free zinc ion bioavailability may apply despite plasma zinc levels within the normal range. Zinc supplementation studies in old people and in Down's syndrome subjects have indeed suggested improvement of some parameters of immune function (88-90). One reason for this may be the zinc-enhancement of otherwise age-associated lowered levels of interferon-alpha production in the aged (91). Experimental zinc depletion and repletion of healthy humans revealed that secretion of the Th1-type cytokines IFN-(??and IL 2 was decreased during zinc deficiency, whereas Th2-type cytokines (IL 4, IL 6, IL 10) were not affected (92). Zinc supplementation for one month at a physiological dose (12 mg/Zn++/day) in old people increases CD4+ cell number and prevents the decrease in CD4+ cells, which is the main risk factor for severe infectious lung disease (90). The same is true in Down's syndrome, where zinc supplementation decreases infectious episodes with subsequent better quality of life, as well as in AIDS (90), acrodermatis enterophatica, leprosy and herpes (93) Therefore, zinc supplementation can improve immune efficiency with a clear benefit against infections. In animals, Mocchegiani *et al*. (84) confirmed that oral zinc supplementation resulted in a recovery of thymic function (and also demonstrated its influence on extrathymic T cell differentiation pathways (94)] and showed that thymic regrowth was associated with a partial reconstitution of peripheral immune function (as measured by mitogen stimulated proliferation and NK activity). Moreover, low levels of activity of the zinc-dependent hormone thymulin were not dependent on the state of the thymus itself, but on decreased zinc saturation of the synthesized hormone. The

authors concluded that age-dependent thymic involution and compromised thymic hormone function were not pre-programmed but were caused by the decreased bioavailability of free zinc ions. Indeed, zinc supplementation in old mice also prolongs survival with a significant decrease in infections and degenerative diseases, confirming the crucial role played by zinc in responses of T cells of both thymic and extrathymic origin (86). T cell apoptosis may also be blocked or partially blocked by zinc (95). There is a wide range of possible mechanisms involved in the immune restoring effects of zinc from direct effects on DNA-RNA polymerases to indirect effects via thymulin activation etc. Among the latter, zinc-bound metallothioneins (Zn-MT) play a pivotal role because the task of these proteins is mainly to regulate zinc homeostasis during transient oxidative stress. Zn-MT synthesis is affected by pro-inflammatory cytokines (IL-1, IL-6). Zn-MT sequester zinc, but in old mice, in the elderly and in Down's syndrome patients they may be less able to release the zinc again because levels oxidative stress are higher in the aged (96). The resulting low free zinc ion bioavailability depresses immune responsiveness (80,81). Indeed, MT gene expression is greatly increased in T lymphocytes from old people and Down's syndrome subjects in comparison with young-adults and with exceptional aged individuals, such as centenarians (81). Consistent with this, transgenic mice overexpressing metallothioneins undergoing constant stress (10 days darkness) display a reduced thymic cortex, low zinc ion bioavailabilty, depressed NK activity (96). These findings have lead to the suggestion that Zn-MT are potential biological and genetic markers of immunosenescence (96). Therefore, zinc turnover, via Zn-MT homeostasis, is crucial for immune efficiency at all ages but dysregulated in the elderly. In this context it is also interesting to note the claim that the beneficial effects of melatonin supplementation or pineal grafting are associated with increased plasma zinc levels in old mice in the absence of exogenous zinc supplementation (97-100) (although melatonin may have direct effects on lymphocytes, which express melatonin receptors (101), ligation of which results in signal transduction and diacylglycerol production (102)). *In vitro* experiments on thymic explants from old mice have clearly shown that it is zinc, rather than melatonin itself, which restores thymic efficiency, suggesting that the immunomodulatory effect of melatonin also occurs via regulation of the zinc pool (100). This is consistent with the finding of an identically prolonged survival in old mice either after melatonin treatment or after zinc supplementation (100). Moreover, grafting of pineal glands from old mice to young decreases the life span of the latter, paralleled by decreased plasma zinc levels, as well as decreased numbers of lymphocytes and decreased thyroid function. Other hormones with an immunomodulatory role, such as growth hormone (GH) and triiodothyronine (T3), may also affect the immune system by means of the zinc pool. *In vitro* and *In vivo* experiments in old mice have shown that also here, zinc, rather than GH or T3, affects the immune system (103). A recent study on transferrin supplementation of old mice, in which several immunosenescent parameters were reported to be reversed, was also suggested to be due to effects on zinc (104). It has been argued by Fabris *et al*. that the common pathway of several life-extending endocrinological manipulations is in fact via zinc bioavailability (105). Thus, even such a well-established concept as the inevitability of the age-associated process of thymic involution and the resulting perturbation of T cell generation may not be immutable. However, the beneficial effects of zinc supplementation are still controversial and others have found no benefit of zinc replacement even in elderly populations confirmed to show serum zinc deficiency (106,107). Moreover, thymopentin alone *In vitro* may increase the precursor frequency of proliferating T cells from old subjects (108). In some studies, even inhibitory effects of zinc supplementation have been reported (109,110). It has also been found that the degree of decrease of lymphoproliferative responses observed in the elderly does not correlate with decreased levels of plasma zinc (or vitamin E, retinol or ß-carotene) (111). Other recent studies have also found little or no effect of supplementation over a year with minerals such as zinc and selenium, either alone or combination with vitamins (C, E, ß-carotene) on lymphocyte proliferation or subset distribution (112). The situation is therefore not yet fully clarified. However, the fact that physiological zinc supplementation for short periods and in periodical cycles is of benefit in the elderly for improving immunity with prolonged survival in old mice and removal of CD4+ risk factors for severe infections in old people (90,100), support the careful application of zinc supplementation to reverse immune damage in aging. Indirect evidence in myasthenia gravis may also support this interpretation. High zinc, enhanced numbers of activated T-helper cells and increased IL-6 are observed in myasthenic patients. Thymectomy normalizes zinc levels and immune functions (113). Moreover, direct evidence showing low Zn-MT, normal zinc bioavailability and good immune responses in centenarians in comparison with old people support the necessity of a good zinc ion bioavailability for immune efficiency and subsequent successful aging (114).

In mice, T cells from old animals stimulated by CD3 + CD4 ligation mobilize less calcium ions than T cells from young animals. They also perform less tyrosine phosphorylation of phospholipase C gamma 1 and other phosphoproteins. Moreover, these events appear to be sensitive to anti-oxidant levels, such that Grossmann *et al*. suggested that one reason for decreased PLC gamma-1-dependent signaling was the decrease in antioxidant levels in old cells in rats (115). The general importance of anti-oxidant systems is illustrated by the report that although resting young and old rat splenocytes did not differ in their content of the important anti-oxidant reduced glutathione, in proliferating cells from old animals, the expected increase in glutathione was delayed. This correlated with an increasing number of cells showing evidence of mitochondrial dysfunction in terms of depolarized membrane potential and decreased mitochondrial mass (116). Similar findings on mitochondrial dysfunction have been reported for enhanced activation of permeability transition and decreased energy metabolism in old mice (117). Long-lived animals have lower steady-state levels of oxidative damage in mitochondrial DNA (of post-mitotic cells) compared to short-lived animals (118,119), and there

is a correlation between maximum species lifespan and the rate of mitochondrial oygen radical production (120). There may be compensatory mechanisms in the elderly, at least in humans, where increased amounts of mitochondrial DNA in lung tissue have been reported, possibly stimulated by oxidative stress (121). The above impairment in rats was completely prevented by addition of extra glutathione to the medium (116). Consistent with this finding, addition of glutathione or its precursor N-acetyl-L-cysteine, to culture medium also restores the CD3-stimulated proliferation of T cells from old mice (122). An *In vivo* relevance for these findings is suggested by the fact that reduced, but not oxidized, glutathione levels in the plasma are decreased in elderly compared to young donors (123). Moreover, the degree of oxidative damage to mitochondrial DNA in heart and brain in 6 mammalian species tested is said to be inversely related to their maximum lifespan (118). On the other hand, *In vivo* treatment of old rats with acetyl-L-carnitine reverses the age-associated decline of mitochondrial membrane potential and increases ambulatory activity; however, perhaps as a result of improved mitochondrial function, levels of oxidative stress were increased (124). Therefore, treatment with both acetyl-L-carnitine and anti-oxidants might be successful, but has not yet been reported. A range of novel anti-oxidants is currently being developed and screened *In vitro* with a view to application *In vivo*. For example, PBN, a spin trap, scavenges hydroxyl radicals and delays senescence *In vitro* in human cells and *In vivo* in mice, apparently via an effect on mitochondria (125). Additionally, treatment with co-enzyme Q(10) may reverse some of the age-associated decline in mitochondrial function (126).

Another report has also documented age-associated decreases in plasma GSH levels and confirmed that this is more oxidized in the elderly than in the young (127). As glutathione levels decrease, those of homocysteine increase (128). Measurements of concentrations of glutathione actually within human lymphocytes have found age-associated decreases, with GSH levels in lymphocytes from both male and female 60 - 80 year-olds being significantly lower than in 20 - 40 and 40 - 60 year-old groups (129). Further studies have shown that not only the level of glutathione in human lymphocytes is inversely correlated with age, but also levels of Vitamin C and that levels of both show seasonal variation, being higher in the summer (130). Further, as glutathione levels decrease, those of homocysteine in the cells as well as plasma increase (128). Increased homocysteine and deceased glutathione will lead to increased lipid peroxidation and oxidative stress. In mice, however, the age-associated reduction of GSH levels did not correlate with increased susceptibility of lymphocytes to oxidative damage. This was found to be due to a predominance of memory cells in the aged animals and the fact that memory cells, despite lower GSH, were more resistant (in young and old mice) to oxidative damage (131). Measurements of anti-oxidant function in rats indicated that levels of anti-oxidant activity in several tissues decreased with age (132). Some evidence consistent with a decrease of anti-oxidant activity with age was also found in human plasma, but only in males and then only over the age of 74 (133). Others have also noted similar anti-oxidant status in healthy elderly men with good nutritional parameters; indeed, serum glutathione peroxidase was increased (134). Hack *et al*. reported a significant age-associated increase in plasma cysteine levels and decrease in plasma thiol levels in 205 donors of both sexes (135). Another study has confirmed decreased antioxidant activity in aged rat plasma but failed to show the same in man (136). Measurements of superoxide dismutase (SOD) in red blood cells in humans revealed a possible age-associated slight decrease in males but not females (137). Centenarians show decreased levels of SOD in plasma and RBC, but have high levels of Vitamins A and E (138). Selected long-lived strains of Drosophila possess enhanced anti-oxidant defences including SOD compared to short-lived strains (139). Strikingly, transgenic fruit flies expressing enhanced levels of superoxide dismutase enjoyed a lifespan up to a third longer than wild-type flies (140), but this degree of benefit had not been seen with transgenic mice (141,142). Indeed, transgenic expression of 2- to 5-fold increased levels of copper/zinc superoxide dismutase slightly decreased murine lifespan (143). Nonetheless, using synthetic small molecule superoxide dismutase/catalase mimetics significantly extended C. elegans lifespans (144). Sublethal oxidative stress leads to rapid acquisition of a state similar to replicative senescence (145,146), consistent with the idea of "oxidative aging". However, certain other agents which successfully reduce indicators of oxidative damage in old worms, and which do extend mean lifespan, have had no effect on increasing the maximum lifespan (147). This may be related to the finding that at least one particular C. elegans mutation extending lifespan is dependent on external supplies of coenzyme Q, essential for ubiquinone synthesis and hence respiration (148).

Recent studies comparing global gene expression in Drosophila as a function of age or exposure to paraquat, a free-radical generator, have revealed many similar changes, but also some as a result of age but not paraquat-treatment (149). This suggests that free-radicals and oxidative stress play a major part in aging but are not the only factors. Strikingly, reducing oxidative stress in houseflies by the mere expedient of preventing them from flying, may greatly increase lifespan (150). Moreover, lifespan-extending mutations to the daf-2 gene in C. elegans, which encodes an insulin receptor-like molecule, may function via the Mn-superoxide dismutase system (151). This may make the argument for using anti-oxidants more compelling. Intriguingly, daf-2-knockouts re-expressing daf-2 only in neurons, but not in muscle or intestine, reverted to a normal shorter lifespan. However, the metabolic defects of daf-2 loss were recovered by daf-2 expression only in muscle. This de-couples regulation of lifespan from metabolism in C. elegans (152). If generally applicable, this is therefore a key finding, because it suggests that lifespan is regulated by the nervous system and not by metabolic rate.

The above finding may contribute to the emerging picture that the results of anti-oxidant supplementation are thus far not very positive even in rodents (153,154), although recent studies using N-acetyl-

cysteine may be more encouraging (135). N-acetyl-cysteine also restores responsiveness to synovial lymphocytes from RA patients (155) and T cells of old mice (122). Modern approaches involving gene therapy to over-express certain proteins may prove more successful; for example over-expression of the enzyme peptide-methionine sulphoxide reductase can protect human T cells from oxidative stress (156). A note of caution may need to be sounded regarding such interventions, namely, that reactive oxygen species are required for many biological processes, possibly including T cell activation, IL 2 secretion and proliferation, which can be blocked by anti-oxidants (157). Similarly, responses to hematopoietic growth factors such as GM-CSF and IL 3 also depend on ROS generation and can be blocked by anti-oxidants, eg. NAC (158). This may apply in certain circumstances even to fibroblasts (159). Nonetheless, an essential component of the T cell response, namely IL 2 production, does seem to be very sensitive to oxidative stress and irradiation, and NAC is a potent protective agent for both stressors (160). Therefore, interventionist approaches with antioxidants remain attractive because of their cheapness and easiness. There is therefore a significant ongoing effort to develop novel classes of antioxidant which are not primarily screened for their activity on the immune system, but which may exert some of their effects by this mechanism (161,162). An interesting recent report described protection of lymphocytes from apoptosis by C60 carboxyfullerene (163).

However, one recent finding that needs to be borne in mind here is that in mice, an antioxidant-rich diet can encourage tumor cell growth, whereas depletion of antioxidants results in increased tumor cell but not normal cell apoptosis and decreased tumor growth (164). Were this to be the case in humans, obviously antioxidant supplementation might be dangerous. However, mice seem to lack many antioxidant defences found in humans and probably human tumors would not be affected anyway by increasing free radical flux due to reducing antioxidants in the diet.

Conjugated linoleic acid has been suggested to have immunoenhancing properties and was therefore tested in young and old mice (165). IL 2 production and T cell proliferation was significantly enhanced in old mice. Other substances may have unexpected effects such as the recently reported ability of the non-steroidal anti-inflammatory agent ibuprofen to protect low-density lipoproteins against oxidative damage, and hence possibly reduce atherogenesis (166). Aspirin, in the same family of drugs, may have similar effects. This could be important in an immunological context because oxidized LDL have been reported to suppress CD4+ T cell proliferation and IL 2R expression *In vitro* (167). Interestingly, the cytokine IL 10 has also been reported to possess anti-atherosclerotic properties in mice (168). What this might mean in the context of increased IL 10 levels in the elderly is unclear.

Sulphydryl compounds such as 2-Mercaptoethanol (2-ME) have been described to be effective *In vivo* and *In vitro* in restoring impaired immune functions in aging mice (169,170). In particular, in aging mice, 2-ME has been described to be a potent mitogen which synergistically acts with other mitogens, to restore antibody responses to T-dependent antigens, to enhance hematopoiesis, as measured by CFU activity of bone marrow cells, and to induce IL 2 production (171-174). The immunopotentiating activities of 2-ME seem to be related to its effect on cyclic nucleotides and prostaglandin metabolism (175). Thus, it appears a promising chemical compound in preventing not only the aging of the immune system but also the diseases associated with death and reduced lifespan.

7.2. Hormones

Aging of the immune system will most likely affect other organ systems and eventually impact upon the lifespan of the individual (176). Manipulations said to increase lifespan of mice by injecting melatonin or by transplanting pineal glands are accompanied by maintenance of T cell immune responsiveness (as measured by DTH) and prevention of thymic involution (177). As mentioned above (7.1), melatonin may have direct effects on CD4 but not CD8 cells because of a direct effect on gene regulation via binding the putative nuclear melatonin receptor (178). It may also reduce DNA damage to lymphocyte DNA caused by radiation (179). However, *In vivo* treatment with melatonin is reported not to reconstitute age-associated impairment of NK activity or lymphoproliferative responses in mice (180), although it has been found to reconstitute depressed humoral responses in aged rats (181). Consistent with these results *In vitro* supplementation also failed to reconstitute proliferation or IL 2 production in old rat cells (182) or old mouse cells (183). Other results have shown that melatonin is able to enhance the antibody response to a T-dependent antigen, when chronically injected into mice immunosuppressed by aging or by cyclophosphamide (184). This enhancement of the antibody response is associated with increased induction of T helper cell activity and IL 2 production. The effects of melatonin as immunotherapeutic agent are in line with the observation that the inhibition of melatonin synthesis by administration of β-blockers leads to a significant depression of humoral and cell-mediated responses in mice (185,186) and that surgical pinealectomy leads to decreased IL 2 production, which is restored by melatonin administration in mice (187). Melatonin may be one of many hormones the levels of which are commonly decreased in the elderly (188). This is also seen in rhesus monkeys, and can be prevented by caloric restriction (189). Like caloric restriction, melatonin administration also reduced body temperature (190,191). However, plasma melatonin levels are not necessarily reduced in the very healthy elderly (192), and it is in any case unclear whether and how melatonin influences immunosenescence. For a critical review of the "anti-aging" effects of melatonin, see (193).

Age-associated changes in secretion of growth hormone (GH) and related hormones, releasing factors and binding factors may contribute to immunosenescence. Thus, GH substitution may reverse some immune defects in humans and primates, as reviewed in (194). Administration of low-dose GH to elderly adults for 6 months resulted in an increase in IGF-1 levels (which are reduced in aging

(195) even in centenarians (196)) and an improvement in some physiological parameters, such as muscle strength (197). Immunological parameters were not reported. However, it has been known for some time that GH and/or prolactin supplementation can improve some parameters of immune function in old rats, albeit not to the level seen in young rats (198). In humans, a comparison of plasma IGF-1 levels with T cell (but not B cell) proliferative responses in 34 healthy young and 41 healthy elderly donors revealed a significant correlation between the two (199). However, levels of free IGF-1 are reported not to decline with age; indeed the very elderly showed increases in IGF-1 levels, possibly again suggesting selective pressure to maintain levels of this hormone (200). However, other studies do indicate decreased IGF-1 plasma levels in both sexes, independent of alterations in body mass index (201). Increased IGF-1 availability may also increase thymic cellularity and presumably thymic output in some animal models (202). On the other hand, higher titers of IGF may be associated with cancer susceptibility and progression in mice (203). In C. elegans, the insulin/IGF system appears to act as a pivotal control of the aging rate of the whole animal, which is influenced by the environment (equivalent to "calorie restriction"?) and by reproduction, where the gonads directly affect lifespan (204). The same also seems to apply to Drosophila (205,206). However, in general, findings with GH supplementation do not favor a major benefit from use of this factor in the elderly (207). In fact, overexpression of GH in transgenic mice is associated with reduced life expectancy and symptoms of premature aging (208), although supplementation of mice or rats did not have this effect (209). However, this latter study also found no benefits of GH supplementation in animal aging (209). The same kind of paradox is seen in dwarf mice - deficiency of GH, prolactin and other factors (eg. TSH) is associated with increased lifespans; levels of glutathione and ascorbate were lower in the livers of these animals (210). These results are reciprocal to expectations; however, in long-lived dwarf mice, catalase activity in liver and kidney was significantly increased, whereas it was decreased in short-lived GH-transgenics (210,211) suggesting perhaps that this is a factor more critical to longevity. Moreover, KO-mice with targeted disruption of GH receptor genes show a ca. 50% reduction in body size, resistance to the effects of GH, decreased plasma IGF-1 levels – and increased longevity (212). However, prolactin levels are increased in GH-R-KO mice (213) Small body size may be positively associated with longevity, as illustrated in a long-lived strain of mice (214) and possibly even in humans (215). Therefore, given also that increased IGF-1 is associated with increased cancer, there is decreasing enthusiasm for growth factor supplementation therapy (216). On the other hand, it may be that bolus application of GH is an inappropriate mode of treatment; rather, it has been suggested that more physiological circadian pulsatile rythms would be more effective (217). Nonetheless, the critical role of loss of GH in extending murine longevity suggests pleiotropic effects of GH, obviously required for development but thereafter deleterious. Studies have shown that even on a relatively long-lived genetic background, GH loss-of-function mutants live up to 40% longer, show delayed age-dependent collagen cross-linking, and delayed alteration of several age-related parameters of immunity (218). Differences in gene expression being monitored by DNA-array technology will reveal further differences, as in eg. IGF-1 and possibly IGF-binding proteins, heat shock proteins, and p38 MAPK (219).

The same considerations may apply to other factors, eg. the native steroid DHEA or DHEA sulfate (DHEAS), which, like most steroids, has immunomodulating activity. Whereas levels of cortisol increase with age in both men and women (220), and may induce suppression (221), in general the levels of DHEA decline with age (222). However, long-term longitudinal studies suggest a great deal of inter-individual variation, and can even show age-associated increases in DHEAS levels in a sizeable proportion of the population (223). Some of the variation may be due to confounding factors such as smoking or obesity (224). As with changes in other hormones with age, there may also be gender differences (225) that have not always been taken into account. There are also differences between DHEA and DHEAS which may complicate matters if not specifically recognized (226). A 5-year longitudinal study in 3 age groups failed to find a significant association between DHEAS levels and morbidity or mortality (227). However, interest has been maintained in DHEA because it has been suggested that decreases of DHEA could be associated in some way with immunosenescence. This derived from findings that treatment of old mice with DHEA augmented the otherwise decreased capacity of T cells to produce IL 2 and IFN-γ. It also decreased the spontaneous secretion of IL 6 (228) and IL 10 observed in old mice and reversed their hypersensitivity to endotoxin-stimulated release of both IL 6 and IL 10 (229), as well as enhancing lymphocyte activation (230), although not in the mucosal immune system (231). On the other hand it increases IL 10 serum levels (232). Analogously, it prevents the retrovirus-induced increased IL 6 and IL 10 secretion seen in old mice, prevents decreases of IL 2 and IFN-gamma production and enhances their T and B cell proliferative responses (233). IL 6 may be the critical cytokine here, because treating aged mice with IL 6- but not IL 1-neutralizing antibody resulted in a reversion of their cytokine production pattern to that characteristic of young animals (234). Some of the effects of DHEA may be mediated through its ability to minimize damage associated with elevated oxidation and loss of anti-oxidants in aging and retroviral infection (235), and restoration of the redox balance via activation and reconstitution of PPAR as mentioned above (236). DHEA reverses the senescent phenotype (as defined by the pattern of cytokine secretion) in mice and enhances the effects of vaccination of old mice to hepatitis B (237). Application of DHEA together with melatonin may have a limited additive effect (238). DHEA together with oral anti-oxidants in mice may also show an increased benefit compared to DHEA alone (239).

There is a reported association in human as well as mice between decreased DHEA and increased IL 6 in the aged; furthermore, DHEA was shown to inhibit IL 6 secretion from mononuclear cells of the elderly (240). In

women, DHEAS levels decreased with age in a cross-sectional study, in correlation with decreased IGF-1 and increased IL 6 (241). DHEA also enhances IL 2 production (242). There is continued interest in DHEA supplementation trials which have been going on for over a decade and are still continuing (eg. see (243-245)), but there are few immunological studies thus far. Khorram *et al*. (246) found that DHEA administration to men resulted in a significant augmentation of serum IGF-1 and decreased IGFBP-1, which may contribute to immune enhancement. They also found an increase of monocytes during treatment, as well as increases in mitogenic responses of both T cells and B cells. The numbers and activity of NK cells were also enhanced. Increases in NK activity had been found in women after a shorter period of DHEA supplementation (247).

In contrast to DHEA, dihydrotestosterone (DHT) downregulates IL 4, IL 5 and IFN-γ production but does not affect IL 2 (248). DHT levels also decrease with age (249), and a recent cross-sectional study found that bioavailable testosterone correlated best with significantly age-associated cognitive and physical parameters (250). Testosterone-supplementation trials have shown that 3 years of treatment of men over the age of 65 years resulted in significant improvement of fat mass/lean mass ratio, but no immunological studies were done (251). However, a recent longitudinal study found no correlation between entry-point testosterone levels and death rates over the 15 year follow-up period in 77 men (252). Together, DHEA and DHT supplementation alter the cytokine profile of old mice such that it again resembles that of young mice; such an activity could be measured *In vivo* as well as *In vitro* (248). Exogenous hormone supplementation might correct age-associated defects insofar as these are dependent upon cytokine profiles. This has been tested in a mouse model of influenza virus vaccination. Danenberg *et al*. (253) reported that DHEA supplementation resulted in a reversal of the age-associated decline in immune responsiveness in mice, reflected by increased humoral responses in treated mice and increased resistance to challenge with live virus. In another study, Ravaglia *et al*. (254) reported on the relationship between DHEA levels and health in free-living people over the age of 90. They found five-fold lower levels of DHEA in both males and females aged 90-106, compared to young controls. Thus, even "successfully" aged persons had greatly reduced levels, leading to the question of whether this matters. Ravaglia *et al*. demonstrated that it can matter, because within the old male group at least, the level of DHEA correlated with their health, as measured on the ADL scale. On the other hand, DHEA levels are clearly reduced in the aged although the degree of reduction fails to correlate with health status as assessed by the strict SENIEUR protocol (255). A supplementation trial to assess the effects of DHEA on responses to tetanus and influenza vaccination in man did not yield as dramatic effects as seen in mice (256): there was a trend toward increased antibody titers to influenza but not tetanus, and even this failed to reach significance (256). Danenberg *et al*. even reported a decrease in attainment of protective antibody titer in elderly volunteers given DHEA in a prospective randomized placebo-controlled double-blind study of the effects of DHEA on influenza vaccination (257). Thus, the decreased flu response in elderly humans, unlike that in mice, could not be reversed by DHEA, and a higher baseline level of DHEA was also not found to be predictive of better flu vaccination outcome (257).

Many years ago, it was demonstrated that T cell functions of old mice can be improved by the administration of natural (258-260) or synthetic thymic peptides (258,261-263). In one of these studies, it was found that injection of a few nanograms of Thymic Humoral Factor (THF)-γ2 into immunodeficient old mice raised the frequency of mitogen-responsive T cells in the thymus and in the spleen as well as the frequency of cytokine-producing splenic T cells, up to the level observed in young mice. Moreover, injection of THF-γ2 was found to restore IL 2 production by mitogen-stimulated spleen cells. Also, the helper activity of splenic T cells was enhanced by this treatment and increased with the THF-γ2 increasing dose over a wide range. Similarly, the effects of two other synthetic thymic factors, Thymopentin and Thymosin-α1, on T helper cell activity increased with increasing injected dose, but the efficiency of THF-γ2 was greater than that of Thymopentin and Thymosin-α1 (264). It has also been demonstrated that the effect of thymosin-α1, a 28 amino acid peptide identified in the bovine thymus extract Thymosin Fraction V (265), on the recovery of T cell functions in aging mice is restricted to the N-terminal half of the molecule (N-14), whereas the C-terminal peptide (C-14) is devoid of biological activity (262,266). The immunorestorative activity of Thymosin-α1 may depend not only on the increase in the precursor frequency of mitogen-responsive T cells but also on the increased number of IL 2R and on the enhanced production of IL 2 that, in turn, favors the expression of IL 2R (266). Chronic injections of Thymosin-α1 or N-14 (active peptide) or C-14 (inactive peptide), weekly, for 12 months, starting at the age of 3 months, were able to enhance T cell functions during the first 6 months of treatment but, although devoid of demonstrable toxicity, did not prolong the lifespan nor reduce the incidence of lymphomas and solid tumors at death (267). In another longitudinal study (268), mice of 3 different strains were injected with different preparations of thymic hormones (Thymosin Fraction V, Facteur Thymique Sérique or Thymopentin), for 3 weeks, starting at 2 months of age, and followed until spontaneous death. Although none of the thymic hormones used was toxic, neither one was able to prolong lifespan in any of the 3 strains of mice. Other groups have been more successful in slightly extending rodent lifespan using various preparations and peptides; lifespan extension is commonly asssociated with decreased spontaneous cancer occurrence in these models (reviewed in (191)).

Given the known or suspected interactions between the endocrinological and immunological systems and the well-established impact of sex and other hormones on immune responses, it is perhaps surprising that few studies have addressed the question not only of gender differences but also the effects of pregnancies on immunosenescence. Some investigators have begun to

approach this by surveying leukocyte subsets in mice of varied gynecological histories. One such study concluded that both gender and pregnancies affect the age-related distribution of lymphoid and macrophage populations in the spleens of C57Bl/6 mice, for example (269).

7.3. Caloric restriction without malnutrition

Many studies have examined the effects of caloric restriction (CR) on lifespan in different species, even yeast (270) and including, to a more limited extent, primates (271). Lifelong dietary restriction, beginning at 3 to 6 months of age in rodents, performed in accordance with the concept of "undernutrition without malnutrition", has been described to decelerate the rate of aging, increase the mean or maximum lifespan and decrease or delay the occurrence of many spontaneous cancers (272,273). Conversely, food restriction starting at 12-13 months of age was found to increase the mean and maximum lifespan only slightly as compared to control mice on a normal diet (274). Restricted energy intake without malnutrition is the only proven way of extending lifespan not only in rodents, but also very likely in primates. Moreover, it appears that some of the physiological changes observed in rodents and primates as a result of CR may also be seen in humans, according to results from the albeit imperfect Biosphere 2 experiment (275). Other, to a great extent circumstantial, evidence does suggest that a similar effect may apply also to humans (276). Although it might be difficult to put CR without malnutrition into practise in humans, detailed knowledge of the mechanisms of life extension by CR might indicate how to achieve the same effect without starvation (277). This has led to the suggestion that anti-diabetic drugs, improving tissue glucose utilization, might be one way of achieving this (191). There remains perhaps also the possibility that CR-mediated decreased body temperature may contribute (190). Immune functions are extremely sensitive to body temperature variation (278). This finding would suggest that a way of delaying the onset and/or reducing the rate of immunological aging consists of slowing down the general metabolism by periodic mild hypothermia. Liu and Walford have demonstrated that mild hypothermia can prolong the lifespan of poikilothermic annual fresh-water fishes, especially when they are subjected to hypothermia during the last half of their life (279,280).

What then, if any, are the effects of CR on immune responses? Some argue that the main affects of CR are in fact mediated through the immune system (281). Although this an extreme view, clearly CR does have effects on the immune system which might contribute to the phenotype of CR animals. In rodents, it is argued that CR generally enhances immunity (281), although it reduces thymic cellularity but also lymphomagenesis (282). However, the situation in primates may once again be different to rodents. In rhesus monkeys, $CD4^+$ cells from old donors also respond less well to CD3-stimulation, and this is partly associated with a decreased frequency of responding cells and is reflected in lower calcium-mobilization in old cells (283). Here CR failed to alter the depressed calcium-mobilization rates in old monkeys. It did, however, retard the marked age-associated decline of DHEAS levels in rhesus monkeys (284). It also ameliorated the levels of lipid peroxidation of lymphocytes, supporting the view that CR effects are at least partly mediated through reduced free-radical damage (285).

Decreased IL 2 production, which was reversed by CR in rodents, was associated with a reduction in the age-related decline of the transcription factor NFAT, essential for IL 2 gene transcription (286). This group also showed that the age-associated reduced induction of T cell signaling molecules such as those of the Ras/MAPK pathway and also calcineurin could be reversed by CR in rats (287). Moreover, CR reduced levels of a marker of oxidative DNA damage in old rats (288). General metabolic pathways impacted by CR and having wide influence on multiple physiological and immunological process may be based on the effect of CR in reducing the production of free radicals. Thus, CR reduced in parallel the generation of ROS, the activation of COX-2 required for PGE synthesis, and the binding activity of NF-κB (289). In mice, CR protects against cancer occurrence and progression, associated with a decrease in IGF-1 levels, a factor having anti-apoptotic effects on cancer cells. In rats, CR also results in a decrease in IGF-1 together with an increase in GH levels (290). IGF-1 restoration in CR mice reverses the protective effect of CR (203). The age-related decline in melatonin production is, however, not prevented by CR in rats (291), although recent data suggest that it may indeed be in primates (189). Identifying the metabolic pathways critically influenced by CR or finding CR mimics might result in a wider-spread adoption of this approach by humans than is likely for CR itself. Thus, the findings by Troyer *et al*. (albeit in short-lived B/W rats) are of interest in that many of the effects of CR (ie. maintained naive T cell levels, maintained responses to mitogens, decreased breast cancer, decreased autoimmunity, decreased IL 6 levels, etc) could be duplicated by administration of omega-3 fatty acids (292). It was suggested that the mechanism for this involved the enhanced induction of anti-oxidant enzymes in the liver, associated with an increase in lymphocyte apoptosis (292). On the other hand, feeding (young) mice n-6 or n-3 PUFAs reduced the levels of Th1-type cytokines secreted (293); if this were also true for older animals it could be perceived as deleterious to immune function.

CR has multiple effects which are only now being elucidated, eg. by examining gene expression is micro-arrays. One such study recently examined the expression of 588 genes in young and old mouse livers, and revealed 6 differentially expressed genes. One of these was affected by CR; on the other hand, comparing old CR with old ad libitum-fed (AL) mice revealed an additional four differences (294). Studies on rhesus monkeys have also identified age-associated upregulation of skeletal muscle genes involved in inflammation and oxidative stress, and downregulation of those involved in mitochondrial electron transport and oxidative phosphorylation; however, adult-onset CR did not modify these patterns, unlike in the mouse (295). Such studies make a start in analysing age-associated changes in different organs and effects of CR and caution that the results may be markedly different in different

species. Other studies approach the question of mechanisms of action of CR by targeting candidate markers and examining the effects of CR on these. For example, some data show that old CR mice retain better GH receptor function than old AL mice (296). A major mechanism may be via the lowering of nutritionally-driven insulin exposure which lowers overall growth factor exposure (297). In mice, CR decreases enzymatic capacity for glycolysis and increases the capacity for hepatic gluconeogenesis (298). Markers of skin collagen glycation and oxidative damage predict early deaths in CR (as well as non-CR) mice, further suggesting decreases of glucose tolerance contributing to longevity determination (299). One relatively clear finding in monkeys is that CR reduced body temperature, as a result of decreasing energy expenditure, consistent with the "rate of living" theory of aging (190), although there are no changes in the proliferative potential of skin cells in CR monkeys (300). A 30% calorie reduction in rhesus monkeys, even if started when they were already 10 years old, resulted in decreased blood insulin and glucose levels, better insulin sensitivity etc., as in rodents (301,302). CR also prevents accumulation of mitochondria with oxidative damage in mouse skeletal muscle (303). Not all data support the idea that longevity enhancement by CR in rhesus monkeys is correlated with improvement of immune responses. For example, Roecker *et al*. (304) showed that mitogen-stimulated proliferation, NK activity, and antibody production were all reduced in CR monkeys compared to controls, with no effects discernible on cell number or surface markers. On the other hand, the greater levels of IL 6 production caused by oxidative stress in old compared to young rhesus monkeys were reported to be reduced in CR animals (305) and in a long-lived rat strain, CR clearly resulted in improved T cell proliferation after mitogenic stimulation. This, and the cytokine (higher IL 2, lower IL 6 and TNF-α) and surface marker (higher OX-22) profiles of the T cells suggested that the CR animals had a higher fraction of "naive" cells compared to the controls with more "memory" cells (306). However, in two other rat strains, Konno (307) had shown accelerated thymic involution in CR animals, and either a slight decrease or no change in immune function. This suggests that the genetic background has a major impact on the effects of CR. In mice as well, CR results in decreases in the otherwise age-associated increased constitutive serum levels of IL 6 and TNF-α (308), and resulted in preserved thymic cellularity (but not size) coupled with preservation of the levels of naive peripheral T cells (309). In addition, the IL 6-related factor leptin, which regulates food intake but also has effects on T cells, and which, like IL 6, is increased in infection and inflammation, is reduced by CR in mice (310). However it must be noted that leptin levels have been reported to decrease with age in (AL) humans (311). Beneficial effects of CR may also still be obtained after middle-age, at least in mice, where maximum life span could still be increased. Paradoxically, the rate of some cancers increased in CR mice, so that the percentage of animals dying with eg. plasma cell cancers was higher (312). This investigation also included DHEAS administration, which was found not to alter either longevity or cancer occurrence (312). All in all, of the multiple effects of CR, it seems that particularly in primates the main life extending mechanism may not be immunological or not directly immunological. The overall value of CR is still not established. Mathematical modeling of mortality curves in Drosophila have confirmed the importance of free radicals in aging, but in rodents the effects of CR or treatment with anti-oxidants have not been shown to impact on rates of aging (313).

7.4. Mutations and DNA repair; cell cycle control

Large scale studies taking various factors such as smoking into account, have revealed that the amount of chromosomal damage in human lymphocytes shows age-associated variation. Chromosomal abberations increase gradually with age, and frequencies of micronuclei (MN) show dramatic increase peaking at 50 – 60 years and remaining stable thereafter (314). Frequency of the latter is influenced by dietary factors: significant negative correlations have been reported between MN and Vitamin B12 and folic acid levels, but not Vitamins C and E (315). Dietary caloric restriction may slow down the observed age-related increase in mutations (316), such that there is a correlation between energy intake and hprt mutation frequency (317). This possibly occurs via effects on DNA polymerase-alpha (318) and by enhancing nucleotide-excision repair (NER) (319). However, mice with NER defects due to Xpa gene knockout, experienced neither increased tumorigensis not premature aging (320). Even artificially-induced mutations, eg. in hprt caused by bleomycin (known to cause oxidative damage), are reduced by CR in mice (321). There is an age-related increase in basal levels of DNA damage in human lymphocytes (322,323), and an increase in chromosomal abnormalities in the elderly was demonstrated many years ago (324). There are age-associated decreases in the fidelity of DNA polymerase as well as 3´ to 5´ exonuclease activity (325), both of which are retained until just prior to replicative senescence, when they suddenly change (326). However, some studies of cellular senescence *In vitro* have not revealed decreases in either total or gene-specific DNA repair capacity, at least as far as UV-induced pyrimidine dimer induction is concerned (327). Neither do polymerase (beta)-deficient knockout mice show age-associated immunological changes any different from those observed in wild-type littermates (328). However, many components of repair pathways for UV-induced damage to human dermal fibroblasts are markedly reduced with age, including ERCC3, PCNA, RPA, XPA and p53 (329).

Mechanisms for recognition of DNA damage and its repair are important in maintaining cellular integrity; differences therein would obviously contribute to heterogeneous patterns of aging, exactly as is seen. However, analysis of DNA damage repair genes in aging is likely to be complex, as at least 300 of them are already known (330). Nonetheless , if these are affected during aging, this would also contribute to failing function. Moreover, it has been established that contrary to widely-held beliefs, although mitochondrial DNA does not encode DNA repair proteins (331), repair of mitochondrial DNA damage does take place and may also be efficient in compensating for oxidative damage (331,332). Nonetheless, mitochondrial damage does accumulate

during the lifetime of the individual (333). However, there is also a school of thought that these estimates of mitochondrial damage are incorrect - in fact the level of damage is much lower when certain artifacts of mitochondrial preparation are eliminated (334). Given the age-associated mitochondrial defects mentioned above (7.1), and that age-associated dysfunction has also been reported in human lymphocyte mitochondria (335), DNA repair must therefore be considered as possibly having a role here also. There are many reports that this is indeed the case, although the mechanism behind this may also reflect the effects of cumulative exposure to ionising radiation and other environmental factors rather than aging *per se* (336). More recently, these analyses have been refined; eg. Boerrigter *et al*. (337) found that the rate of disappearance of a particular kind of chemically-induced DNA damage was age-dependent in mice, and also varied between strains, with longer-lived strains having better damage repair capacity than shorter-lived strains of the same age. In humans, genetic polymorphisms exist in DNA repair genes, and some of these may be associated with more or less efficient repair, thus contributing to individual differences (338). Early reports had suggested a correlation between DNA repair capacity and maximum species lifespan (339). Cortopassi & Wang recently summarized various publications to survey agreement on rates of DNA repair in different species and the correlation between repair and maximal lifespan (340). They concluded that large differences in DNA repair capacity were found in different species and that the correlation between maximal lifespan and repair was indeed good, although not perfect. Both increased damage and decreased repair are associated with decreased longevity in certain mouse strains, and this holds true for various different tissues (341). Moreover, DNA repair capacity within a particular species may correlate with age of the individual. Thus, there is an age-related decrease in post-UV-irradiation DNA repair capacity in cultured skin fibroblasts from normal human donors, estimated at -0.6% per year up to the age of > 90 years (342). Furthermore, the same group estimated a corresponding increase in mutability of DNA in B cell lines from these donors of + 0.6% per year (342), suggesting that DNA repair decreases with age and this correlates with increased mutability. An underlying mechanism responsible for changes in DNA repair with aging may be decreased expression and function of DNA topoisomerase I, an enzyme that alters the superhelicity of DNA (343).

One key event in the earliest steps leading to DNA repair is the poly(ADP-ribosyl)ation of nuclear proteins by the enzyme poly(ADP-ribose) polymerase (PARP), which binds to single or double-stranded breaks in DNA. The level of activity of PARP measured in different species is related to their longevity, with long-lived animals showing the highest levels of enzymatic activity irrespective of the quantity of PARP protein present (344,345). There may also be structural differences in PARP from short and long lived species (346). Moreover, in B cell lines from centenarians, levels of PARP activity were found to be greater than in younger controls (347) and the specific activity of the enzyme greater (348). PARP may therefore be important in maintaining the integrity of the lymphocyte genome and qualitative and quantitative differences in PARP would therefore impact on immunosenescence. It may do this also by virtue of its association with telomeres, whereby it binds a negative regulator of telomere length maintenance, thus possibly directly helping to prevent telomere loss (349). Indeed, PARP-KO mice have shorter telomeres than wild type (350). One protein with PARP activity, designated tankyrase, interacts with and blocks a negative regulator of telomere length, TRF-1, such that over-expression of tankyrase in telomerase-positive human cells results in telomere elongation (351). In contrast, the expression of ku70/80, also associated with the telomere (352) via binding of TRF1 (353), stabilising broken DNA ends (354) and implicated in early recognition of DNA damage, appears to be similar in T cells from the young or old (355). However, this latter group did find some evidence for changes in ku with age, in that the age-related decreased DNA repair after irradiation of PBMC from old donors was paralleled by a progressive decline in ku70/80 DNA binding (356). One mechanism involved in decreased ku activity may be related to the observation that senescent human fibroblasts contain an elevated ku86 proteolytic cleavage activity (357). Consistent with an important role for ku proteins in this context, deletion of ku86 in mice resulted in early onset of senescence (358).

Another key protein involved in DNA repair processes is the DNA-dependent protein kinase (DNA-PK), a serine/threonine kinase consisting of a 470 kDa catalytic subunit (DNA-PKcs) and a heterodimeric regulatory complex, called ku, which is composed of 70 kDa (ku 70) and 86 kDa (ku 80) proteins (354). The ku heterodimer binds the ends of various types of DNA discontinuity and is involved in the repair of DNA breaks caused by V(D)J recombination, isotype switching, physiological oxidation reactions, ionizing radiations and some chemotherapeutic drugs (354,359-363). The ku-dependent repair process, called non homologous end joining, is the main DNA double strand break (dsb) repair mechanism in irradiated mammalian cells (364-366). The two subunits of the ku heterodimer associate tightly and may form a tetramer when bound to the two DNA ends of the break (367). Moreover, ku is able to translocate along DNA in an ATP-independent way (81,368). The ku heterodimer is probably involved in stabilizing broken DNA ends, bringing them together and preparing them for ligation (369), as well as in preventing digestion of the broken ends by DNA exonucleases (354). As ku has been described to activate mammalian DNA ligases *In vitro* (369), it is possible that ku and a ligase may be sufficient to repair breaks without further processing before ligation. After binding to the DNA ends, ku recruits DNA-PKcs to the breaks, activating its kinase function (370,371). Thus, ku acts as a subunit of DNA-PK and is largely responsible for the DNA-dependent activation of this enzyme. The potential role of DNA-PKcs is to recruit other repair components to the sites of breaks and regulate them by phosphorylation. Moreover, DNA-PKcs may signal the presence of a DNA damage and induce cell cycle arrest or apoptosis (372), suggesting a role of this kinase in p53 activation. However, cells lacking DNA-PKcs or ku can still mediate a p53-dependent block

of the cell cycle in response to ionizing radiations and other DNA-damaging agents (280), indicating that DNA-PKcs is only one of the several molecules that signal DNA damage. DNA-binding of ku is unchanged in normal lymphocytes from aging subjects but progressively declines in X-ray-irradiated lymphocytes from young to adult and elderly subjects (356). The radiation-induced activation of ku in lymphocytes from young subjects results from the increased concentrations of ku 80 and DNA-PKcs in the cytoplasm of lymphocytes from young, but not from elderly subjects, leading to a higher concentration of phosphorylated ku 80 which readily migrates to the nucleus where, after dimerization with ku 70, binds to DNA breaks (373). Recent results have demonstrated that gp130 signaling cytokines have an effect on the DNA-binding activity of ku and on unscheduled DNA repair in X-ray-treated lymphocytes from human subjects of different ages. In particular, they have been shown to be able to significantly increase DNA-binding activity of ku in young but not in elderly subjects, although they are able to enhance unscheduled DNA repair in both, suggesting a novel strategy of intervention to improve the cellular response to DNA damage (374). As to the mechanisms whereby ku and gp130 signaling are coupled in lymphocytes, results from co-immunoprecipitation experiments have shown that ku in the cytoplasm of lymphocytes from young, but not from elderly subjects, is associated with Tyk-2, a kinase involved in signal transduction events after gp130 triggering by IL-6-type cytokines and that this association is independent of cell stimulation. Moreover, after gp130 signaling both Tyk-2 and ku are phosphorylated, suggesting their activation by IL-6-type cytokines (375).

The precise role of ku in gp130 signaling events is unknown. Ku may be involved in Tyk-2-mediated activation by phosphorylation of other molecules operating in gp130 signal transduction events. It has indeed recently been demonstrated (376) that in the IFN-? -responsive human cell line U266, ku functions by favouring the interaction between Tyk-2 and p95vav, a proto-oncogene product, acting as a signal transduction element in hematopoietic cells. Following this interaction, p95vav is phosphorylated by Tyk-2. Moreover, ku may act by protecting phosphorylated Tyk-2 from phosphatase attack, thus leading to prolonged activation of the mitogen-activated protein kinase (MAPK) cascade. In addition, ku associated to Tyk-2 has been described to be phosphorylated, and activated to translocate to the nucleus, by this kinase. In the nucleus, phosphorylated ku exhibits DNA-binding activity. In elderly subjects, a physical association between ku 80 and Tyk-2 has not been demonstrated yet. This may depend on the fact that levels of ku 80 are significantly reduced in lymphocytes from aging subjects, as previously shown (373). It is unknown whether Tyk-2 is also reduced by aging. Nevertheless, lymphocytes from elderly subjects still display DNA-binding activity of ku, suggesting that Tyk-2 represents only one of the different ways of ku activation.

The mismatch repair system (MMR), consisting of at least 6 genes in humans (hMSH2, hMSH3, hMLH1, hPMS1, hPMS2 and hMSH6). Microsatellite stability in human cells is primarily reliant on MMR, and so can be used as a surrogate measure of MMR integrity. Thus, microsatellite instability (MSI) increase with age and this may be associated with alterations in the methylation status of certain MMR genes (377). Increasing MSI with age has now also been observed in human leukocytes (378).

Cross-sectional studies in human lymphocytes suggest an age-associated increase in general DNA damage (as assessed by the Comet assay), which may bear little relationship to the levels of anti-oxidant activity in the donors´ serum (379). Further analysis by this group actually showed a trend towards greater DNA damage in those elderly with normal compared to low levels of plasma anti-oxidants (380). Interestingly, elderly men had higher damage levels than women, and for men there was an association between damage and low anti-oxidant status (380). Again, clearly, gender has to be taken into account. In T cells, studies of mutations (unrepaired DNA damage) revealed that background mutant frequency (MF) at a marker locus, *hprt*, increases with age up to advanced middle age (381,382). Although there is a wide inter-individual variation in mutant frequency, some of which may be due to individuals possessing clonal expansions of T cells with mutator phenotypes (383) and which is also sensitive to a past history of exposure to radiation (384), but apparently not smoking (385), in general this relationship holds up to advanced age. However, when older aged individuals were examined, basal levels of DNA damage in lymphocytes from donors 75-80 years old were similar to those of the 35-39 year-old group (381). There was also no significant difference between frequency of mutation at the hprt locus in the young and more aged populations, nor was there any difference in DNA repair capacity after hydrogen peroxide-induced DNA damage (386). These findings may possibly be explained by donor selection pressures resulting in an association of longevity with retention of DNA repair capacity. T cells with mutations measured at the hprt locus show a reduced proliferation rate *In vitro* and may therefore have a selective disadvantage (387). Together with the increased levels of anti-oxidants glutathione peroxidase, catalase and ceruloplasmin in the elderly (386), these data suggest that those individuals with best retention of DNA repair mechanisms and anti-oxidant defences form a group with extended longevity. Concordant with this idea, treatment with anti-oxidants (see 7.1 above) may also decrease DNA damage in human lymphocytes. Thus, dietary anti-oxidant supplementation was found to reduce hprt mutant frequency in murine lymphocytes (388), and Duthie *et al*. showed that supplementation of 50-59 yr. old men with high-dose vitamin C, vitamin E and ß-carotene for 20 weeks resulted in a protective effect against oxidative DNA damage both by decreasing endogenous oxidative damage and increasing lymphocyte resistance to exogenous oxidative damage caused by hydrogen peroxide (389). These results seem consistent with the observation that the ability of human plasma to protect against oxidative stress caused by ionising radiation is inversely correlated with the age of the plasma donor (390), but not with increased levels of anti-oxidants in the elderly (386). It may be necessary to measure the intracellular levels of such anti-oxidants for a proper picture to emerge; thus, higher levels of intracellular

ascorbate and glutathione have been reported to be associated with lower levels of DNA damage (391). In contrast, no correlation has been found between dietary anti-oxidant intake and hprt mutation frequency (317) and levels of oxidative DNA damage as measured by 8-oxydeoxyguanosine (8-oxo-dG) levels in lymphocytes, were not affected by carotenoid supplementation, nor did they correlate with baseline levels of serum anti-oxidants (392). Interestingly, in this latter study, 8-oxo-dG levels varied greatly in males from five different countries studied (in donors of age 25 - 45 yr). More recently, it was shown that supplementation of the young with vitamin C or vitamin E resulted in decreased peroxide-induced but not endogenous lymphocyte DNA damage at the same time as decreasing plasma glutathione peroxidase and superoxide dismutase levels (393). Another study, however, has shown that supplementation with 500 μg of Vitamin C per day reduces levels of 8-oxo-dG in serum and in lymphocyte DNA, correlating well with the levels of Vitamin C achieved in the plasma (394). This marker of oxidative damage of DNA, 8-oxo-dG, was also decreased in subjects fed a controlled experimental diet rich in anti-oxidants (395). In elderly humans, general lymphocyte DNA damage assessed by the Comet assay was decreased as a result of 80 days-supplementation with fruit and vegetable extracts (396). However, in a study comparing a Northern with a Southern European population, the latter had higher levels of plasma vitamin E and carotenoids (natural Mediterranean diet) but nonetheless significantly higher 8-oxo-dG levels (397). Of course, it is well established that a Mediterranean-type diet is associated with reductions in overall mortality (eg, see ref. (398)) but the reasons for this are not completely clear.

Not only is the frequency of the various hprt mutations increased with age, but possibly also in situations of chronic antigen stimulation resulting in clonal expansion. Thus, the hprt mutant frequency was estimated to be five times higher in peripheral blood T cells of RA patients compared to normal controls. This increased to 10-fold higher in T cells obtained from synovial tissue (399). In genetic diseases with a progeriac component, such as Werner´s syndrome and, more strikingly, Bloom´s syndrome, mutations in CD3/TCR which may compromise immune function, are found to be increased (400). Such "segmental" premature aging syndromes may be informative about normal aging, not only in these patients. In this respect, it has been reported that when grown as uncloned lines, T cells from Werner´s patients have an identical proliferative potential to those from normals (401). This demonstrates that the longest-lived T cell clone in the Werner´s cultures possessed equivalent longevity to the longest-lived normal clone, but is not informative on the behavior of the majority of the T cell population.

A genetic polymorphism of the gene, which when mutated causes Werner´s syndrome in homozygotes, may be associated with susceptibility to atherosclerosis in the normal population (402). Additionally, heterozygous carriers also show evidence of the same genetic instability as seen in the patients themselves (403). Indeed, several human genetic disorders with premature aging components share aberrations in the family of DNA helicase genes, which may imply a role for these in normal aging (404), and DNA repair (405). Similarly, Down´s syndrome may also be informative; here immune dysfunction with aging may also be accelerated (406). It has been reported that an oral zinc supplementation, able to correct zinc deficiency and some immune defects in Down's syndrome, is also involved in the maintenance of DNA integrity. DNA damage and repair after gamma-radiation was studied by alkaline elution assay in PHA-stimulated lymphocytes from Down's syndrome children before and after an oral zinc supplementation given for 4 months to correct their immune defects. In Down's subjects the rate of DNA repair was highly and significantly accelerated and reverted to normal values after zinc supplementation (407).

Mutations at several other loci have also been examined in the context of aging (408). Accumulation of mutations with age is likely to be a general finding applicable to different tissues, albeit with different rates (409). There is a significant age-associated increase in the number of CD4+ T cells expressing variant (presumably mutant) TCR, as Kyoizumi *et al*. demonstrated using 127 normal donors from < 10 to > 80 yr. old (410). Because mutations in the TCR can subtly alter T cell responses and divert Th1 towards Th2-like reactivity, this finding may have functional implications (411). Age-related increases in mutations of HLA genes have also been reported (412). Mutations in mitochondrial DNA have been identified in fibroblasts from old but not young individuals in cross-sectional studies, and their age-associated appearance was documented in a longitudinal study (413). In this seminal study, the same point mutations were found in more than one donor and were located in the control region for replication of the mitochondria. Clearly, this would have grave implications for mitochondrial function in cellular aging. However, in yeast, there is a mechanism by which nuclear stress-response genes are upregulated in compensation for mitochondrial dysfunction (414), and there is an association between both mtDNA variability and longevity and between nuclear stress genes and longevity in humans, suggesting that this type of mechanism may exist in humans as well (415). Translocations associated with oncogenesis in younger donors (bcr/abl, bcl-2t(14:18)) also show age-associated increases in frequencies, most of which fail to result in overt tumorigenesis (416-418). In some cases, this may be because of subtle differences between cancer-related and “normal” age-related fusion transcripts, eg. for ALL1 (MLL) possibly due to differential mRNA splicing (419). In mice also, the frequencies of translocations and insertions increase significantly with age (420). However, despite increase with age, the frequency of these events is too low to be causative of immune depression, although they may offer good biomarkers for T cell aging. On the other hand, increased frequencies of measured bcl-2 translocations and hprt mutations may also depend on factors other than age, such as environmental stressors like exposure to sunshine (421). Nonetheless, they may indirectly indicate parameters which do have a causative effect, for example, overall DNA damage. In parallel with the age-related increase in hprt MF, for example, an age-related decrease in DNA repair capacity

for hydrogen peroxide-induced DNA damage has been observed (422). The repair of unavoidable damage may conceivably be facilitated by transfection of DNA repair enzymes, at least under certain experimental conditions (423,424).

Circumvention of growth arrest programs by blocking the action of mitotic inhibitors may extend T cell longevity, and can be accomplished by components of transforming viruses. The clear drawback here is of course the danger of tumorigenesis. Indeed, it is commonly believed that the reason for the emergence of cellular senescence programs at all is to do with tumor suppression (425). However, use of antisense technology to temporarily prevent growth arrest and allow a limited number of extra cell divisions might still be beneficial. Thus, use of combined p53 and Rb antisense was reported to extend the lifespan of fibroblasts by 10 PD (426), and use of $p33^{ING1}$ antisense to extend fibroblast lifespan by 7 PD (427). However, the importance of an intact p53-pathway not only for tumor suppression, is illustrated by the accelerated immune aging of p53-deficient mice, including accumulation of apoptosis-resistant memory T cells (428). Antisense to either $p16^{INK4}$a or the closely related $p19^{ARF}$ also extends the lifespan of primary mouse embryonic fibroblasts, underlining the importance of the Rb-mediated pathway (429). Retinoic acid extends the lifespan of human keratinocytes in culture, associated with decreased p16 expression, and maintained telomerase activity (430). Enforced expression of helix-loop-helix protein Id-1 is also reported to extend the lifespan of keratinocytes by affecting p16 expression, but without leading to immortalisation (431). Induction of p16 for more than a brief period results in commitment of the cells to senescence or apoptosis (432). Therefore, the p16 / Rb pathway plays an important role, and the p53 / p21 pathway provides a second critical regulatory pathway. Indeed, enforced expression of p21 causes growth arrest and acquisition of a senescent phenotype in human fibroblasts (although at the same time the cells are stimulated to secrete factors with growth-promoting properties for other cells) (433). DNA damage recognition requires Chk2 activation to trigger the p53 cascade; Chk2-knockout mouse thymocytes are resistant to DNA damage-induced apoptosis, do not stabilize p53 transcripts or upregulate p21 (434).

Recent work has demonstrated that transfection of telomerase into certain fibroblasts and epithelial cells can result in their so-called immortalization (435-437) In lines expressing $p16^{INK4}$, this may have to be inactivated beforehand (438). However, inactivation of p16 together with telomerase transfection may result in the generation of immortalised lines with chromosomal abnormalities and altered differentiation characteristics (439). Moreover, “immortalisation” by hTERT expression may not result in truly permanent lines; they may still experience a (much delayed) growth crisis or only a subset of cells in the line may really be immortalised (440). These findings obviously invite caution when applying telomerase transfectants clinically. However, none of these approaches has yet been attempted with lymphocytes, although expression of a p16-specific ribozyme, which downmodulates p16 expression, has been found to accelerate cell cycle progression in a mouse erythroleukemia cell line (441). Although it was previously thought that p16 was not expressed in T cells, more recent work by Erickson *et al*. showed that both p16 and p15 proteins (p15 behaves similarly to p16 in many respects (442)) accumulate as PHA-stimulated T cells age in culture, and that there was increased binding of p16 to its target Cdk6 kinase (443). In contrast, p21 levels were only slightly elevated (443) and p53 levels were unchanged in resting T cells from the elderly but decreased in PHA-activated old T cells (444). Therefore, p16 may play the most important role in growth control of lymphocytes as well as fibroblasts etc. and could possibly be a target for manipulation in immunosenescence. However, once again, in the experiments of Erickson *et al*., PHA was used to stimulate T cells which were subsequently grown with IL 2 until proliferation ceased. Thus, like those of Pan *et al*. (445), these experiments were not measuring T cell senescence but quiescence, despite the decrease of surface CD28 expression measured, and, interestingly, an increase in ß-galactosidase (ß-gal.) (443). Both of these are taken to be markers of senescence, but here appear at quiescence, as previously argued for ß-gal. by Rubin (446,447). Erickson *et al*. believed that they had proven their cultured T cells to be senescent by restimulating them with PHA and IL 2; however, failure to stimulate precultured T cells under these conditions merely reflects the lack of accessory cells or APC required for presentation of PHA. Unfortunately, others have followed this lead, resulting in some confusion in the literature about what T cell senescence is.

Other mitotic checkpoints such as p27kip may also be important in T cell senescence; CD4 cells from old mice, for instance, failed to downregulate p27 on activation (448). P27 is a cyclin-dependent kinase inhibitor which is upregulated by TGF-ß. In anergic T cells, p27 blocks AP-1 and IL 2 transcription (449). As well as failure to downregulate p27, anergic cells also upregulate p21(sdi) (450), and given the similarity between anergic T cells and old T cells, it is likely that the same mechanism applies to old cells.

Cell cycle analyses of PHA-stimulated cells from aged donors indicate a decreased frequency of cells entering S-phase with this age-related impairment of G1 progression correlating with decreased expression of c-jun, c-myc, c-myb, IL 2 and CD25 (451-454). The proportion of cells expressing c-myc (G0 to G1 marker and telomerase activator (455)) and c-myb (G1 to S marker) was decreased after PHA stimulation of old T cells, but the amount per cell seemed to remain the same as in young T cells (454). CD3-stimulated T cells from elderly donors showed both G0/G1 and G1/S-phase blocks associated with decreased phosphorylation of the Rb regulatory protein by cdk6, due to the low level cdk6 activity (456). Low cdk6 activity is possibly caused by binding of the inhibitory protein p16*ink4* (443), the level of which is increased in old T cell clones (457). T cells retaining antigen recognition and effector function, yet apparently in a post-mitotic senescent or pre-senescent state have been described (458). These investigators also demonstrated that aged human T cells paralleled the senescent phenotype of fibroblasts in that on

restimulation, fewer cells responded by entering the cell cycle, the remainder being arrested before S-phase. The cell cycle was also prolonged in those ca. 20% of senescent cells which could be restimulated (459). At least some of these results may reflect the situation *In vivo*, where PHA stimulation resulted in an earlier accumulation of cells in S phase in young donors´ T cells, and a significant delay, but eventually equivalent level, of S phase cells in the elderly (460). The postponement of progression of T cells through G(2)/M appears to be associated with low cdk1 activity caused by low levels of cdk1 protein, the associated cyclin B1 and incomplete dephosphorylation of what kinase there is still present (461).

7.5. Recombinant cytokines

The regulation of Th1 (IL 2 and IFN-γ) and Th2 (IL 4) cytokine production by addition to cultures of mitogen-activated spleen cells from young, adult and old mice of recombinant cytokines (IL-1β, IL 2, IL 3, IL 4, IL 12, or IFN-γ) has been reported (462). In this study, the production of IL 2, as protein in culture supernatant and mRNA extracted from cultured CD4+ cells, is profoundly depressed by aging, whereas that of IFN-γ as protein and mRNA, firstly declines and then increases with age. The production of IL 4, as protein, monotonically declines with aging whereas, as mRNA, firstly decreases and then increases above the level in young mice. When spleen cells in culture were incubated with mitogens and with a recombinant cytokine, cytokine production was enhanced when the level induced by mitogens alone was low. This conclusion applies to IL 2 and IFN-γ production as protein and mRNA. The enhancing effect on IL 2 production is more pronounced upon addition of recombinant IL-12, which is involved in Th1 cell amplification. Also the addition of IL 4 increases IL 2 production, a finding that may result from the network of interactions among different cells and a variety of different cytokines. It may be envisaged that IL 4-induced amplification of the Th2 cell pool leads to increased release of IL 6, a pleiotropic cytokine with multiple effects on lymphocytes and accessory cells. IL 6 may, indeed, activate macrophages to release cytokines, such as IL 12 and IL 1, involved in Th1 cell maturation. On the other hand, there may be a positive feedback whereby IL 6 inhibits Th1 cell differentiation (463), which would fit with the idea of a shift from Th1 to Th2 phenotype with age, as IL 6 is commonly reported to be increased in aged individuals. Also the level of IL 2-specific mRNA may be effectively upregulated by recombinant cytokines when spleen cells are derived from adult but, to a much lesser extent, when spleen cells are derived from old mice, suggesting that changes in post-transcriptional regulation of IL 2-specific mRNA occur during aging. The stability of this mRNA may be increased in CD4+ cells from old as compared to young and adult mice leading to increased protein release in the culture supernatant, as also demonstrated by the age-related increase in the half-life of IL 2-specific mRNA, as found when purified CD4+ cells were activated by anti-CD3 and anti-CD28 mAbs (464,465). The production of IFN-γ-specific mRNA and IFN-γ protein is lower, but can be increased by recombinant cytokines, in spleen cells from adult as compared to young and old mice. Thus, unlike IL 2, the IFN-γ production seems to be independent from age-related differences in mRNA at the post-transcriptional level. As to IL 4, the production of this Th2-type cytokine continuously decreases with increasing age and could be enhanced by addition of recombinant cytokines only when spleen cells were derived from old mice. Conversely, the expression of IL 4-specific mRNA was decreased to a minimum at the intermediate age, but could be enhanced by *In vitro* treatment with recombinant cytokines only when spleen cells were derived from young mice, suggesting age-related changes in transcription rate as well as in post-transcription half-life and translation kinetics. These results altogether demonstrate the possibility to enhance the synthesis and release of cytokines when their production is deficient, suggesting that recombinant cytokines may be considered appropriate immunomodulators of clinical relevance.

7.6. Stress

It has been argued that one of the unifying factors shared by life-extension manipulations and mutations is the adjustment of the organism to low levels of stress. It is hypothesized that activation of stress-protective mechanisms early in life may result in their better function later in life and that stress-resistance is a determining factor of longevity (466). In man, senescent T cells do show a reduced stress response as reflected by decreased production of hsp70 after heat shock, associated with decrease in binding of nuclear extracts to the consensus heat shock element. The progressive decline in hsp70 response with increasing age of T cells in culture was found to correlate with the percent of proliferative lifespan already completed (467). This finding has since been confirmed for hsp 70 and also for many other heat shock proteins (468). An age-dependent decrease of heat shock factor-1 (HSF-1) binding in isolated human lymphocytes ex vivo, as well as gradual loss of heat-inducible HSF-1 in cultured T cells as they age has also been observed by Jurivich *et al.* (469). Similarly, in rat splenocytes ex vivo, hsp70 expression and HSF-1 binding activity also decrease with age (470). A member of the hsp70 family, mortalin, has been proposed as a marker for cells committed to apoptosis. An hsp70 family member designated mot-2 is associated with an immortal phenotype in fibroblasts, and transfection of normal human lung fibroblasts with mot-2 extended lifespan by 12-18 PD, associated with p53 transcripional inactivation (471). Hsp70 may protect against apoptosis (472), both caspase-dependent and -independent (473), possibly by suppressing the stress-induced protein kinase JNK (474) as well as blocking late events downstream of caspase-3-like proteases (475). Transduced hsp70 can also protect chondrocytes from heat stress (476). On the other hand, others have shown that overexpression of transfected hsp70 can enhance AICD in T cells (477). The expression not only of the hsp70 family, but also hsp90 family stress proteins has been reported to be reduced after PHA stimulation of aged T cells directly ex vivo, suggesting that results with cultured cells are relevant to the *In vivo* situation (478). There is now some evidence that hsp90 plays a part in CD28-mediated T cell activation (479), suggesting that reduction of hsp90 might further reduce the already compromised function of CD28

in aging and help to explain lack of function even of the CD28 still expressed on old (mouse) cells (see section 4.3.3.1). Moreover, hsp90 (as well as the p23 chaperone) may be required for the assembly of active telomerase (480), so that progressively decreasing telomerase activity during T cell aging *In vitro* (see 5.2) may partly be caused by decreasing hsp90 levels.

Interventions which would enhance the stress response may therefore also be directly relevant to delaying senescence (466,481). Repeated mild heat stress may do so via induction of heat shock proteins (482). Even low-level irradiation may fall into this category. Thus, multiple low-dose irradiation of human fibroblast cultures extended their lifespan by one-quarter (483); this was not accompanied by specific chromosome abberations or activation of telomerase (483). However, the same group also showed that although low-dose irradiation also prolonged the lifespan of cultured human embryo cells, this was accompanied by chromosome instability (484). Therefore, the effects and mechanisms of irradiation may differ according to the type of tissue treated. Furthermore, in a large study involving 900 mice, Caratero *et al*. (485) demonstrated that low-dose irradiation (25 - 50-fold background) resulted in a significant increase in longevity compared to the control group (673 days compared to 549 days for 50% survival of the starting population). The same group later investigated some simple immunological parameters, such as numbers of CD4 and CD8 cells in thymus and spleen, but found no effect of low-dose chronic irradiation (486). Moreover, in C. elegans, the mutations conferring extended longevity also confer resistance to stress; and the same is true for the recently discovered trk-1 gene, overexpression of which can increase lifespan by up to 100% (487). This tyrosine kinase receptor gene may have homologies amongst the numerous such receptors expressed by lymphocytes and in this way be relevant to immunosenescence. Finally, even the mechanism of action of caloric restriction may be associated with inducing resistance to stress, because multiple parameters of older CR animals are consistent with their already experiencing a state of enhanced oxidative stress, successfully resisted (488). Application of new molecular technologies continues to shed light on these questions. In a seminal paper, gene expression profiling examined skeletal muscle of young and old mice, testing the expression of >6000 genes. It was found that aging was associated with an upregulation of stress-induced genes and a downregulation of metabolic and bioysnthetic genes (489). Moreover, CR partially or completely prevented these changes in old mice. This type of study has not yet been reported in lymphocytes.

7.7. Other approaches – gene therapy

In fact, the emerging association of CMV infection with immunological remodelling in the elderly may also be partly attributable to the recently demonstrated presence of a viral IL 10 moiety in CMV as well as EBV (490). Gene therapeutic approaches may also allow selective diversion of immune response away from type 2, an approach with broad applicability in disease states as well as aging; for example, targeted disruption of the transcription factor Stat 6 has been reported to have this effect (491).

One of the hallmarks of old T cell is that they fail to secrete IL 2 which is necessary for their clonal expansion and maintenance of viability. However, they still respond to signals via the TCR in an antigen-specific fashion and continue to secrete other cytokines such as GM-CSF. Normally, they do not respond to these cytokines themselves. It is possible to engineer T cells such that they do respond to GM-CSF, by transducing them with a chimeric GM-CSF/IL 2R gene. This has been accomplished so that the T cells proliferated strongly *In vitro* in the absence of exogenous cytokines (492). This is a very significant finding, because even when T cell clones are young enough still to make IL 2, they require exogenous factors for clonal propagation, due to insufficient autocrine production.

Other possible approaches to extend the functional lifespan of T cells *In vitro* for the purpose of producing sufficient cells for adoptive immunotherapy includes telomerase transfection to immortalise cells without causing tumorigenicity, and this has just been published for T cells. These results deserve some discussion because one group reported success (493), the other failure (493,494). Whether this discrepancy is caused by p16 status, or some other factor (possibly, eg. the presence of negative regulatory elements such as MZF-2 (495)), is as yet unclear. Most other immortalising agents carry a high risk of carcinogenesis but might be considered a possibility if engineered to respond to inhibitory agents. Methods are being developed to employ retroviral components for temporary transformation by engineering their transient, controlled expression (496). Using this type of approach, reversibly immortalised hepatocytes have been applied to prevent acute liver failure in rats (497). Delivery of telomerase may also act in the same way; this has been successfully tested already in a rat model of liver cirrhosis (498). Other approaches for transient lifespan extension include protein rather than gene transduction, for example, using the HSV-1 protein VP-22 to introduce SV-40 large T antigen into target cells (499).

Experimental approaches being considered also include the search for new genes which affect aging processes, even without knowledge of their function or even their nature, using the following strategy (500): on the assumption that some of the genes expressed in cultured cells from long-lived organisms will differ from those of related short-lived organisms (at least after the application of stressors) in a way which is relevant to longevity, these can be isolated and transferred to the short lived species to investigate effects on longevity.

8. CONCLUSION

As the organism ages, the output of T cell precursors from the BM decreases. Those precursors that enter the progressively involuting thymus are doubly compromised in their ability to generate new T cells: firstly because of their intrinsic deficiencies and secondly because

of the reduced thymic function. There is therefore a quantitative and qualitative component to the dysregulated generation of naive T cells which becomes greater the older the individual is. In addition, naive cells produced by the thymus of the individual when young and surviving for extended periods in the periphery, themselves age even in the quiescent state. T cells which have been activated at some time during the life of the individual may remain present as memory cells and respond to rechallenge by antigen. However, because memory cells are maintained in a proliferative state even in the absence of antigen, they are subject to the aging limitations of proliferating cells and eventually undergo "replicative senescence". Even before they reach this terminal state, their function is altered and impaired compared to young cells, for example in terms of their altered cytokine secretion patterns and susceptibility to activation-induced cell death. Since these cells cannot be so easily replaced by freshly activated naive cells as efficiently in old as in young individuals, the resulting immune response is reduced and generation of memory compromised in the elderly.

9. ACKNOWLEDGEMENTS

This is an updated version of a review originally conceived under the aegis of the European Union Concerted Action on the Molecular Biology of Immunosenescence (EUCAMBIS; Biomed 1 contract CT94-1209, 1994 - 1997). This updated version is produced as part of the work of the current EU “Thematic Network” *Immunology and Aging in Europe* (ImAginE; contract QLK6-CT-1999-02031). For support of laboratory work over the years, GP is grateful to the Deutsche Forschungsgemeinschaft, the Deutsche Krebshilfe, the European Commission, the Sandoz Foundation for Gerontological Research, the Mildred Scheel Foundation, the Dieter Schlag Foundation, the VERUM Foundation and the Fortüne Program of the University of Tübingen Medical School. RBE is supported by grants from the NIH (#AG10415) and the Seigel Life Fund. RS is supported by the Fondo de Investigación Sanitaria (FIS 95/1242). YB is supported by DevR Funding and the Department of Health and Social Services, Northern Ireland. The studies of CC have been supported by grants from MURST, Rome (ex 40%, Immunogenetics of Longevity). We thank Beatrix Grübeck-Loebenstein, Innsbruck, Martin Aringer, Vienna, and Anders Wikby, Jönköping, for their critical comments.

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Key Words: T cells, aging, Immunosenescence, Immune Response, Immunogerontology, Review

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