T CELLS AND AGING

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1. ABSTRACT

Deterioration of the immune system with aging ("immunosenescence") is believed to contribute to morbidity and mortality in man due to the greater incidence of infection, as well as possibly autoimmune phenomena and cancer in the aged. Dysregulation of T cell function is thought to play a critical part in these processes. Factors contributing to T cell immunosenescence may include a) stem cell defects, b) thymus involution, c) defects in antigen presenting cells (APC), d) aging of resting immune cells, e) disrupted activation pathways in immune cells, f) 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.

2. INTRODUCTION: WHAT IS IMMUNO-SENESCENCE?

T cell function is altered in vivo and in vitro in elderly compared to young individuals. These changes are generally perceived as reflecting a deterioration of immune function. This state, designated "immunosenescence", occurs in both long and short-lived species as a function of their age relative to life-expectancy rather than chronological time. 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 (Bradley et al., cited in (1) and in murine models of systemic lupus erythematosis (2). The same may be true for acute rejection in human liver transplantation (3).

T cell immunosenescence

Most tests of T cell function are depressed in elderly individuals (4), including even such very strong reactions as the rejection of allogeneic skin transplants in rats (5), consistent with the results in human, mentioned above. However, aside from these special cases, several studies have suggested a positive association between good T cell function in vitro and individual longevity (6-8), and between absolute lymphocyte counts and longevity (9). A high proportion of centenarians have relatively well-preserved immune functions compared to the less elderly (10), and a recent longitudinal study of the very old indicated that two-year non-survival was associated with poor T cell proliferative responses to mitogen, high CD8 (cytotoxic/suppressor cell) cell counts, and low CD4 (helper/delayed-type hypersensitivity [DTH] cell) and CD19 (B) cell counts. It was found that no single parameter was predictive for survival, but that a cluster of the above parameters was predictive (11). These data suggest that well-preserved immune function is associated with extended longevity.

The earliest observations on immunosenescence showed that cytotoxic T cells were compromised in old mice (12); 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 (13). Many studies mostly performed in mice, rats and man but also including monkeys and dogs (14) have established that age-associated immune decline is characterized by decreases in both humoural 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 (15). 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 (16). 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 (17). 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 (18), and as a chemoattractant factor for CD4 cells, produced by mast cells and fibroblasts (19,20).

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 (9, 21-23). 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 decrease again (24). Reciprocally, increased numbers of apparently activated T cells (HLA-DR$^+$, CD25$^+$), as well as increased numbers of natural killer (NK) cells (25) are seen. 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 (26). The "homing environment" in murine spleen seems to deteriorate with age (27). 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 (28). There are strong arguments for the extrathymic nature of such NK-1+ T cells (29), which may therefore increase in compensation for decreased thymic output of conventional T cells.

Apparently paradoxically, despite declining immune function, aging is also associated with increased autoimmune phenomena. There are increased levels of autoantibodies in the aged; because some such antibodies can penetrate living cells and activate them (30), this may have functional consequences, perhaps helping also to explain some aspects of dysregulated T cell function in the elderly. In centenarians, the increased levels of autoantibodies found do not include disease-associated antibodies such as anti-ENA and anti-dsDNA antibodies (31), or the thyroid autoantibodies which cause problems in the less elderly (32) but this could be because of selection pressures in the extremely old. In mice, old animals have a quantitatively but not qualitatively altered autoantibody pattern (33) 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 (34). 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 controversial, but some are consistent with decreased cellular suppressive activity with age (35-37) or with increased resistance to suppressive influences in the aged (38). Decreased specific suppressor cell activity in aged mice is associated with the appearance of MHC unrestricted T helper cells (39). The appearance of the same kind of MHC unrestricted helper activity has been observed in elderly humans (40), suggesting that altered suppression in aging may also occur in man. This has not been systematically investigated. 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 (41). Although many widespread autoimmune diseases commonly occur in the young, there is a set which show late onset (42); a contribution of age-associated dysregulated immune function to these autoimmune diseases remains to be explored. The occurence of cancer also increases in the elderly, of course, 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 (43).

Given its potential importance and the increasing proportion of elderly people the world over, a better understanding of the causes of immunosenescence may offer the possibility of therapeutic intervention. Amelioration of the

effects of dysregulated immune responses in the elderly by supplementation 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.

3. FACTORS CONTRIBUTING TO IMMUNO-SENESCENCE

3.1. Hematopoiesis

Dysregulated hematopoiesis is seen in elderly individuals, raising the possibility that this could contribute to altered immune function in the aged. Hematopoiesis in man may be compromised because of a severely reduced capacity to produce colony stimulating factors (44) and increased production of pro-inflammatory factors such as IL 6 (45), because lower numbers of progenitor cells are present in the BM (46) and because of an age-related decline in proliferative potential of putative haematopoietic stem cells (HSC) (47). In mice, the repopulating potential of murine fetal liver-derived HSC is higher than that of adult BM-derived stem cells (48), suggesting possible aging of the HSC themselves. Normal cells with shorter telomeres 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 (49). Telomere lengths decreased on culture despite low level expression of telomerase in these cells (50,51), 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 (52). 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 (53). On the other hand, ESC from telomerase-knockout mice show gradual telomere shortening over many population doublings (PD) resulting in slowing and eventual cessation of growth (54). Certain animals which do not downregulate telomerase manifest a permanent growth phase and little or no senescence (55,56).

A survey of 500 BM autotransplant patients (57) 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). 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 (58). 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). 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 (59). These findings are 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 may be applicable therapeutically in this and other contexts (60,61). Oxidation-resistant vitamin C formulations may be one possible way to accomplish this (62).

The recent finding that CD34$^+$ cells mobilise less effectively in cytokine-treated elderly compared to young donors is also of direct clinical significance (63). Moreover, in mice, the capacity of progenitor T cells from old BM to develop in the young thymus may also be compromised (64). However, this has not been found in all models (65) 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. By studying thymocytes generated in vitro from young and old donor-derived progenitor cells, co-cultured in the presence of lymphoid-depleted fetal thymus, decreased generation of CD4/8-double negative thymocyte progenitors was demonstrated, along with a developmental arrest at the transition from CD44+ CD4/8-double negative to CD44-negative, CD4/8 double positive cells. This does suggest an intrinsic change in the stem cells with age (66).

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 (67). Some of the previously published inconsistencies in the data may be resolved by the study of de Haan *et al.* (68), 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) (69). In outbred 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 (70).

3.2. Thymus

It is fairly well accepted that one of the reasons that T cell differentiation is compromised with age is thymic involution. However, this assumption may not be universally applicable to all individuals under all circumstances. 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 (71). 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. In mice, T cell export to the periphery from the thymus peaks in the young animal (ca. 4 weeks old) but by "middle age" (6 months) this has strikingly decreased (by >95%). Until recently, it has 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). It has been proposed that episomal DNA circles generated during excisional rearrangements of TCR genes may provide stable markers for RTEs (72). These TCR-rearrangement excision circles (TRECs) are not duplicated during division and because they are stable they are diluted out as cells divide (73). In the case of TCR2 cells, TRECs generated during the deletion of TCR-δ locus are identical in about 70% of the T cells (74,75). 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 (76). 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 "naive" T cells 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 (76). As Rodewald pointed out (77), 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 (77). Nonetheless, measurement of TRECs in T cell subsets of the elderly will enable the contribution of freshly generated (thymic or extrathymic) T cells-versus-old T cells to immune responses to be assessed for the first time in the aged.

There may be a strong genetic contribution influencing thymic involution; for example, rats of the Buffalo strain do not experience thymic involution and in parallel do not manifest decreased T cell function with age (78). Again, therefore, genetic heterogeneity in outbred populations might be expected to contribute to marked inter-individual differences. Careful experiments in the rat have shown that thymic involution cannot be viewed simply as a progressive shrinkage, but as complex remodelling dependent on unknown factors (79), 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 T cell antigen receptor (TCR)-transgenic mice, leading to the conclusion that successfully matured T cells can maintain thymic integrity (80,81). 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 (82). This was associated not 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.

Reconstitution experiments indicate that the observed accelerated maturation of T cells to a memory phenotype in old mice is largely due to the aged environment and involves interactions via the TCR which are, however, not antigen-specific [(83) and M. Thoman, cited in ref. (1)]. T cells also affect the thymus itself via a feedback effect and provide survival signals for the medullary microenvironment. An important survival signal of this type may be IL 4 (M. Ritter, cited in (1)). Thus, the aging of stem cells and/or T cell precursors may directly influence processes of thymic involution. 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 [(84) and A. Globerson, cited in (1)). Signals controlling thymic status may also be derived from the nervous system, either directly from sympathetic innervation or indirectly via the hypothalamic-pituitary axis (85). There are increased numbers of noradrenergic sympathetic nerves and 15-fold increases in concentration of norepinefrine in the thymi of 24 month-old mice (86).

The phenomenon of thymic involution begins very early in life, even before puberty, and progressively continues. However, despite the assumption that thymic involution is essentially complete in adulthood, this may not be the case, at least not with all individuals, as discussed above. In addition, there are also data in human to suggest 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 (87). Moreover, the amount of non-fatty material in the thymus may not decrease further after the age of about 30 years (87). Secretion of the important immunoactive hormone, thymulin, continues throughout life, although blood thymulin levels do decrease with age (88). There is evidence here, however, that lower levels of thyroid hormones and insulin, rather than thymus dysfunction, are responsible for lower thymulin levels (88). 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 man. There is evidence to suggest that even in the very old, sufficient thymic function may be retained to allow for naive T cell differentiation (89). It has been estimated that complete thymic atrophy in humans would not occur until the age of about 120 years (90). 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 (91).

T cell immunosenescence

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, leading to the suggestion that thymic atrophy is an energy-saving process according to the disposable soma theory of aging (92). According to this view, 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 (92) 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 (93).

Whatever the reason, the effects of thymic changes associated with increased age on the immune system in mice, at least, are marked. As mentioned above, the situation in humans may not be so clear. Moreover, in mice, age-related changes in the thymus may influence other organ systems in some manner, as has been reported for effects on the liver (94). In mouse, the number of T cells exported from the thymus decreases with age, as does the ability of thymic epithelium from old animals to support the differentiation of T cells from young animals´ BM. The type of T cell produced is affected by aging. There may be a developmental block which results in an increase in the frequency of CD3$^+$ CD4/8-DN thymocytes (95). This may result in higher proportions of apparently immature T cells being present in old individuals (consistent with decreased thymic function) (96-98). 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. One group specifically tested this and concluded that such cells were indeed functionally active NK cells (99). Other important changes related to altered thymic function may include changed restriction repertoires of the T cells generated, such that even TCR2 (TCR-alphaβ) cells acquire responsiveness to antigen presented by non-self MHC in man (40) and mouse (39). Evidence has also been presented for increased levels of extra-thymically-differentiated T cells in elderly humans, as well as increased NK-phenotype (but not NK-functional) cells (100). The increase in extra-thymically-differentiated T cells may also represent some sort of compensatory mechanism for decreased thymic integrity.

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 (101). 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 (102). The ability to generate new T lymphocytes after chemotherapy is inversely related to the patients´ age, probably an indirect indication of thymic involution (103). 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 (104). Recovery of CD4 cells was inversely related to age of donor and was enhanced in patients with thymic enlargement after chemotherapy (105). 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 apoptosis on the part of these cells (106). These data are reminiscent of events noted in tissue culture, where CD4 clones become more susceptible to apoptosis as they age (107). 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. 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 (105). 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 were present before initiation of therapy (108). 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 (CD45RAlo, CD29hi, CD11ahi) were abundant in these patients and many of these were CD28-negative (see section 4.2.1). Moreover, there was a negative correlation between the ability to produce naive T cells after BMT and patient age (109), 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 (110). A bone marrow-transplanted young thymectomized patient mimicked this phenotype (111). 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 (111). 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 (112).

3.3. Post-thymic aging

Finally, mature T cells are also subject to aging processes, either of the type affecting post-mitotic cells (when quiescent) or of the "replicative senescence" type (during clonal expansion for effective immune responses). This will be discussed at length in the following sections.

In human, 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 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 (113). 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 immobilised CD3 mAb may not be reduced (114). The reason for this discrepancy 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.

4. ALTERATIONS IN T CELL STIMULATION

4.1. Accessory cells

Decreased T cell responses in the elderly may be due to decreased T cell function, decreased accessory cell function, or both. There is some evidence for age-associated changes at the level of the accessory cell (115). For example, in human, the decreased cloning efficiency of T cells from elderly individuals was found to be caused not only by a defect in the T cells but also by a defect in accessory function of old PBMC, whereas the proliferative ability of the T cells after establishment was equivalent in young and old (116). In mice, the precursor frequency of memory cytotoxic T cells which respond to influenza is entirely dependent upon the age of the antigen presenting cell donor. Specifically, these studies clearly demonstrated that memory T cells from influenza-primed old mice showed a significantly higher response in limiting dilution cultures when stimulated with influenza-infected splenocytes from young-versus-old mice (117). Additional studies on age-associated decreased proteosome function (essential for generation of antigenic peptides in APC) may yield information on the molecular basis of altered antigen processing / presentation (118,119).

In other respects also, APC may function suboptimally in the aged. For example, monocytes are compromised in their function in the elderly in that they secrete less IL 1, and show decreased cytotoxicity and protein kinase translocation (120). A more recent analysis suggested that lipopolysaccharide (LPS)-stimulated monocytes from the elderly produced less G-CSF, GM-CSF, IL 8, TNF-alpha, and MIP-1-alpha as well as less IL 1ß compared to those from young donors (121), although they may secrete equivalent amounts of the critical Th cytokine IL 12 (122). 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 (123). 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 (124). 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 esthers, mAb), Beckman *et al.* (125) 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. Thus, signalling 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 esther, suggesting multiple deficiencies in the naive cells but not the memory cells of old donors.

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 (126). The same group also reported that DC from the elderly were able to inhibit apoptosis and stimulate proliferation in pre-senescent cultured T cells (127). Thus, Steger *et al.* (126,127) 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 (128). However, Steger *et al.* were able to generate larger numbers of DC from the elderly than from the young, apparently providing them with an APC advantage over the young. Nonetheless, this group also reported that DC from the elderly may fail to cross tissue barriers properly and have an impaired capacity to trigger IFN and IL 10 production by influenza-specific T cells in vitro, suggesting that they may manifest highly selective lesions in normal functioning (129). 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 (130,131).

4.2. COSTIMULATORY PATHWAYS

4.2.1. CD28

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. Aberrations in these molecules and/or their receptors would also lead to compromised T cell responses. CD28 is an important costimulatory receptor, the expression of which is decreased in aging in man (132-134) and monkeys (135). This has functional consequences. It has been suggested that 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 (136). These findings are reminiscent of our own, where we established that the age-associated decrease in density of expression of CD28 on CD4+ T cell clones in culture correlated with their proliferative capacity (137). 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 (138).

In mice, Dobber *et al.* (139) reported that aged mouse CD4$^+$ cells stimulated with Con A or anti-CD3 + 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 (140). However, CD28-signalling, at least as far as the induction of Raf-1, appears not to be compromised in CD4+ cells of aged mice (Kirk & Miller, pers comm). Engwerda *et al.* have also more recently shown that activation-induced cell death (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 (141) (although there are also other mechanisms of protection against apoptosis, some of which may not involve CD28 (142) and conversely under certain circumstances CD28 costimulation may actually enhance apoptosis by upregulating apoptotic mediators (143)). However, CD28 costimulation most often protects against apoptosis, even that caused by ligation of CD95 (fas) on the T cell surface (144), and CD28-negative cells are more susceptible to apoptosis (145). 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 (Caruso et al., unpublished results). Emphasising its importance, this "biomarker" of aging, CD28 downregulation, is accompanied by the presence of shorter telomeres in CD28-negative cells (133,146,147) (see section 5.2).

Decreased signalling 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 (148) may be able actively to suppress other cells in a mixed population, cells which otherwise would be capable of proliferation (149). 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 delivers "off" signals to the T cell when ligated by the same structures as CD28 (CD80, CD86) (150). Old T cells may express increased amounts of CD152 (CTLA-4), which may therefore make them harder to turn on even if CD28 functions normally (151). This is another property shared with young anergic T cells (A. Engel & G. Pawelec, unpublished results).

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. However, there is very little data thus far on age-associated changes of costimulatory molecule 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 (152). In contrast, DC in germinal centers of aged mice may lack expression of CD86 (153) which would encourage the induction of anergy or apoptosis in the antigen-specific T cells with which they interacted. This clearly an area where more data are needed.

4.2.2. 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.* (154) reported defects in cell-cell binding amongst healthy elderly donors, which was associated with altered activation capacity of the integrin LFA-1. 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 (122,155). 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 (156). Thus, 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 signalling (157). Consistent with the in vivo observations, the level of expression of CD154 (CD40-ligand) was also reduced (156).

4.3. T cell receptor signal transduction

That early events in T cell activation are compromised in the elderly is reflected in findings that calcium influx is reduced (158,159) and the earliest cell surface alterations associated with activation are decreased, eg. CD69 and CD71 (160). 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 signal transduction through either or all of the TCR components, or costimulatory receptors (section 4.2), or growth factor receptors (section 4.4). There is evidence for age-associated alterations at all three of these levels.

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 remain the same, their degree of phosphorylation after T cell activation declines with age in mouse and human (161,162). In mice, initial biochemical events following TCR triggering are compromised. There is decreased formation of second messengers such as IP3 and DAG, although the activity of PLC (which is responsible for IP3 and DAG generation) appears conserved in old T cells (163). However, the actual amount of PLC present in freshly

isolated cells may be decreased with aging (164). In human T cells, selective reduction of one isoform of protein kinase C (165) might contribute to decreased T cell proliferation. This applies to both resting and activated human T cells (166). Moreover, 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, signalling 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 (167). 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 (168). They further found that TCR-associated p59fyn enzymatic activity 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 (168). 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 (169). 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 (170). This was associated with defects in IL 2 but not IFN-gamma production (170).

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 (171). 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 signalling 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 (172), and in man, CD3-stimulated T cells from 50% of old subjects were found to show reductions in MAPK activation (173). 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 (173), suggesting signalling 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 (174).

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 (175-178). The proportion of cells expressing c-myc (G0 to G1 marker) 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 (178). T cells retaining antigen recognition and effector function, yet apparently in a post-mitotic senescent or pre-senescent state have been described (179). 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 (180). 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 (181). 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 (182).

4.4. Cytokine production and response

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 (183,184). Specific defects in AP-1 activation have been reported in young T cell clones rendered anergic in vitro (185). 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). AP-1 activation may be impaired in in vivo-aged human T cells as well (186). 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 (186). Thereafter, the same group reported that both AP-1 and NF-AT were reduced in elderly donors´ stimulated T cells (187). 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.* (177) 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 (188). 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 (189), 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 (190) and is thought to be important for IL 2 gene transcription (191) as

well as for IL 2R (CD25) transcription (192). Additionally, there is evidence in old mice for differential expression of the negative transcriptional regulator for IL 2, Nil-2a (193).

Amongst other transcription factors of known importance for IL 2 production, CD3-stimulated induction of NF-kappa B was also found to be decreased in old mice (194) and humans (195). One reason for insufficient NF-kB activation may be that the natural inhibitor I-kB is not adequately degraded because of compromised proteosome function (Ponnappan, cited in ref. (1)). Age-associated inactivation of proteosome function has been independently reported and attributed to the effects oxidative damage, which can be partly prevented by hsp90 (118). Hsp90 levels are themselves decreased with age in PHA-stimulated T cells (196). Whisler *et al.* (187) also found reduced NF-kappa B 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 (197). Finally, in rats, Pahlavani *et al.* reported that the induction of AP-1, NF-kappa B 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 (198). On the other hand, constitutive NF-kappa B activation in lymphoid tissue of old animals (which can be corrected by dietary anti-oxidants) may contribute to dysregulated cytokine synthesis (199). 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 sulphate (DHEAS) and WY-14,643. This results in restoration of the cellular redox balance and reduction of spontaneous inflammatory cytokine production (200). Ageing in mice is accompanied by decreased levels of PPAR transcripts, but this decrease can also be reversed by DHEAS administration (200).

Thus, one result of poorer T cell function is decreased cytokine production. 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 (201). Many studies have confirmed that T cells from aged humans can also show defects in IL 2 secretion, IL 2R expression and DNA synthesis after stimulation with mitogens like PHA. It has also been argued that changes in cytokine secretion patterns are determined by the relative proportions of different T cell subsets. Thus, several investigators who confirmed the decrease in IL 2 production in old mice concluded that this was solely a result of different subset composition. Engwerda *et al.* (202) 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-gamma). Kirman *et al.* (203) 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. However, Kurashima *et al.* (204) 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, age-associated alterations in cytokine production are not determined solely by the subset changes, but by alterations within each of those subsets.

Data on cytokine secretion in human are inconsistent, partly because early studies employed apparently healthy elderly donors without rigorously excluding underlying illness, or assessing nutritional or psychological status. In addition, cytokine secretion may be affected by other (non-pathological) parameters such as exercise, as reported by Shinkai *et al.* (205), or possibly by time of day of blood collection because of circadian rythm in cytokine secretion (206). It was nonetheless anticipated that when data became available using donors selected according to the strict standard criteria of the SENIEUR protocol (207)), 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. However, 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 (208). 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 (208). 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-gamma production, but significantly decreased IFN-gamma and soluble IL 2 receptor secretion has been reported in German SENIEUR donors (209). Equivalent levels of IL 2 secretion by cells from SENIEUR and JUNIEUR donors has also been reported by others (210). 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- gamma production but unaltered IL 4 and IL 6 secretion after PHA stimulation in Sicilian donors (211). Both IL 2 and IL 4 secretion have been reported to be reduced in other Italian SENIEUR donors (212). 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 (213). Other changes in cytokine secretion patterns have also been established. Increased IFN- gamma production (25), and, as also shown in mouse, enhanced IL-10 production have been reported (214,215). *In vivo* studies of plasma levels of factors such as IL 6 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 (216). For example, increased levels of IL 6 (and in this case, also TNF-alpha and CRP) are associated with senile osteoporosis in both females and males (217). This may offer a possibility for pharmacological intervention by IL 6R blockade (218). Even

for IL 6, however, not all investigators have found elevated serum levels in perfectly healthy elderly humans (219).

It therefore seems that despite the use of the SENIEUR protocol, discrepancies in cytokine production data still arise. The reasons for this are not yet clear. Given the strict parameters of the SENIEUR protocol, it is perhaps unlikely to be caused by the unrecognised presence of underlying disease, but this remains a possibility. The care with which selection procedures are applied may thus greatly influence the results obtained. Thus, for example, even in SENIEUR donors, further subtle differences such as serum folate levels between individuals may affect IL 6 production (210). In the context of psychoneuroendocrinological considerations, the SENIEUR protocol may not offer the best selection procedure. That age-associated decreased immunological function may be linked to psychological factors has been rarely considered (210,212). Such factors may need to be taken more into account in studies of immunosenescence (220), and 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 (221). Other psychosocial factors may also play a significant role in clinically-relevant situations, eg. stress (222). The humoral response to influenza vaccination was altered by stress in one study, where the effect of chronic stress (caring for a demented spouse) resulted in significantly lower antibody titers, as well as IL 1 and IL 2 production in the elderly caregivers (223). Some data are beginning to emerge on the regulatory effects of neuropeptides on cytokine secretion in young and elderly donors (224). The expression of dopamine D3 receptors on human lymphocytes is decreased precipitously between 40 - 50 years of age (225). Conversely, increasing levels of expression of cellular amyloid precursor protein by human lymphocytes are significantly associated with age (226). 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-gamma and other cytokines to modulate the production of melatonin (227). 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 (228).

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 (229). Particularly the MHC class II alleles, which are mostly responsible for presenting foreign antigen to CD4+ helper T cells, may influence longevity (230) 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 (231). 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-alpha and IL 6 secretion by CD3+ cells have been confirmed (232).

On the other hand, the IL 2 secretion defect seen in many studies in vitro 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 (233). 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 immunisation (233). If these results are confirmed, which seems still not to be the case, 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 (234). 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 (235,236). Not only might these activated cells explain the data of Huang et al (233), but since they might eventually arrive at a post-mitotic state, this would also explain their eventual loss from the system altogether.

Direct measurements of IL 2 levels in the serum of SENIEUR donors have also failed to detect age-associated decreases (237), although a second study suggested that serum IL 2 levels were reduced in the very old (238). 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 (239). 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 (240). Other defects in IL 2R expression by aged T cells have also been noted; in particular, they are defective in their upregulation of the high affinity receptor for IL 2 (241), and even that lower proportion of cells expressing the receptor and retaining their ability to internalise the IL 2 still fail to respond properly (242). 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 (243), aged T cells may still proliferate less vigorously than young cells, even in the presence of exogenous IL 2 (243), although this has not been seen in all studies (244). The

reasons for any such suboptimal proliferation are still not clear. Such intrinsic decreased proliferative capacity has even been seen in T cell clones established from aged SENIEUR donors´ cells, particularly for CD8^{+} cells (245). Few other studies have compared the behaviour of T cells from young and old donors at the clonal level. Paganelli *et al.* (246) 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-gamma 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.

Other cytokines are also beginning to be examined now, eg. spontaneous production of IL 8 by monocytes 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 (247). 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-ß and MIP-1alpha) than young donors (121), while other studies suggested increased production of IL 6, IL 8 and TNF-alpha by the elderly (209,248) and unchanged IL 12 (122). 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 (249). Thus, production of cytokines such as TNF-alpha may increase rather than decrease with age (250), although serum titers in the healthy elderly may not (219). Increases in TNF-alpha may be directly relevant for decreased T cell responses, because TNF-alpha can inhibit proliferation of some human TCC (251) and can attenuate TCR-signalling in vivo in mice (252).

As well as altered cytokine levels in aging, altered levels of cytokine antagonists might also influence cytokine networks. These possibilities are now beginning to be explored (for factors in addition to sIL 2R mentioned above). Thus, Catania et al. (253) 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. 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-alpha-receptor and IL 1Ralpha. 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 (253). Indeed, the production of factors such as IL 6 is clearly influenced by health status, even in "near-SENIEUR" donors (254). In fact, as mentioned above, it has been proposed that IL 6 levels may be a good overall biomarker of general health in aging because plasma levels are correlated with functional status (216). This may in turn be related to subclinical disease status. IL 1 and TNF-alpha, as well as IL 6, together with IL 3 and IL 4, the levels of which are increased in aged mice and humans (248,255), 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. IL 6 may also influence the responsiveness of T cells to other factors such as PDGF, and hence alter their cytokine production profiles (256). These observations emphasise 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.

At present, the Th1/Th2 paradigm is an important conceptual tool to characterise T helper cells, since cytokine profiles appear to be skewed towards these two main types, and some of the data discussed above may be viewed in terms of Th1 and Th2 shifts with age. The Th1 cells are characterised by their ability to secrete IFN-gamma, whereas Th2 cells are characterised by IL-4 secretion. The Th1 cells are considered pro-inflammatory cells, which are important for defence against intracellular pathogens, requiring cell-mediated immunity, whereas Th2 cells are considered anti-inflammatory cells, and mediate humoral responses (257,258). However, because the Th1/Th2 paradigm has been defined using clones of CD4^{+} T cells in mouse, this model does not necessarily reflect cytokine regulation at all levels. It may be more useful to consider responses in terms of type 1 cytokine predominance (primarily stimulating cellular responses, i.e. IL 2, IFN-gamma, TNF-ß, IL 12 and IL 15) and type 2 cytokines (primarily supporting humoral responses, i.e. IL 4, IL 5, IL 6, IL 10 and IL 13). Some of these cytokines are cross-regulatory in that they not only upregulate one immune component but also downregulate the other. Infant humans exhibit impaired cellular but strong humoral immunity, and are probably in a type 2-dominant state. Soon thereafter, a type 1-state becomes dominant and persists in healthy humans until mid-to-later life, at which time a dominant type 2 cytokine profile may again emerge (259). Accordingly, in the elderly, an impairment of in vitro production of type 1 cytokines has been described whereas the ability of mitogen-stimulated PBMC from old healthy subjects to produce type 2 cytokines may be unchanged or even increased, as discussed above. Naive CD4+ T cells when stimulated produce IL 2 as their major cytokine. Upon priming, these cells develop either into Th1-type or Th2-type cells. The dominant factors that determine the differentiation towards the Th1 or Th2 patterns are other cytokines. For example, if type 2 cytokines are present during the priming period, the resulting CD4^{+} T cells produce IL 4 upon restimulation; the development of IFN-gamma-producing cells is strikingly inhibited by type 2 cytokines, although this inhibitory effect is blunted in the presence of IL 12. In the absence of type 2 cytokines, priming for IFN-gamma production occurs, but this is markedly enhanced by IL 12 (260). Although other factors may play a role in the determination of cytokine-producing phenotype, such as antigen dose, MHC type of antigen-presenting cell (261), and expression of accessory molecules and hormones, these effects appear to be secondary to the dominant role of cytokines.

The question of which mechanism results in type 2 dominant state in the elderly has yet to be definitively answered. A key role might be played by the decreased production of IL 12, now considered the key cytokine for the induction of a cellular response. IL 12 production by APC is induced through two different pathways of stimulation (262,263). One is associated with their exposure to a variety of pathogens or their derived products and is not impaired in the elderly. The other is activated by interaction of APC with T cells responding to nominal antigens or to mitogens and it is impaired in the elderly because of a reduced efficiency of costimulatory signals, as already discussed. To gain further insight into the mechanisms of this impairment, experiments were performed to evaluate the production of type 1 cytokines by PBMC from old and young donors in the presence of IL 2. Results showed that the impaired type 1 cytokine production was reversed by adding IL 2 to the culture medium, suggesting that IL 2 treatment restores the production of Type 1 cytokines by partially overcoming the costimulation deficit (Lio et al., submitted for publication). At the molecular signal transduction level, the preferentially decreased amounts of c-jun in stimulated T cells from elderly donors might be involved in skewing the response because recent data suggest that in jnk1-knockout mice, T cell stimulation results in preferential Th2 responses.

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 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. Therefore, limits to the proliferative capacity of the T cell clones might impact deleteriously on the overall response. T cell clones in vitro may provide a good model for investigating age-associated changes in a longitudinal manner. T cells can be maintained for extended periods in tissue culture, but in most cases they have finite lifespans, eg. see refs. (133,179,245,264-269). 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 (270). 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 after a single stimulation on the part of old T cells. 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. (264) achieved far greater T cell expansion ranging from 62 - 172 PD (as calculated from their figures of cell expansion). There were no differences in this work 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 (271). 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. This is more physiologically relevant than merely establishing a record holder. McCarron et al. also examined longevity of T cell clones in their seminal paper (264). Here, their 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 a comparison of the in vitro longevity of TCC derived from mature peripheral T cells and those derived from T cell progenitors of the same donor it was found that both manifested limited lifespans. Progenitor-derived cells survived on average 55 PD, whereas those derived from cells with a mature T cell phenotype averaged 35 PD, in good agreement with McCarron´s earlier study (137).

There may nonetheless be rare exceptions to T cell 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 immortalised (272). 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 (273).

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 fibroblasts (274,275). Conversely, increasing the oxygen content from 20% to 40% rapidly rendered young fibroblasts senescent and gene expression changed accordingly (276). Whether T cells behave similarly and whether they are more sensitive to oxidative damage, has not yet been reported. 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 (277). 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 (278). 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, anti-sense oligonucleotides for p53 and Rb, high albumin concentrations, additional growth factors and other hormones (reviewed in ref. (279)). 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 (280).

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. (281)). However, skin-infiltrating cells cultured in the presence of IL 4 in addition to IL 2 but in the absence of antigen presenting cells have been found to grow apparently indefinitely (282). These cells were found not to harbour 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 (283). 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 generalised 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, personal communication, May, 1998). Even such T cells also showed decreasing levels of CD28 expression with increasing age; they also expressed telomerase (284). 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 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 (285). Loss of telomeric repeats is not an in vitro phenomenon as it is also observed in human cells in vivo (286). 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 (287). Telomere shortening might therefore act as a mechanism counting the number of cell divisions that a cell population

has experienced. Telomere length in human blood cells in vivo was shown to be related to donor age (288). The correlation between shortening of telomeric repeats and age of the donor is not confounded by differences in white blood cell count (289). Telomere attrition occurs more rapidly in premature aging syndromes, eg. Hutchinson-Gilford progeria (290) or trisomy-21 (291). The overall rate of telomere shortening is not necessarily linear with age (292,293), but this may reflect differential activation-induced upregulation of telomerase activity (294) or genetically regulated differences between donors in telomerase activity which may further complicate cross-sectional comparisons between donors of different ages (295). 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 (296). It must also be borne in mind that the different human chromosomes may not all experience telomere shortening at the same rate and therefore an averaging of all chromosomes´ telomeres (as usually reported in the literature thus far) may not provide a true picture of critical chromosomal damage (297). 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 (291). Weng et al. (298) 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 (137), 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 (290,299). Indeed, telomerase, an enzyme responsible for maintaining telomere length in unicellular eukaryotes, had, in fact, been previously demonstrated in immortalised 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 (55). Using more sensitive assays, it became clear that the strict dichotomy between tumor and somatic cells was not as straightforward as had been previously thought (300). For example, Hiyama *et al.* (294) have shown that telomerase activity is detectable at very low levels in normal human T and B cells and that it increases greatly after mitogenic stimulation. The level of telomerase activity in freshly isolated CD4+ cells from normal donors is said not to change with age (301). However, optimal telomerase induction requires optimal stimulation via CD3 and CD28 (302), 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 (303). 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 (304). 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 (305). In CD8 cells, Monteiro *et al.* (147) 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 (306). 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 (306). 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 (301). 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 (Valenzuela & Effros, manuscript in preparation). Whether the situation is comparable in vivo is open to question. In acute infectious mononucleosis it was found that CD8 cells upregulate telomerase and maintain or even increase telomere lengths until resolution of infection (Maini et al., manuscript submitted for publication), so other mechanisms may be involved here, which are lacking in vitro. 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 (284). 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. Strahl & Blackburn reported (307) that inhibitors of retroviral reverse transcriptase (telomerase itself is a specialized cellular reverse transcriptase) could cause progressive telomere shortening of immortalised 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. More recent data have confirmed and taken this idea further (308). On the other hand, certain tissues appear to undergo senescence correlated to decreased telomerase activity despite their ability to retain telomere lengths (309). However, that both telomerase activity and telomere length can be absolutely critical in determining longevity of cells in culture has been unequivocally demonstrated by two independent laboratories (310,311) and indirectly confirmed by a third (312). Using telomerase-negative cell types, they 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. This is the first evidence for a causal relationship between telomere shortening and cellular senescence in vitro. However, these experiments have been criticised on the basis that a proper negative control was lacking. It was pointed out that a vector-only control lacking any inserted genes was used but a vector replacing the telomerase-encoding portion with an irrelevant gene should have been used (313). It was noted that similar experiments not using telomerase in the past had indeed resulted in similar lifespan extensions (313). Nevertheless, the duplication of the telomerase experiment by two independent groups provides strong support for the critical role of telomere shortening in the phenomenon of replicative senescence.

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. 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 visualised 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 (236). 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 (314). 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 (315). A direct approach to whether memory cells continue to cycle in vivo has been taken using transgenic mice (316). 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) (317). 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 (318). 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 (319)). 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 (320). 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 (321). 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 (146). Evidence suggests that the CD28-negative cells are indeed derived from originally CD28+ cells (322). 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 (323). There is also an increase in CD4$^+$ CD28-negative cells in HIV infection, albeit not so marked as in the CD8 subset (324). 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 (325). 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 (326). 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 (327). 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 (328). However, virally infected cells may conceivably express dysregulated telomerase. 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 (329), 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.

For non-viral antigens, and thymus-independent regeneration of T cells in general, it is probable that antigen-driven peripheral expansion commonly occurs (330), 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 sort of TCR cross-reactivity responsible for maintaining numbers of T cells, eg. in the case of CD4 cells via class II molecules regardless of their peptide loading (331).

5.4. Activation-induced cell death and aging

Further light has been cast on the aging of human CD4$^+$ T cells using the mAb PD7/26 specific for the CD45RB isoform. Salmon et al (332) 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 (333). 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 (334).

Studies on CD45RB in vivo in elderly humans confirmed that CD4$^+$ cells in old donors showed significantly decreased CD45RB expression (335). In agreement with the in vitro data, the percentage of human CD4 and CD8 fas+ cells also increased with age in vivo (336,337). The majority of these expressed CD45RO, and CD25 and CD69 activation markers (338). It must be mentioned, however, that one study has reported an age-associated decrease in both CD4+ CD45RA+ (but not CD45RO+) and CD45RA+ and RO+ CD8+ cells coexpressing fas (CD95); only this latter study actually examined subjects at an advanced age (339). Hence, the frequency of CD95+ cells may rise through adulthood, but go on to decrease in certain but not all subsets in old age (339). A decrease of CD95+ cells in advanced age following a rise through adulthood has also been observed by others (Caruso et al., unpublished results). On the other hand, another recent study examining CD95 expression in the elderly found increased levels of expression of both fas and fas ligand on CD4 and CD8 cells, and these did not seem to decrease at more advanced age (340). Thus, in the Aggarwal study, 6% of CD4+ CD45RA+ cells in the young were CD95+ but 17% of the old, whereas in the Aspinall study these figures were 5% and 1% respectively. In the CD4+ CD45RO+ subset in the young-versus-old these figures were 25% and 40% respectively in the Aggarwal study, but 11% and 9% respectively in the Aspinall study (both with unstimulated cells). In the CD8 subsets, differences between the two data sets are just as extreme. The reasons for these differences are unknown, although the percentages of CD95+ cells in unstimulated cells of the Aggarwal study do seem rather high, possibly due to their use of donors not selected by strict criteria such as the SENIEUR protocol. The two studies each employed only one single CD95 mAb (UB-2 and DX-2), perhaps contributing to these disparate data. If so, this would be a serious problem in interpreting such results, as most studies do not use multiple mAb to the same CD antigen. The 6th CD workshop determined that at least three CD95 epitopes were detected by the submitted CD95 mAb, one of which was DX-2 (341). In the Aggarwal study but not in the Aspinall study the CD95 changes were noted on CD45RO+ cells as well, together with decreased levels of bcl-2 and correspondingly increased levels of CD95 antibody-triggered apoptosis in all subsets with increased CD95 expression (340). In a different system, in which T cells were stimulated by PHA and cultured with IL 2 for up to 6 days, apoptosis (not induced by CD95 mAb) was reported to be increased in CD45RO-negative but not RO-positive cells in the elderly compared to the young (342). It was therefore suggested that differential susceptibility to apoptosis late after T cell stimulation might contribute to explaining the preponderance of CD45RO+ cells in the elderly, although the differences actually measured in these studies were minimal (46.0 +/- 4.5% versus 57.6 +/- 6.1, P < 0.01, but n = only 7 in each group, with wide inter-individual variation and no information given on donor selection criteria). Lack of enhanced susceptibility of CD45RO+ cells to apoptosis is also contrary to findings noted above in vitro and in vivo (107,332,340), and careful inspection of the data in the above publication revealed that there was in fact a greater degree of susceptibility to apoptosis by CD45RO+ cells in the elderly as well (27%-versus-36% respectively, a difference of 9% compared to 11% in the RO-negatives; hardly an impressive difference).

The amount of soluble fas in the blood of elderly donors was also reported to be significantly increased compared to young donors (343). Moreover, several studies have shown increased CD3- or PHA-mediated AICD and hence decreased proliferation in the elderly (344-346). This was found not to be due to IL 2 deprivation, nor was it associated with decreased bcl-2 expression (344). 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 (347) as well as adult T cells in an IL 2-independent, fas/fas-ligand-dependent manner (348). 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, CD4+ T cell clones aged in culture also become increasingly susceptible to AICD (107); 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 (345). In mice in vivo, evidence for increased apoptotic death of superantigen-stimulated T cells has been forthcoming (349). 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 (350). 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 (351). 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 (352). This could be interpreted to mean that stronger proliferative responses, while neuroprotective (352), result in more rapid clonal exhaustion and therefore disease progression.

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. (353) 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 (353). 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 (354). 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 <u>protect</u> against cell death (355), and thereby result in enhanced responsiveness. Spaulding *et al.* (356) 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.* (357) 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 (Spaulding, Wei & Effros, manuscript submitted for publication). These data are reminiscent of fibroblast cultures, which show similar senescence-associated changes (358). Some further supporting data for the concept of decreased apoptosis in aged cells may be found in the report of Lechner *et al.* (345) 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 (345) 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 (107). 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 6). 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 (359), and expression of CD95 by CD4 cells of old mice infected with M. tuberculosis is not decreased (360). Other tissues may also show increasing susceptibility to apoptosis with age, as is the case with hepatocytes (361). Here, caloric restriction also reverses the age-associated effects and reduced apoptosis, the opposite of its effect on lymphocytes.

6. ALTERATIONS IN T CELL SUBSETS, REPERTOIRE AND MARKERS WITH AGING

6.1. T cell subsets

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 and may be influenced by underlying disease (362), although not all studies agree on this (363). It is probably true to say that all in all, the consensus is that age-associated alterations in T cell numbers are minimal (364). 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 (365), and the same may be true in humans, although the reduction in CD4 cells is also associated with poor nutritional status (210).

There are clearly more CD4$^+$ CD45RO$^+$ "memory-phenotype" cells and less CD45RA$^+$ "naive-phenotype" cells in PBMC from elderly individuals (24,366,367). These changes are also observed in strictly selected elderly populations and seem to occur independently of health and nutritional status (210). If the CD45RO$^+$ cells represent 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. However, not only does the proportion of memory-phenotype cells increase with aging, but in mice at least the memory cells themselves function less well in old than in young donors (368-370). Moreover, in the oldest old, decreases in memory cell phenotype RO$^+$ cells have also been recorded (238) and in whole blood analyses, a relative decrease of CD45RO$^+$ cells may also be seen in the CD8 but not in the CD4 population (214). 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 (371). 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 (372). More subtle analyses may 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 (Pgp^{hi}) and therefore able to extrude rhodamine 123. Pgp is known also to participate in the transportation and secretion of cytokines including IL 2, IL 4 and IFN-gamma (373), although Pgp-knockout mice appear to proliferate and produce cytokines normally (Eisenbraun & Miller, pers. comm., December, 1998). Nevertheless, mAb against Pgp block IL 2 release in PHA-activated T cells, demonstrating a role for this molecule in T cell function (374). Despite this, high Pgp expression in old T cells is associated with dysfunctional status. Possibly the Pgp itself is dysfunctional and over-expression represents an attempted compensation. There is an age-associated increase in the expression of MHC class I molecules on these PGP^{hi} CD4+ memory cells. In spite of this, the levels of TAP1 decrease in old mice (TAP1 transporter is usually required for class I peptide loading and antigen presentation). The Pgp and TAP1 molecules are related, so Pgp may be taking over the function of TAP1 in old cells and increasing the level of expression of MHC class I molecules. This question remains to be clarified (375). Recent data in the human system, however, suggest that Pgp is unable to substitute for TAP as a peptide transporter and that it does not enhance MHC class I expression in T2 cells (376). The failure of the murine Pgp^{hi} cells to respond to

TCR-mediated stimulation cannot be overcome by CD28 signalling, PMA, IL 4 or IL 12 or combinations of these, and may therefore be considered "anergic". Not only do Pgp^{hi} cells fail to secrete IL 2 but also show impaired IL 5 and IL 10 production and proliferation. Oddly, however, their ability to secrete IFN-gamma increases with age (377).

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 (378). 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 (379).

In human, most T cells are CD7+, but the frequency of CD7-negative cells increases with age (380) and although isolated T cell clones retain stable expression of their CD7-positive or negative phenotype (381), repeated stimulation and propagation of uncloned lines results in accumulation of CD7-negative cells in the CD4 but not CD8 subset (382). Increased proportions of CD7-negative cells are found in situations of chronic antigenic stimulation in vivo, eg. in rheumatoid arthritis (383) and in kidney transplant recipients (384). Such CD7-negative cells show low proliferative responses to CD3-stimulation, low IL 2 secretion but high IL 4 and IL 10 secretion (385). 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 (386). Effros *et al.* were the first to observe a decreasing percentage of CD8 cells carrying CD28 in the elderly, paralleling their observations in T cell lines aging in culture (133). Others have confirmed that particularly the CD8 subset shows progressively decreasing CD28 expression with age (134). CD4+ T cells, almost all of which are CD28+ in young adults, also show an increasing CD28-negative fraction in the elderly (136). The fraction of CD8+ CD28-negative cells in centenarians is somewhat higher than in the elderly (70-90 year-old) population (136). 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 (387) (see section 5.2). This type of proliferative senescence may therefore be responsible for the commonly observed accumulation of CD28-negative oligoclonal populations in elderly people (388). 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 (389). Similar observations on clonal expansions in CD4 populations have also now been made in mouse (364). It may well be that the origin of at least some of these clonal expansions resides in anti-viral immunity (390).

In diseases with chronic antigenic stimulation, further circumstantial evidence in favour 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 (391); both CD4 and CD8 cells show decreased CD28 expression in chronic B lymphocytic leukemia (392) and in hairy cell leukaemia (393); in Crohn´s Disease, the ability of CD28 to mediate costimulation of CD4 cells is compromised (394). 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 (395) and this was accompanied by reduced proliferation in vitro by graft recipent lymphocytes stimulated by donor cells (396). 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 (397). In rheumatoid arthritis, the percentage of CD4 cells carrying CD28 is reduced (398) and in both RA patients and normal controls the CD4$^+$ CD28-negative cells show TCRVB oligoclonality (399). Such CD4+ CD28-negative cells appear also to be CD7-negative (399). This phenomenon is influenced by HLA type, however, with RA-associated HLA-DRB1*0401+ donors having higher proportions of CD4+CD28-negative cells (400), 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 (399,400). These cells differ from in vitro expanded CD4+CD28-negative cells in that they are more not less resistant to apoptosis (401). In systemic lupus erythematosus (SLE), T cells also show increased levels of bcl-2 (402). The relationship of these CD28-negative cells to ageing 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 leukaemia, the CD28-negative cells while retaining normal functions such as cytotoxicity and cytokine release, show decreased clonal expansion capacity and increased susceptibility to apoptosis (393). 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 (156). However, the in vivo expression of CD11a, CD49e, CD54 and CD62L has been reported to show age-related alterations in mice (403).

6.2. 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 characteriazed 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 (404). 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 (388). 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 (405). Within the $CD8^+$ cells, these oligo- or monoclonal populations are prevalent in the CD28-negative subset (388) and the CD57-positive subset, which essentially overlaps with the CD28-negatives (406). 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 (407). 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 (132). 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 (408). 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 (409). Moreover, the realisation that mature thymus-derived T cells can re-acquire sensitivity to positive and negative selection outside the thymus, in germinal centers (410), indicates that in theory the generation and selection of T cells may take place even in the absence of a functional thymus. 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 (411).

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% (412). Moreover, this was clearly the case to the same extent for the CD4 as well as CD8 cells (412). 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 (412), 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 (413). In human, although not observed by all investigators (414), it may be the rarer CD4 expansions which are observed at increasing frequency in the aged (415). CD4 expansions may be easier to find in the CD45RO memory-phenotype population in the oldest old (416). In contrast, in some disease states, eg. rheumatoid arthritis, CD4 cells may show striking oligoclonal expansions (417). 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 (417). As with the CD8+ cells, in normal donors, the CD4 cell expansions were found in the CD28-negative population (418); 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 (418). 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.

7. CLINICAL RELEVANCE OF IMMUNO-SENESCENCE

7.1. 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. (419-424). 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 (425). Autopsy data on the very old suggest that the accepted prime age-associated causes of death in the "younger old" (ie. cardiovascular, cancer) do not necessarily apply to 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). An extensive study on major causes of death 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 (426). According to some data, a major predictor of mortality in the elderly is lung function (427). Immunosenescence may also play an important role here, since the defence of this most common pathogen entry portal is critical. In support of this concept, Meyer *et al.* (428) 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 (429). 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-alpha induction, than in young mice (430). 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 IFN-gamma production and decreased IL 5 in old rats T cells (431).

However, despite the findings discussed above, it remains the case that the precise clinical relevance of T cell immunosenescence is hard to define. Indeed, there are studies suggesting that the NK status of subjects may also be important or 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 (432). 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 (433). Moreover, inclusion of T cell functional parameters has been shown to predict mortality in a Swedish prospective study (11). 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 (434). 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 (435). 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 (436). 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-gamma and decreased amounts secreted compared to young immune donors, while the production of IL 4 in the same donors was unchanged (437). Correspondingly, antibody levels to VSV are maintained in the elderly, but this is clearly not always enough to prevent reactivation of infection.

7.2. Vaccination (see also section 4.1)

Decreased T cell function in the elderly is shown most clearly in vivo by DTH tests to recall antigens (438) as well as to clinically relevant immunisation procedures where T cell-dependent antibody production is depressed, eg. see refs. (439,440). 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 immunisations 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 (441). 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 (442). Even when initially successful, vaccination may have a less long-lasting effect in older donors (443). 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 (444). Consistent with this, the majority (62%) of elderly individuals vaccinated with tetanus 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 (445). 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.* (446).

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 (447,448). 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 (449-453). 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 (449-453). These findings are probably due to pre-exposure of the elderly and young to different strains of flu viruses (454). PBMC from the healthy elderly may produce lower levels of IFN-gamma both pre- and post-vaccination compared to the young, although levels do go up after vaccination in both young and old (455). Moreover, underlining the importance of IFN-gamma, these investigators found that although elderly individuals often responded only with either a cellular or a humoral response to vaccination, production of IFN-gamma was correlated with the presence of both (456). The production of a potentially inhibitory cytokine, IL 10, was also found to be greater in the young, whereas IL 6 production was similar (455). Others have found increased IL 10 production in the elderly (457); 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 (457). The same group had previously shown that the elderly may also have lower IL-2 production following in vitro stimulation with flu vaccine (458), although this is also partly dependent on the strain used (457). Accordingly, subcutaneous low-dose IL 2 treatment prior to vaccination has been reported to enhance protection of the elderly to ´flu (459). Similar findings concerning antibody titer (but <u>not</u> lymphoproliferative responses) have been reported for tetanus toxoid vaccination (460). 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 (461). However, it could also be that the 4 - 6 week period examined in the latter study after immunisation is

not long enough to be informative. For example, old mice did not show a decline in antibody responses after an immunisation with Streptococcus pneumoniae, but nonetheless, after a second immunisation, 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 (462). Another recent study of the efficacy of ´flu vaccinations in the elderly found 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 (463). 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 (464) and in a study on responses to hepatitis B vaccination (465).

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 randomised double-blind placebo-controlled clinical trial using aspirin has been carried out on 281 donors >65 yr. immunised with influenza vaccine. Aspirin did not affect post-immunisation 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 (466).

Other approaches to enhance responses recently explored may be to use "naked" DNA vaccines, as illustrated by studies in old mice (467,468), or to use technical variations on the theme of viral inactivation-versus-attenuation in elderly humans (469). Further approaches currently being assessed include the use of ISCOM preparations in an effort to enhance immunogenicity of the vaccine (470) or the use of highly active dendritic cells as "professional antigen presenters" to provide a stronger stimulus to the T cells (471). Experimentally, stress also reduces the efficacy of influenza vaccination, theoretically also offering the possibility of enhancing vaccination efficacy by stress reduction approaches (472).

8. PREDICTORS OF MORTALITY AND LONGEVITY (SEE ALSO SECTION 2)

Lifespan is influenced by genetic makeup, most clearly demonstrated so far for the association between longevity and MHC type in mice (473) and possibly humans (230,474-476). One mechanism accounting for this association may be a relationship between MHC alleles and rate of thymic involution (477). Another may relate to the differences in DNA repair capacity in lymphocytes observed between mice of different MHC types (478). However, data from twin studies suggest that in humans the genetic component accounts at most for only one-third of the variance in longevity (479).

The lifespan of allophenic mice produced from a long-lived and a short-lived strain (480) is positively correlated with the proportion of lymphocytes derived from the long-lived strain, showing the importance of the immune system to overall lifespan (481), perhaps via the effect of mature T cells on maintenance of the thymic environment alluded to above (see section 3). 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 lifespans caused by increase incidence of lymphomas (482). The CD4 memory correlation holds regardless of whether the mice died of lymphoma, fibrosarcoma, mammary carcinoma or other terminal disease (483). 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 (484). 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 (485). Increased levels of IL 6 in old mice may play a role in the increased occurrence of lymphoma (486). 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 lifespans, and this was also associated with lower incidence of lymphoma (487). 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 (487). 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 (488). What these results may mean in longer-lived, less tumor-prone species like man is unclear.

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. For example, the CD4:CD8 ratio, which is also affected by age, is different in males and females and in family segregation analysis has been shown to be under genetic control (489). The MHC type of the individual may also affect the absolute numbers of lymphocytes in the periphery; for example, it has been reported that HLA-B8, DR3-haplotype-bearing donors have lower levels of peripheral lymphocytes than other

donors, possibly because of increased levels of spontaneous apoptosis (490). It may be interesting to note that this HLA-B8, DR3 haplotype was one of those found very rarely in a recent study of elderly Greeks (in 1.6% of SENIEUR elderly versus 10% of young controls (476)). Several groups are searching for genetic markers of longevity, and some data are now beginning to appear, eg. the finding that a particular allele, C2, of the proto-oncogene transcription factor ETS-1 is represented at significantly higher levels in the elderly compared to teenagers (491). The relevance to mammalian aging of the recently defined mutations in "gerontogenes" in roundworms and other invertebrate species is unclear, but will be an exciting area of investigation (492).

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 (493). 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). Studies of the very old (ie. the survivors) may also be informative. Centenarians may be considered to be that very small 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 (10), T cell proliferative responses are well-maintained (albeit taking place more slowly), the T cell repertoire still contains all Vß families, hematopoiesis is maintained, autoantibodies are absent, and interestingly, there is a high level of lymphocyte genomic stability (low spontaneous breaks etc., which otherwise are thought to increase with age in average, non-centenarian, donors (494)). 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 (89).

9. POSSIBLE APPROACHES TO INTERVENTIONIST MANIPULATIONS

9.1. Vitamins and minerals; anti-oxidants
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 (495). 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 (496).

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. However, some factors are easily manipulated and have encouraged interventionist approaches simply because these are feasible. Even in carefully selected donors, for example, nutritional status may play a significant role in exacerbating immunological differences (210,497-499). However, studies have also concluded that there is an age-increment which influences immunological status independently of nutritional effects (210). Nonetheless, nutritional status does seem to contribute significantly to immune status and may be relatively amenable to correction by dietary supplementation. "Correction" of immunological parameters coupled with a beneficial effect on resistance to infectious disease has been reported in some studies (500) but not others (501). 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 (502) and has even been reported to slow down the rate of telomere attrition in dividing cells (62). 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 (503). 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 (504). 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 (505). 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 (506). Parameters of immune function in vivo, such as DTH responses, may also normalise in the nutritionally-deficient elderly following appropriate dietary vitamin and mineral supplementation for shorter periods (507). 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 (508). 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 (509). However, it is not known whether these effects of vitamin E are attributable to its anti-oxidant or some other function. 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 (510,511).

A poor vitamin D status is also frequently encountered in the elderly (512). 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 IFN-alpha, one of the cytokines involved in modulating NK activity (513). 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 angiogensis induced by tumor cells (514). Therefore, it is possible that maintained vitamin D3 levels and NK activity help to protect individuals from cancer in old age.

Another supplement commonly believed to enhance immunity is often taken along with other factors, namely ß-carotene. 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 randomised 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 (515).

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 (516) and selenium supplementation has been reported to reverse low levels of proliferation and CTL generation in aged mice (517). In elderly people, selenium supplementation was reported to enhance lymphocyte proliferative responses to pokeweed mitogen (PWM) (518). 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 (519). Another very important mineral factor may be zinc (520,521). Zinc is necessary for the function of many hormones and enzymes, including those known to affect immune responses (eg. testosterone (522)). The elderly have lower zinc levels and the disabled elderly, lower still. 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 (523). This has practical implications, because even if absorption is compromised in the elderly, sufficient supplementation might still overcome the problem (524). In rats, there is also a decrease in serum zinc, and the levels found in the thymus are lower compared to young animals as well (525). These observations on zinc levels may not be limited to rodents, because in humans, zinc supplementation studies have indeed suggested improvement of some

parameters of immune function (526,527). One reason for this may be the zinc-enhancement of otherwise age-associated lowered levels of interferon-alpha production in the aged (528). Experimental zinc depletion and repletion of healthy humans revealed that secretion of the Th1-type cytokines IFN-gamma and IL 2 was decreased during zinc deficiency, whereas Th2-type cytokines (IL 4, IL 6, IL 10) were not affected (529). In animals, Mocchegiani *et al.* (530) confirmed that oral zinc supplementation resulted in a recovery of thymic function (and also demonstrated its influence on extrathymic T cell differentiation pathways (531)) 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 preprogrammed but were caused by the decreased availability of zinc. T cell apoptosis may also be blocked or partially blocked by zinc (359). In this context it is 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 (532) (although melatonin may have direct effects on lymphocytes, which express melatonin receptors (533), ligation of which results in signal transduction and diacylglycerol production (534)). It is argued by Fabris *et al.* that the common pathway of several life-extending endocrinological manipulations is in fact via zinc bioavailability (535). 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 controversial and others have found no benefit of zinc replacement even in elderly populations confirmed to show serum zinc deficiency (214,536). Moreover, thymopentin alone in vitro may increase the precursor frequency of proliferating T cells from old subjects (537). In some studies, even inhibitory effects of zinc supplementation have been reported (538). 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) (539). 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 (540). The situation is therefore not yet clarified.

In mice, T cells from old animals stimulated by CD3 + CD4 ligation mobilise 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 signalling was the decrease in antioxidant levels in old cells in rats (541). 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 depolarised membrane potential and decreased mitochondrial mass. Impairment was completely prevented by addition of extra glutathione to the medium (542). An in vivo relevance for these findings is suggested by the fact that reduced, but not oxidised, glutathione levels in the plasma are decreased in elderly compared to young donors (543). Another report has also documented age-associated decreases in plasma GSH levels and confirmed that this is more oxidised in the elderly than in the young (544). 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 (545). 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 (546). Measurements of anti-oxidant function in rats indicated that levels of anti-oxidant activity in several tissues decreased with age (547). 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 74 (548). Hack *et al.* reported a significant age-associated increase in plasma cystine levels and decrease in plasma thiol levels in 205 donors of both sexes (549). Another study has confirmed decreased antioxidant activity in aged rat plasma but failed to show the same in man (550). Despite the above data, interventionist approaches with antioxidants remain attractive because of their cheapness and easiness. Results thus far, however, are not very positive even in rodents (551,552), although recent studies using N-acetyl-cysteine may be more encouraging (549).

9.2. Hormones

Aging of the immune system will most likely affect other organ systems and eventually impact upon the lifespan of the individual (553). 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 (554). 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 (555). However, in vivo treatment with melatonin is reported not to reconstitute age-associated impairment of NK activity or lymphoproliferative responses in mice (556). Consistent with these results in vitro supplementation also failed to reconstitute proliferation or IL 2 production in old rat cells (557) or old mouse cells (558). On the other hand, melatonin is one of many hormones the levels of which are decreased in the elderly (559). It is, however, unclear whether and how melatonin influences immunosenescence. For a critical review of the "anti-ageing" effects of melatonin, see (560).

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 (561). Administration of low-dose GH to elderly adults for 6 months resulted in an increase in IGF-1 levels (which are reduced in aging (562)) and an improvement in some physiological parameters, such as muscle strength (563). 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 (564). 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 (565). 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 (566). Increased IGF-1 availability may also increase thymic cellularity and presumably thymic output in some animal models (567). However, in general, findings with GH supplementation do not favour a major benefit from use of this factor in the elderly (568). In fact, overexpression of GH in transgenic mice is associated with reduced life expectancy and symptoms of premature ageing (569), although supplementation of mice or rats did not have this effect (570). However, this latter study also found no benefits of GH supplementation in animal ageing (570).

The same considerations may apply to other factors, eg. the native steroid DHEA or DHEA sulphate (DHEAS), which, like most steroids, has immunomodulating activity. Whereas levels of cortisol increase with age in both men and women (571), and may induce suppression (572), in general the levels of DHEA decline with age (573). 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 (574). Some of the variation may be due to confounding factors such as smoking or obesity (575). It has been suggested that decreases of DHEA could be associated in some way with immunosenescence, because treatment of old mice with DHEA augments the otherwise decreased capacity of T cells to produce IL 2 and IFN-gamma. It also decreases the spontaneous secretion of IL 6 (576) and IL 10 observed in old mice and reverses their hypersensitivity to endotoxin-stimulated release of both IL 6 and IL 10 (577), and enhances lymphocyte activation (578). 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 (579). 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 (580). 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 (581), and restoration of the redox balance via activation and reconstitution of PPAR as mentioned in Section 4.4 above (200). 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 (582). Application of DHEA together with melatonin may have a limited additive effect (583). 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 (584). In man, DHEA also enhances IL 2 production (585) and DHEA supplementation trials have been carried out (eg. see (586)), but there are few immunological studies. Khorram *et al.* (587) 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 (588).

In contrast to DHEA, dihydrotestosterone (DHT) downregulates IL 4, IL 5 and IFN-gamma production but does not affect IL 2 (589). DHT levels also decrease with age (590), and a recent cross-sectional study found that bioavailable testesterone correlated best with significantly age-associated cognitive and physical parameters (591). 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 (592). 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 (589). 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.* (593) 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.* (594) 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 (595). 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 (596): there was a trend toward increased antibody titers to influenza but not tetanus, and even this failed to reach significance (596). Danenberg et al. even reported a decrease in attainment of protective antibody titer in elderly volunteers given DHEA in a prospective randomised placebo-controlled double-blind study of the effects of DHEA on influenza vaccination (597). 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 (597).

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 (598).

9.3. Caloric restriction

Many studies have examined some aspects of the biochemical changes associated with T cell activation and their alteration with aging. In some animal models other than mouse, results comparable to those in humans have been obtained; thus, in rhesus monkeys, CD4+ cells from old donors respond less well to CD3-stimulation, and this is partly associated with a decreased frequency of responding cells and is reflected in lower calcium-mobilsation in old cells (599). The same investigators also reported that one of the major strategies to prolong rodent life, which is associated with improved immune function, namely caloric restriction (CR), did not alter the depressed calcium-mobilsation rates in old monkeys. It did, however, retard the marked age-associated decline of DHEAS levels in rhesus monkeys (600). 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 (601). Moreover, CR reduced levels of a marker of oxidative DNA damage in old rats (602). Limited biochemical studies employing SENIEUR donors have indicated that membrane lipid alterations in the elderly may be important for altered immunological function (603). 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. CR has multiple effects which are only now being elucidated, eg. some data show that old CR mice retain better GH receptor function than old ad libitum-fed mice (604). A major mechanism may be via the lowering of nutritionally-driven insulin exposure which lowers overall growth factor exposure (605). 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 (606). Further evidence that any longevity enhancement by CR in rhesus monkeys is not correlated with improvement of immune responses, stems from the study of Roecker et al. (607). They 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, 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-alpha) 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 (608). However, in two other rat strains, Konno (609) 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-alpha (610), and resulted in preserved thymic cellularity (but not size) coupled with preservation of the levels of naive peripheral T cells (611).

9.4. Mutations and DNA repair

Dietary caloric restriction may have many physiological effects which are only indirectly relevant to immunity; for example, CR slows down the age-related increase in mutations (612), perhaps via effects on DNA polymerase-alpha (613). There is an age-related increase in basal levels of DNA damage in human lymphocytes (614,615), and an increase in chromosomal abnormalities in the elderly was demonstrated many years ago (616). Mechanisms for recognition of DNA damage and its repair are important in maintaining cellular integrity. If these are reduced during aging, this would also contribute to failing function. There are many reports that this is indeed the case, as reviewed some time ago by Rattan (617). More recently, these analyses have been refined; eg. Boerrigter *et al.* (618) 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. Early reports had suggested a correlation between DNA repair capacity and maximum species lifespan (619). Cortopassi & Wang recently summarised various publications to survey agreement on rates of DNA repair in different species and the correlation between repair and maximal lifespan (620). 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 excellent. 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 (621). Furthermore, the same group estimated a corresponding increase in mutability of DNA in B cell lines from these donors of + 0.6% per year (621), suggesting that DNA repair decrease 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 (622).

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 (623,624). Moreover, in B cell lines from centenarians, levels of PARP activity were found to be greater than in younger controls (625). 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 (626).

In T cells, studies of mutations (unrepaired DNA damage) revealed that background mutant frequency (MF) at an oft-studied marker locus, the hprt locus, increases with age up to advanced middle age (494,627), 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 (628). 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 (494). 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 (629). 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 (630). Together with the increased levels of anti-oxidants glutathione peroxidase, catalase and ceruloplasmin in the elderly (629), 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 may also decrease DNA damage in human lymphocytes. Thus, dietary anti-oxidant supplementation was found to reduce hprt mutant frequency in murine lymphocytes (631), 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 (632). However, Collins *et al.* reported that levels of oxidative DNA damage as measured by 8-oxydeoxyguanosine (8-oxo-dG) levels in lymphocytes, were not affected by carotenoid supplementation, not did they correlate with baseline levels of serum anti-oxidants (633). 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). Another study 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 (634).

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 (635).

Mutations at several other loci have also been examined in the context of ageing (636). 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 (637). Age-related increases in mutations of HLA genes have also been reported (638). Translocations associated with oncogenesis in younger donors also show age-associated increases in frequencies, most of which fail to result in overt tumorigenesis (639,640). However, despite increase with age, the frequency of these events is too low to

be causative of immune depression, even though they may offer good biomarkers for T cell aging. 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 (641). The repair of unavoidable damage may conceivably be facilitated by transfection of DNA repair enzymes, at least under certain experimental conditions (642).

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. 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 (643), and use of p33^{ING1} antisense to extend fibroblast lifespan by 7 PD (644). Recent work has demonstrated that transfection of telomerase into certain fibroblasts and epithelial cells can result in their immortalization (310,311,645), but only if p16^{INK4} is inactivated beforehand (646). 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 erythroleukaemia cell line (647). 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 accumulate as PHA-stimulated T cells age in culture, and that there was increased binding of p16 to its target Cdk6 kinase (648). In contrast, p21 levels were only slightly elevated (648) and p53 levels were unchanged in resting T cells from the elderly but decreased in PHA-activated old T cells (649). 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.* (306), 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.) (648). Both of these are taken to be markers of senescence, but here appear at quiescence, as previously argued for ß-gal. by Rubin (279,313). 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.

9.5. 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 hypothesised 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 (492). 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 (650). 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-iducible HSF-1 in cultured T cells as they age has also been observed by Jurivich *et al.* (651). A member of the hsp70 family, mortalin, has been proposed as a marker for cells committed to apoptosis. Some recent data implicate hsp70 as a protector against apoptosis (652), others show that overexpression of transfected hsp70 enhances AICD in T cells (653). 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 (196). There is now some evidence that hsp90 plays a part in CD28-mediated T cell activation (654), 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 4.2.1).

Interventions which would enhance the stress response may therefore also be directly relevant to delaying senescence (492). Even low-level irradiation may fall into this category. Thus, multiple low-dose irradiation of human fibroblast cultures extended their lifespan by one-quarter (655); this was not accompanied by specific chromosome abberations or activation of telomerase (655). Furthermore, in a large study involving 900 mice, Caratero *et al.* (656) 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). 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% (657). This tyrosine kinase receptor gene may have homologies amongst the numerous such receptors expressed by lymphocytes and in this way be relevant to immunosenescence.

10. PERSPECTIVES

We suggest the following simple interpretation (figure 1):

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
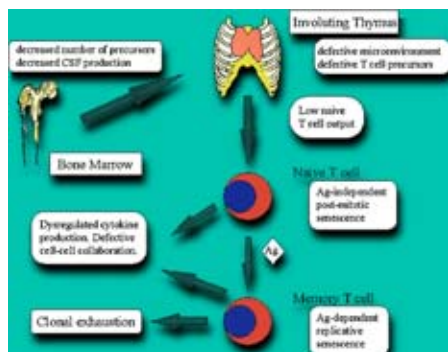
Figure 1. Senescence affects T cell differentiation and function at different levels. First, there is a decreased number of bone marrow precursors migrating to the thymus. In the involuting thymus the function of both the thymocytes and the cells from the microenvironment is compromised in the ability to support T cell differentiation, resulting in a decreased output of new naive T cells in the elderly. Long lived naive T cells suffer antigen-independent post-mitotic senescence even in their quiescent state. T cells which have been activated and become memory cells are maintained in a proliferative state and are, therefore, subjected to replicative senescence and clonal exhaustion. These changes result in a defective capacity of T cells to collaborate in other aspects of the immune response, hematopoiesis and lymphopoiesis.

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 increased 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.

11. ACKNOWLEDGEMENTS

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