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Biochemical and genetic analyses of T cell aging in mice

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Introduction

The aged immune system shows defects in cellular and humoral immune responses to vaccines [37], infectious agents [7], and tumors [10], deficiencies whose root causes are not yet well understood. Among the several cellular elements whose collaborations lead to protective immunity, the T cell population shows the most consistent and dramatic sensitivity to aging, although defects in B cells and antigen-presenting cells (APC) have also been documented [28]. Declines with age in thymic production of new T cells may ultimately be shown to underlie T cell immune senescence, although from some perspectives [43] age-associated changes in the homeostatic mechanisms that regulate T cell survival and turnover in the periphery seem at least equally critical and are much less well studied. Developmental changes – whether caused by alterations in thymic or post-thymic pathways – lead to alterations in the relative proportions of several cell types, including a replacement of naive by memory T cells [9, 24] and an increase in an anergic CD4 memory cell pool marked by cell surface P-glycoprotein [2, 48]. Aging also leads to accumulation of non-transformed T cell clones, easily documented by Vβ quantitation among CD8 cells [4], but also demonstrable by more sensitive methods within the CD4 population [36]. The provenance and putative functional implications of this loss of clonal heterogeneity are still uncertain.

The net result of these developmental processes is a population of T cells, many of which fail to respond to antigenic and mitogenic stimulation. Limiting dilution assays [5, 25], followed by flow cytometric analyses of cell responses [18, 40], have consistently shown that the aged mouse immune system includes a mixture of T cells, some of which seem to respond as strongly as cells from younger donors, and others of which are much harder to activate from their resting state. Because it is difficult to follow activation of single T cells in intact animals, much of what we know of immune senescence at the level of cell biology and biochemistry has emerged from analyses, in vitro, of cells freshly removed from the donor and then quickly exposed to an antigen or to a polyclonal activator. The result of these encounters was, in early experiments, typically evaluated 2–5 days later using radio-thymidine to quantitate cell division.

Development of assays for cytokines and their receptors made it possible to "trace back" the cause of the proliferative failure by showing age-dependent declines in production of IL-2 and its receptor [15, 45, 46], first at the level of proteins and then soon also at the level of RNA expression. These defects are likely to reflect the age-related decline in activation of genes, such as c-myc [3], that are turned on at still earlier stages of the activation process.

Studies of early stages in T cell activation

The first hour after T cells encounter an antigen (or, in model systems, a polyclonal activator) is a busy time, characterized by changes in the activity, compartmentalization, and interactions of multiple enzymes, substrates, and scaffolding proteins, along with their products and regulators. The details of these early activation steps have been much studied in convenient lymphoma models [1, 41, 47, 50], and are also gradually being sorted out in freshly isolated lymphocytes in which the activation process conforms to that of lymphomas and long-term cell lines in some ways but not in others. It has been clear for over a decade that T cells from old mice and old humans show defects in the activation cascade that are detectable within the first minute or two after encounter with a stimulus. The first part of this essay focuses on the mechanism of T cell activation: what, in biochemical terms, goes wrong in the first few minutes, and which of the many early activation defects should be considered primary causes of later downstream events that go awry in T cells from old donors?

Initial studies of this problem have been reviewed in detail earlier [26, 31], and are summarized here only to provide a context for more recent work. Aging leads to a decline in the proportion of T cells, from spleen or blood, that can elevate intracellular calcium free ion concentration after encounter with mitogens like concanavalin A or after stimulation through the CD3 components of the TCR [32, 33]. The change is in part attributable to the age-dependent accumulation of $CD44^{hi}$ memory cells, of both CD4 and CD8 lineages, and the intrinsic resistance of memory T cells, at any age, to elevation of Ca^{2+} levels, even when induced by TCR-independent ionophores [40, 42]. This decline in calcium signal generation has functional implications, in that isolation, either by flow cytometry or by a buoyant density method, of cells that do not generate a calcium signal also enriches correspondingly for cells that cannot proliferate or produce cytokines in short-term in vitro cultures [11, 39].

Protein kinase signals are also disrupted in T cells from aged donors, whether these are detected as anonymous proteins on two-dimensional electrophoretic gels [38], or by assessment of activation of the MAP kinase ERK and its MAPKK activator MEK [l6], Raf-1 [22], or JNK [21]. These defects in activation of kinases are detectable in the first 2–15 min after activation, and do not represent a decline with age in the amount of the enzyme, but instead reflect a diminution of the level of activation of the kinase involved.

The JNK experiments are particularly provocative, in that they reveal unexpected complexity in the pathways leading to enzyme activation. JNK, also referred to as "stress-associated protein kinase" or SAPK, can be triggered in T cells either by joint stimulation of CD3 and CD28 signals, or by a variety of cellular stresses including oxidizing agents, ultraviolet light, and hyperosmotic conditions. CD4 T cells from old mice exhibit a twofold decline, compared to cells from young donors, in activa-

Table 1. Effects of donor age and pharmacologic inhibitors on JNK activation in murine T lymphocytes

Stimuli	Inhibited by age	Inhibited by forskolin and by cyclosporin
CD3+CD4+CD28	Yes	Yes
UV light	Yes	No
H_2O_2	No	Yes
Hyperosmotic sorbitol	No	No
Ceramide	No	Yes

(Data from [23])

tion of JNK function by CD3/CD28 cross-linking as tested by an in vitro kinase method, and there is a corresponding deficit in the ability to produce phosphorylated c-Jun, the product of JNK action, in CD3/CD28-stimulated intact cells [21]. Similarly, JNK activation by UV light shows a twofold decline in aged CD4 cells [23]. In contrast, however, there is no effect of donor age on JNK responses to stimulation by H_2O_2 or by hyperosmotic sorbitol solutions (see Table 1). These two pathways, one age-sensitive and one not altered by aging, may actually reflect four distinct modes of activation, because the calcineurin inhibitor cyclosporin A and the cAMP inducer forskolin inhibit the responses to CD3/CD28, and to H_2O_2, but do not alter responses to hyperosmotic sorbitol or UV light. Responses to intracellular mediators (PMA plus ionomycin, or a membrane soluble ceramide isoform) are also sensitive to cyclosporin and to forskolin [23], consistent with models in which these agents mediate one age-sensitive (TCR/CD28) and one age-insensitive (UV) pathway to JNK activation. The molecular links in these activation chains are still obscure, although our data have shown that of the two proposed JNK activating kinases, MKK7 is not detectable, and MKK4 is not age-sensitive in mouse CD4 T cells.

To what age-sensitive upstream factors can these early changes in calcium and protein kinase signals be connected? Our initial studies of CD3 phosphorylation by the T cell-specific Syk-family kinase Zap-70 provided some clues, but also raised new questions. Separation of immunoprecipitated CD3ξ on nonreducing acrylamide gels, followed by immunoblotting with anti-phosphotyrosine antibodies, showed that the major species of ξξ dimer, at 36 kDa, was heavily phosphorylated in freshly isolated resting CD4 T cells, and that the level of phosphorylation diminished by a factor of three between 6 and 18 months of age [12].

Stimulation of the CD4 cells by CD3/CD4 cross-linking did not alter the tyrosine-specific phosphorylation levels of the 36-kDa ξξ dimer, but did induce phosphorylation of ξξ dimers at higher molecular masses, and the level of induced phosphorylation was a good deal higher (about threefold) in T cells from young mice compared to cells from 18-month-old animals. Binding of Zap-70 to CD3ξ is known to depend upon tyrosine-specific phosphorylation of immunoreceptor tyrosine activation motif (ITAM) domains within the CD3ξ sequence, and it was thus surprising to discover that aging led to an increase in association of Zap-70 to CD3ξ in resting CD4 T cells despite the decline in phosphotyrosine content [13]. This age-related increase in Zap-70/CD3ξ association was seen in two separate series of experiments, each using a different source of anti-CD3ξ antibody for immunoprecipitation, and each involving different sets of young and old donor mice. This paradoxical increase with age in the level of Zap-70 bound to CD3ξ might in principle reflect changes in the pattern of

distribution of phosphotyrosine residues within the CD3ξ ITAMs, or alterations in the level of other ITAM-binding proteins that could interfere with Zap-70/CD3ξ association in the young T cells. We also consider below a third model, in which aging alters association of CD3ξ partitioning between plasma membrane and cytoskeletal pools.

To see if the age-related changes in CD3ξ phosphorylation and Zap-70 association led to alterations in Zap-70 activation and kinase function, we took two, complementary, approaches: analysis of Zap-70 kinase function in vitro, and measurement of Zap-70 phosphotyrosine content. Two distinct methods for in vitro kinase function, one based on incorporation of radiophosphate into the exogenous substrate erythrocyte Band III protein and the other exploiting anti-phosphotyrosine antibodies to monitor Band III phosphorylation, both led to the same result: a small (25–40%) increase with age in Zap-70 function in resting cells, and a small, though statistically significant response of young (but not old) T cells to stimulation. These two, contrary, effects balance each other out, and there was thus no measurable change with age in the level of Zap-70 activity in activated CD4 cells as measured with the in vitro kinase methods [13]. Measurements of the level of tyrosine-specific phosphorylation of Zap-70 – a necessary step in activation of this kinase – produced parallel results: a slight increase with age in baseline phosphorylation, a slight response to stimulation in young but not old CD4 cells, and thus no net change with age in Zap-70 tyrosine phosphate content in activated T cells.

How, then, to reconcile the clear and consistent decline with age in activation of Ca^{2+}, Raf-1, MEK, Erk, and JNK signals with the absence of any parallel alteration in Zap-70 function, thought to be the key initial step in T cell triggering? We considered the idea that aging might lead to changes in the function of the src-family kinase Lck, thought to play a role in phosphorylation of both Zap-70 and other kinase substrates, but found no change with age in Lck activity in resting or in activated cells, and no effect of age on association of Lck with CD4, through which Lck affiliates with the plasma membrane (Garcia and Miller, unpublished data).

We turned instead to the idea that Zap-70, though activated appropriately in aged T cells, might be restricted to specific cellular compartments or plasma membrane domains and thus not be able to gain access to key substrates needed for transmission of the activation signal.

To address this issue we turned to immunofluorescence methods that can detect relocalization of specific signaling molecules in individual T cells by confocal microscopy, a method developed by Monks, Kupfer and their colleagues [34, 35]. In good agreement with previous studies with T cell lines and lymphomas, we found that a proportion of freshly isolated mouse CD4 T cells, when conjugated to a stimulatory APC, would form conjugates in which a wide range of signaling molecules migrated to the interface between the T cell and the APC, a specialized membrane structure termed the “immune synapse” by other investigators [17]. We obtained qualitatively similar results using either of two systems: one in which anti-CD3 hybridoma cells were used to form conjugates with T cells independent of the specificity of the TCR, and the other in which CH-12 lymphoma cells were used to present peptides, derived from pigeon cytochrome c (PCC) sequences, to T cells from TCR-transgenic mice whose naive CD4 cells were largely PCC specific. There was no effect of aging on the proportion of CD4 T cells that could form two-cell conjugates with either variety of APC, but among conjugates there was a clear effect of age on the proportion that could develop a mature (and presumably functionally active) immune synapse. When

T cells came from young donors, approximately 45–60% of the CD4 cells in conjugates would form immune synapses containing either the coupling protein LAT or the guanine nucleotide exchange factor Vav in responses to anti-CD3 hybridomas, and about 70% of TCR-transgenic T cells from young mice form LAT- and Vav-containing synapses in response to PCC-derived agonist peptides [44]. The confocal images using the peptide-specific system are consistent with other analyses of the organization of the supramolecular activation complex (SMAC) at the synapse, with evidence that LAT and Zap-70 colocalize in a small central patch, surrounded by a more peripheral ring containing PLCγ, c-Cbl, Lck, Vav, Grb-2, and Fyn [14].

Aging diminishes the proportion of responding cells by about twofold, i.e., from about 50% to about 20–30% (over a false-positive background, using rabbit IgG in place of detecting antibody, of 10–20%) in both of these systems [14, 44]. In the CD3 system we have examined Vav, LAT, and the T-cell-specific iso-enzyme PKCθ [44, 49]; in the PCC-specific system we have similar results for Vav, LAT, Grb-2, PKCθ, Zap-70, c-Cbl, PLCγ, Lck, Fyn, and CD3ε [14]. Two-color experiments, in which different secondary antibodies are used to detect different components of the SMAC, show that this age-sensitive response is “all or nothing” at the level of the individual T cell. In other words those T cells, in young or old mice, that respond to peptide or anti-CD3 stimulation by movement of one of the tested proteins to the immune synapse seem to move all of the others (with an exception, discussed below, of c-Cbl in certain anergic memory cells). This blockage at the stage of synapse formation is sufficient to account for the defects in production of downstream signals, which are also typically on the order of twofold when comparing 6-month to 18-month-old mice. The inability to move LAT to the synapse may be a key element in the interruption of signal transduction: our own data [44] show that LAT phosphorylation is diminished about twofold in T cells from aged donors, and others have shown [51] that phosphorylated LAT serves as a nucleation site for accumulation of other members of the signal transduction cascade at the SMAC site. Phosphorylation of PLCγ at the early stages of T cell activation has also been reported to decline with age [19], again consistent with our observations of altered migration of this kinase to the synapse. The data on PKCθ are of particular interest, in view of published evidence [35] that movement of this kinase occurs only when T cells are stimulated by agonist, but not by closely related antagonist peptides.

We have assessed the functional implications of these observations in two sets of experiments. The first of these has used two-color confocal microscopy to examine both membrane assembly of SMACs and nuclear accumulation of the transcription factor NFAT in individual cells. Interestingly, the CD3 and PCC systems behave differently with respect to NFAT migration. Anti-CD3 provides a high-affinity, persistent stimulus, and with this activator synapse formation is almost invariably followed by NFAT migration in CD4 cells of both young and old mice [44]. Thus, the proportion of old CD4 cells that show NFAT migration after anti-CD3 is about 50% of that seen in CD4 cells from young donors, but those old cells that do produce SMACs typically proceed to NFAT migration as well. In the lower affinity PCC system, however, only about half of the aged CD4 cells that generate synapses proceed on to NFAT migration; the proportion of NFAT-responsive cells is thus only about 25% of that seen in young mice [14]. The molecular basis for this second-stage defect, the defect preventing NFAT migration in synapse-positive cells, is at present unknown, but could involve alterations in the calmodulin/calcineurin signals required for NFAT assembly in the nucleus.

The second functional test involves analysis of a subset of CD4 memory T cells, marked by high expression of the multi-drug resistance pump P-glycoprotein, which we have in previous work shown to accumulate with age in mice and to be functionally anergic [2, 48]. CD4 memory cells with P-glycoprotein function ("Pgp-hi" cells) are rare in young mice and more common in old mice, but regardless of donor age were found to be essentially unable to form synapses or to translocate NFAT to the nucleus in responses using the anti-CD3 system [8]. The inhibition could be overcome by addition of ionomycin, suggesting that the alterations involved steps prior to activation of PLCγ and calcium signals. Conversely, the Pgp-lo subset of CD4 memory cells, which are common in young mice but rare in old mice, were at either age found to be able to form functional synapses and to induce NFAT migration at high frequency. These findings suggest that poor synapse formation may be responsible for the relative inability of Pgp-hi cells, at any age, to activate CD69, proliferate, and secrete cytokines. Interestingly, the hyporesponsive Pgp-hi cells tended to form synapses that had high levels of c-Cbl despite low levels of LAT; whether the retention of c-Cbl is a cause or merely a marker of the formation of non-functional synapses is still to be determined.

Our most recent efforts have focused upon the hypothesis that age-sensitive alterations in associations between CD3ξ isoforms and elements of the T cells' cytoskeleton might contribute both to defects in synapse formation and to age-dependent changes in CD3ξ association with Zap-70 in resting T cells. Others [35] have previously shown that TCR-transgenic T cells will respond to antagonist peptides by producing an early precursor of the immune synapse containing the cytoskeletal protein talin at the T cell/APC interface, although only agonist peptides induce subsequent stages characterized by accumulation of LAT and other coupling proteins and kinases. We have confirmed these observations, and found in addition (Garcia and Miller, submitted) that CD4 T cells from old mice do not move talin to the APC contact area in response to antagonist peptides. This observation suggests strongly that the age-dependent defect in synapse formation affects very early stages of the cascade, stages involving cytoskeletal rearrangements prior to accumulation of other elements of the SMAC. A model system in which T cells are induced to form lamellopodia by adherence to anti-CD3-coated plastic surfaces also shows an age-dependent increase in unresponsive CD4 cells, again consistent with models that implicate early changes in cytoskeletal rearrangements. The pattern of binding of CD3ξ isoforms to the T cell cytoskeleton can be shown to shift with age (Garcia and Miller, submitted), and it thus seems possible that sequestration of CD3ξ into compartments relatively accessible to Zap-70 and its substrates may play a central role in T cell anergy in older mice.

T cells as indicators of age-related vulnerability

In parallel with the analysis of T cell failure at the cellular and biochemical level, our group has also embarked on a series of studies to see if age-related changes in T cell status can serve as a harbinger of systemic vulnerability to illness in mice. Such an association, if it existed, could reflect either a cause-and-effect relationship in which prematurely aged T cell immunity led to late-life illness, or alternately reflect the ability of T cell changes to serve as an indicator, a biomarker, of the underlying pace and extent of aging, which then in turn contributes to disease risk. To reduce the

Table 2. Summary of T cell subsets used for biomarker and gene mapping studies

Subset	Description	Defined as	Age effect (blood)
CD4	Class II-specific helper T cells	CD3, CD4 as % of CD3	Down
CD8	Class I-specific killer T cells	CD3, CD8 as % of CD3	Stable
CD4V	Virgin CD4	CD4, CD45RB-hi as % of CD4	Down
CD4M	Memory CD4	CD4, CD44-hi as % of CD4	Up
CD8M	Memory CD8	CD8, CD44-hi as % of CD8	Up
CD4P	CD4 with P-glycoprotein	CD4, R123-lo as % of CD4 [a]	Up
CD8P	CD8 with P-glycoprotein	CD8, R123-lo as % of CD8	Up

[a] cells with active P-glycoprotein are detected by their ability to extrude the P-glycoprotein substrate R123

chances that our findings might apply only to a single mouse genotype, we conducted this work using a genetically heterogeneous mouse stock, called UM-HET3, bred as the progeny of CB6F1 females and C3D2F1 males. This cross produces a population of mice in which no two are genetically identical, but in which any mouse shares half its genes with any other in the population; thus all mice in the test group are, in a genetic sense, full sibs.

We had shown previously [27] that aging leads to declines in the proportions of CD4 and CD4V cells in the blood, and increases in CD4M, CD8M, CD4P, and CD8P cells, in this stock as in all previously tested mouse strains (see Table 2 for abbreviations and definitions of the seven tested T cell subsets). We thus proceeded to a study in which the levels of each subset were measured at the ages of 8 months and again at 18 months, and each tested mouse then followed until it died or became severely ill and was killed. We found that four of the tested subsets, CD4, CD4V, CD4P, and CD4M, when measured at 18 months of age, had a statistically significant ability to predict remaining longevity [29], with P values ranging from 0.003 for CD4 cells to 0.00004 for the CD4M cell subset. UM-HET3 mice live, on average, about 26 months, and only about 7% die prior to the age of 18 months; it therefore seems unlikely that the subset differences seen at 18 months are the result of serious illness. In each case the direction of the correlation was consistent with the previously observed age effect, so that subsets that increase with age (CD4P, CD4M) showed negative correlations with remaining longevity, and those that decrease with age (CD4, CD4V) showed positive correlations with life expectancy. In other words those middle-aged mice whose T cell subset patterns resembled those of younger animals tended to have long lives, while those whose T cell patterns resembled those of older mice tended to die relatively soon. We also found a relationship between high CD8M levels and short life-span that reached $P<0.04$ both at 8 months and at 18 months of age. These associations were tested in a group of mice that included virgin males, virgin females, and mated females, and the results were independent of group except that the CD4P correlation was strong in males only.

We then used the statistical method of “cluster analysis” to see if our mice could be sorted, based on T cell subset patterns, into groups that differed in longevity [29]. The cluster analysis method uses the information in all seven tested subsets to divide the mice into two groups (“clusters”) such that the members of each cluster resemble one another in subset pattern but are as far away as possible from mice in the opposite cluster. Figure 1 (left panel) shows the outcome of the clustering routine: the 182

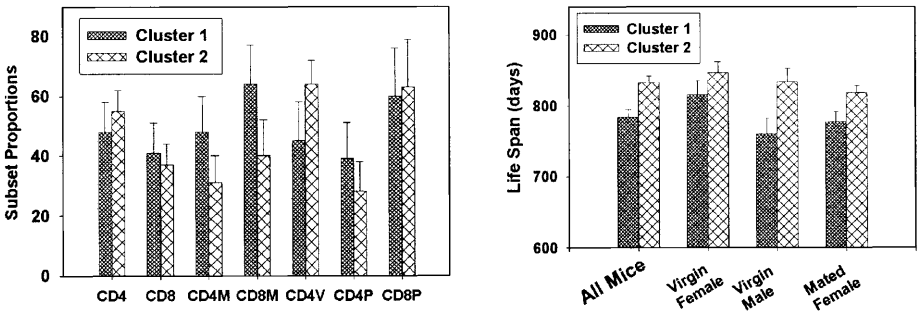

Fig. 1. Cluster analysis of T cell subset patterns. T cell subsets were measured in blood of 18-month-old genetically heterogeneous mice, and a k-means clustering procedure was used to sort the animals into two clusters based on subset distributions. The *left panel* shows differences between the two clusters in each of the seven subsets employed by the clustering routine. The *right panel* shows mean longevity (with standard error of the mean) for all mice (*left bars*), and also for three subgroups of mice (*right bars*), i.e., for virgin females, virgin males, and mated females (from [29])

mice in Cluster 1 tend to have higher levels of CD4M, CD8M, and CD4P than the 320 mice in Cluster 2, but have lower levels of CD4 and CD4V cells. Thus, mice in Cluster 1 appear to be "older" in their immune subset patterns than their brothers and sisters in Cluster 2. Figure 1 (right panel) then addresses the question of whether mice in the two clusters had different life-spans, and indeed those in Cluster 2 tend to outlive Cluster 1 animals by 50 days (P=0.0007). The relationship between cluster assignment and longevity is about equally strong in virgin males, virgin females, and mated females (Fig. 1, right panel).

These results establish that, at least in this four-way cross population, mice with old-looking T cell patterns tend to die young, but they do not address the issue of whether the T cell changes produce late-life disease, or instead identify mice in which the aging process is proceeding more rapidly. In favor of the latter interpretation is our previously published report that middle-aged mice with "old" T cell subset patterns tend also to have lower muscle strength [30], because it is difficult to imagine how T cell subset patterns could themselves lead to muscle weakness.

To provide a further test of these two alternate hypotheses, we turned to necropsy analysis to see if the relationship between T cell subset patterns and mortality risk applied to groups of mice dying of different causes. Our results (Miller, Chrisp, et al., submitted) employed another statistical method, principal component analysis, to calculate an index of immunological age for each mouse. This index, called "Immune Factor l," represents a combination of each mouse's T cell subset results, weighted in such a way as to generate the maximum variation in Immune Factor 1 across the entire mouse population. Thus, mice with high values of Immune Factor 1 tend to have high levels of CD4M, CD8M, and CD4P cells, and low levels of CD4 and CD4V cells. Once we calculated a value for Immune Factor 1 for each mouse, we could then show a strong relationship between Factor 1 values (at 18 months of age) and mortality risk (P<0.0001), consistent with the previous cluster analysis. Of greater interest was the observation that there was a significant relationship between Factor 1 and mortality risk in separate subgroups of the mice, i.e., those dying of lymphoma, of fibrosarcoma, of mammary adenocarcinoma, or of all other causes combined. The proportion of variance predicted (measured as R-squared) was modest, ranging from 6% for all-cause mortality to 12–13% for lym-

phoma and fibrosarcoma: unsurprisingly there are many factors that influence mortality risk, not all of which can be assessed by measurement of T cell subsets. These results represent an important extension of the regression and cluster analysis work, because they show that the relationship between T cell subset patterns and life expectancy influences each of the three most common causes of death in this mouse population. Lymphoma, mammary adenocarcinoma and fibrosarcoma are all neoplastic diseases, although they represent neoplastic variants of three different cell types with different patterns of spread and different mechanisms of oncogenesis. At worst, our data indicate that T cell subset patterns have prognostic value (in mice) for multiple forms of neoplasia; new data will be needed to determine whether mice with old looking T cell systems also show acceleration of multiple forms of non-neoplastic pathology.

Genetic analysis of age-sensitive T cell subsets

The four-way cross mice used for the analyses of T cells as prognostic factors for late-life illnesses are, conveniently, a set of full sibs, making them suitable for mapping polymorphic genetic variations that might contribute to differences among the mice in T cell subsets at different ages. Each of the first 174 mice in the population was genotyped using a set of 83 simple sequence length polymorphism (SSLP) markers whose position in the mouse genome was known, to evaluate association between inherited chromosomal segments and resulting T cell subset levels among the mice [20]. The significance of each gene/trait association was evaluated using a permutation approach [6] that adjusts the acceptance threshold to account for the many different genetic segments examined. Some of the associations were detected using an approach that examines only one genetic marker at a time ("point" mapping) and others using a method that considered multiple genetic markers ("interval" mapping). These results are collected in Table 3. Each of the associations listed in the table meets or exceeds an experiment-wise probability criterion of $P<0.05$. We found at least one polymorphism with effects on each of the seven listed subsets; follow-up studies, now in progress, make use of equivalent data from more than 500 mice and should extend this initial analysis by providing greater statistical power.

It is noteworthy that some of the detected quantitative trait loci (QTL) are detectable (i.e., achieve statistical significance) at 8 months of age, but that others have detectable effects only at 18 months of age; these latter alleles may be age specific in their modes of action. Figure 2 shows some illustrative examples, selected from the QTL listed in Table 3. The three examples in the top row show genes whose effects are apparent in 8-month-old mice, and remain obvious when these mice are retested at 18 months of age. In all three cases shown the T cell subset is itself age sensitive, increasing or decreasing between 8 and 18 months of age, but the genetic effect is fully apparent at the earliest time point. These alleles may well be exerting an effect on the pace of T cell maturation or development within the first third of the life-span. The first two examples on the bottom row (Fig. 2) show genes for which the effect on the T cell subset appears sometime between 8 and 18 months of age. The lower right hand panel shows a more complex pattern, in which a specific combination of alleles (the BALB/c allele from the mother together with the CH3/He allele from the father) leads to a transient increase in CD4M cells, established within the first 8 months of life but not apparent in 18-month-old animals. Thus, the evidence to date

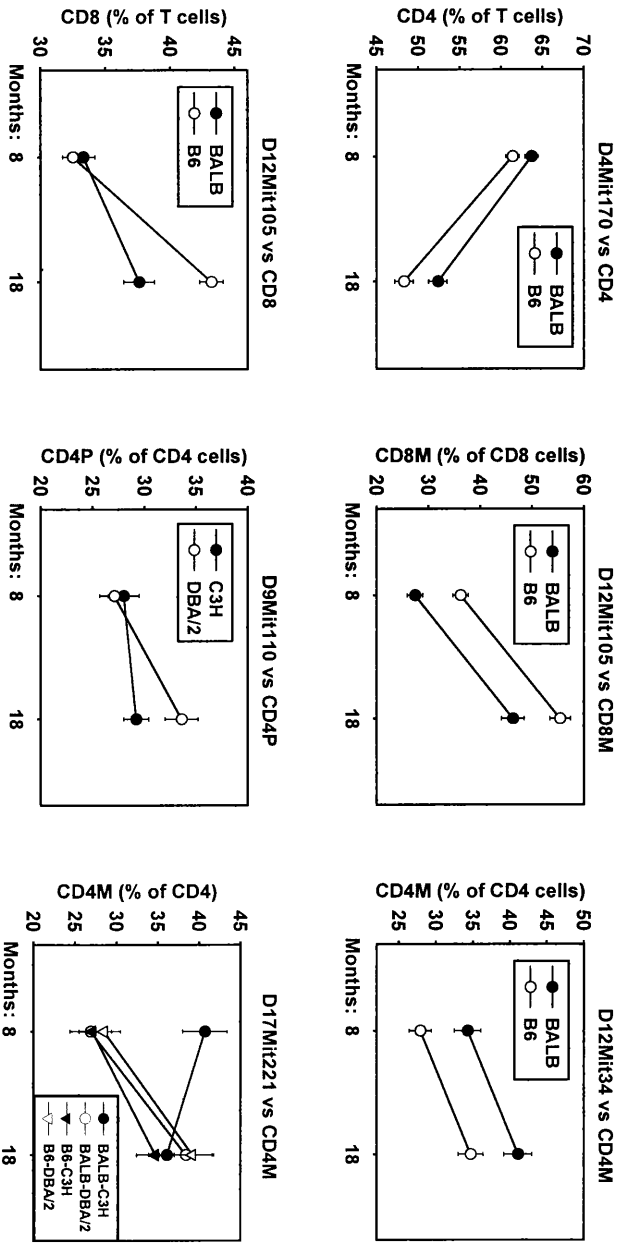

Fig. 2. Quantitative trait locus analyses of polymorphic genes controlling T cell subset levels at 8 and/or 18 months of age. The name of the marker locus is shown at the *top* of each panel; D4Mit170, for example, is a marker on mouse chromosome 4. T cell subsets are abbreviated as shown in Table 2. Each connected pair of symbols shows mean T cell subset levels in the mice that have inherited the indicated allele. In the first panel, for example, the *closed circles* show mean levels of CD4 cells (at 8 and at 18 months) in mice that have inherited the BALB/c allele at locus D4Mit170. Each of the associations shown has experimentwise significance at $P<0.05$ (from [20])

Table 3. Summary of gene mapping for T cell subsets in mice

Trait	Chromosome location (cM)	Method	Age
CD4	Chl, 105	Interval	8
CD4	Ch7, 11	Interval	8
CD4	Ch9, 31	Both	8
CD4	Ch15, 28	Both	18
CD4M	Ch11, 4	Interval	Both
CD4M	Ch12, 23	Point	Both
CD4M	Ch17, 52	Point	8
CD4P	Ch4, 69	Interval	8
CD4P	Ch9, 44	Point	18
CD4V	Ch4, 48	Point	18
CD8	Ch12, 6	Interval	18
CD8M	Ch1, 98	Interval	Both
CD8M	Ch5, 18	Interval	Both
CD8M	Ch5, 41	Interval	8
CD8M	Ch11, 8	Interval	18
CD8M	Ch12, 6	Point	Both
CD8M	Ch12, 13	Point	Both
CD8M	Ch17, 17	Interval	Both
CD8M	Ch17, 23	Interval	Both
CD8P	Ch9, 17	Both	Both
CD8P	Ch9, 34	Interval	8
CD8P	Ch12, 16	Interval	18

Excerpted from [20]

documents at least two kinds of genetic effect relevant to immunogerontology: (a) effects, early in life, on T cell subsets that change with age (including some, shown above, to provide an indicator of pathology and longevity); and (b) effects that are not seen until the mice become middle-aged.

Conclusions: synopsis and prospectus

The genetic data available show that the four-way cross mouse system is able to provide useful information about genetic controls of T cell subset levels and their changes with age, and the biomarker data show that variation in some of these age-sensitive T cell subsets is relevant, either directly or indirectly as an index of underlying aging rate, to late-life diseases. Further work comparing immune phenotypes to age-related changes in bone, muscle, eyes, brains, and marrow will test the hypothesis that common elements time the pace of aging in multiple tissues and cell types, while further work on the genetics of age change in these tissues will define the extent to which such correlations are based upon common genetic control elements. Selection of groups of mice based on genes that predict life-span and disease resistance will help us to clarify the extent to which these inherited polymorphisms alter the developmental patterns that lead to age-related changes in T cell subset abundance and to deficits in the signal transduction cascade.

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References

1. Alberola-Ila J, Takaki S, Kerner JD, et al. (1997) Differential signaling by lymphocyte antigen receptors. Annu Rev Immunol 15:125
2. Bining N, Miller RA (1997) Cytokine production by subsets of CD4 memory T cells differing in P-glycoprotein expression: effects of aging. J Gerontol A Biol Sci Med Sci 52A:B137
3. Buckler A, Vie H, Sonenshein G, et al (1988) Defective T lymphocytes in old mice: diminished production of mature c-myc mRNA after mitogen exposure not attributable to alterations in transcription or RNA stability. J Immunol 140:2442
4. Callahan JE, Kappler JW, Marrack P (1993) Unexpected expansions of CD8-bearing cells in old mice. J Immunol 151:6657
5. Candore G, Di Lorenzo G, Caruso C, et al (1992) The effect of age on mitogen responsive T cell precursors in human beings is completely restored by interleukin-2. Mech Ageing Dev 63:297
6. Doerge RW, Churchill GA (1996) Permutation tests for multiple loci affecting a quantitative character. Genetics 142:285
7. Effros RB, Walford RL (198 3) Diminished T-cell response to influenza virus in aged mice. Immunology 49:387
8. Eisenbraun MD, Tamir A, Miller RA (2000) Altered composition of the immunological synapse in an anergic, age-dependent memory T cell subset. J Immunol 164:6105
9. Ernst DN, Hobbs MV, Torbett BE, et al (1990) Differences in the expression profiles of CD45RB, Pgp-1, and 3 G11 membrane antigens and in the patterns of lymphokine secretion by splenic $CD4^+$ T cells from young and aged mice. J Immunol 145:1295
10. Flood PM, Urban JL, Kripke MI, et al (1981) Loss of tumor-specific and idiotype-specific immunity with age. J Exp Med 154:275
11. Flurkey K, Stadecker M, Miller RA (1992) Memory T lymphocyte hyporesponsiveness to non-cognate stimuli: a key factor in age-related immunodeficiency. Eur J Immunol 22:931
12. Garcia GG, Miller RA (1997) Differential tyrosine phosphorylation of zeta chain dimers in mouse CD4 T lymphocytes: effect of age. Cell Immunol 175:51
13. Garcia GG, Miller RA (1998) Increase in Zap-70 association with CD3 in CD4 T cells from old mice. Cell Immunol 190:91
14. Garcia GG, Miller RA (2001) Single-cell analyses reveal two defects in peptide-specific activation of naive T cells from aged mice. J Immunol 166:3151
15. Gillis S, Kozak R, Durante M, et al (1981) Immunological studies of aging. Decreased production of and response to T cell growth factor by lymphocytes from aged humans. J Clin Invest 67:937
16. Gorgas G, Butch ER, Guan KL, et al (1997) Diminished activation of the MAP kinase pathway in CD3-stimulated T lymphocytes from old mice. Mech Ageing Dev 94:71
17. Grakoui A, Bromley SK, Sumen C, et al (1999) The immunological synapse: a molecular machine controlling T cell activation. Science 285:221
18. Grossmann A, Ledbetter JA, Rabinovitch PS (1989) Reduced proliferation in T lymphocytes in aged humans is predominantly in the $CD8^+$ subset, and is unrelated to defects in transmembrane signaling which are predominantly in the CD4+ subset. Exp Cell Res 180:367
19. Grossmann A, Rabinovitch PS, Kavanagh TJ, et al (1995) Activation of murine T-cells via phospholipase-C gamma 1-associated protein tyrosine phosphorylation is reduced with aging. J Gerontol A Biol Sci Med Sci 50:B205
20. Jackson AU, Fornes A, Galecki A, et al (1999) Longitudinal QTL analysis of T cell phenotypes in a population of four-way cross mice. Genetics 151:785
21. Kirk CJ, Freilich AM, Miller RA (1999) Age-related decline in activation of JNK by TCR and CD28-mediated signals in murine T lymphocytes. Cell Immunol 197:75
22. Kirk CJ, Miller RA (1998) Analysis of Raf-1 activation in response to TCR activation and co-stimulation in murine T-lymphocytes: effect of age. Cell Immunol 190:33
23. Kirk CJ, Miller RA (1999) Age-sensitive and insensitive pathways leading to JNK activation in mouse CD4 T cells. Cell Immunol 97:83
24. Lerner A, Yamada T, Miller RA (1989) PGP-1^{hi} T lymphocytes accumulate with age in mice and respond poorly to Concanavalin A. Eur J Immunol 19:977
25. Miller RA (1984) Age-associated decline in precursor frequency for different T cell-mediated reactions, with preservation of helper or cytotoxic effect per precursor cell. J Immunol 132:63
26. Miller RA (1996) Calcium signals in T lymphocytes from old mice. Life Sci 59:469

27. Miller RA (1997) Age-related changes in T cell surface markers: a longitudinal analysis in genetically heterogeneous mice. Mech Ageing Dev 96:181
28. Miller RA (1999) Aging and immune function. In: Paul WE (ed) Fundamental immunology. Lippincott-Raven, Philadelphia, pp 947–966
29. Miller RA (2001) Biomarkers of aging: prediction of longevity by using age-sensitive T-cell subset determinations in a middle-aged, genetically heterogeneous mouse population. J Gerontol A Biol Sci Med Sci 56:B180
30. Miller RA, Bookstein F, Meulen JH van der, et al. (1996) Candidate biomarkers of aging: age-sensitive indices of immune and muscle function co-vary in genetically heterogeneous mice. J Gerontol Biol Sci 52A:B39
31. Miller RA, Garcia GG, Kirk CJ, et al. (1997) Early activation defects in T lymphocytes from old mice. Immunol Rev 160:79
32. Miller RA, Jacobson B, Weil G, et al (1987) Diminished calcium influx in lectin-stimulated T cells from old mice. J Cell Physiol 132:337
33. Miller RA, Philosophe B, Ginis I, et al (1989) Defective control of cytoplasmic calcium concentration in T lymphocytes from old mice. J Cell Physiol 138:175
34. Monks CR, Freiberg BA, Kupfer H, et al (1998) Three-dimensional segregation of supramolecular activation clusters in T cells. Nature 395:82
35. Monks CR, Kupfer H, Tamir I, et al (1997) Selective modulation of protein kinase C-theta during T-cell activation. Nature 385:83
36. Mosley RL, Koker MM, Miller RA (1998) Idiosyncratic alterations of TCR size distributions affecting both CD4 and CD8 T cell subsets in aging mice. Cell Immunol 189:10
37. Padgett DA, MacCallum RC, Sheridan JF (1998) Stress exacerbates age-related decrements in the immune response to an experimental influenza viral infection. J Gerontol A Biol Sci Med Sci 53:B347
38. Patel HR, Miller RA (1992') Age-associated changes in mitogen-induced protein phosphorylation in murine T lymphocytes. Eur J Immunol 22:253
39. Philosophe B, Miller RA (1989) T lymphocyte heterogeneity in old and young mice: functional defects in T cells selected for poor calcium signal generation. Eur J Immunol 19:695
40. Philosophe B, Miller RA (1990) Diminished calcium signal generation in subsets of T lymphocytes that predominate in old mice. J Gerontol Biol Sci 45:B87
41. Qian D, Weiss A (1997) T cell antigen receptor signal transduction. Curr Opin Cell Biol 9:205
42. Rajasekar R, Augustin A (1994) Antigen-dependent selection of T cells that are able to efficiently regulate free cytoplasmic calcium levels. J Immunol 153:1037
43. Stutman O (1986) Postthymic T cell development. Immunol Rev 91:159
44. Tamir A, Eisenbraun MD, Garcia GG, et al (2000) Age-dependent alterations in the assembly of signal transduction complexes at the site of T cell/APC interaction. J Immunol 165:1243
45. Thoman ML, Weigle WO (1982) Cell-mediated immunity in aged mice: an underlying lesion in IL 2 synthesis. J Immunol 128:2358
46. Vie H, Miller RA (1986) Decline, with age, in the proportion of mouse T cells that express IL-2 receptors after mitogen stimulation. Mech Ageing Dev 33:313
47. Wange RL, Samelson LE (1996) Complex complexes: signaling at the TCR. Immunity 5:197
48. Witkowski JM, Li SP, Gorgas G, et al (1994) Extrusion of the P-glycoprotein substrate rhodamine-123 distinguishes CD4 memory T cell subsets that differ in IL-2-driven IL-4 production. J Immunol 153:658
49. Yang D, Miller RA (1999) Cluster formation by protein kinase C during murine T cell activation: effect of age. Cell Immunol 195:28
50. Zhang W, Samelson LE (2000) The role of membrane-associated adaptors in T cell receptor signalling. Semin Immunol 12:35
51. Zhang W, Trible RP, Samelson LE (1998) LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. Immunity 9:239