

Genetic polymorphisms in mouse genes regulating age-sensitive and age-stable T cell subsets

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To see whether genetic polymorphisms regulate inter-individual differences in T cell subset levels, we have conducted a genome scan in two populations of mice, bred as the progeny of a cross between CB6F1 females and C3D2F1 males. The data document quantitative trait loci (QTL) with statistically significant effects on CD4, CD8, and CD8 memory T cells, and on subsets of CD4 and CD8 T cells that express P-glycoprotein. Some of the loci detected were robust, in the sense that they produced effects of similar size both in mated female mice, and in a population that included male and female virgin animals. Some of the effects were stable, in that they were apparent at both 8 and 18 months of age, but others were age-specific, showing effects either at 8 or at 18 months but not at both ages. Genes that had an effect on the same T cell subset were in almost all cases additive rather than epistatic, and their combined effects could produce large overall effects, leading in the most dramatic case to a two-fold difference in CD8 memory cells. The analysis also documented two QTL, on chromosomes 4 and 13, that regulate an age-sensitive composite index of T cell subset pattern which has been shown previously to be a predictor of life expectancy in these mice. The analysis thus reveals both subset-specific genes and others which modulate the overall pattern of age-sensitive changes in T cell subset distributions.

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Introduction

The processes that regulate the relative proportions of T cell subsets are still only dimly understood.¹ In normal young mice, the ratio of CD4 ('cluster of differentiation 4') to CD8 cells seems to be carefully regulated, in that the ratio of CD4 to CD8 cells generated in athymic nude mice does not depend on the relative proportions of CD4 and CD8 cells used for reconstitution.² In genetically abnormal mice, however, in which the absence of Class I or Class II MHC molecules precludes the usual pathways for development of (respectively) CD8 or CD4 cells, the total number of T cells is nonetheless similar to that in normal mice, suggesting that each of these two cell types is able to reach abnormally high cell numbers in the absence of the other cell type.^{3–5}

The relative proportions of naïve and memory T cell subsets are also under complex homeostatic control, potentially involving output of thymic emigrants,⁶ transient preferential survival of recent thymic emigrants,⁷ and antigen-stimulated expansion of specific naïve T cells to an activated compartment followed by loss of 90% or more of the activated cells and retention of 10% or fewer as antigen-specific memory T cells.^{8–10}

Although it seems likely that the appearance of new thymic emigrants to the naïve T cell pool plays a critical role in regulation of the relative proportions of naïve and memory T cells, the number of thymic emigrants, even in young adult mice, is too small to account for the rate of loss in the periphery,^{2,11} and thus most peripheral T cells are presumably produced in the peripheral lymphoid tissues from precursors whose identity and properties are not well-documented. Proliferation induced by cytokines in the absence of antigen-specific signals^{12,13} may also help to regulate naïve and memory cell proportions.

Aging has a gradual, but eventually dramatic, effect on the relative proportions of several of the T cell sub-populations. The proportion of cells with the surface markers of memory T cells increases with age, with a corresponding decrease in the proportion of naïve T cells, both in mice^{14,15} and in humans.^{16–18} Studies of the proportions of CD4 and CD8 cells are less consistent, but there are numerous reports of age-related decline in CD4 cell numbers in mouse blood (reviewed in Miller¹⁹). Age also leads, in mice, to an increase in the proportion of T cells expressing the multiple drug resistance pump P-glycoprotein,²⁰ a marker for anergic cells within the CD4 memory population.^{21,22} The increase with age in the P-glycoprotein positive CD4 pool apparently does not depend on antigenic stimulation, because it is also seen within the naïve population of CD4 cells in mice transgenic for a T cell receptor specific for pigeon

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cytochrome C.²³ Some of these transitions are likely to reflect the decline with age in the number of new thymic emigrants,⁶ and indeed the ability of intact thymic glands to support T cell reconstitution declines early in life both in mice^{24,25} and in humans.^{26,27} We know very little, as yet, about the way in which altered thymic output interacts with cytokine-dependent and antigen-dependent processes, as well as with clone-specific cell-autonomous epigenetic changes,^{28–30} to change the balance of naïve and memory T cells, to lead to accumulation of P-glycoprotein positive cells, and more generally to reduce protective immune reactions in old age. Although the functional implications of these subset changes are still uncertain, it is noteworthy that mice whose immune systems appear to be relatively old — ie have higher than normal levels of CD4 memory cells and CD4 P-glycoprotein cells, and lower levels of CD4 and CD4 naïve cells — have shorter life expectancies than littermates with younger T cell subset patterns.^{31,32}

There is a wide variation among individual mice in the levels of any one T cell subset.³³ Among 8-month-old mice of a genetically heterogeneous population, for example, 5% of the animals have CD4 percentages that are either higher than 76% or lower than 50%. Similarly, 5% of the animals have CD4 memory proportions that exceed 56% (as a proportion of CD4 cells) or are lower than 7%, and other T cell subsets show a similar degree of inter-animal variability. To see if these differences are under the control of polymorphic alleles that vary among stocks of laboratory mice, we have conducted a whole genome scan to look for associations between specific chromosomal segments and the levels of seven T cell subsets. The mice were tested at two ages, 8 and 18 months, to determine whether the gene/subset association was stable over time. Two groups of mice were tested, one consisting of mated females, and one consisting of virgin mice, to see to what extent the gene/trait associations could be reproduced in mice differing in gender and in reproductive history. The results show that T cell subset levels are indeed regulated by genetic polymorphisms, some stable and some age-sensitive, and that specific combinations of additive alleles can have major influences over the proportions of several T cell subsets.

Results

Genetic alleles that influence T cell subsets in mated mice

To seek evidence for polymorphic alleles that have significant effects on T cell subset proportions at either

8 or at 18 months of age, we conducted a genome scan in a population of mated female mice of the UM–HET3 stock (see Table 1). Table 2 summarizes key results of these QTL mapping calculations. Two methods were used to test the strength of the locus/trait association: a single locus method and an interval mapping (IM) approach. To reduce the chance of reporting false-positive results, the table includes only those associations where the single locus experiment-wise *P*-value, derived from permutation calculations, was *P* < 0.10, supplemented by results for QTL detected by IM at *P* < 0.05 if these were not significant in the initial single locus screen. Although calculations were carried out independently for the 8-month and the 18-month data, they are assembled together in this table for convenient comparison. The calculations showed at least one association at *P* < 0.10 by the single locus method for each of the seven tested subsets, and significant effects, based on the experiment-wise criterion *P*(*e*) < 0.05, were noted for CD4, CD8, CD8M, CD4P, and CD8P cell subsets. All 11 of the associations noted in the single locus method were confirmed using the IM method at the 5% significance level, and in addition the IM approach detected QTL on chromosomes 4, 7, and 12 that did not achieve statistical significance in the single locus scan. The IM analysis suggested the possibility of two loci on chromosome 7, flanking marker D7Mit91, with effects on the CD4P subset, but further work at higher resolution will be needed to confirm this suggestion.

Genes with an effect on T cell subsets in a population of virgin male and female mice

Table 3 shows the results of a similar linkage study conducted using a population of virgin mice. The number of animals for which both genotype and phenotype were available ranged from 143 to 242; in each case about two-thirds of the mice were female. The ANOVA calculation adjusted for potential effects of gender on the T cell subset, but did not adjust for potential (gender × marker) interaction effects. The table lists 10 QTL where the experiment-wise *P*-value < 0.05 in the single locus analysis, plus six others for which *P*(*e*) < 0.10 but *P*(*e*) > 0.05. In this population of mice, significant effects (using *P* < 0.05 as the criterion) are noted for CD4, CD8, and CD8M T cell subsets. Of the 10 gene/trait associations for which *p*(*e*) < 0.05, seven were also detected by the IM approach, and in addition the IM calculations suggested the presence of two QTL, one near D9Mit12 with an effect on CD4V levels, and one near D1Mit221 with an effect on CD8M levels, that did not reach significant levels in the single locus approach.

Table 1 T cell subsets tested in 8- and 18-month old HET3 mice

Subset designation	Description	Calculation
CD4	Class II restricted helper T cells	CD4(+),CD3(+) as % of CD3
CD8	Class I restricted cytotoxic T cells	CD8(+),CD3(+) as % of CD3
CD4M	Memory CD4 cells	CD4(+),CD44(high) as % of CD4
CD8M	Memory CD8 cells	CD8(+),CD44(high) as % of CD8
CD4V	Virgin CD4 cells	CD4(+),CD45RB(high) as % of CD4
CD4P	P-glycoprotein-positive CD4 cells	CD4(+), R123-extruding as % of CD4
CD8P	P-glycoprotein-positive CD8 cells	CD8(+), R123-extruding as % of CD8

Table 2 Genetic effects on T-cell subset proportions in mated female mice

Trait	Age ^a	Marker ^b	IM Position ^c	IM sig ^d (%)	P(F) < ^e	P(e) < ^f	Allele effect	Effect size	
								% points ^g	S.E. ^h
CD4	8	D4Mit84	-4	5	0.0003	0.05	D2>C3	3.3	0.9
CD4M	8	D1Nds2	+0	5	0.0006	0.09	C>B6	5.9	1.7
CD4P	8	D7Mit91	-2	5			C>B6		
CD4P	8	D7Mit91	+14	5			C>B6		
CD4P	8	D13Mit64	+2	5	0.0001	0.01	C>B6	4.9	1.2
CD4P	18	D6Mit268	+4	5	0.0001	0.02	D2>C3	6.9	1.8
CD4V	18	D4Mit55	-2	5			D2>C3		
CD4V	8	D17Mit46	+12	5	0.0005	0.06	C>B6	4.7	1.3
CD8	18	D5Mit79	+9	1	0.0001	0.02	D2>C3	4.7	1.2
CD8M	8	D1Nds2	-3	5	0.0001	0.01	C>B6	9.0	2.0
CD8M	8	D17Mit46	+6	1	0.0001	0.01	B6>C	10.2	2.0
CD8M	18	D1Mit206	-3	5	0.0001	0.01	D2>C3	10.3	2.4
CD8M	18	D12Mit105	+11	5			B6>C		
CD8M	18	D17Mit46	+3	1	0.0001	0.01	B6>C	12.7	2.6
CD8P	8	D5Mit25	+0	5	0.0002	0.03	B6>C	7.7	2.0

^aTime point (8 or 18 months) at which association was noted.

^bGenotypic markers with strongest association. Markers where $P(e) < 0.10$ in the ANOVA calculations are shown in this table, supplemented by four QTL that were detected as significant only by using the interval mapping approach.

^cPosition of QTL estimated by interval mapping, stated in cM with respect to the indicated marker; a negative sign indicates an estimate closer to the centromere and a positive sign indicates a location further from the centromere.

^dSignificance level using the interval mapping method.

^eNominal (ie uncorrected) P -value of the F -statistic using the single-marker (ANOVA) analysis.

^fExperiment-wise probability, calculated using permutation method with 1000 permutations.

^gMean difference, in percentage points, between the two alleles indicated.

^hStandard error of the effect size

Table 3 Genetic effects on T cell subset proportions in virgin male and female mice

Trait	Age ^a	Marker ^b	IM position ^c	IM sig ^d (%)	P(F) < ^e	P(e) < ^f	Allele effect	Effect size	
								% Points ^g	S.E. ^h
CD4	8	D3Mit25	+0	10	0.0002	0.02	C>B6	3.6	0.9
CD4	8	D3Mit73	+9	5			C>B6		
CD4	8	D8Mit51	+1	5	0.0001	0.01	C>B6	3.7	0.9
CD4	8	D9Mit104	-3	5	0.0003	0.02	C3>D2	3.4	0.9
CD4	8	D15Mit100	ND		0.0014	0.09	D2>C3	2.8	0.9
CD4	18	D12Mit105	ND		0.0011	0.09	C>B6	4.3	1.3
CD4	18	D13Mit57	ND		0.0009	0.07	D2>C3	4.4	1.3
CD4P	8	D16Mit5	ND		0.0011	0.08	C>B6	5.0	1.5
CD4V	8	D4Mit155	ND		0.0009	0.07	D2>C3	5.0	1.5
CD4V	8	D9Mit12	-4	5			C>B6		
CD8	8	D8Mit190	ND		0.0006	0.04	B6>C	3.3	1.0
CD8	8	D15Mit100	ND		0.0003	0.02	C3>D2	3.5	1.0
CD8	8	D19Mit10	ND		0.0004	0.03	C3>D2	3.5	1.0
CD8M	8	D1Mit206	+7	5	0.0005	0.05	D2>C3	5.4	1.5
CD8M	8	D11Mit168	-1	10	0.0005	0.05	D2>C3	5.3	1.5
CD8M	8	D12Mit105	+0	5	0.0004	0.04	B6>C	5.7	1.6
CD8M	18	D1Mit221	-8	5			D2>C3		
CD8M	18	D2Mit58	ND		0.0007	0.06	B6>C	7.9	2.3
CD8M	18	D12Mit105	+0	5	0.0001	0.02	B6>C	8.5	2.2

^aAge (8 or 18 months) at which association was noted.

^bGenotypic markers with strongest association. Markers where $P(e) < 0.10$ in the ANOVA calculations are shown in this table, supplemented by three QTL that were detected as significant only by using the interval mapping approach. Number of mice used for each calculation varied between 143 and 242.

^cPosition of QTL estimated by interval mapping, stated in cM with respect to the indicated marker; a negative sign indicates an estimate closer to the centromere and a positive sign indicates a location further from the centromere. ND = not detected by the interval method.

^{d-h}As in Table 2.

Table 4 Stability of genetic effects at different ages

Trait	Time	Locus	Effect Size ^a (% Points) at	
			8 months	18 months
<i>Mated female mice</i>				
CD4	8	D4Mit84	3.3	1.9
CD4M	8	D1Nds2	5.9	3.3
CD4P	8	D13Mit64	4.9	2.5
CD4P	18	D6Mit268	1.0	6.9
CD4V	8	D17Mit46	4.7	6.9
CD8	18	D5Mit79	0.7	4.7
CD8M	8	D17Mit46	10.2	12.7
CD8M	18	D17Mit46	10.2	12.7
CD8M	18	D1Mit206	4.0	10.3
CD8M	8	D1Nds2	9.0	7.7
CD8P	8	D5Mit25	7.7	2.1
<i>Virgin males and females</i>				
CD4	8	D3Mit25	3.4	1.4
CD4	8	D8Mit51	3.7	3.4
CD4	8	D9Mit104	3.4	2.0
CD4	8	D15Mit100	2.7	2.0
CD4	18	D12Mit105	1.5	4.3
CD4	18	D13Mit57	1.0	4.5
CD4P	18	D16Mit5	3.7	5.2
CD4V	8	D4Mit155	4.1	6.4
CD8	8	D8Mit190	3.3	0.6
CD8	8	D15Mit100	3.5	3.3
CD8	8	D19Mit10	3.4	0.6
CD8M	8	D1Mit206	5.2	6.6
CD8M	8	D11Mit168	4.9	1.2
CD8M	8	D12Mit105	5.8	8.6
CD8M	18	D2Mit58	2.7	7.8

^aEffect size is computed as the difference, in percentage points, in mean level of the T-cell subset, at the indicated age, between mice differing at the indicated locus. The specific alleles (B6 vs BALB or C3H vs DBA/2) are given in Table 2. Boldface type is used for those effects where $P < 0.05$ by *post hoc t*-test.

Stability of QTL effects at different ages

To determine if the genes detected by the genome scanning approach had effects that varied depending on the age of the mouse, we carried out a *post hoc* analysis of the effect size at both 8 and 18 months of age. Table 4 summarizes these calculations for those QTL, detected by the single locus method, listed in Tables 2 or 3. Using $P < 0.05$ as an arbitrary criterion, four of the QTL tested in mated mice, and six of those detected in virgin mice appear to influence T cell subset values at both 8 and 18 months of age, ie have an effect that is established in adult mice and remains roughly equivalent throughout middle age. Two of these loci are illustrated in the top panel of Figure 1. Four of the loci in mated females and six of those detected in virgin females have effects that reach nominal $P < 0.05$ at 8 months, but show a smaller effect (and in some cases little or no effect) at 18 months of age; these loci may produce a transient influence on T cell subset proportions. Five loci (two with effects in mated mice, and three with effects in virgin mice) appear to have an influence on T cell subsets that appears only in the 18-month-old animals. Two of these late-acting QTL, with negligible effects at 8 months and large effects at the 18-month time point, are illustrated in the bottom panels of Figure 1. It is possible that alleles in this class

Table 5 Post hoc analyses of QTL effects in distinct mouse populations

Trait	Time	Locus	Effect size ^a (% points) in	
			Mated	Virgin
<i>Loci with significant effects in mated mice</i>				
CD4	8	D4Mit84	3.3	2.3
CD4M	8	D1Nds2	5.9	1.6
CD4P	8	D13Mit64	4.9	4.8
CD4P	18	D6Mit268	6.9	1.4
CD4V	8	D17Mit46	6.9	4.6
CD8	18	D5Mit79	4.7	0.2
CD8M	8	D17Mit46	12.7	3.3
CD8M	18	D17Mit46	12.7	4.5
CD8M	18	D1Mit206	10.3	6.6
CD8M	8	D1Nds2	9.0	1.4
CD8P	8	D5Mit25	7.7	5.5
<i>Loci with significant effects in virgin mice</i>				
CD4	8	D3Mit25	0.1	3.4
CD4	8	D8Mit51	2.2	3.7
CD4	8	D9Mit104	1.5	3.4
CD4	8	D15Mit100	0.8	2.7
CD4	18	D12Mit105	2.3	4.3
CD4	18	D13Mit57	0.9	4.5
CD4P	18	D16Mit5	3.4	5.2
CD4V	8	D4Mit155	1.6	6.4
CD8	8	D8Mit190	1.2	3.3
CD8	8	D15Mit100	1.7	3.5
CD8	8	D19Mit10	0.7	3.4
CD8M	8	D1Mit206	4.0	6.6
CD8M	8	D11Mit168	0.5	4.9
CD8M	8	D12Mit105	4.3	8.6
CD8M	18	D2Mit58	1.5	7.8

^aEffect size is computed as the difference, in percentage points, in mean level of the T cell subset, at the indicated age, between mice differing at the indicated locus. For QTL with significant effects in mated mice, the age selected is the same as that shown in Table 2, and for QTL with significant effects in virgin mice, the age is the same as that shown in Table 3. Boldface type is used for those effects where $P < 0.05$ by *post hoc t*-test.

may affect some aspect of the aging process that modulates T cell production or turnover.

Comparison between mated and virgin mice

Our data set also allows us to search for genetic alleles whose effects on T cell subset patterns may be robust, ie in this case, detectable even in mouse populations that differ in gender and in reproductive history. Table 5 presents a *post hoc* analysis of the effect size of each QTL (from the single locus method in Tables 2 and 3) as examined in the alternate mouse population, ie in a population different from that in which the original effect was detected. It is important to recognize that the mated and virgin populations do not represent an attempt to replicate the original findings — not only do the two populations differ in gender composition and mating history, but also the statistical model used for experiment-wise significance testing in the virgin group included a term for gender effect that could not be used in the mated female group. Nonetheless, it is of interest to note that of the 10 QTL detected in the mated female group, six achieve a nominal $P < 0.05$ in the population of virgin mice (counting D17Mit46 once). Similarly, of the

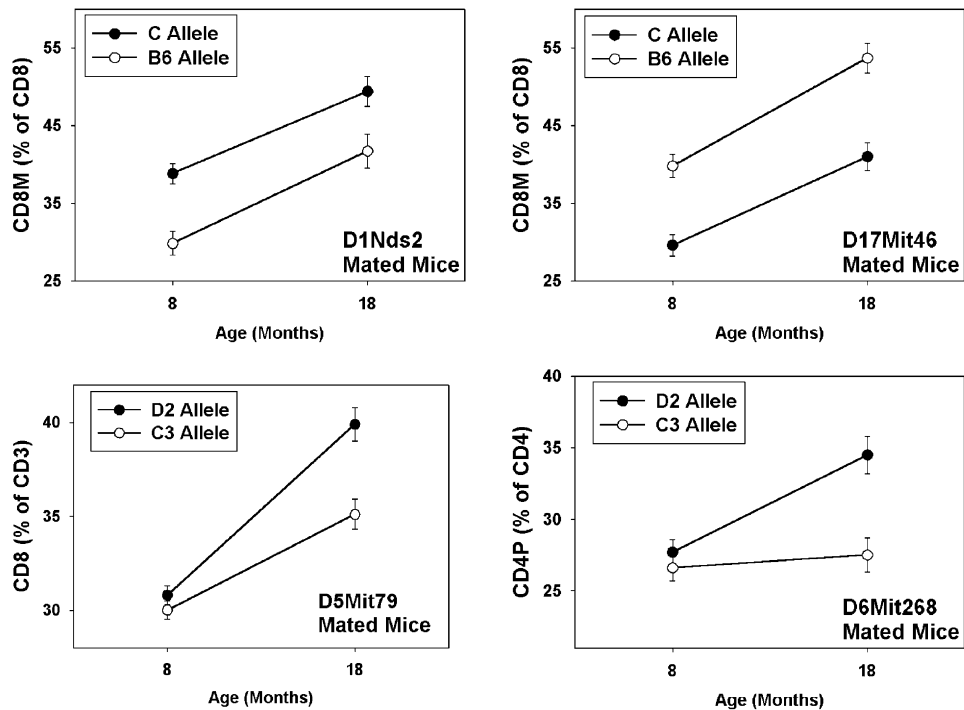


Figure 1 Genetic effects on T cell subset levels: four examples. Each connected pair of symbols shows levels of the indicated T cell subset in mice at either 8 or 18 months of age, as mean \pm standard error. Filled symbols represent mice with one allele, and open symbols indicate mice with the counterallele at the indicated loci. The upper left panel, for example, shows that mated female mice inheriting the C (BALB/c) allele at D1Nds2 have higher levels of CD8M cells, at both ages, than their sisters who inherit the B6 (C57BL/6) allele at this locus. The top two panels show QTL whose effect is similar at 8 and 18 months. The bottom two panels show QTL whose effect is not apparent until 18 months of age.

Table 6 Absence of epistatic interactions among QTL with effects on T cell subset proportions

Trait	Age	Population	Loci	Alleles	Highest value ^a	Value for counteralleles ^b	Delta ^c	Expected ^d
CD8M	8	Mated	D1Nds2 + D17Mit46	C + B6	44 \pm 2	27 \pm 2	17	19
CD8M	18	Mated	D1Mit206 + D17Mit46	D2 + B6	59 \pm 3	37 \pm 2	22	23
CD4P	8	Mated	D7Mit91 + D13Mit64	C + C	31 \pm 1	23 \pm 1	8	9
CD8	8	Virgin	D8Mit190 + D15Mit100 + D19Mit10	B6 + C3 + C3	36 \pm 1	29 \pm 1	7	10
CD4	8	Virgin	D3Mit25 + D8Mit51 + D9Mit104 + D15Mit100	C + C + C3 + D2	69 \pm 1	55 \pm 2	14	14
CD4	18	Virgin	D2Mit285 + D12Mit105 + D13Mit57	C3 + C + D2	56 \pm 2	48 \pm 2	8	13
CD8M	8	Virgin	D1Mit206 + D11Mit168 + D12Mit105 + D13Mit26	D2 + D2 + B6 + C	44 \pm 3	29 \pm 3	15	22
CD8M	18	Virgin	D1Mit206 + D2Mit58 + D12Mit105 + D15Mit100	D2 + B6 + B6 + C	70 \pm 5	32 \pm 4	38	30
CD4V	8	Virgin	D4Mit155 + D12Mit46 ^e	D2 + C3	76 \pm 2	67 \pm 2	9	10

^aPercentage of the indicated T cell subset in mice with the composite genotype associated with highest subset levels. In the first line, for example, this is the percentage of CD8M cells in 8-month-old mated mice with the C + B6 alleles at the D1Nds2 and D17Mit46 loci, respectively.

^bPercentage of the indicated T cell subset in mice with the composite genotype associated with the opposite set of alleles. In all cases but one (CD8 cells in 8-month-old virgin mice), this allele combination was in fact associated with the lowest level of the indicated subset.

^cDelta=difference between the previous two columns, ie the difference in subset between the two most disparate genotypes.

^dExpected=sum of the effect size indicated in Table 2 or Table 3, corresponding to the expected difference between the two most disparate genotypes if the effects were strictly additive.

^eToo few cases to test three-way interaction; D9Mit12 omitted.

15 QTL detected among virgin mice, five reach $P < 0.05$ when re-examined in the group of mated female mice. In these cases, it appears that the association between QTL and T cell subset pattern is sufficiently robust to be detectable even in a second population including mice with different gender and reproductive experience.

Tests for additivity and epistatic interactions

Genes with effects on the same trait can in principle have additive effects, in which the level of the trait in any composite genotype is close to the sum of the individual gene effects, or can exhibit epistasis, in which the effect of a specific allele is dependent on which allele has been

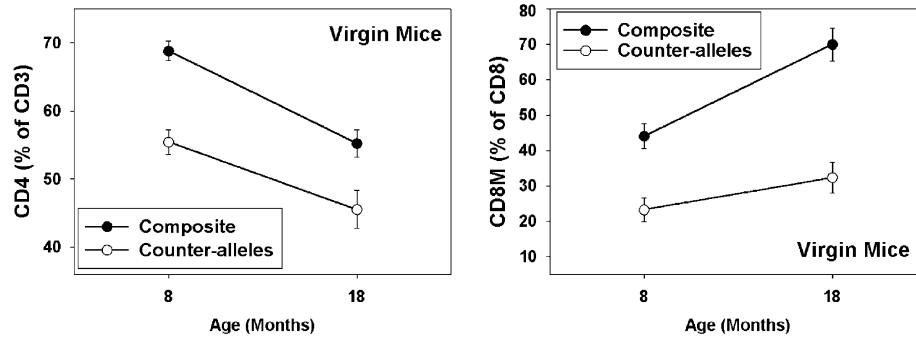


Figure 2 Additive effects of genes influencing CD4 (left) or CD8M (right) subsets. For the left panel, the filled circles refer to mice that inherit the C, C, C3, and D2 alleles at D3Mit25, D8Mit51, D9Mit104, and D15Mit100, respectively; the open circles reflect mice with the complementary set of alleles. For the right panel, the filled circles refer to mice that inherit the D2, B6, B6, and C alleles at loci D1Mit206, D2Mit58, D12Mit105, and D15Mit100, and the open circles show mice with the opposite allele combination.

inherited at one or more of the other loci. For each of the traits in Tables 2 and 3 for which there was more than one QTL at a given time point (detected by the single locus method), we conducted an analysis of variance (ANOVA) to see if there was evidence for significant inter-allele interaction. In all cases but one, we found no evidence for gene/gene interaction, as indicated by the significance test of the two-way and higher-order interaction terms in the ANOVA results (not shown). (The exception was due to a significant interaction, at $P=0.009$, between the D8Mit51 and D15Mit100 markers that are associated with differences in CD4 T cells in 8-month-old virgin mice.) This analysis also allowed us to calculate the differences in T cell subset levels between mice that differ at two or more of the QTL that are associated with differences in T cell subsets. Table 6 shows the results of this analysis. In all cases, the observed difference in subset levels between the most disparate genotypic combinations was close to the sum of the individual gene effects, consistent with the lack of statistically significant epistasis noted in the ANOVA. The 'delta' column, which shows for each allele combination the difference in subset levels between the group with the most extreme phenotype and the group with the opposite allele pattern, illustrates that these genetic differences, when taken in combination, can have large effects on T cell subset values. Figure 2 illustrates two of these multi-gene composite effects for age-sensitive subsets (CD4 and CD8M) in the virgin mice. In each case, the combined genetic effect seen at 8 months of age is similar in magnitude to the effects of aging itself over the 8 to 18-month interval; in this sense, mice with one set of alleles appear to resemble, in their subset patterns, animals that are 10 months older or younger.

Mapping QTL for an age-sensitive composite index of T cell subset status

With a few exceptions, most of the QTL listed in Tables 2 and 3 appear to influence only a single T cell subset. Nonetheless, we have shown in previous work that many of the age-sensitive T cell subsets are correlated with one another when tested in 18-month-old mice,³³ and that those 18-month-old mice whose T cell subsets resemble those of younger animals tend to be relatively

long lived.³¹ To see if there were a genetic influence on the overall pattern of T cell subsets in 18-month-old mice, we used a principal component method to calculate, for each animal, a first principal component, ie a linear combination of T cell subset levels that captures the maximum amount of inter-animal variation in a single composite index. This principal component, termed 'Immune Factor 1', has an eigenvalue of 2.7, indicating that it encapsulates 2.7-fold as much inter-animal variation as the average T cell subset by itself. Figure 3 shows a plot of the correlations between Immune Factor 1 and each of the seven T cell subsets used for its calculation. There are strong positive correlations between the factor and CD4M, CD8M, and CD4P, all subsets that increase with age, and strong negative correlations between the factor and the levels of CD4 and CD4V cells, subsets that tend to decline with age in mice. We interpret this pattern of factor loadings as evidence that the values of Immune Factor 1 can be used as a measure of the overall effect of aging on the immune subset pattern. We have shown elsewhere (Miller, submitted) that mice with high levels of Immune Factor 1 have a shorter life expectancy than mice with low

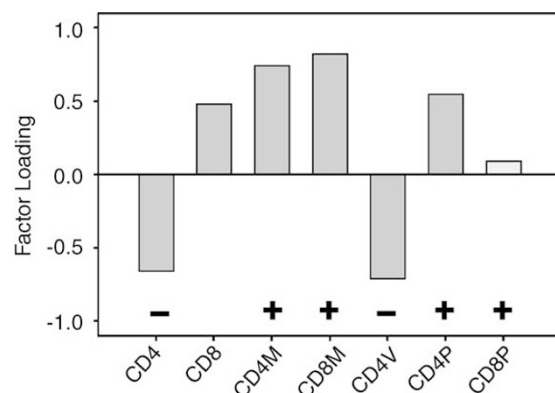


Figure 3 Factor loadings for Immune Factor 1. Each bar shows the correlation between the indicated subset and the calculated first principal component (Immune Factor 1) for mice at age 18 months. The + and - signs at the bottom of the graphic show the direction of the age effect for each subset: a + sign indicates that the subset in question tends to increase with age.

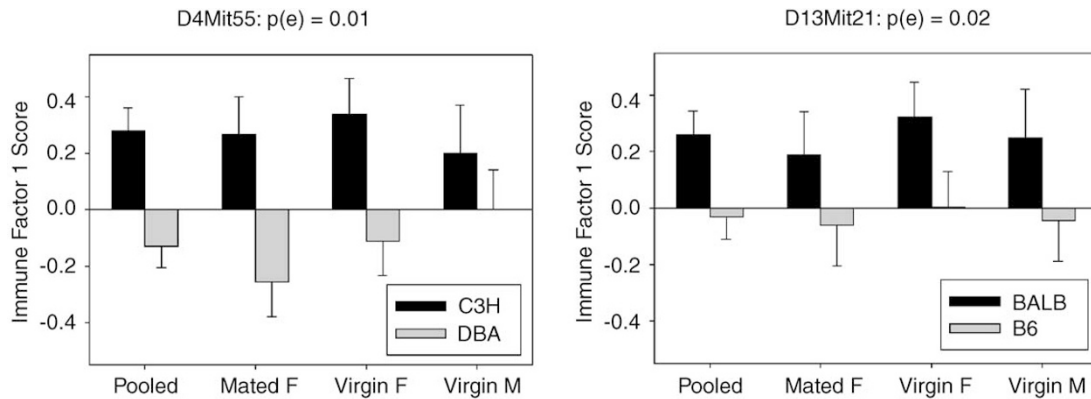


Figure 4 Means and standard errors of the means for Immune Factor 1 scores. Each bar shows the mean level of Immune Factor 1 in 18-month-old mice with the indicated allele, either (left panel) C3H *vs* DBA allele at D4Mit55 or (right panel) BALB *vs* B6 allele at D13Mit21. Error bars show standard error of the mean. In each panel the left pair of bars shows the results for all mice combined ('pooled'), and the other three pairs of bars show data for subpopulations of the mice differing in gender and mating history. The *P*-values given at the top of each panel are experiment-wise probabilities for the pooled data set.

levels, consistent with the interpretation of this factor as an index of immunological age.

Figure 4 shows the results of a QTL analysis of Immune Factor 1 levels. There were two markers, D4Mit55 at $P=0.01$ and D13Mit21 at $P=0.02$, for which we found a significant level of association between marker and factor level for the entire pooled data set. Figure 4 also shows mean Immune Factor 1 levels for each of the three mouse populations that were pooled for the mapping calculation. The association between genotype and Immune Factor 1 level is apparent and of roughly equal magnitude in each of these three independent groups of mice for each of the loci in question.

Discussion

As far as we know, our data represent the first systematic attempt to map genes that influence T cell subset levels in either young or old mice. The genome scan approach we chose is unbiased, in the sense that it does not depend on prior information about strain-specific differences in one or more of the T cell subsets of interest. This permits the same genotypic information to be used efficiently to search for gene/trait associations for a wide range of traits of interest. By examining each test mouse twice, once at 8 and then again at 18 months of age, we were able to search for genes whose effect on T cell subsets was transient, permanent, or late-acting with respect to the life span. This approach allowed us to document statistically significant associations, at an experiment-wise $P < 0.05$, for genes that influence CD4, CD4P, CD8, CD8M, and CD8P T cell subsets in mated female mice. A similar approach using a population of virgin male and female mice documented QTL with effects on CD4, CD8, and CD8M subsets. Many other loci had suggestive evidence for linkage; those where $P < 0.10$ are included in Tables 2 and 3 and a complete list of QTL where $P < 0.20$ can be obtained by request from the authors. *Post hoc* analyses showed that 10 of the loci with $P < 0.10$ led to stable effects on their respective T cell subsets, with

significant effects at both 8 and 18 months of age. Five other alleles had effects that were significant only in the older mice, and thus may be influencing age-dependent elements of T cell homeostasis. Eleven of the QTL effects were sufficiently robust that they were apparent, by *post hoc* analyses, both in the mated female population and in the mixed-sex virgin population. We found no evidence for epistatic interactions; genes with an effect on a common T cell subset seemed to act independently of one another. Our search for epistatic interactions involved only those QTL identified as significant in the single-locus scan; it is thus possible that epistatic interactions might exist between pairs of genes that were not individually detectable in our population.

In some cases, the net effect of the multiple, additive genetic differences could be quite large: in the most dramatic case, mice with a specific combination of alleles were found to have more than twice as many CD8M cells as mice with the opposite allele combination. Lastly, we found that an age-sensitive composite index of T cell subset pattern, Immune Factor 1, previously found to be a predictor of all-cause mortality risk, was itself regulated by genes on chromosomes 4 and 13, with equivalent effects in mated females and in virgin male and female mice.

These data, though meeting accepted standards of statistical significance for genome scans, nonetheless need to be considered preliminary in several ways. We do not know how many of the associations we have documented will be replicated in subsequent analyses, now in progress, of additional mice generated using the same breeding system. Nor is it clear to what extent these QTL will be detectable in other segregating combinations of these or other grandparental strains. Those QTL that are seen in both the mated and virgin populations seem likely to prove replicable in additional sets of mice, but among those QTL seen in only one of these two studies it is not possible to determine without more data whether the disparity represents a real effect of gender or reproductive history, or instead reflects a false positive or unstable result.

There have been only a few previous studies of genetic effects on T cell subset levels. One group³⁴ has reported a QTL on rat chromosome 9, and two others (chromosomes 10 and 20) for which evidence was only suggestive, which modify the balance between CD45RC^{high} and CD45RC^{low} CD4 T cells in a cross between Lewis and Brown-Norway rats. The region containing the rat chromosome 9 locus is syntenic with a portion of mouse chromosome 17, but is about 20 cM away from the D17Mit46 locus associated with differences in CD4V and CD8M levels in our mouse data set. The rat chromosome 20 region is also syntenic to the same area of mouse chromosome 17, ie about 20 cM distant from D17Mit46. The rat CD45RC^{high} subset of CD4 cells resembles the mouse CD4V subset in some of its properties, and it is plausible, though by no means certain, that both the studies may have mapped a region that influences T cell development through similar biochemical pathways.

A second group³⁵ has reported a mutation (nkt) at 35 cM on mouse chromosome 13 that can, when homozygous, lead to severe depletion of CD4 cell numbers in the thymus and periphery as well as alopecia. D13Mit57, which we report to be associated with altered CD4 cells, maps to position 1.1 cM on chromosome 13, and thus seems unlikely to represent a variant of the nkt locus. D13Mit21, which we find to be associated with the age-sensitive Immune Factor 1, maps to position 35 cM on chromosome 13, and we cannot rule out the idea that this genetic area may contain several loci with effects on T cell homeostasis.

A third group³⁶ has shown that differences between B6 and BALB/c mice in the proportion of CD4 to CD8 cells reflect differences that map to the region of the T cell receptor alpha chain locus at 21 cM on mouse chromosome 6. In our own genetic combination, we see no significant effects of chromosome 6 loci on CD4 or CD8 levels in either tested population at either age.

Our new data can serve as a springboard to several sorts of follow-up studies. Data on genetic controls of T cell subsets may help to guide searches, either in mouse or in humans, for genes that may modify susceptibility to infectious illnesses or lead to alterations in immune competence. Our own group is currently using the same genetic system to map QTL with effects on T cell function and on antibody response, and it will be of interest to see if some regions influence both subset levels and immune responsiveness.

Some of the QTL we have detected exert effects that are not seen in young mice, but become manifest only in middle age. It will be of interest to see if any of these loci modulate the pace of age-related change outside the immune system. The observations that several of the T cell subsets, as well as the principal component derived from them, are predictors of longevity are consistent with two explanatory models. One of these models attributes the differences in mortality risk to alterations in immune function *per se*; the other model views the immune status variations as an index of underlying pathological processes that influence both immunity and disease risk. Follow-up studies to see if the QTL implicated in our study exert an influence on age-sensitive aspects of bone, muscle, and other organ systems will help to distinguish among these ideas.

Materials and methods

Mice

All animals were bred at the University of Michigan using CB6F1 mothers and C3D2F1 fathers obtained from the Jackson Laboratories (Bar Harbor, ME, USA). Mice were weaned at age 3–4 weeks into same-sex cages containing 3–4 pups, and the cages maintained without replacement of animals that died. On a quarterly basis, sentinel mice (not part of the experimental groups) were exposed to pooled spent bedding material from the study population and then later tested serologically for evidence of immunity to murine Sendai, mycoplasma and coronavirus (mouse hepatitis virus (MHV)). In addition, the animals were examined for pinworm. All such tests were negative during the course of this study. In some cages of female mice, a male mouse was introduced at approximately 8 weeks of age to create a group of 'mated females'. Litters were removed from these cages within the first week after birth, and the male mouse was removed when the females reached 6 months of age. Cages of virgin males, virgin females, and mated females were all housed within the same room.

Exclusion criteria

Fighting in cages of male mice caused serious wounding in approximately 25% of the cages; in these cases all males in the cage were culled, always prior to 12 months of age and typically much earlier. The few mice dying at ages less than 8 months were not included in the study, because they did not reach the age at which the first immune assessment was conducted. The study population thus included 292 mated female mice, of which 267 survived to be re-examined at 18 months: 146 virgin females of which 136 were re-examined at 18 months, and 121 virgin males of which 91 were tested at 18 months of age.

Assessment of T cell subset levels in peripheral blood

Two color flow cytometry analyses were conducted as previously described³² on samples of peripheral venous blood obtained at 8 and then again at 18 months of age. Table 1 shows the definition of the seven T cell subsets that form the subject of this report.

Genotyping

Genotyping was performed by standard PCR amplification of genomic DNA from each animal using marker loci obtained from the Mouse Simple Sequence Length Polymorphism Database, Whitehead/MIT Center for Genome Research (carbon.wi.mit.edu:8000/ftp/distribution/mouse_sslp_releases/may99).³⁷ Polyacrylamide gels were scored using the ALFExpress automated sequencer as described.³⁸ Analyses at marker loci were performed on 272 virgin mice (males = 125, females = 147) and 213 mated females. For the virgin mice, 79 markers were fully informative for four grandparental alleles, and 20 markers were informative for either the maternally transmitted (C or B6) or paternally transmitted (C3 or D2) alleles only. For the mated mice, 73 markers were four-way informative and 11 markers were partially informative. The average distance between markers in our scan is 15–20%, depending on how one treats sex-specific differences in recombination frequencies, and excluding the region between the most distal

marker and the telomere for each chromosome. About 5% of the genome lies more than 20 cM from any of the markers used in the survey.

Statistical analysis

Genome-wide searches for quantitative trait loci were performed separately for virgin mice and mated mice, using a single point locus scan for each phenotype measured at 8 or 18 months. To make the model consistent for differentially informative markers, four-way informative markers were split into two sets of biallelic markers informative for either the maternally or paternally transmitted alleles. The total number of biallelic markers was 178 for the virgin mice, and 157 for the mated mice. Analysis of variance was performed for single biallelic loci using PROC MIXED in SAS version 8.0 (SAS Institute). For virgin mice, the following statistical model was used:

$$y_k = \mu + s + g + \varepsilon_k,$$

where y_k is the phenotype for the k th individual, μ is the overall mean, s is the sex effect, g is the biallelic marker effect, and ε_k is the error term with $N(0, \sigma_\varepsilon^2)$. The model for the mated mice did not include the sex term. Phenotype data were not transformed. Significance was assessed by permutation testing performed essentially as described,³⁹ with 1000 permutations of the data.

Interval mapping was conducted using the software package Map Manager QTXb13 on the same data set using the methods described in Manly *et al.*⁴⁰ Sex was included as a covariate for the virgin mice. Significance was assessed by permutation analysis, using 1000 permutations at intervals of 2 cM.

Post-hoc analyses

For alleles shown, by permutation analysis, to be unlikely to reflect chance associations, differences in mean level of T cell subsets were evaluated by Student's t -test in other groups of mice or at other ages, using $P < 0.05$ as the significance criterion.

Principal component analysis

A principal components method was used to combine data from the seven tested T cell subsets into a single composite index. The method calculates weighting factors, one for each subset, such that the sum of the weighted, standardized subset values achieves the maximum possible variance among the mice. The specific algorithm used, implemented in NCSS statistical software (NCSS Statistical Software, Kaysville, UT, USA) uses multivariate regression to estimate missing values. Factor loadings — the correlation between each of the PC factors with each of the original trait measurements — were calculated as guides to the biological interpretation of each of the extracted factors. The value of the first principal component was calculated for each mouse for which data were available for each of the seven subsets at 18 months of age, and these values were then used for QTL mapping as described above for the individual T cell subset levels.

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