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T Cell Development in Mice That Lack the ζ Chain of the T Cell Antigen Receptor Complex

Paul E. Love,* Elizabeth W. Shores, Mark D. Johnson,† Michel L. Tremblay,‡ Eric J. Lee, Alexander Grinberg, Sing Ping Huang, Alfred Singer, Heiner Westphal

The ζ subunit of the T cell antigen receptor complex is required for targeting nascent receptor complexes to the cell surface and for receptor-mediated signal transduction. To examine the significance of the ζ subunit in T cell development, mice deficient for ζ expression were generated by gene targeting. These $\zeta^{-/-}$ mice had few $CD4^+CD8^+$ thymocytes, and the generation of $CD4^+$ and $CD8^+$ single positive T cells was impaired but not completely abrogated. Peripheral T cells were present but were unusual in that they expressed small amounts of CD5 and few T cell receptors. Thus, ζ chain expression influences thymocyte differentiation but is not absolutely required for the generation of single positive T cells.

The T cell antigen receptor (TCR) is a complex multisubunit structure that consists of at least six different protein chains (TCRα and TCRβ; CD3γ, CD3δ, and CD3ε; and ζ) that are assembled in the endoplasmic reticulum and transported to the cell surface (*1*). The α and β chains are clonotypic and confer ligand specificity; the other subunits are invariant and function as signal-transducing molecules. The best characterized of the invariant subunits is ζ, which is assembled either as a disulfide-linked homodimer or as a disulfide-linked heterodimer with another member of the ζ family of proteins (η or the γ chain of the type I Fcε receptor) (*2*). The presence of ζ is required both for efficient transport of assembled TCR complexes to the cell surface and for efficient signal transduction (*1*, *3*). Signaling through the TCR antigen complex is thought to trigger intrathymic selection events during T cell development, particularly the negative and positive selection events that affect immature $CD4^+CD8^+$ thymocytes and regulate their differentiation into mature $CD4^+$ and $CD8^+$ T cells. Consequently, ζ expression should be critical for T cell development and for the generation of the mature T cell repertoire. We evaluated the importance of ζ in the maturation of T cells by assessing T cell differentiation in mice made deficient for ζ expression.

The ζ-deficient mice were derived from embryonic stem (ES) cells in which one of the ζ genes was specifically mutated by homologous recombination (*4*). Disruption of the ζ coding sequence was detected by screening independent ES clones for homologous integration of pζ-Δ/CT94 after electroporation of the targeting construct. Positive clones contained an inactivated ζ allele that lacked exon II (encoding the ζ transmembrane domain) and carried an insertion of the neomycin phosphotransferase (*neo*) gene in exon IV (Fig. 1A). This disruption of the ζ locus interferes with expression of both the ζ and η proteins because they are derived from the same transcript by alternative splicing (*5*). Two clones, J52 and J66, were used to generate chimeric mice that transmitted the mutated allele to their offspring. Southern (DNA) analysis of tail DNA from the progeny of heterozygote intermatings revealed the expected restriction pattern for the wild-type ($\zeta^{+/+}$), heterozygous mutant ($\zeta^{+/-}$), and homozygous mutant ($\zeta^{-/-}$) genotypes (Fig. 1B).

Expression of the endogenous and mutant ζ alleles was examined by Northern (RNA) blotting of total thymocyte and lymph node RNA. Thymocytes and lymph node cells from $\zeta^{+/-}$ heterozygous mice contained approximately 50% correctly sized (1.7 kb) transcripts when compared to $\zeta^{+/+}$ littermates and additionally contained small amounts of a mutant transcript of approximately 2.5 kb that may represent either an aberrantly or incompletely spliced mRNA species or a partial ζ transcript that contains the inserted *neo* gene (Fig. 1C). Thymocytes from $\zeta^{-/-}$ mice did not contain the wild-type ζ transcript and expressed only the mutant ζ transcript (Fig. 1C). Lymph node cells from $\zeta^{-/-}$ mice did not contain detectable ζ transcripts, either wild-type or mutant. Consistent with the absence of mature ζ transcripts in $\zeta^{-/-}$ mice, metabolic labeling studies of $\zeta^{-/-}$ thymocytes revealed that they did not synthesize ζ protein as determined by immunoprecipitation with a combination of polyclonal antibodies to ζ (anti-ζ 528 and anti-ζ 551), which are specific for the NH_2- and COOH-terminal sequences of the ζ cytoplasmic domain, respectively (Fig. 2) (*6*). Synthesis of η, which would additionally

P. E. Love, M. D. Johnson, M. L. Tremblay, E. J. Lee, A. Grinberg, S. P. Huang, H. Westphal, Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, National Institutes of Health (NIH), Bethesda, MD 20892.
E. W. Shores and A. Singer, Experimental Immunology Branch, National Cancer Institute, NIH, Bethesda, MD 20892.

*To whom correspondence should be addressed at Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, NIH, Building 6B, Room 210, Bethesda, MD 20892.
†Present address: Department of Gynecology and Obstetrics, Stanford University Medical Center, Palo Alto, CA 94305.
‡Present address: Department of Biochemistry, McGill University, Montreal, Quebec H3G 1X6, Canada.

Table 1. Numbers of T cells in thymuses and lymph nodes from $\zeta^{+/-}$ and $\zeta^{-/-}$ mice. Numbers shown are means × 10^{-6} (for thymuses, $n = 4$ mice; for lymph nodes, $n = 3$ mice). Values in parentheses represent the percent of cells within quadrants as shown in Fig. 3. Thymocytes and lymph node cells (inguinal, axillary, and mesenteric) were obtained from 4- to 8-week-old $\zeta^{+/-}$ and $\zeta^{-/-}$ littermates.

Genotype	Source	Total T cells	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^+$
$\zeta^{+/-}$	Thymus	194	7 (4)	168 (87)	13 (7)	7 (4)
$\zeta^{-/-}$	Thymus	28	8 (28)	19 (68)	<1 (2)	<1 (1)
$\zeta^{+/-}$	Lymph node	23	–	–	10 (43)	5 (22)
$\zeta^{-/-}$	Lymph node	17	–	–	2 (12)	1 (6)

have been precipitated by antibody 528 (5, 6), was also not detected (Fig. 2).

We next assessed the synthesis of other TCR components by metabolic labeling and found that each of the other chains was synthesized in $\zeta^{-/-}$ thymocytes, although steady-state amounts were lower than in $\zeta^{+/+}$ thymocytes possibly because partial TCR complexes that lack the ζ subunit are rapidly degraded (Fig. 2). Consistent with a ζ requirement for efficient surface expression of αβ TCRs, thymocytes and lymph node T cells from $\zeta^{-/-}$ mice expressed little or no CD3ε and TCRβ as assessed by staining with anti-CD3ε [monoclonal antibody (mAb) 145-2C11] and anti-TCRβ (mAb H57-597), respectively (Fig. 3, A and B). To document that the observed effect on αβ TCR surface expression was due to the absence of ζ, we introduced a ζ transgene (*7*) into $\zeta^{-/-}$ mice ($\zeta^{-/-;Tg+}$). The expression of ζ in $\zeta^{-/-;Tg+}$ mice restored CD3ε and TCRβ surface expression in both thymocytes and lymph node cells (Fig. 3, A and B).

To assess T cell development in the absence of ζ, we examined CD4, CD8, and CD5 expression on thymocytes of $\zeta^{-/-}$ mice. Thymocyte populations from $\zeta^{-/-}$ mice expressed small amounts of CD5 ($CD5^{lo}$) and contained $CD4^-CD8^-$ and $CD4^+CD8^+$ cells, but were deficient in single positive (SP) ($CD4^{hi}CD8^-$ and $CD4^-CD8^{hi}$) thymocytes (Fig. 3A and Table 1). The $CD4^{lo}CD8^-$ and $CD4^-CD8^{lo}$ thymocytes that were present in $\zeta^{-/-}$ mice were probably precursors of immature $CD4^+CD8^+$ thymocytes (*8*). Thymic cellularity was also affected. The average number of total thymocytes in $\zeta^{-/-}$ homozygotes was only 14% (range, 1 to 32%; n = 10) of that in either wild-type $\zeta^{+/+}$ or heterozygous $\zeta^{+/-}$ littermates, which were phenotypically identical (Fig. 3A and Table 1). When total thymic cellularity was taken into account, $\zeta^{-/-}$ thymocyte populations contained equivalent numbers of $CD4^-CD8^-$ thymocytes, approximately 10% of the total number of $CD4^+CD8^+$ thymocytes, and approximately 10% of the total number of SP thymocytes (most of which were precursors of $CD4^+CD8^+$ cells) compared to $\zeta^{+/-}$ littermates (Table 1 and Fig. 4). However, the lymph nodes of $\zeta^{-/-}$ mice contained significant, albeit reduced, numbers of SP ($CD4^+$ and $CD8^+$) T cells (Table 1 and Fig. 3B). These $CD4^{hi}$ and $CD8^{hi}$ lymph node cells were uniformly Thy-1.2^+ but were unlike SP peripheral T cells from $\zeta^{+/+}$ or $\zeta^{+/-}$ mice in that they expressed only barely detectable surface levels of CD3ε and TCRβ (Fig. 3B). These cells, which were undetectable in the periphery of normal littermates, were also distinguished by their low surface expression of CD5, as peripheral T cells in $\zeta^{+/+}$ or $\zeta^{+/-}$ mice are uniformly $CD5^{hi}$ (Fig. 3C). The atypical $TCR^{lo}CD5^{lo}$ phenotype of $\zeta^{-/-}$ peripheral T cells was caused by the

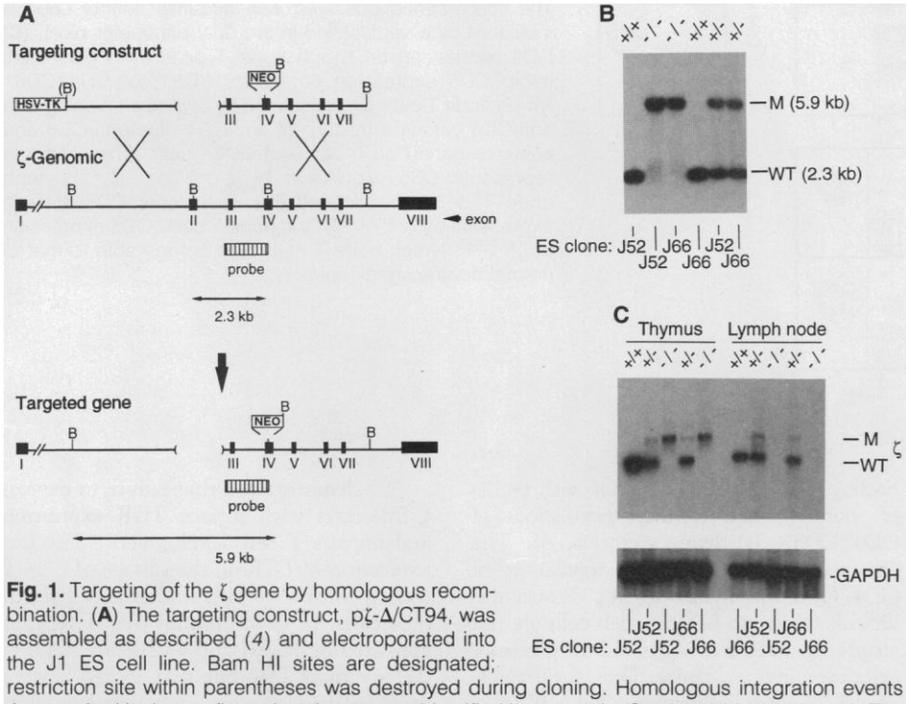


Fig. 1. Targeting of the ζ gene by homologous recombination. (**A**) The targeting construct, pζ-Δ/CT94, was assembled as described (*4*) and electroporated into the J1 ES cell line. Bam HI sites are designated; restriction site within parentheses was destroyed during cloning. Homologous integration events that resulted in the configuration shown were identified by genomic (Southern) blotting (*4, 15*). The targeted allele should produce nonfunctional ζ and η proteins because exon II, which encodes the ζ/η transmembrane domain, was deleted (depicted as open parentheses) and exon IV, which encodes part of the cytoplasmic domain, was disrupted by insertion of the neomycin phosphotransferase (*neo*) gene. The probe used for hybridization in these experiments and the predicted sizes of fragments generated by the endogenous and targeted alleles after digestion with Bam HI are depicted. (**B**) Germline transmission of the targeted ζ chain allele as verified by Southern analysis. Chimeric mice were generated from two independently derived ES cell clones, J52 and J66. Representative $\zeta^{+/+}$, $\zeta^{+/-}$, and $\zeta^{-/-}$ littermates from intermatings of F_1 $\zeta^{+/-}$ mice are shown. (**C**) Northern blot analysis (*15*) of ζ transcripts in thymocytes and lymph node cells from $\zeta^{+/+}$, $\zeta^{+/-}$, and $\zeta^{-/-}$ mice. The ζ transcripts were detected with probes generated from full-length ζ complementary DNA by random primer labeling. To assess loading consistency, we hybridized identical samples with a probe derived from the gene for the protein glyceraldehyde phosphate dehydrogenase (GAPDH) (*16*). WT, wild-type transcripts; M, mutant transcripts. The ES cell lines used to generate founder chimeric mice are indicated.

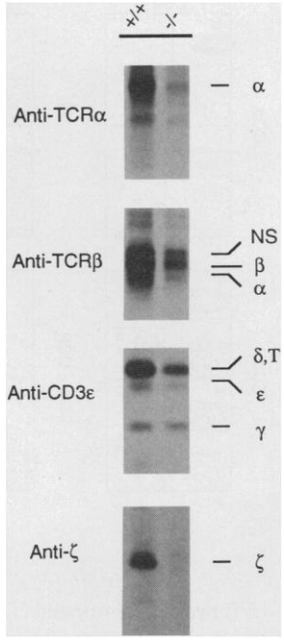
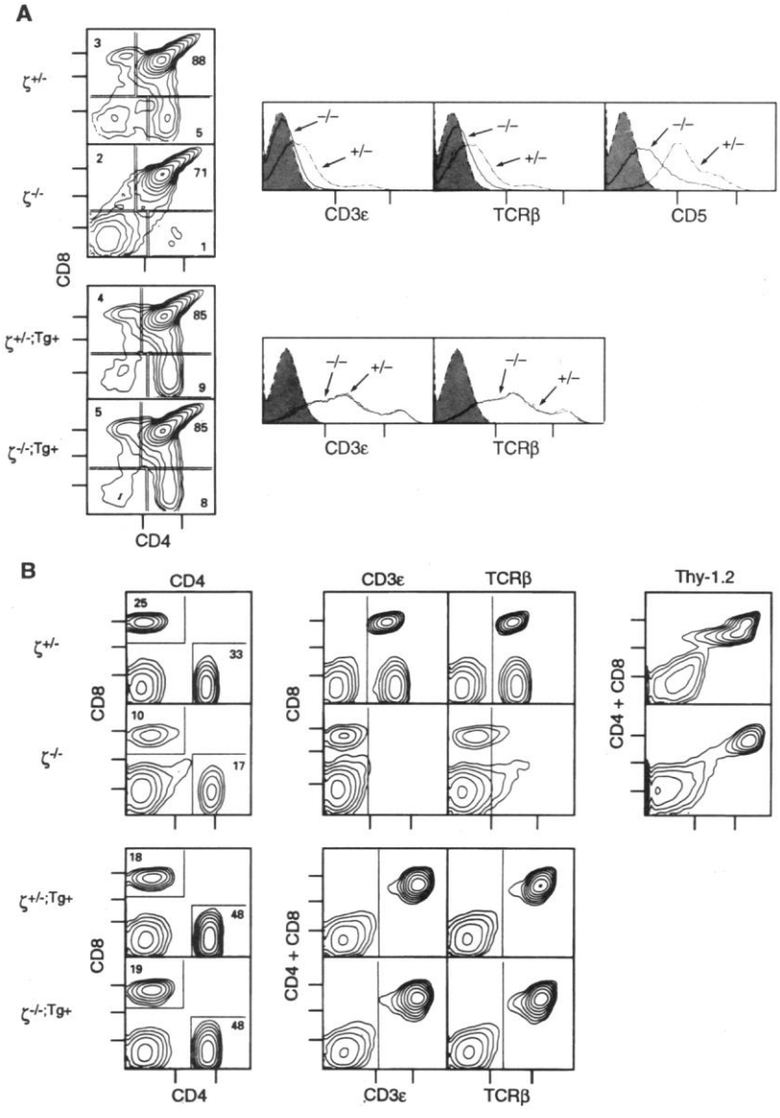


Fig. 2. Synthesis of TCR subunits in wild-type ($\zeta^{+/+}$) and homozygous mutant ($\zeta^{-/-}$) mice. Total thymocytes were metabolically labeled with [^{35}S]methionine (*17*), solubilized with 1% NP-40, precleared, and immunoprecipitated with mAbs to TCRα [H28-710 (*12*)], TCRβ [H57-597 (*13*)], CD3ε [145-2C11 (*14*)], or ζ [mixture of mAbs 528 and 551 (*6*)]. The precipitated proteins were then analyzed under reducing conditions by SDS-PAGE (13%) and visualized by autoradiography. In metabolically labeled NP-40 lysates, anti-TCRα precipitates nascent α protein; anti-TCRβ precipitates nascent β and associated α proteins; anti-CD3ε precipitates nascent ε and associated γ, δ, and TRAP(T) proteins; and anti-ζ precipitates nascent ζ and or η proteins. Nonspecific (NS) bands were present in immunoprecipitates without antibody. The faint bands present in anti-ζ immunoprecipitates were also nonspecific, as they were present in immunoprecipitates without antibody.



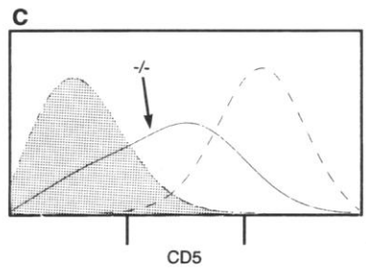


Fig. 3. Analysis of thymocytes and lymph node T cells in $\zeta^{-/-}$ mice. Immunofluorescence and flow cytometric analysis of cells from young adult (4- to 8-week-old) heterozygous $\zeta^{+/-}$ and homozygous $\zeta^{-/-}$ mice and from similar mice ($\zeta^{+/-;Tg+}$, $\zeta^{-/-;Tg+}$) into which was bred a ζ transgene (founder line C721) that expresses two to four times the amount of endogenous ζ under the control of the human CD2 regulatory elements (*7, 18*). Cells were stained with directly labeled mAb to CD4 (Rm4-5), CD8 (53-6-72), CD3ε (145-2C11), TCRβ (H57-597), Thy-1.2 (30-H12), or CD5 (53-7.3) as indicated (*19*). Shaded areas represent staining with control antibody (Leu4) to human cells. Numbers in quadrants represent the frequency of cells contained within that quadrant. (**A**) Effect of the absence of ζ on thymic subpopulations. (**B**) Lymph node T cells in ζ-deficient mice. Lymph nodes were from the mice whose thymocytes were analyzed in (A). The fluorescence observed with negative control Leu4 is indicated by a vertical line in the dual parameter plots. (**C**) CD5 expression on lymph node T cells from ζ-deficient mice. CD5 expression on $CD4^+$, $CD8^+$, or $CD4^+CD8^+$ lymph node T cells was assessed by two-color staining with anti-CD5 versus a mixture of anti-CD4 plus anti-CD8 and software gating on $CD4^+$ and $CD8^+$ cells. The solid line represents CD5 expression by ζ-deficient ($\zeta^{-/-}$) lymph node T cells; the dashed line represents CD5 surface expression by $\zeta^{+/-;Tg+}$ lymph node T cells. CD5 expression in $\zeta^{+/-;Tg+}$ lymph node T cells was comparable to that of normal nontransgenic mice (*7*).

absence of ζ because peripheral T cells in $\zeta^{-/-;Tg+}$ mice were phenotypically normal (Fig. 3B). Interestingly, the ratio of $CD4^+$ to $CD8^+$ peripheral cells in $\zeta^{-/-}$ mice (2:1) was identical to that in $\zeta^{+/-}$ mice (Table 1), which suggests the possibility that these cells were of thymic origin.

The presence of $CD4^{hi}$ and $CD8^{hi}$ SP T cells in the periphery of young adult $\zeta^{-/-}$ mice caused us to reexamine the thymuses of mice for $CD4^{hi}$ and $CD8^{hi}$ SP thymocytes. Each $\zeta^{-/-}$ thymus did contain a small (<1% of normal) but distinct population of $CD4^{hi}CD8^-$ SP thymocytes (Fig. 4). The presence of the reciprocal population of $CD4^-CD8^{hi}$ SP thymocytes in $\zeta^{-/-}$ mice was difficult to discern because such cells are not clearly separated from $CD4^-CD8^{lo}$ precursor cells, even in normal mice. Thus, the thymuses of $\zeta^{-/-}$ mice did contain $CD4^{hi}$ SP cells, although only 1% of the total number of such cells present in $\zeta^{+/-}$ mice (Table 1).

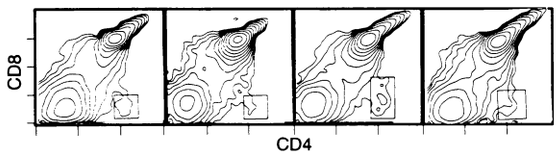


Fig. 4. Analysis of $CD4^{hi}CD8^-$ thymocytes from $\zeta^{-/-}$ mice. Thymocytes from four separate 4- to 8-week-old $\zeta^{-/-}$ mice were analyzed for CD4 and CD8 expression by staining with directly conjugated antibodies specific for CD4 (Rm4-5) and CD8 (53-6-72). $CD4^{hi}CD8^-$ cells were specifically quantitated by software gating on cell populations shown within insets. The average number of $CD4^{hi}CD8^-$ thymocytes in $\zeta^{-/-}$ mice was 0.1×10^6 (0.35% of the total thymocytes).

We demonstrated that failure to express ζ interferes with surface TCR expression and impairs T cell development. The low expression of TCR on the surface of $\zeta^{-/-}$ T cells is consistent with studies in T hybridoma cells, in which partial αβ TCRs that consisted of TCRα and TCRβ and CD3γ, CD3δ, and CD3ε but that lacked ζ were degraded in lysosomes such that few complexes are transported to the cell surface (*1*). Whether partial ζ-deficient TCR complexes such as those described previously (*9*) are expressed on the surface of $\zeta^{-/-}$ thymocytes or whether ζ is replaced by another member of the ζ family dimers (that is, the γ chain of the type I Fcε receptor) remains to be determined.

The decrease in numbers of $CD4^+CD8^+$ thymocytes in $\zeta^{-/-}$ mice was unexpected, as TCR expression and TCR-mediated signaling are not known to be required for the generation of $CD4^+CD8^+$ thymocytes. In

the absence of TCRα, the number of $CD4^+CD8^+$ thymocytes is normal (*10*, *11*), whereas decreased numbers of $CD4^+CD8^+$ thymocytes were reported for $TCR\beta^{-/-}$ mice (*10*), which suggests that efficient entry of $CD4^-CD8^-$ thymocytes into the CD4-CD8 developmental pathway or, alternatively, expansion of the pool of $CD4^+CD8^+$ thymocytes may require expression of both β and ζ subunits.

We also did not expect to find SP T cells in the thymus and periphery of $\zeta^{-/-}$ mice. These cells were not γδ T cells: they did not express surface TCRδ (*7*). The generation of SP T cells is thought to require positively selecting TCR signals that are induced in $CD4^+CD8^+$ thymocytes that are TCR^{hi} and $CD5^{hi}$. The $CD4^+CD8^+$ thymocytes in $\zeta^{-/-}$ mice are $CD5^{lo}$ and express only barely detectable quantities of surface TCR; it was therefore expected that T cell development would be arrested at the $CD4^+CD8^+$ stage of differentiation. We do not yet know whether the unusual peripheral SP T cells detected are derived from the SP cells in the thymus or whether they are generated extrathymically by a cryptic developmental pathway. However, their normal CD4/CD8 ratio is most consistent with their being of thymic origin.

In conclusion, ζ performs a previously unappreciated role in quantitatively promoting the generation or expansion of $CD4^+CD8^+$ thymocytes and is critical for the efficient generation of SP T cells. Surprisingly, some SP T cells can be generated in the thymus and appear in the periphery despite low expression of surface TCR complexes that are devoid of ζ (or η). Understanding the mechanism by which such SP T cells are generated may provide additional insight into the mechanism of positive selection in the thymus.

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Gene Dose of Apolipoprotein E Type 4 Allele and the Risk of Alzheimer's Disease in Late Onset Families

E. H. Corder, A. M. Saunders, W. J. Strittmatter, D. E. Schmechel, P. C. Gaskell, G. W. Small, A. D. Roses, J. L. Haines, M. A. Pericak-Vance*

The apolipoprotein E type 4 allele (*APOE*-ε4) is genetically associated with the common late onset familial and sporadic forms of Alzheimer's disease (AD). Risk for AD increased from 20% to 90% and mean age at onset decreased from 84 to 68 years with increasing number of *APOE*-ε4 alleles in 42 families with late onset AD. Thus *APOE*-ε4 gene dose is a major risk factor for late onset AD and, in these families, homozygosity for *APOE*-ε4 was virtually sufficient to cause AD by age 80.

Alzheimer's disease (AD) is a devastating neurologic disorder that affects millions of individuals of all races and ethnic backgrounds. Onset before age 60 is infrequent and caused by either a mutation in the amyloid precursor protein (APP) gene located on chromosome 21 or, more commonly, by an unidentified gene on chromosome 14 (*1–4*). Previous evidence of the involvement of chromosome 19 in late onset AD (*5*) has been confirmed by the finding of an association between AD and the apolipoprotein E locus (*APOE*) on chromosome 19 (*6–8*). *APOE* has three alleles: *APOE*-ε2, *APOE*-ε3, and *APOE*-ε4. A total of 80% of familial and 64% of sporadic AD late onset cases have at least one *APOE*-ε4 compared to 31% of control subjects. This finding implicates *APOE*-ε4 as an important factor in the etiology of more than half of all AD. Here we examine the effect of *APOE*-ε4 gene dose and show that it is correlated with increased risk and earlier onset.

Four of the 46 families tested to date are early onset families; of these, two have chromosome 21 APP mutations and two have the disease state linked to chromo-