ri_map.html). In this regard, the gene order in the previous report (*4*) is corrected to *mto1-hy2-abi3*. The CGS gene was mapped about 4 cM north of *nga172* (*22*).
14. We amplified a 6651–base pair region covering the CGS gene from the *mto1-1* mutant by polymerase chain reaction (PCR) and compared the nucleotide sequence of the amplified DNA with the wild-type sequence (GenBank database accession no. AB010888). The primers were designed after the wild-type sequence. Sequencing was carried out with an ABI PRISM dye terminator cycle sequencing kit and model 377 DNA sequencer (Perkin-Elmer). Additional alleles of *mto1* mutants were isolated as described in (*4*), and *mto1-2* through *mto1-5*, which are independent of each other, were used for further study after they were back-crossed three times to wild type. Because the *mto1* mutation is semidominant over wild type (*4*), complementation tests were not applicable. Mapping with a SSLP-type marker in the 5′-upstream region of CGS (*22*) indicated that they all mapped within a few centimorgans from *mto1-1*. These mutants were sequenced for the exon 1 region.
15. The CGS gene has 11 exons encoding 563 amino acids (*23*).
16. The level of CGS mRNA in *mto1-4* mutant plants was lower than in other *mto1* mutants (*10*), which suggests that the *mto1-4* mutation is leaky. The fact that the reporter activity of the *mto1-4* mutant construct in the presence of Met was also lower than that of the *mto1-1* mutant construct supports the idea that the reporter activity reflects the response to Met at the mRNA level.
17. The first four amino acids were included to provide the same context for the translational start site as the other constructs.
18. The MTO1 region is not necessary for enzyme activity (*23*).
19. Although CGS is transported to chloroplasts (*24*), synthesis of Met and most of its direct metabolites occurs in the cytosol (*5, 24*).
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27. Plants were cultured as described in S. Naito *et al.* [*Plant Physiol.* **104**, 497 (1994)]. Application of Met to plants was as described in M. Y. Hirai *et al.* [*Plant Cell Physiol.* **35**, 927 (1994)] except that the treatment was started 12 days after sowing.
28. The (full-length) CGS cDNA [GenBank accession no. X94756; L. Le Guen, M. Thomas, M. Kreis, *Mol. Gen. Genet.* **245**, 390 (1994)] was a gift from M. Thomas. The 5′ probe (nucleotide position −20 to 183 relative to the translation start site) and the 3′ probe (1693 to 1870) of CGS were prepared by PCR with primers 5′-CATTGAGAAACGAAACAACA-3′ and 5′-GATCCTGGAGGATAATCCAC-3′, and 5′-AAATGACACATCACACAAAAAC-3′ and 5′-GAACTAAACAGAATTTATTATATAGCACAAC-3′, respectively. The ubiquitin probe was the *UBQ5* gene of *Arabidopsis* [E. E. Rogers and F. M. Ausubel, *Plant Cell* **9**, 305 (1997)] (*21*). ^{32}P-labeled probes were prepared with a multiprime DNA labeling system (Amersham).
29. We determined CGS activity by phosphate release as described in (*25*) except that Mops replaced Tricine in the extraction buffer and the assay mixture contained 2 mM cysteine and 6 mM *O*-phosphohomoserine.
30. We prepared protoplasts from liquid callus cultures (*8*) as described in (*21*). Transfection of protoplasts by electroporation was carried out as described in (*21*) except that capacitance and voltage were 100 μF and 475 V cm^{-1}, respectively. Reporter activities were determined as described [Y. Sakata *et al., Biosci. Biotech. Biochem.* **58**, 2104 (1994)].
31. We followed standard procedures for DNA manipulations (*26*). Wild-type and *mto1* mutant exon 1 were amplified by PCR with respective genomic DNA and primers Ex1P1 (5′-CGAATCTAGAATGGCCGTCTC-3′) and Ex1P2 (5′-ATCTAGGATCCACCGGCATG-3′), which carried Xba I and Bam HI recognition sequences, respectively (mismatched bases are underlined). We constructed exon 1 with silent mutations as described in I. Mikaelian and A. Sergeant [*Nucleic Acids Res.* **20**, 376 (1992)]. The mutagenic primers used to construct G84G-1, G84G-2, S81S-1, S81S-2, S81S-3, and R77R-1 were 5′-CTGTAGCAACATCGGAGTTG-3′, 5′-CTGTAGCAACATCGGCGTTG-3′, 5′-AGAAACTGTCAAACATCGG-3′, 5′-AGAAACTGTCTAACATCGG-3′, 5′-AGAAACTGTCGAACATCGG-3′, and 5′-GCATTAAAGCCGGGAGAAAC-3′, respectively. The internal primer was 5′-GACGTAGTGAGGCTCCCATC-3′, and the external primers were Ex1P1 and Ex1P2. Synthetic oligonucleotides (5′-CTAGAATGGCCGTCTCAG-3′ and 5′-GATCCTGAGACGGCCATT-3′) were used to construct Δ5-183, which carries the first four amino acids of exon 1. To obtain plasmids carrying the GUS reporter gene [R. A. Jefferson, T. A. Kavanagh, M. W. Bevan, *EMBO J.* **6**, 3901 (1987)], we ligated the exon 1 DNA with Xba I– and Bam HI–digested pTF33 [T. Fujiwara, S. Naito, M. Chino, T. Nagata, *Plant Cell Rep.* **9**, 602 (1991)]. The control plasmid 221-LUC$^+$ carries the firefly luciferase (LUC) gene under control of the CaMV 35*S* RNA promoter (K. Hiratsuka, personal communication). To obtain plasmids carrying the LUC reporter, we excised the LUC coding region from pT3/T7-LUC (Clontech, Palo Alto, CA) by Bsm I and Sac I digestion and ligated it with Xba I– and Sac I–digested pTF33 along with exon 1 DNA. The exon 1 sequences were verified by sequencing.
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Defective Thymocyte Maturation in p44 MAP Kinase (Erk 1) Knockout Mice

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The p42 and p44 mitogen-activated protein kinases (MAPKs), also called Erk2 and Erk1, respectively, have been implicated in proliferation as well as in differentiation programs. The specific role of the p44 MAPK isoform in the whole animal was evaluated by generation of p44 MAPK-deficient mice by homologous recombination in embryonic stem cells. The p44 MAPK$^{-/-}$ mice were viable, fertile, and of normal size. Thus, p44 MAPK is apparently dispensable and p42 MAPK (Erk2) may compensate for its loss. However, in p44 MAPK$^{-/-}$ mice, thymocyte maturation beyond the CD4$^+$CD8$^+$ stage was reduced by half, with a similar diminution in the thymocyte subpopulation expressing high levels of T cell receptor (CD3^{high}). In p44 MAPK$^{-/-}$ thymocytes, proliferation in response to activation with a monoclonal antibody to the T cell receptor in the presence of phorbol myristate acetate was severely reduced even though activation of p42 MAPK was more sustained in these cells. The p44 MAPK apparently has a specific role in thymocyte development.

Erk1 or p44 MAP kinase was the first mammalian MAPK to be characterized and cloned a decade ago (*1*). This MAPK together with its isoform p42 MAPK (Erk2) are commonly expressed in most, if not all, tissues and are activated through the small guanosine triphosphatase Ras and sequential activation of the protein kinases Raf and MEK upon stimulation of cells with a broad range of extracellular signals (*2*). This Ras-MAPK module appears to be as central to cellular signaling as the Krebs cycle and glycolysis are to energy metabolism. Indeed, the Ras-dependent MAPK signaling cascade functions in control of cell fate, differentiation, proliferation, and cell survival in various invertebrates and

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mammalian cells (*3*). Although both the p42 and p44 MAPK isoforms translocate to the nucleus upon stimulation, phosphorylate common substrates, and share an apparently identical pattern of spatio-temporal activation, their 17% divergence in amino acid sequence might signify functional specificity. To evaluate their potential specific roles in mouse development, we generated p44 MAPK-deficient mice through homologous recombination in embryonic stem cells (*4*). Targeted disruption of the p44 MAPK gene (*5*) was confirmed by restriction fragment length polymorphism analysis of genomic DNA (Fig. 1, A and B) and determination of mRNA and protein expression in tissue extracts from progeny animals. The p44 MAPK-deficient mice were viable, fertile, and of normal size. In all parental tissues examined, both p42 and p44 MAPK were expressed. Specific ablation of p44 MAPK did not influence the expression of the remaining p42 MAPK isoform in embryo fibroblasts, sciatic nerve, thymus, or spleen (Fig. 1C).

The pivotal role of nuclear translocation of p42 and p44 MAPKs (*6*) and persistent activation during the G_1 phase of the cell cycle is critical for control of fibroblast proliferation (*7*). We therefore analyzed the temporal activation of p42 and p44 MAPKs and reinitiation of DNA synthesis in wild-type and p44 MAPK-deficient mouse embryo fibroblasts (MEFs). In serum-starved MEFs, reinitiation of DNA synthesis was unimpaired by the ablation of p44 MAPK gene (*8*). The dose-response to serum or individual growth factors (α-thrombin or platelet-derived growth factor B), the magnitude of stimulation of DNA synthesis, and the time of reentry into S phase were unaffected in p44 $MAPK^{-/-}$ MEFs (*8*). Although the amount of p42 MAPK was unchanged in p44 $MAPK^{-/-}$ MEFs, the time course of p42 MAPK activation, in particular at 2, 4, and 8 hours, revealed a more sustained activation than in wild-type MEFs (Fig. 2A). Quantitation of total MAPK activity showed identical long-term (>200 min) activation in wild-type and MAPK-deficient MEFs (Fig. 2B). This result indicates that (i) the intensity of the MAPK signal may matter more than the particular MAPK isoform which initiates the long-term signaling and that, in this instance, (ii) p42 MAPK can fully substitute for the lack of p44 MAPK. No difference in growth rate of MEFs derived from wild-type or p44 $MAPK^{-/-}$ mice was detected at early or late passages.

Experiments with transgenic mice in which dominant negative mutants (DN) of members of the Ras → Raf → MEK kinase cascade (*9*) and with thymocytes that express a gain-of-function p42 MAPK mutant (*10*) indicated that p42 and p44 MAPKs may contribute critically to thymocyte differentiation. We therefore examined the percentage of cells in each thymocyte subset by measuring the surface expression of CD4 and CD8 antigens and αβ–T cell receptor (TCR) in p44 MAPK-deficient mice and their normal littermates. Flow cytometric analysis of thymocytes from 6-week-old p44 $MAPK^{-/-}$ knockout mice revealed a reduction in the number of mature $CD4^+CD8^-$ and $CD4^-CD8^+$ single-positive (SP) thymocytes from 11.8 to 6.3% and 1.6 to 0.8%, respectively, together with a reciprocal increase in cells that expressed both CD4 and CD8 (Fig. 3A).

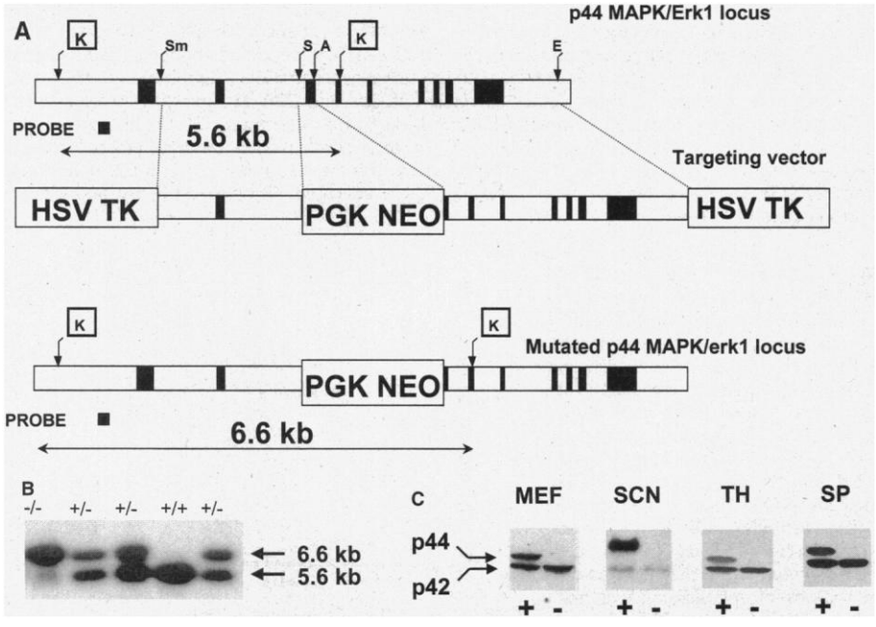

Fig. 1. Generation of p44 MAPK (Erk1)-deficient mice. (**A**) Schematic representation of the genomic p44 MAPK locus, the targeting vector, and the mutated p44 MAPK locus. An internal region between a Sac I site located in the second intron and the Afl II site located in the third exon was replaced by the PGK NEO (Neomycin resistance gene). This exon deletion removed the essential and conserved kinase subdomain V and VI. The knockout construct was electroporated into embryonic stem cells, and three targeted clones gave germ line transmission of the disrupted allele. Heterozygous mice (−/+) were intercrossed to generate homozygous wild-type and mutant mice. The probe used for Southern (DNA) analysis and the restriction enzyme sites are shown (K, Kpn I; S, Sac I; A, Afl II; E, Eco RI; Sm, Sma I). (**B**) Southern blot analysis of genomic DNA from five littermates issued from p44 MAPK heterozygotes crosses. Wild-type (5.6 kb) and disrupted (6.6 kb) Kpn I fragments are visualized together with genotypes. (**C**) MAPK immunoblot analysis confirmed the absence of p44 MAPK protein. Extracts of embryo fibroblasts (MEF), sciatic nerve (SCN), thymus (TH), and spleen (SP) from wild-type (+) or knockout (−) animals were examined by immunoblot analysis with a polyclonal antibody directed against both p42 and p44 MAPKs (*14*).

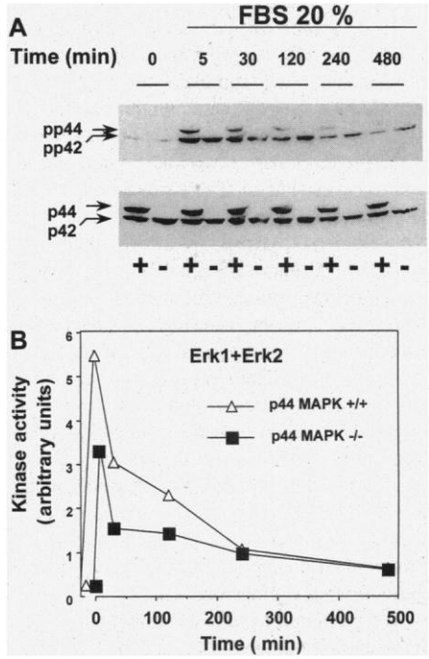

Fig. 2. Time course of serum-stimulated p42 and p44 MAPK activity in wild-type and p44 MAPK-deficient MEFs. MEFs from early passages (3 to 5) were serum-deprived for 24 hours and subsequently stimulated with 20% FBS for the times indicated. (**A**) Total cell extracts from wild-type (+) or knockout (−) animals were separated by SDS-PAGE (7.5% gels) and examined by immunoblot using either a polyclonal antibody directed against (top) phosphorylated MAPKs (Promega) or (bottom) the polyclonal antibody that recognizes the COOH-terminal domain of both MAPK isoforms (*14*). The Promega antibody recognized only dually phosphorylated p42 and p44 MAPKs (not less phosphorlyated forms) and bound phosphorylated p42 and p44 MAPK with equal affinity; the same results were obtained with the monoclonal antibody directed against anti–phospho-Erk (New England Biolabs). (**B**) Quantification of the active forms of p42 and p44 MAPKs in wild-type and p44 MAPK-deficient MEFs.

There was a decrease in the number of $CD3^{high}$ thymocytes in p44 $MAPK^{-/-}$ knockout mice (from 10.3 to 4.3%) and in the number of lymphocytes that express CD69 (Fig. 3B). Despite this reduction in the number of mature thymocytes, the thymuses in p44 $MAPK^{-/-}$ mice displayed normal cellularity. Similarly, overexpression of a DN MEK1 mutant (Lys^{97} → Ala^{97}) decreased the number of single-positive mature thymocytes in young adult transgenic mice (*9*). Moreover, flow cytometric analysis revealed that the proportion of $CD3^{high}$ CD4 SP and $CD3^{high}$ CD8 SP thymocytes is reduced in DN-Raf transgenic mice (*11*). Positive and negative selection in the thymus is dependent on the Ras-MAPK module and on pathways mediated by the related p38 and JNK protein kinases (*12*). Because activation-induced apoptosis in vitro of thymocytes from p44 $MAPK^{-/-}$ mice was unimpaired, it is likely that positive selection is affected.

To determine whether lack of expression of p44 MAPK affected TCR-mediated signaling in thymocytes, we stimulated thymocytes by cross-linking the TCR with immobilized monoclonal anti-CD3ε in the presence or the absence of the protein kinase C activator, phorbol myristate acetate (PMA). There was an 80 to 90% decrease in the proliferation capacity of thymocytes from p44 $MAPK^{-/-}$ mice treated with anti-CD3 and anti-CD3 plus PMA, compared to thymocytes from control animals (Fig. 4A). When the number of single-positive cells present in each culture was normalized, a decrease in the proliferation capacity (60 to 70%) of p44 MAPK-deficient thymocytes was still observed. This reduced capacity of p44 MAPK-deficient thymocytes to proliferate in response to an anti-CD3ε in either the presence or the absence of PMA cannot be accounted for by a decrease in interleukin 2 production nor by a diminution in CD25 (interleukin-2 receptor) expression upon stimulation.

We measured MAPK activation in vitro in thymocytes from wild-type and p44 MAPK-deficient mice. Thymocytes were stimulated for various times in the presence of a combination of immobilized anti-CD3 plus PMA, and the amounts of active phosphorylated p42 and p44 MAPKs were assessed (Fig. 4B). The p44 MAPK accounted for ~50% of the total MAPK activity in thymocytes treated with anti-CD3 plus PMA. Maximal phosphorylation of p42 MAPK in thymocytes isolated from control or p44 $MAPK^{-/-}$ mice was detected after 5 min of stimulation. The amount of phosphorylated p42 MAPK remained unchanged during the first 4 hours and then decreased after 6 hours. Lack of p44 MAPK in knockout mice did not appear to modify the phosphorylation status of p42 MAPK except that a more sustained activation was observed after 6 hours of stimulation. This compensation in the long-term activation of p42 MAPK is similar to that observed in MEFs (Fig. 2A).

Our results demonstrate that p44 MAPK is critically required for (i) the differentiation of double- to single-positive thymocytes and (ii) for thymocyte proliferation. The inhibition of thymocyte positive selection and of in vitro proliferation observed in p44 $MAPK^{-/-}$ mice is in agreement with the results obtained in mice expressing dominant negative Ras (*13*), Raf (*12*), or MEK1 (*9*), but is inconsistent

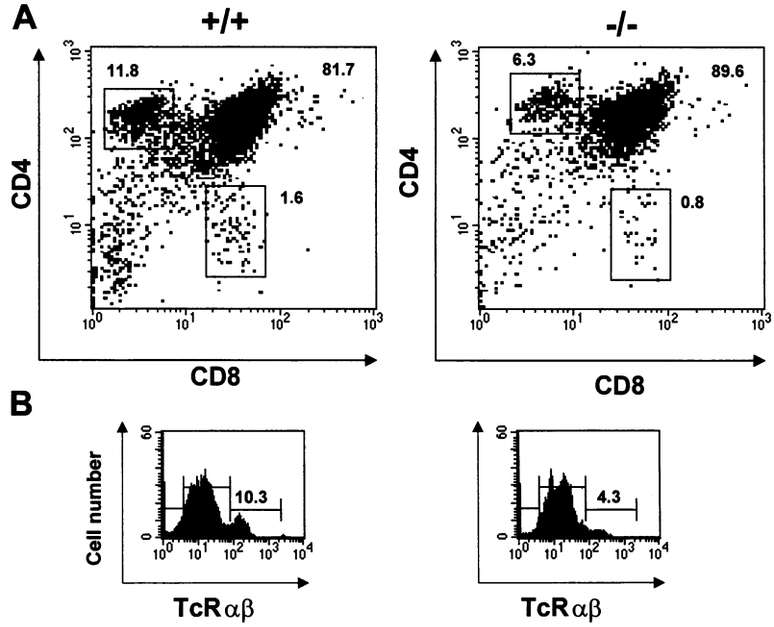

Fig. 3. Inhibition of thymocyte development in p44 MAPK-deficient mice. (**A**) Thymocytes from 6-week-old mice were stained with anti-CD4–PE, anti-CD8–FITC monoclonal antibodies and analyzed by flow cytometry. Thymocytes were stained with saturating concentrations of antibodies at 4°C for 30 min. Cells were then examined for surface expression of CD4 and CD8 (Pharmingen). Analyses were performed using a FACscan flow cytometer (Becton-Dickinson) as described (*15*). On histograms, the percentage of cells in each quadrant is indicated. The total number of thymocytes present in each animal was similar. (**B**) Thymocytes stained with the Cy-Chrome–anti-mouse αβTCR-Cy monoclonal antibody (*15*). The percentage of cells expressing TCR is indicated. The staining in (A) and (B) are representative of at least six experiments.

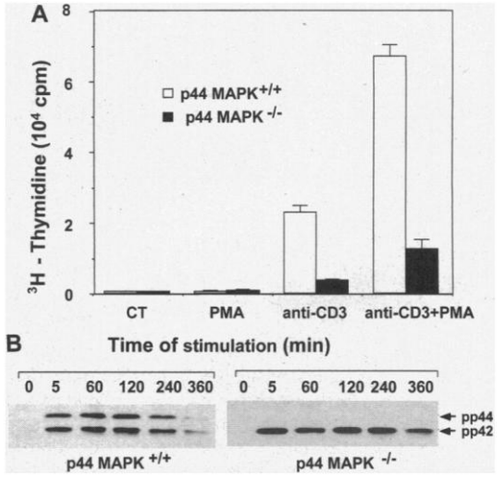

Fig. 4. Defect in proliferation of p44 $MAPK^{-/-}$ thymocytes. (**A**) Reduced proliferative responses in p44 MAPK-deficient thymocytes. Total thymocytes from control or p44 MAPK-deficient mice were isolated and stimulated with cross-linked monoclonal antibody to CD3ε alone or in combination with PMA in 96-well microplaques. Thymocytes (2×10^5) were plated in 200 μl of medium [RPMI 1640 containing FBS (10%), penicillin (100 U ml^{-1}), and streptomycin (100 μg ml^{-1})]. Cells were stimulated with the indicated concentrations of mitogens: 10 ng ml^{-1} PMA or rat anti-mouse CD3 (plated at 10 μg ml^{-1}), or both, for 72 hours. We added [^{3}H]thymidine (1 μCi) for the last 18 hours, then measured its incorporation. Results are the mean ± SEM of three different experiments made in quadruplicate. In each case, an 80 to 90% inhibition of thymocyte proliferation was observed. Normalization to the numbers of single-positive cells present in the culture, as determined by flow cytometry, indicates that there was at least a 60% decrease in thymocyte proliferation capacity in p44 $MAPK^{-/-}$-deficient mice compared to that of normal littermate controls. (**B**) Activation of p42 and p44 MAPKs in wild-type and p44 MAPK-deficient thymocytes. Thymocytes were stimulated with anti-CD3 plus PMA for the time indicated. Proteins from cell lysates were separated by SDS-PAGE and transferred to immobilon membranes for protein immunoblotting with antibodies to phosphorylated MAPK (New England Biolabs) (*14*). Immunoreactivity was detected by enhanced chemiluminescence.

with the observation that thymocyte proliferation is not affected in mice expressing dominant negative MEK1 (*9*). Ablation of p44 MAPK appeared not to affect the long-term total MAPK activity in MEFs or thymocytes. This result suggests that both isoforms might compete with each other for the upstream MEK activator. These findings indicate that there may be a physiological distinction between p42 and p44 MAPK isoforms.

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8. MEFs issued from wild-type and p44 MAPK-deficient mice were analyzed for reinitiation of DNA synthesis following serum starvation for 24 hours, and for doubling time at low (2–3) and high passages (6–8). Reinitiation of DNA synthesis by 10% fetal bovine serum (FBS) (3.5- to 4.5-fold stimulation) and doubling time (18 to 24 hours) were indistinguishable between the two types of fibroblasts (G. Pagès and J. Pouysségur, unpublished data).
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Persistence of Memory CD8 T Cells in MHC Class I–Deficient Mice

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An understanding of how T cell memory is maintained is crucial for the rational design of vaccines. Memory T cells were shown to persist indefinitely in major histocompatibility complex (MHC) class I–deficient mice and retained the ability to make rapid cytokine responses upon reencounter with antigen. In addition, memory CD8 T cells, unlike naïve cells, divided without MHC–T cell receptor interactions. This "homeostatic" proliferation is likely to be important in maintaining memory T cell numbers in the periphery. Thus, after naïve CD8 T cells differentiate into memory cells, they evolve an MHC class I–independent "life-style" and do not require further stimulation with specific or cross-reactive antigen for their maintenance.

Immunological memory is the ability of the immune system to respond with greater vigor upon reencounter with the same pathogen. Many currently used vaccines and most natural infections induce long-term T cell memory as assessed by rapid anamnestic responses (*1–3*). These accelerated recall responses are due to increased numbers of antigen-specific T cells and to qualitative changes in memory T cells that allow them to develop into effector cells more rapidly than naïve T cells (*2–4*). There has been considerable interest in determining whether continued presentation of specific or cross-reactive antigens by MHC molecules is necessary for maintenance of memory T cells and for retaining their "response ready" mode (*1–6*). We have addressed this issue by analyzing the survival, proliferation, and functional characteristics of memory CD8 T cells under conditions of MHC class I deficiency.

Adult mice resolve an acute lymphocytic choriomeningitis virus (LCMV) infection within 2 weeks and then exhibit long-term CD8 T cell memory (*2*, *3*). To determine the requirement of MHC class I molecules in maintaining CD8 memory, we obtained fluorescence-activated cell sorter (FACS)–purified CD8 T cells (>99% pure) from Thy1.1$^+$ LCMV immune mice and transferred the cells into either MHC class I–positive ($\beta_2M^{+/+}$) or MHC class I–deficient ($\beta_2M^{-/-}$) congeneic Thy1.2$^+$ mice. Before adoptive transfer, the FACS-purified CD8 T cells were tested for the presence of virus and found to be free of any detectable viral material (*7*). The transferred CD8 T cells initially expanded in the irradiated recipient mice, reaching a plateau at around day 20, and were then maintained indefinitely in both $\beta_2M^{+/+}$ and $\beta_2M^{-/-}$ mice (Fig. 1A). Although the initial expansion of CD8 T cells was less in $\beta_2M^{-/-}$ mice, by day 22 the numbers were similar in both groups of mice. The LCMV-specific memory CD8 T cells were CD44^{hi}, Ly6C^{hi}, CD25^{lo}, and CD69^{lo} before transfer, and this phenotype was retained in MHC class I–deficient mice. The total Thy1.1$^+$ CD8 T cell population initially consisted of equal numbers of CD44^{lo} (naïve) and CD44^{hi} (memory) cells. However, by day 50 nearly all of the donor Thy1.1$^+$ CD8 T cells were CD44^{hi}, suggesting preferential survival of memory cells in $\beta_2M^{-/-}$ mice (*8*).

Peripheral T cells undergo homeostatic proliferation under lymphopenic conditions to reconstitute the empty immune system (*5*). When lymphoid homeostasis is reached, naïve cells cease to proliferate, whereas memory cells continue to divide, although at a much slower rate, in order to maintain the pool of memory cells (*9*). We sought to determine whether memory CD8 cells persisting in MHC class I–deficient mice at relatively constant numbers after reaching lymphoid homeostasis (between day 22 and day 310) (Fig. 1A) were undergoing proliferation or surviving without any cell division. The turnover rate of LCMV-specific memory CD8 T cells (identified by staining with MHC class I tetramers) was similar in $\beta_2M^{+/+}$ and $\beta_2M^{-/-}$ mice (Fig. 1B). Likewise, the total population of donor Thy1.1$^+$ CD8 T cells, which consist predominantly of non-LCMV–specific CD44^{hi} CD8 T cells, proliferated equally well in +/+ and $\beta_2M^{-/-}$ mice. Thus, not only do memory CD8 T cells persist in $\beta_2M^{-/-}$ mice, but their homeostatic proliferation is not compromised in a heavily MHC class I–deficient environment.

In the experiments described above the donor CD8 T cells were derived from $\beta_2M^{+/+}$ mice. It is unlikely that survival of memory CD8 T cells in $\beta_2M^{-/-}$ mice was due to the transferred CD8 T cells seeing MHC class I on each other because the transferred cells comprised only about 0.5 to 1% of the total splenocytes [$\sim 5 \times 10^5$ Thy1.1$^+$ CD8 T cells in (5 to 10) $\times 10^7$ $\beta_2M^{-/-}$ spleen cells]. Nevertheless, to test this possibility, we performed adoptive

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References and Notes

[1] **Insulin-Stimulated Microtubule-Associated Protein Kinase is Phosphorylated on Tyrosine and Threonine in vivo**

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