ERK1-Deficient Mice Show Normal T Cell Effector Function and Are Highly Susceptible to Experimental Autoimmune Encephalomyelitis[1]

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T cell activation engages multiple intracellular signaling cascades, including the ERK1/2 (p44/p42) pathway. It has been suggested that ERKs integrate TCR signal strength, and are important for thymocyte development and positive selection. However, the requirement of ERKs for the effector functions of peripheral mature T cells and, specifically, for T cell-mediated autoimmunity has not been established. Moreover, the specific requirements for ERK1 vs ERK2 in T cells have not been resolved. Therefore, we investigated the role of ERK1 in T cell immunity to foreign and self Ags and in the induction of experimental autoimmune encephalomyelitis. The results show that in ERK1-deficient ($ERK1^{-/-}$) mice, the priming, proliferation, and cytokine secretion of T cells to the self Ag myelin oligodendrocyte glycoprotein peptide 35–55 and to the prototypic foreign Ag OVA are not impaired as compared with wild-type mice. Furthermore, $ERK1^{-/-}$ mice are highly susceptible to experimental autoimmune encephalomyelitis induced with myelin oligodendrocyte glycoprotein peptide 35–55. Finally, thymocyte development and mitogen-induced proliferation were not impaired in $ERK1^{-/-}$ mice on the inbred 129 Sv and C57BL/6 backgrounds. Collectively, the data show that ERK1 is not critical for the function of peripheral T cells in the response to self and foreign Ags and in T cell-mediated autoimmunity, and suggest that its loss can be compensated by ERK2. *The Journal of Immunology,* **2005, 175: 2374–2380.**

Mitogen-activated protein kinases are composed in mammalians of five evolutionary conserved families of serine-threonine protein kinases that are activated by a range of different stimuli, including growth factors, cytokines, cellular stress, and cell adherence (1–3). Of the mammalian MAPKs, ERK1 and ERK2 (p44/p42) are studied the best. ERK1 and ERK2 are expressed in most tissues, including hemopoietic cells such as T, B, and dendritic cells.

ERK1 and ERK2 share >80% sequence homology, and are thought to be functionally redundant (4, 5). ERK1/2 are activated by extracellular stimuli that result in activation of the ras-raf-MEK1/2 signaling cascade, and, in T cells, they are essential elements of the TCR-mediated signaling pathway. Activated MEK1/2 phosphorylate ERK1/2 at two positions of the activation loop, which increases ERK activity ~1000-fold (1, 2). Targets of activated ERK1/2 are numerous and include transcription factors and several cytoplasmic proteins, including phospholipase A_2 (1, 2).

Recently, ERK1 was implicated in the thymic development of T cells, raising the intriguing possibility that ERKs could regulate thymic selection and peripheral effector function of pathogenic T cells in autoimmunity (6, 7). However, the role of ERKs in autoimmunity and Ag-specific T cell immunity has not been investigated stringently. Moreover, little is known regarding the specific requirement for ERK1 or ERK2 in T cell function, and how much they overlap functionally. In this respect, some evidence suggests that ERK1 and ERK2 may be independently regulated under some circumstances. For example, ERK2 null mutations result in embryonal lethality, whereas $ERK1^{-/-}$ mice are viable (8, 9).

In this study, we have applied a genetic approach to examine the requirement for ERK1 in the immune response of peripheral mature T cells to the prototypic foreign Ag OVA and the self Ag myelin oligodendrocyte glycoprotein (MOG)[3] 35–55 in ERK1-deficient mice. We show that T cell proliferation, cytokine production, and clonal sizes are unaltered in $ERK1^{-/-}$ mice on the inbred 129 Sv and C57BL/6 backgrounds and are comparable to that of wild-type (WT) mice. Moreover, we found that $ERK1^{-/-}$ mice are highly susceptible to experimental autoimmune encephalomyelitis (EAE).

Taken together, the data show that ERK1 is not critical for the function of peripheral T cells, and suggest that ERK2 may compensate for the lack of ERK1.

Materials and Methods

Targeted disruption of the erk1 *gene*

The mouse genomic 129 Sv library (Stratagene) (3.5×10^6 original plaques) was screened with a ERK1 rat cDNA probe. Multiple phage plaques were isolated from the library after the first screening. Second and third rounds of screening were performed with a 1.3-kb mouse ERK1 intron probe. Five independent λ phage clones were isolated and mapped by restriction enzyme digestion and found to contain the entire mouse *erk1* gene. To mutate ERK1 in mouse embryonic stem (ES) cells, we generated a vector that deleted exons 1–6 of the ERK1 protein coding sequence,

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[3] Abbreviations used in this paper: MOG, myelin oligodendrocyte glycoprotein; EAE, experimental autoimmune encephalomyelitis; ES, embryonic stem; PT, pertussis toxin; SI, stimulation index; SP, single positive; WT, wild type.

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including protein kinase domains I-X, replacing them with a neomycin-resistance expression cassette flanked by loxP sites (pPGKneolox). The 5′ and 3′ arms of the targeting vector were 4 and 4.5 kb in length, respectively, and the neo expression cassette was placed in the opposite transcriptional orientation compared with the *erk1* gene, and a diphtheria toxin expression cassette was inserted for negative selection. The construct was electroporated into the 129 Sv-derived ES cells. A total of 98 G418-resistant colonies was selected and screened using Southern blot hybridization with an internal probe. Two targeted clones were identified, indicating a targeting frequency of 1 in 49 G418-resistant clones. ES cells from each clone carrying the targeted ERK1 mutation were injected into CD1 blastocysts and implanted into pseudopregnant CD1 females. Chimeras of both sexes were bred to CD1, and germline transmission was achieved for one clone and homozygous ERK1 mutants were obtained. Subsequently, the mutation was transferred to 129 Sv and C57BL/6 backgrounds by 10 backcrosses. ERK1 mutant mice were maintained in a pathogen-free environment. All experiments were done according to guidelines of the Institutional Care and Use Committee of Case Western Reserve University.

Animals, Ags, and treatments

Female ERK1$^{-/-}$ and WT (ERK$^{+/+}$) control mice on the 129 Sv or C57BL/6 background were injected at 6–10 wk of age with the Ags in CFA. Pertussis toxin (PT, 200 ng; List Biological Laboratories) was injected i.p. in 500 μl of saline at 0 and 24 h after injection, as indicated in the text. MOG peptide aa 35–55 (MOG35–55, MEVGWYRSPFSRVVH LYRNGK) was synthesized by Princeton Biomolecules. OVA was purchased from Sigma-Aldrich. IFA was purchased from Invitrogen Life Technologies, and CFA was made by mixing *Mycobacterium tuberculosis* H 37 Ra (Difco Laboratories) at 5 mg/ml into IFA. Ags were mixed with the adjuvant to yield a 2 mg/ml emulsion, of which 100 μl was injected s.c.

Proliferation assay

Proliferation assays were performed, as previously described (10, 11). Briefly, single cell suspensions of popliteal lymph node cells were prepared, and 5×10^5 cells were plated per well in flat-bottom 96-well microtiter plates in serum-free HL-1 medium (BioWhittaker) supplemented with 1 mM L-glutamine. Ags were added at 7 μM. During the last 18 h of a 4-day culture, [^{3}H]thymidine was added (1 μCi/well) and incorporation of label was measured by liquid scintillation counting. Stimulation index (SI) was calculated by dividing the cpm cytokine spots detected in wells pulsed with relevant Ag by the number of cpm in wells without Ag (medium only).

Cytokine measurements by ELISPOT and computer-assisted ELISPOT image analysis

Cytokine ELISPOT assays were performed, as described (12). ELISPOT plates (Multiscreen IP; Millipore) were coated overnight with specific cytokine capture Ab (IFN-γ, AN-18, 2 μg/ml; IL-2, JES6-1A12, 4 μg/ml; IL-5, TRFK5, 4 μg/ml; all eBioscience; and IL-4, 11B11, 4 μg/ml; IL-17, TC11-18H10, 2 μg/ml; BD Pharmingen) diluted in 1× PBS. The plates were blocked with 1% BSA in PBS for 1 h at room temperature, then washed four times with PBS. Cells from draining lymph nodes were plated at 5×10^5 cells/well alone or with MOG35–55 peptide or OVA (7 μM) in serum-free HL-1 medium supplemented with 1% L-glutamine and cultured for 24 h. Subsequently, the cells were removed by washing four times with PBS and four times with PBS/Tween-20, and the respective biotinylated detection Ab (IFN-γ, R4-6A2, 2 μg/ml; IL-2, JES6-5H4, 2 μg/ml; IL-4, BVD6-24G2, 2 μg/ml; IL-5, TRFK4; all eBioscience; and IL-17, TC11-8H4, 0.125 μg/ml; BD Pharmingen) was added and incubated overnight. The plate-bound second Ab was then visualized by adding streptavidin-alkaline phosphatase (DakoCytomation) and NBT/5-bromo-4-chloro-3-indolyl phosphate substrate (Bio-Rad/Sigma-Aldrich). Image analysis of ELISPOT assays was performed on a Series 1 ImmunoSpot Image Analyzer (Cellular Technology), as described previously (11, 12). In brief, digitized images of individual wells of the ELISPOT plates were analyzed for cytokine spots based on the comparison of experimental wells (containing T cells and APC with Ag) and control wells (T cells and APC, no Ag). After separation of spots that touched or partially overlapped, nonspecific noise was gated out by applying spot size and circularity analysis as additional criteria. Spots that fell within the accepted criteria were highlighted and counted. The spot number in unimmunized or control mice (irrelevant Ag) was in the same range as the medium controls shown.

Evaluation of clinical disease in mice

Mice were monitored daily for 30$^+$ days after injection of neuroantigen, and the severity of disease was recorded according to the following scale (13): grade 0, no abnormality; grade 1, limp tail; grade 2, moderate hind limb weakness; grade 3, complete hind limb paralysis; grade 4, quadriplegia or premoribund state; grade 5, death. Statistical analysis was performed with the paired *t* test or the Mann-Whitney rank sum test using SigmaStat software.

Western blot analysis

For Western blot analysis, cells or tissues were rinsed in ice-cold PBS twice and lysed in cell lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 50 mM NaF, 2 mM Na_3 VO_4, 2 mM EGTA, 2 mM EDTA, and 0.25 mM PMSF) for 30 min. Samples were sonicated for 10 s and centrifuged for 20 min at 13,000 × *g* at 4°C. Samples were electrophoresed on a 7–12% SDS-polyacrylamide gel and electrophoretically blotted on polyvinylidene difluoride or nitrocellulose membrane. Membranes were blocked in TBS/Tween-20 with 3% BSA and incubated with primary Abs diluted in TBS/Tween-20/BSA overnight at 4°C. Primary Abs specific for the following Ags were used: phospho-ERK (1/1000; New England Biolabs) and pan-ERK (1/5000; UBI). All blots were incubated with secondary Abs conjugated to HRP (1/2000; Amersham) and developed using the ECL method (Pierce). Protein concentration was determined using bicinchoninic acid assay (Pierce).

Results

ERK1 is not critical for the effector function of peripheral T cells

To investigate the function of ERK1 in mature T cells and its role in T cell-mediated autoimmunity, we generated ERK1-deficient mice by homologous recombination in ES cells (Fig. 1*a*). We confirmed that the targeted mutation of the *erk1* locus resulted in a null allele by analysis of genomic DNA, and by Western blotting of proteins isolated from various tissues of WT and ERK1$^{-/-}$ animals (Fig. 1, *b* and *c*, respectively). As expected, ERK1 protein was not expressed in the knockout mice (Fig. 1*c*). Moreover, as

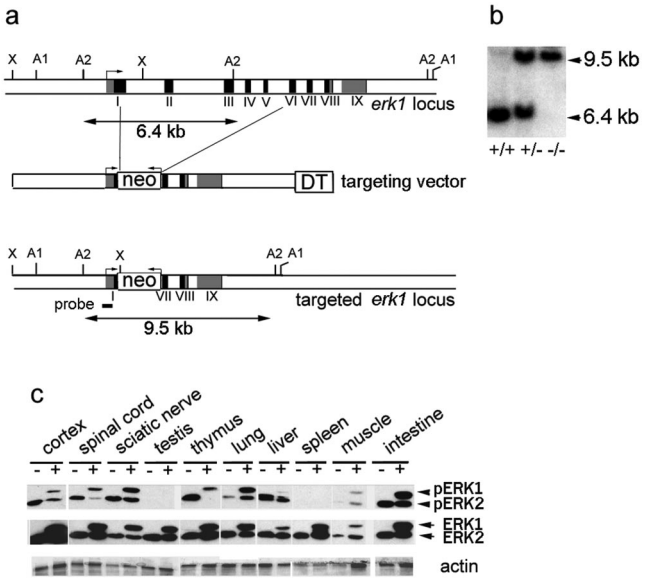

FIGURE 1. Targeted inactivation of the mouse *erk1* gene. *a*, Genomic organization of the mouse *erk1* gene. A 5.8-kb fragment containing exons 1–6 of *erk1*, with the exception of exon I sequence encoding the first 39 aa, was replaced with pPGKneolox cassette to generate targeting vector. Black horizontal lines represent position and size of restriction fragments used for genotypic analysis. A1, *Ase*1; A2, *Afl*II; X, *Xba*I; DT, diphtheria toxin expression cassette. *b*, Southern blot analysis of DNA obtained from WT (+/+), heterozygous (+/−), and ERK1 (−/−) mice. *c*, Western blots of indicated tissues from ERK1$^{-/-}$ or ERK1$^{+/+}$ mice probed with anti-pan-ERK Ab (*lower panel*) and with anti-phospho-ERK Ab, which detects the active form of the enzymes. Lanes containing samples from ERK1$^{-/-}$ mice are indicated as −, and ERK1$^{+/+}$ positive controls as +.

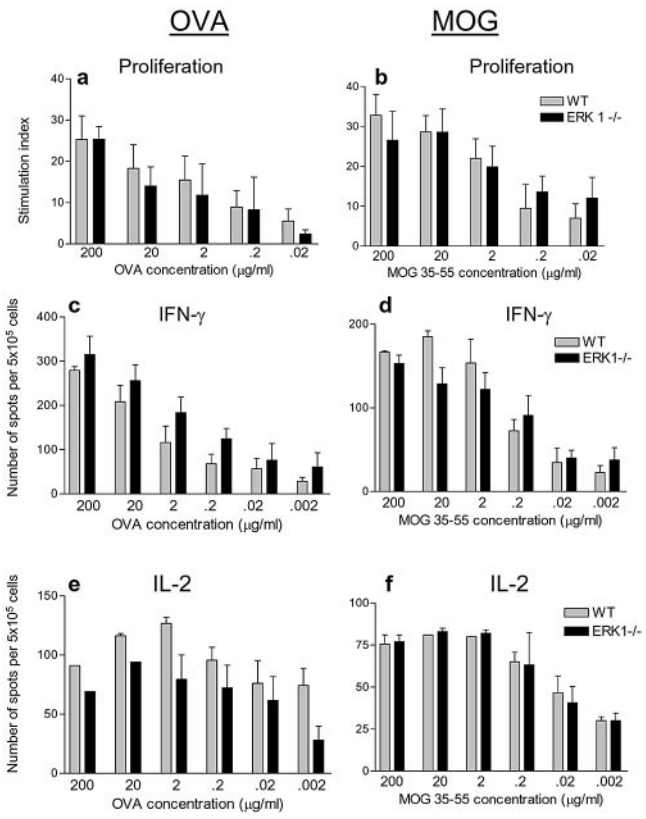

FIGURE 2. T cell effector function is maintained in ERK1$^{-/-}$ mice. The 129 Sv ERK1$^{-/-}$ or WT mice (6–8 wk old) were immunized with OVA (*left panels*) or MOG35–55 (*right panels*) in CFA, and single cell suspensions of lymph node cells were tested 10 days later for [^{3}H]thymidine incorporation or cytokine production by ELISPOT assay. *a* and *b*, Ag-induced proliferation of OVA or MOG35–55-specific T cells from ERK1$^{-/-}$ (■) or WT mice (▨). An SI of > 3 is considered positive. *c–f*, Frequencies and cytokine profiles of OVA or MOG35–55-specific ERK1$^{-/-}$ (■) or WT (▨) T cells. Shown are the numbers of cytokine spots per 5×10^5 cells. Shown are the results of groups of four mice, and the values shown represent means + SD of triplicate wells. Similar results were obtained in three independent experiments.

shown in Fig. 1*c*, the absence of the ERK1 protein had no effect on the expression levels of ERK2 in any tissue tested. However, the activity of ERK2 was increased in some tissues, including the thymus, as indicated by enhanced levels of phosphorylated ERK2 (Fig. 1*c*, *top panel*). Phenotypically, ERK1$^{-/-}$ mice were viable and fertile, and no histologic abnormalities were detected. Furthermore, the numbers and percentages of CD4$^+$ and CD8$^+$ T cells, B cells, and macrophages in the spleens of the ERK1$^{-/-}$ mice were similar to WT mice (see Fig. 7, *b* and *c*).

To investigate the requirement of ERK1 for the effector functions of peripheral mature T cells, we examined Ag-induced T cell proliferation and cytokine production in ERK1$^{-/-}$ and WT mice on the 129 Sv and C57BL/6 background upon immunization with the prototypic foreign protein Ag OVA and the self Ag MOG35–55.

As shown in Fig. 2, *a* and *b*, lymph node cells from 129 Sv ERK1$^{-/-}$ mice immunized with OVA or MOG35–55 in CFA (■) showed vigorous [^{3}H]thymidine incorporation when recalled 10 days later with the respective Ags. The magnitude of the Ag-induced T cell proliferation was similar to that of WT mice (▨), and no difference was seen when the Ag was titrated over a wide range of concentrations. Similar results were obtained in ERK1$^{-/-}$ mice on the C57BL/6 background (Fig. 3*a*).

TCR-induced ERK activity has recently been implicated in the Th1 and Th2 cytokine differentiation of T cells (14), but the individual contribution of ERK1 vs ERK2 to this process has not been investigated. Therefore, to provide information on the cytokine differentiation and frequencies of OVA and MOG35–55-specific T cells in ERK1$^{-/-}$ and WT mice, we examined Ag-induced cytokine production by cytokine ELISPOT assay. As shown in Fig. 2, T cells from ERK1$^{-/-}$ 129 Sv mice showed vigorous production of IFN-γ (Fig. 2, *c* and *d*, ■) and IL-2 (Fig. 2, *e* and *f*, ■) upon

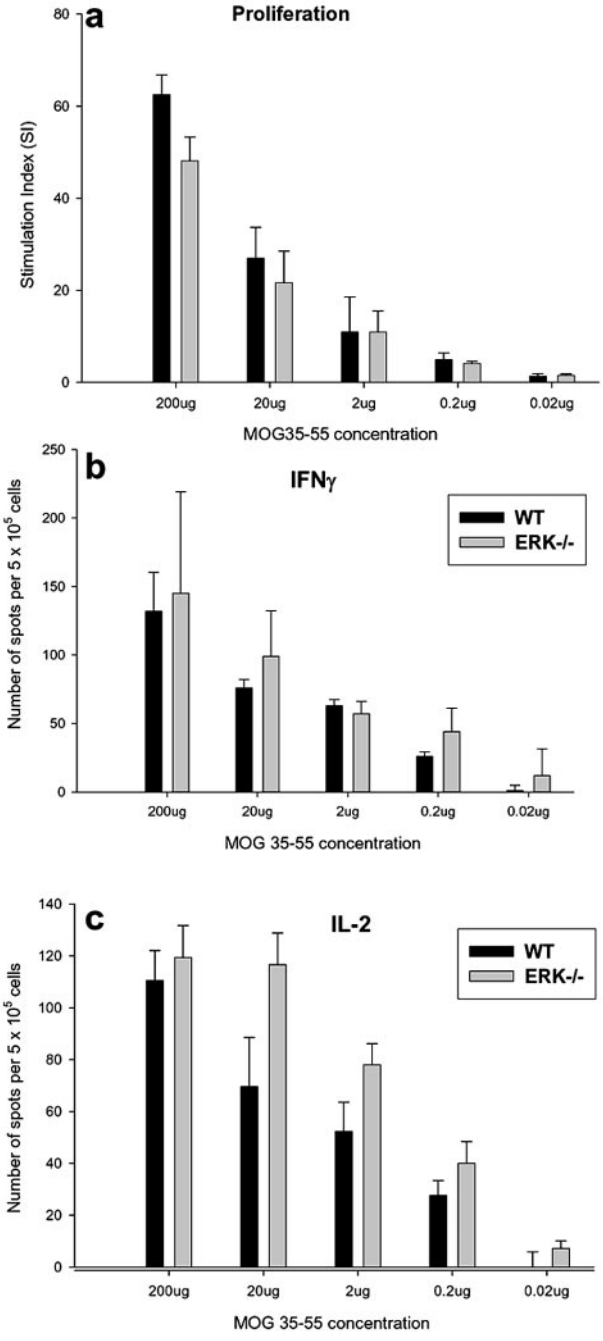

FIGURE 3. T cell effector function is maintained in C57BL/6 ERK1$^{-/-}$ mice. Experiments were performed, as outlined in Fig. 2. Mice used for these experiments were ERK1$^{-/-}$ mice backcrossed 10 generations to the C57BL/6 background or C57BL/6 WT mice. Shown are MOG35–55-induced proliferation (*a*) and production of IFN-γ (*b*) or IL-2 (*c*) by cytokine ELISPOT assay. Shown are the means ± SD of triplicate wells of groups of three to four mice. Similar results were obtained in three independent experiments.

recall with OVA or MOG35–55, respectively. Importantly, the frequencies of Ag-specific T cells producing either IFN-γ or IL-2 in ERK1$^{-/-}$ mice were comparable to those observed in ERK$^{+/+}$ 129 Sv mice (Fig. 2, *c–f*, ▨). Furthermore, OVA- and MOG-reactive T cells from ERK1$^{-/-}$ mice had comparable activation thresholds for cytokine production as T cells from WT mice when the Ag was titrated over a wide range of concentrations (Fig. 2, *c–f*). Similar results were obtained when ERK1$^{-/-}$ mice on the C57BL/6 background were tested (Fig. 3, *b* and *c*). Therefore, the results show that ERK1 deficiency did not impair the differentiation of T cells toward the Th1 cytokine profile.

To address whether Th2 differentiation was impaired in ERK1$^{-/-}$ mice, we injected 129 Sv WT or ERK1$^{-/-}$ mice with MOG35–55 in IFA and coinjected PT, a protocol that was shown previously to result in vigorous production of Th1 and Th2 cytokines (12). As shown in Fig. 4 (*top panel*), spleen cells from 129 Sv ERK1$^{-/-}$ mice showed vigorous Ag-induced production of IFN-γ, IL-2, IL-6, and IL-4, comparable to the cytokine production observed in WT mice. Similar results were observed in ERK1$^{-/-}$ mice on the C57BL/6 background (Fig. 4, *bottom panel*). Interestingly, the production of IL-17 was increased and that of IL-5 decreased in 129 Sv ERK1$^{-/-}$ mice as compared with 129 Sv WT mice, but no difference was noted in mice on the C57BL/6 background. Thus, the increase in EAE susceptibility of 129 Sv ERK1$^{-/-}$ mice (Fig. 5) could be due to an increased production of IL-17 (15), but this remains to be formally tested.

Collectively, the results unequivocally show that ERK1 is not critical for the effector function and cytokine differentiation of peripheral, mature T cells in the response to foreign and self Ags.

ERK1-deficient mice are highly susceptible to MOG35–55-induced EAE

ERK1$^{-/-}$ mice were not significantly impaired in terms of T cell proliferation and cytokine differentiation/production. However, as another, sensitive readout for T cell effector function, we investigated the potential of ERK1-deficient T cells to induce EAE in ERK1$^{-/-}$ mice on the C57BL/6 or 129 Sv background.

As shown in Fig. 5*a*, C57BL/6 WT mice (Fig. 5*a*, open symbols) developed EAE upon injection with the MOG35–55 peptide in CFA and PT. Importantly, C57BL/6 ERK1$^{-/-}$ mice (Fig. 5*a*, filled symbols) were also highly susceptible to EAE, and the animals developed disease with similar incidence and severity as compared with the WT mice. ERK1$^{-/-}$ mice on the 129 Sv background also developed EAE (Fig. 5*b*, filled symbols), whereas 129 Sv WT mice showed less susceptibility to the disease, consistent with previous reports (16). Histologic examination of the brains of ERK1$^{-/-}$ and WT mice with EAE by H&E and immunofluorescence staining showed comparable degrees of inflammation in mutant and WT mice on the C57BL/6 background, whereas inflammatory infiltrates were mildly increased in ERK1$^{-/-}$ mice on the 129 Sv background as compared with 129 Sv WT mice (Fig. 6). Analysis of the CNS infiltrates by immunofluorescence staining showed that the inflammatory cells consisted predominantly of CD4$^+$ T cells (Fig. 6) and Mac-1$^+$ microglia (data not shown) in both ERK1$^{-/-}$ and the WT mice.

Taken together, the data show that ERK1-deficient neuroantigen-specific T cells are pathogenic and mediate EAE. Thus, ERK1 is not critical for disease mediated by autoreactive T cells, and is also dispensable for the proliferation and cytokine differentiation of T cells.

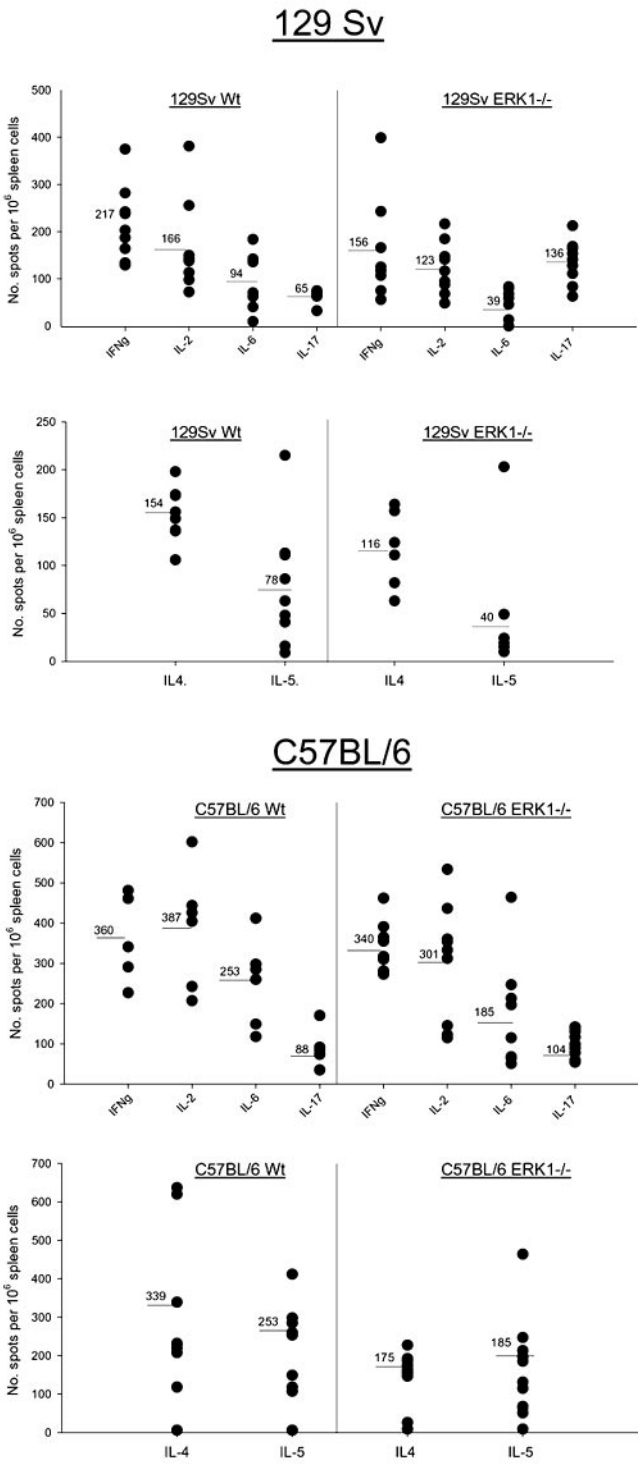

FIGURE 4. Th1/Th2 cytokine differentiation is independent of ERK1. ERK1$^{-/-}$ (*right panels*) or WT (*left panels*) mice on the 129 Sv (*top half*) or C57BL/6 (*bottom half*) background were immunized with MOG35–55 in IFA s.c. and PT to induce Th1 and Th2 responses, as previously described. Shown are the mean frequencies of cytokine-producing cells per million spleen cells of individual mice (n = 6–9 mice) tested in three independent experiments.

Thymocyte development is normal in ERK1$^{-/-}$ mice on the 129 Sv and C57BL/6 inbred background

In the present study, ERK1$^{-/-}$ mice exhibited no obvious defects in the function of peripheral T cells. Moreover, the numbers and distribution of peripheral CD4$^+$ and CD8$^+$ T cells in ERK1$^{-/-}$

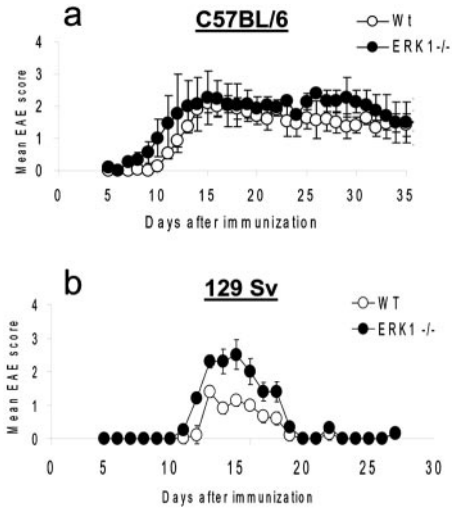

FIGURE 5. ERK1-deficient mice are susceptible to EAE. *a*, The 6- to 8-wk-old C57BL/6 ERK1-deficient mice (●) or WT mice (○) were immunized with MOG35–55 in CFA and PT and observed for clinical signs of EAE. Shown are the mean EAE scores for a representative experiment ($n = 10$ mice per group). The experiment was repeated three times with similar results. *b*, Induction of EAE in 129 Sv ERK1$^{-/-}$ or WT mice. EAE was induced with MOG35–55, and disease was monitored, as described in *Materials and Methods*. Shown is one representative experiment ($n = 10$ mice per group) of three experiments performed.

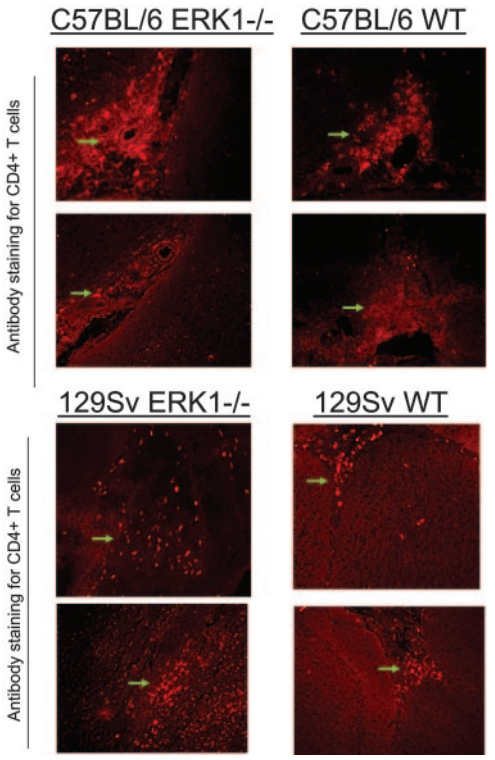

FIGURE 6. CD4$^+$ T cells infiltrate into the CNS to a similar extent in ERK1$^{-/-}$ and WT mice. The 6- to 8-wk-old C57BL/6 or 129 Sv ERK1$^{-/-}$ or WT mice were immunized with MOG35–55 in CFA and PT and observed for clinical signs of EAE. At the peak of disease, brains of the mice were removed and stained with anti-CD4-PE-labeled mAbs and evaluated by immunofluorescence microscopy. Shown are representative sections of ERK1$^{-/-}$ (*left panels*) or WT brain tissue (*right panels*) of C57BL/6 (*top half*) or 129 Sv (*bottom half*) mice with EAE. CD4$^+$ T cells are indicated by arrows.

mice were comparable to that of WT mice (Fig. 7*b*), which is interesting in light of the previously suggested defect in thymocyte maturation (6). Therefore, we revisited the question of thymocyte maturation in ERK1$^{-/-}$ mice, specifically, in mice on the inbred 129 Sv or C57BL/6 background.

Shown in Fig. 7, *a* and *b*, are the percentages of single-positive (SP) CD4$^+$ and CD8$^+$ T cells in the thymus and spleen of age-matched ERK1$^{-/-}$ (■) and WT control 129 Sv mice (▨). Interestingly, no significant difference in CD4$^+$ and CD8$^+$ SP T cells was noted between the ERK1$^{-/-}$ mice and WT mice. Moreover, the percentage of double-positive thymocytes was comparable (data not shown). Furthermore, no significant difference in SP or double-positive thymocytes was noted in ERK1$^{-/-}$ mice on the C57BL/6 background (data not shown). Finally, no significant difference was noted in the percentage of CD19$^+$ spleen B cells or Mac-1$^+$ macrophages (Fig. 7*c*).

Because we did not detect differences in the distribution of thymocyte subsets, contrary to previous reports (6), we investigated whether proliferation of thymocytes induced with anti-CD3 or anti-CD3 plus PMA was impaired in ERK1$^{-/-}$ mice (6).

As shown in Fig. 8*a*, thymocytes from 129 Sv WT mice (*left panels*) proliferated strongly upon stimulation with anti-CD3 or anti-CD3 plus PMA. However, thymocytes from 129 ERK1$^{-/-}$ mice (Fig. 8*a*, *right panels*) also proliferated vigorously upon stimulation, and no significant difference in proliferation was noted as compared with the WT mice. Similarly, mitogen-induced proliferation of C57BL/6 WT or ERK1$^{-/-}$ thymocytes was not significantly different between WT and mutant mice (Fig. 8*b*). Moreover, the kinetic of thymocyte proliferation was similar in ERK1$^{-/-}$ and WT thymocytes (data not shown). Finally, anti-CD3 or anti-CD3 plus PMA induced vigorous proliferation of both WT and ERK1$^{-/-}$ spleen cells of similar magnitude (data not shown).

Collectively, ERK1$^{-/-}$ mice on two different genetic backgrounds, i.e., 129 Sv and C57BL/6, did not show evidence of impaired thymocyte maturation and mitogen-induced proliferation. Our results do not support a critical requirement for ERK1 in thymocyte development.

Discussion

In this study, we show that ERK1-deficient mice exhibit unaltered Ag-specific proliferation, cytokine production, and clonal sizes of CD4$^+$ T cells as compared with WT animals. Moreover, we found that ERK1$^{-/-}$ mice were highly susceptible to EAE. Our results show that the ERK1 null mutation did not affect the percentage of peripheral CD4$^+$ or CD8$^+$ T cells, B cells, and macrophages, and that the percentage of SP CD4$^+$ or CD8$^+$ thymocytes and their proliferation upon mitogen stimulation were not impaired.

Collectively, the data show that ERK1 is not critical for the maturation, activation, and differentiation of T cells and their effector function in the immune periphery. Furthermore, the data suggest that ERK2 may compensate for the lack of ERK1 function in T cells.

ERK1 and ERK2 are ubiquitously expressed in most tissues, including hemopoietic tissues, and are activated by a variety of signals, including mitogenic stimuli, differentiation signals, and cytokines (1, 2). In T cells, ERKs are critically important because they are embedded in many cellular signaling pathways such as TCR signaling, CD28-mediated costimulation, cytokine signaling, and cell adhesion. However, the exact role of each ERK isoform, specifically the extent of functional redundancy of the closely related MAPK family members ERK1 and ERK2 in thymocytes and in mature peripheral T cells, has not been resolved.

ERKs are believed to be critical for thymocyte development, specifically for the process of positive selection (17). The role of

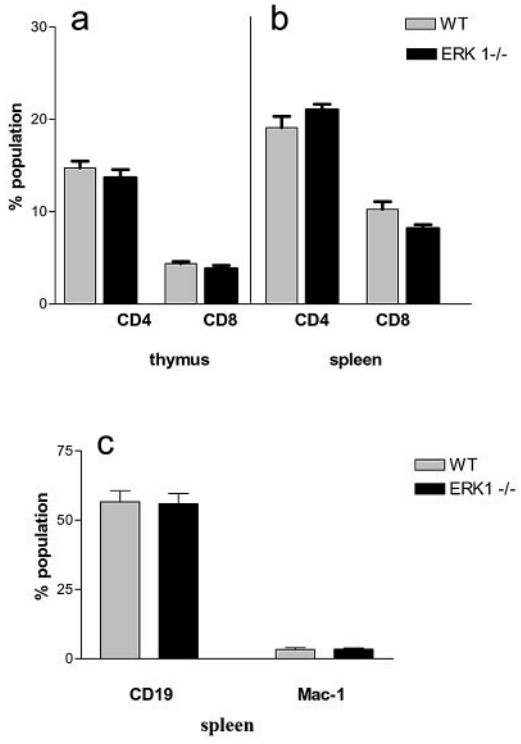

FIGURE 7. Similar distribution of lymphocytes in the thymus and spleen of ERK1$^{-/-}$ and WT mice. Thymus and spleen of 6- to 8-wk-old ERK1$^{-/-}$ and WT mice were removed and analyzed by flow cytometry for the percentage of CD4$^+$ or CD8$^+$ SP T cells (*a* and *b*), CD19$^+$ B cells, or Mac-1$^+$ APCs (*c*), as outlined in *Materials and Methods*. Shown are the mean ± SD of groups of three mice. Similar results were obtained in three independent experiments.

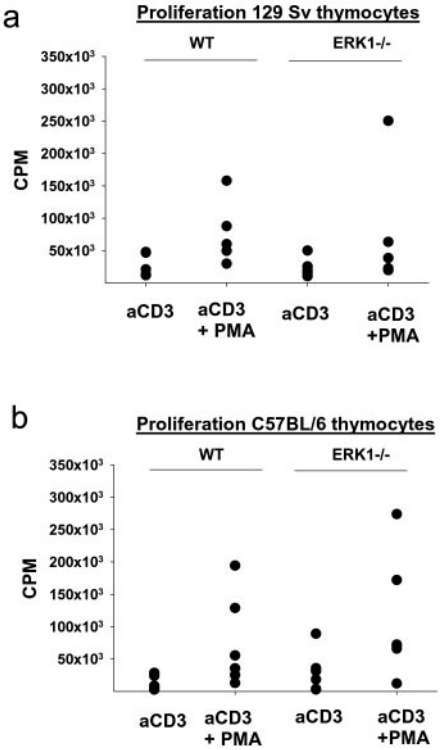

FIGURE 8. Mitogen-induced proliferation of ERK1$^{-/-}$ thymocytes is not impaired. Thymus and spleen of 129 Sv and C57BL/6 WT and ERK1$^{-/-}$ mice were removed, and single cell suspensions were prepared. Cells were incubated with anti-CD3 or anti-CD3 + PMA, as outlined in the text, for 48–72 h, and [^{3}H]thymidine incorporation was tested by scintillation counting. *a*, Shown is the mitogen-induced proliferation of 129 Sv WT (*left half of panel*) or ERK1$^{-/-}$ thymocytes (*right half of panel*). Symbols represent results for individual mice. *b*, Shown is the proliferation of C57BL/6 WT or ERK1$^{-/-}$ thymocytes. Experiments were performed, as outlined above.

ERKs in negative selection has remained questionable (7, 17). Recently, it was shown that mice with a null mutation of ERK1 exhibited impaired thymocyte development with decreased percentages of SP CD4$^+$ and CD8$^+$ T cells and impaired thymocyte proliferation upon mitogen stimulation (6). Our own studies could not confirm this observation (Figs. 7 and 8). However, several possibilities could account for this discrepancy, including strain background differences, environmental differences such as animal housing, and differences in the gene-targeting strategy due to the use of different constructs. Along these lines, targeting of the ERK2 gene by several investigators using different constructs inevitably resulted in an embryonally lethal phenotype (8, 9, 18). However, significant developmental and phenotypic differences were present, dependent on which part of the coding sequence of ERK2 was targeted (8, 9, 18). Our targeting strategy of ERK1 resulted in deletion of most of the gene (exons 1–6), whereas the approach taken by Pages et al. (6) resulted in the deletion of exon 3 of the ERK gene. Furthermore, differences in knockout phenotypes can also depend on the genetic background of the mice. Studies performed over the past few years have clearly illustrated that phenotypes caused by specific genetic modification are strongly influenced by genes unlinked to the targeted locus (19–21). Therefore, it is important to study genetically modified mice on congenic (>99% identical) genetic backgrounds, which was done in the present study, but not in the study by Pages et al.

Finally, it is conceivable that parameters of thymocyte development were affected in our ERK1$^{-/-}$ mice that we did not investigate. For example, the T cell repertoire could have been altered in terms of the positively and negatively selected T cell receptors, as compared with WT mice.

Nevertheless, our findings show that, overall, thymocyte development into mature CD4$^+$ and CD8$^+$ T cells seems to proceed normally in the absence of ERK1.

Although ERKs have been studied extensively in the context of thymocyte development, less information is available on the role of these protein kinases in the biology of peripheral T cells in vivo. As pointed out earlier, ERKs are placed at many critical signaling junctions in T cells, and it can be assumed that they are important for T cell effector function. For example, ERKs have been implicated in the integration of TCR-mediated signals in peripheral mature T cells (22, 23). Moreover, ERKs may play a role in the protection of T cells from apoptosis, and in the regulation of cell proliferation (24). Finally, ERKs have been implicated in Th1 and Th2 cell cytokine differentiation (14, 25). However, there is also evidence that ERK1 and ERK2 can be independently regulated and may have different functions. For example, ERK1$^{-/-}$ mice are viable and phenotypically normal, whereas the ERK2 null mutation is embryonally lethal (8). Thus, while current evidence supports an important role for ERKs in T cell biology, their exact contribution has remained unresolved, in particular in respect to the individual contributions of ERK1 vs ERK2. Moreover, ERKs could have different functions during intrathymic T cell development as compared with their role in peripheral mature T cells.

Investigating Ag-specific T cell immunity in ERK1$^{-/-}$ mice, we found no defect in multiple parameters of T cell effector function, including proliferation, cytokine production, and potential to mediate autoimmune disease. Interestingly, while not statistically significant, we observed a tendency for ERK1$^{-/-}$ mice to develop

more severe disease as compared with WT mice. For example, mice on the 129 Sv background that are generally less susceptible to EAE developed more frequently and more severe EAE after introduction of the ERK1 null mutation. One possible explanation for this observation comes from a previous report that noted enhanced secretion of proinflammatory IL-12 and decreased production of anti-inflammatory IL-10 by ERK1-deficient dendritic cells (26), which could facilitate the development of pathogenic Th1 cells. Along these lines, we have observed that T cells from ERK1$^{-/-}$ 129 Sv mice showed enhanced production of IL-17 as compared with WT mice (Fig. 4), a cytokine that was recently implicated in the pathogenesis of EAE (15). Another possibility is that increased phosphorylation of ERK2 could impair T cell apoptosis, which could result in enhanced survival of pathogenic T cells (24, 27–29). Together, these findings raise the critical question as to whether allelic variation of ERKs in humans could contribute to the genetic basis of T cell-mediated autoimmune diseases such as multiple sclerosis, as has been previously suggested for other components of the TCR signaling cascade (30).

Currently, most of the information regarding ERK1 and ERK2 has been derived from studies of T cells using MEK1/2 inhibitors that do not discriminate between the two ERK isoforms. Thus, our experiments provide novel insights into the specific requirement for ERK1 in T cells in vivo. Because the ERK2 null mutation is embryonally lethal, the investigation of the specific role of this molecule awaits generation of mice with a T cell-specific deletion of ERK2.

Our studies also shed some light on the requirement for ERK1 in hemopoietic cells other than T cells. The numbers of B cells in ERK1$^{-/-}$ mice were normal, as was the percentage of macrophages. Moreover, constitutive levels of serum Abs in ERK1$^{-/-}$ mice were normal (data not shown). Thus, ERK1 does not appear to be critical for the development and maturation of these cell types. However, we have also observed higher levels of IL-12 secreted by ERK1$^{-/-}$ spleen cells upon mitogen stimulation (data not shown), confirming the report by Dillon et al. (26).

Finally, the data show that the expression of ERK1 and ERK2 protein in WT mice is comparable in most tissues, including the thymus and the spleen (Fig. 1). However, there are significant variations between the activated form of ERK1 and ERK2 in certain tissues (Fig. 1), suggestive of differential posttranscriptional regulation. Using a genetic approach, we found that ERK1-deficient mice showed an increase in ERK2 activity in the thymus without a compensatory increase in ERK2 protein expression.

In conclusion, ERK1 seems to be dispensable for the development and effector function of T cells. The data suggest that enhanced phosphorylation of ERK2 may have compensated for the lack of ERK1.

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Disclosures

The authors have no financial conflict of interest.

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