

ERK1 is important for Th2 differentiation and development of experimental asthma

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ABSTRACT The ERK1/2 signaling pathway regulates a variety of T-cell functions. We observed dynamic changes in the expression of ERK1/2 during T-helper cell differentiation. Specifically, the expression of ERK1/2 was decreased and increased by IL-12 and IL-4, respectively. To address this subject further, we examined the specific role of ERK1 in Th2 differentiation and development of experimental asthma using ERK1^{-/-} mice. ERK1^{-/-} mice were unable to mount airway inflammation and hyperreactivity in two different models of asthma, acute and chronic. ERK1^{-/-} mice had reduced expression of Th2 cytokines IL-4 and IL-5 but not IL-17A or IFN- γ . They had reduced levels of allergen-specific IgE and blood eosinophils. T cells from immunized ERK1^{-/-} mice manifested reduced proliferation in response to the sensitizing allergen. ERK1^{-/-} T cells had reduced and short-lived expression of JunB following TCR stimulation, which likely contributed to their impaired Th2 differentiation. Immunized ERK1^{-/-} mice showed reduced numbers of CD44^{high} CD4 T cells in the spleen. *In vitro* studies demonstrated that Th2 but not Th1 cells from ERK1^{-/-} mice had reduced numbers of CD44^{high} cells. Finally, CD4 T cells from ERK1^{-/-} mice expressed higher levels of BIM under growth factor-deprived conditions and reduced Mcl-1 on stimulation. As a result, the survival of CD4 T cells, especially CD44^{high} Th2 cells, was much reduced in ERK1^{-/-} mice. We conclude that ERK1 plays a nonredundant role in Th2 differentiation and development of experimental asthma. ERK1 controls Th2 differentiation and survival through its effect on JunB and BIM, respectively.—Goplen, N., Karim, Z., Guo, L., Zhuang, Y., Huang, H., Gorska, M. M., Gelfand, E., Pagés, G., Pouyssegur, J., Alam, R. ERK1 is important for Th2 differentiation and development of experimental asthma. *FASEB J.* 26, 000–000 (2012). www.fasebj.org

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THE EXTRACELLULAR SIGNAL-REGULATED kinase (ERK)1/2 signaling pathway regulates a variety of cellular processes, including growth, proliferation, differentiation, cytokine/mediator secretion, and cell motility (1). Defining the role of ERK1/2 in T-cell growth, differentiation, and function has been quite challenging (2–4). This is likely because ERK1/2 functions as a modular switch. Its biological response depends on the magnitude of the signaling output, duration, and subcellular location (5, 6). ERK2 knockout (KO) is embryonically lethal (7), but ERK1 KO is not (8). ERK1 has been shown to be important for growth and function of certain tissues, *e.g.*, adipocyte growth (9) and retinal cell survival (10). Reports are conflicting regarding the role of ERK1 in thymocyte development (8, 11). Although it was initially reported to affect thymocyte maturation beyond the double-positive stage, the current consensus is that ERK1 is not essential but is synergistically important for positive selection when ERK2 is also deficient (11). In mature T cells, ERK1 deficiency promotes Th1 differentiation and aggravates experimental autoimmune encephalitis (EAE; refs. 12, 13). The role of ERK1 in Th2 differentiation is less clear. One study demonstrated reduced IL-5 production by ERK1^{-/-} T cells *in vitro* (13). Whether this translates into a generalized impairment in Th2 differentiation and a failure to mount Th2 inflammation is unknown.

ERK1/2 is activated in the airway tissue from human patients with asthma (14) and from the mouse model of asthma (15, 16). Pharmacological inhibition of MEK1/2, the upstream activator of ERK1/2 blocks airway inflammation in a mouse model of acute asthma (17). A similar pharmacological inhibition of MEK1/2

Abbreviations: AEH, airway epithelial hypertrophy; DRA, dust mite, ragweed, and *Aspergillus*; EAE, experimental autoimmune encephalitis; ERK, extracellular signal-regulated kinase; H&E, hematoxylin and eosin; KO, knockout; MBP, major basic protein; TCR, T-cell receptor; WT, wild type.

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abrogates the differentiation of CD8 effector memory T cells and the development of experimental asthma in a model of adoptive CD8 T-cell transfer (18). However, the pharmacological inhibition studies did not define the specific role of ERK1 and ERK2. To address this matter, we examined the role of ERK1 in T-helper cell differentiation and survival. We also evaluated the importance of ERK1 in 2 mouse models of asthma, acute and chronic (15).

MATERIALS AND METHODS

Human T-cell studies

Healthy subjects were recruited from the National Jewish Hospital staff. The study was approved by the National Jewish Health institutional review board. Written consent was obtained from all study subjects. Peripheral blood mononuclear cells were isolated by density centrifugation using Histopaque (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. CD4⁺ T cells were purified by negative selection using the Miltenyi CD4⁺ T-Cell Isolation Kit according to the manufacturer's recommendations (Miltenyi Biotec, Auburn, CA, USA) as described previously (19). CD4⁺ T cells were >97% pure, as determined by flow cytometry. CD4⁺ T cells were cultured with and without stimulation, as indicated in the text, in RPMI 1640 (Irvine Scientific, Santa Ana, CA, USA) supplemented with 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, 1.3 mg/ml sodium pyruvate, 50 μ g/ml gentamicin, and 10% FBS.

Animals

ERK1^{-/-} mice on a SV129 background were obtained from Dr. Gilles Pagès (University of Nice, Nice, France; ref. 8). The mice were crossed to C57BL/6 for >4 generations before study. These mice had a normal number of single-positive CD4 cells in the periphery. The protocol for the mouse studies was approved by the National Jewish Health institutional animal care and use committee.

Allergens and adjuvant

Allergens used included extracts of dust mite (*Dermatophagoides farinae*), ragweed (*Ambrosia artemisiifolia*), and *Aspergillus fumigatus* (Greer Laboratories, Lenoir, NC, USA). Adjuvant was aluminum and magnesium hydroxide (Imject alum; Pierce, Cheshire, UK; 1:1 v/v with allergen). Quantities of allergens for subcutaneous sensitization (100 μ l behind the ear) and intranasal (20 μ l in saline) allergen challenges were as follows: ragweed (50 μ g); and 3-allergen mixture chronic model (DRA; dust mite, 5 μ g; ragweed, 50 μ g; and *Aspergillus*, 5 μ g). The LPS content and protease activity of these extracts has been described elsewhere (15).

Asthma models

For chronic asthma, female mice were immunized at 8–12 wk of age, 2 $\times$ 1 wk apart, with DRA mixture in alum, as described above. Beginning at 1 wk after the second immunization, intranasal challenges were performed 2 $\times$ /wk for 5 wk with the immunizing allergens (15). A control group of littermate wild-type (WT) mice were immunized with saline in alum and intranasally exposed to saline. The mice were rested for 3 wk before analyses. The protocol for acute sensitization was

similar to the first 2 wk above, but only ragweed was used in conjunction with alum. These mice were challenged intranasally as above 2 $\times$ /wk for 2 wk and studied 3 d later.

Morphometric measurements

Mice were immunized and challenged as stated above and rested for 3 or 21 d. Right lung lobes were fixed in formalin by instilling it gently through a tracheal cannula, sectioned (5 μ m), and stained with hematoxylin and eosin (H&E). Images were captured at $\times$ 200 view, and inflammation on H&E-stained lung sections was quantified with Metamorph image acquisition and analysis software (Molecular Devices, Eugene, OR, USA). Airway inflammation was measured by color thresholding the 24-bit H&E image for nuclei of the inflammatory infiltrates. The area covered by the infiltrate's nuclei was divided by the perimeter of the airway's basement membrane. Thus, a quantitative airway inflammation score of 1 is indicative of an airway that has 1 μ m² of inflammatory infiltrate (nuclear area) per micrometer of basement membrane. Airway epithelial hypertrophy (AEH) was also scored quantitatively and is defined as the area of epithelium (in square micrometers) divided by the perimeter of the lamina propria (in micrometers). Thus, AEH is a combinatorial measure of the increased airway size as well as growth of the lamina propria, which is primarily due to smooth muscle hypertrophy in these models. Ten randomly selected inflamed airways from each mouse were analyzed to obtain a mouse average. Data obtained from 4–6 mice were used for statistical analyses.

Collagen deposition in the lamina propria was visualized by staining lung sections with Sirius red (Sigma) and inducing fluorescence in the tetramethyl rhodamine isothiocyanate (TRITC) channel (perivascular regions of collagen deposition were excluded from this analysis; ref. 15). The fluorescent intensity of the subepithelium was calculated as integrated fluorescent intensity per micrometer of airway lamina propria and then normalized to saline control values (arbitrarily defined as 100). Images were acquired and analyzed with an epifluorescence microscope (Nikon Eclipse2000; Nikon, Tokyo, Japan) equipped with a Cool-snap CCD camera (Nikon) and Metamorph image analysis software (Molecular Devices).

Airway hyperreactivity measurement

This procedure has been explained in depth elsewhere (15). Briefly, mice were anesthetized with ketamine (180 mg/kg), xylazine (9 mg/kg), and acepromazine (4 mg/kg). After the animal lost the foot-pad pinch response, a tracheotomy was performed, and the subject was connected to a small-animal ventilator with a computer-controlled piston (Flexivent; Scireq, Montreal, PQ, Canada) through an 18-gauge cannula. Subjects were ventilated at a frequency of 150 breaths/min with a tidal volume of 0.2 ml while breathing against an artificial positive end-expiratory pressure of 2.5 to 3 cm H₂O. After this, the lungs were inflated 2 $\times$ to total lung capacity to standardize volume history among subjects. Resistance measurements were then taken to establish baselines for both the single-compartment model (allows measurement of total lung resistance) and the constant-phase model (compartmentalizes total lung resistance into raw and tissue resistance/elastance).

Resistance measurements were taken with a 2.5-Hz sinusoidal piston volume movement of 0.15 ml; subsequently, pressure-volume and flow data were fit to the single-compartment model (16) to obtain values for total lung resistance. Group averages were expressed as fold increases in baseline resis-

tance (mean $\pm$ SE). Statistical significance was established between groups by using both ANOVA and the unpaired Student *t* test (JMP; SAS Institute, Cary, NC, USA; and Microsoft Excel; Microsoft, Redmond, WA, USA) at each methacholine dose.

Eosinophil studies

Rat anti-mouse major basic protein (MBP; from James Lee, Mayo Clinic, Scottsdale, AZ, USA) was used to visualize MBP⁺ cells by flow cytometry. Eosinophilopoiesis was induced in fresh bone-marrow isolates by culturing cells at 6×10^5 /ml in RPMI with 10% FBS supplemented with 1 ng/ml of IL-3 for 3 d (20). Cells were then cultured in IL-3 (1 ng/ml) and IL-5 (6 ng/ml), changing medium every 3 d for 9 d before fixing in 1% paraformaldehyde, intracellular staining for MBP, and assaying by flow cytometry.

Allergen-specific IgE

This assay has been described elsewhere (15). Serum was collected at necropsy and diluted to 1 mg/ml (Bradford) in assay diluent. Briefly, ELISA plate wells (Immulon 4 HBX; Fisher Scientific, Pittsburgh, PA, USA) were coated overnight at 4°C with 10 μ g/ml *Aspergillus* extract in PBS. Plates were washed 3 times with PBS-Tween 20 (0.05%) and blocked in PBS (10% FBS) for 1 h at room temperature. The above diluted samples in a 50- μ l volume (50 μ g of protein) were applied in triplicate for 2 h at room temperature, and plates were washed 5 times. One-step detection was accomplished by means of incubating with biotinylated anti-IgE (BD PharMingen, San Jose, CA, USA) mixed 1:1000 with streptavidin-horseradish peroxidase (BD PharMingen) for 1 h at room temperature. Plates were washed 7 times and developed with TMB substrate (Pierce) for 20 min. The reaction was stopped by means of the addition of 2 N H₂SO₄ and read at 450 nm. Results are reported as raw OD values.

T-helper cell differentiation, proliferation, and cytokine detection assays

Whole splenocytes were isolated from 8-wk-old naive ERK1^{+/+} and ERK1^{-/-} mice. Spleens were removed and

passed through a 70- μ m nylon mesh filter. CD4 cells were purified by negative selection according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). For Th1, Th2, and Th17 differentiation experiments, CD4 T cells were seeded at 10^6 /ml on anti-CD3/28-coated 24-well plates as above and cultured with 20 U/ml of hIL-2 (U.S. National Institutes of Health/National Cancer Institute, Bethesda, MD, USA) in Th1 conditions: 10 μ g/ml anti-IL-4 and 10 ng/ml IL-12; Th2 conditions: 10 μ g/ml each of anti-IL-12 and anti-IFN- γ (eBiosciences, San Diego, CA, USA) and 10 ng/ml mIL-4; or Th17 conditions: 10 μ g/ml each anti-IL-4 and anti-IL-12 plus 20 ng/ml IL-6 and 3 ng/ml TGF β for 3 d as described previously (15, 21). For the remaining 4 d, the cells were cultured with the same concentration of IL-2 but half with the concentration of neutralizing antibodies and cytokines in complete RPMI with 10% FBS in 6-well plates.

For CD4 T-cell cultures, spleens were removed and passed through 70- μ m nylon mesh filters and washed. Red blood cells were lysed with the hypotonic buffer ammonium chloride (ACK), and the cells were cultured in complete RPMI at 2.5×10^6 /ml for 72 h. ³H-thymidine (0.5 μ Ci) was added for the last 16 h of culture before cells were harvested and read on a β counter. In other experiments, these supernatants were harvested after culture and assayed for IL-5 (sensitivity: 63 pg/ml), IL17A (sensitivity: 15 pg/ml), and IFN- γ (sensitivity: 9 pg/ml) according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA).

Flow cytometry staining

Antibodies used for immunofluorescent staining included anti-MBP (James Lee; Mayo Clinic, Scottsdale, AZ, USA), anti-CD4-AF488 (1:99 dilution; BD Biosciences and eBiosciences), anti-mIL-4-PE (1:99 dilution) and anti-mCD44-AF647 (1:299 dilution, eBiosciences). Cell staining was done in 96-well round-bottom plates (Cellstar; Greiner Bio-One, Monroe, NC, USA). Blocking was performed with 10% goat serum in PBS (0.1% saponin for intracellular stained samples) in the presence of 10 μ g/ml rat anti-Fc γ R blocking antibody 2.4G2 (in non-MBP-stained samples; *i.e.*, samples that did not require a rat secondary antibody) at room temperature for 20 min. The unlabeled primary

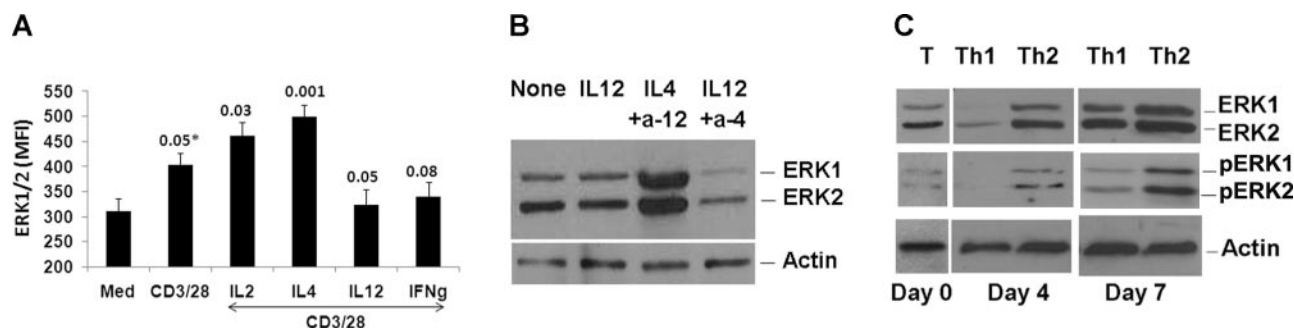


Figure 1. Effect of T-helper cell-inducing cytokines on ERK1 and ERK2 expression. *A*) Human peripheral blood mononuclear cells were cultured in medium alone or in anti-CD3/28-antibody (2 μ g/ml)-coated wells with or without the indicated cytokines (10 ng/ml) for 4 d. Expression of ERK1/2 in CD4 T cells was measured by flow cytometry. Mean fluorescent intensity (MFI) of ERK1/2 was analyzed statistically ($n=6$). Cytokine-stimulated samples were compared with the CD3/28-stimulated sample for calculation of the statistical difference (values of *P* are indicated above bars). CD3/28-stimulated sample (asterisk) was compared with the medium (med) control. *B*) Human CD4 T cells were purified from the peripheral blood by negative selection and then cultured in anti-CD3/28 antibody-coated wells in the presence of the indicated cytokine (10 ng/ml) and antibody (a-4, anti-IL-4; a-12, anti-IL-12; 10 μ g/ml) for 4 d. Cell lysates were Western blotted for ERK1 and ERK2 ($n=3$). *C*) Purified human CD4 T cells were cultured under Th1- and Th2-inducing conditions, and the expression of ERK1/2 and pERK1/2 was examined on d 4 and 7 ($n=4$).

antibodies (1 $\mu\text{g}/\text{ml}$) were then incubated at room temperature for 2 h in PBS (0.05% saponin; labeled antibodies were incubated for 30 min), washed several times in PBS (0.05% saponin) before applying the AlexaFluor-488-conjugated secondary antibody (5 $\mu\text{g}/\text{ml}$) for 1 h at room temperature. Cells were washed several times and assayed by flow cytometer. Staining with propidium iodide was done for 10 min at a concentration of 5 $\mu\text{g}/\text{ml}$ after 30 min room temperature incubation with labeled antibodies on live cells. Cells were washed twice before analysis on either a FacsCalibur or FacsScan (BD Biosciences).

Expression of survival factors and assessment of CD4 T-cell survival

Splenocytes were isolated from 6- to 8-wk-old naive $\text{ERK1}^{+/+}$ and $\text{ERK1}^{-/-}$ mice. Red blood cells were lysed with ACK buffer, and the cells were cultured in 24-well plates in complete RPMI at $2 \times 10^6/\text{ml}$ in presence of anti-CD3/28 antibodies (2 $\mu\text{g}/\text{ml}$ each) or medium alone. After 48 h, cells were fixed in 4% paraformaldehyde for 20 min, washed twice with PBS, and stained with mouse anti-CD4 PE (BD Biosciences), anti-BIM, and anti-Mcl-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For apoptosis assay, splenocytes from immunized $\text{ERK1}^{+/+}$ and $\text{ERK1}^{-/-}$ mice were cultured in the presence of medium alone, *Aspergillus* (3 $\mu\text{g}/\text{ml}$) and *Aspergillus*

with recombinant FasL (10 $\mu\text{g}/\text{ml}$) for 5 d. Then 1×10^6 cells were washed with PBS, centrifuged, and stained with annexin V-APC (BD Biosciences) and mouse anti-CD4 PE (BD Biosciences) in presence of propidium iodide for 15 min. Later, cells were analyzed with cyan within 1 h. For baseline measurement, cells were stained immediately after isolation of splenocytes.

Statistical analyses

Group data are expressed as means $\pm$ SE. We used *t* tests (paired and unpaired) and ANOVA to analyze data statistically. A value of $P < 0.05$ was considered significant.

RESULTS

T-helper cell differentiation-inducing cytokines alter the expression of ERK1 and ERK2

The activation/phosphorylation of ERK1 and ERK2 in differentiated T-helper cells has been studied previously. However, very little information is available on the absolute level of expression of ERK1 and ERK2 in differentiating T-helper cells following cyto-

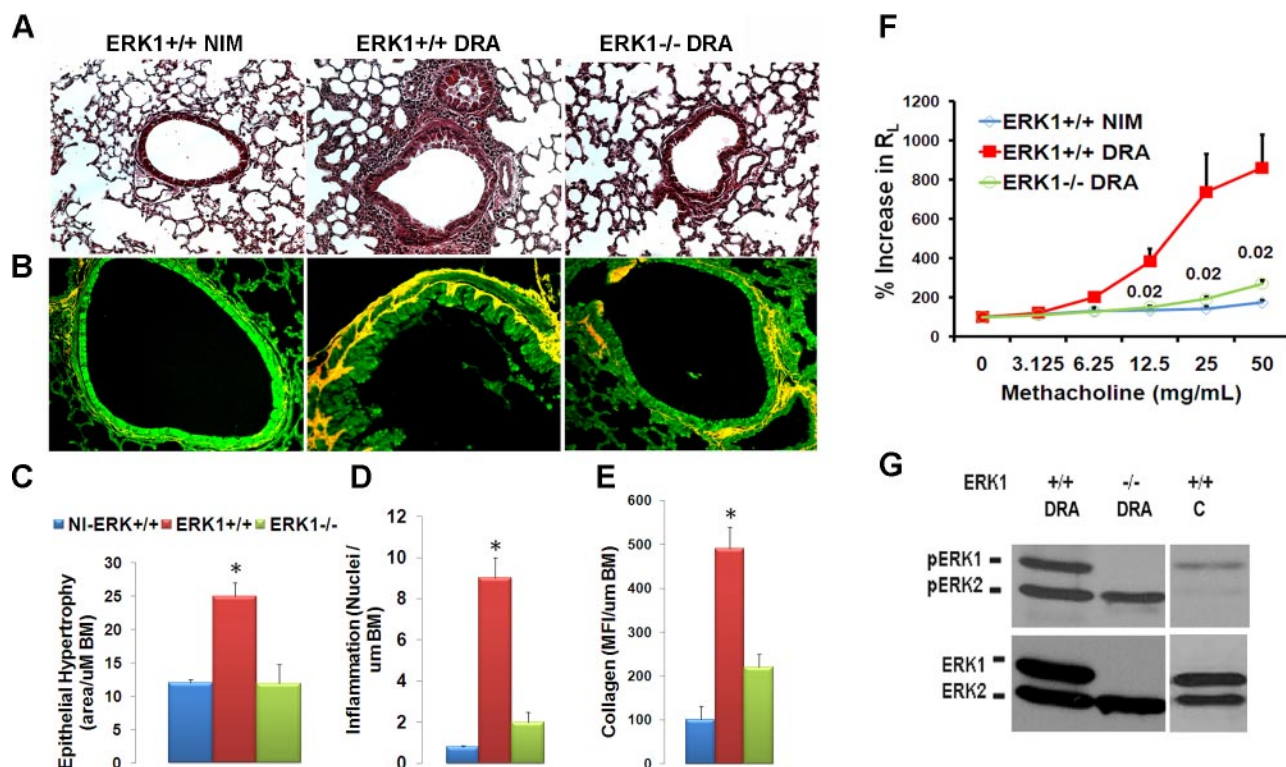


Figure 2. Asthma phenotype in $\text{ERK1}^{-/-}$ mice. $\text{ERK1}^{-/-}$ and littermate control $\text{ERK1}^{+/+}$ mice were immunized and exposed to 3 allergens (DRA: dust mites, ragweed, and *Aspergillus*) according to the chronic asthma protocol as described previously. Mice were rested for 3 wk without any further allergen exposure and then assessed for airway inflammation and response to methacholine provocation. A group of nonimmunized $\text{ERK1}^{+/+}$ (NIM $\text{ERK1}^{+/+}$) mice was used as a negative control. *A*) H&E staining of the lung sections ($\times 200$ view). *B*) Sirius red staining for collagen deposition. *C–E*) Morphometric analysis of epithelial hypertrophy (*C*), airway inflammation (*D*), and collagen deposition (*E*). * $P < 0.04$ vs. $\text{ERK1}^{-/-}$ and NIM; $n = 4$ (NIM), $n = 5$ ($\text{ERK1}^{+/+}$, $\text{ERK1}^{-/-}$ DRA). *F*) Airway resistance (R_L) to nebulized methacholine. Airway resistance was measured by the Flexivent apparatus, as described previously. Values of P (ANOVA) are indicated above doses ($n = 4$ for NIM; $n = 5$ for $\text{ERK1}^{+/+}$ and $\text{ERK1}^{-/-}$ DRA). *G*) Western blotting of the airway tissue from the study mice for phosphorylated ERK1/2. Membrane was reprobbed for ERK1/2. C, nonimmunized control. Western blot is representative of 3 separate experiments.

kine stimulation. We examined the effect of Th1- and Th2-inducing cytokines on ERK1 and ERK2 expression by flow cytometry in human T cells. We found that antibody-mediated stimulation of the CD3 and CD28 receptors for 4 d increased the expression of ERK1/2 in T cells (Fig. 1A). This condition was further enhanced by IL-2, and especially by IL-4. In contrast, IL-12 inhibited the anti-CD3/28 antibody-induced expression of ERK1/2. IFN- γ had a marginal and nonsignificant effect. Next, we cultured CD4 T cells with the Th1- and Th2-inducing cytokines and neutralizing antibody against the opposing cytokine in the presence of anti-CD3/28 antibodies for 4 d. For these experiments, we performed Western blotting to visualize the individual levels of ERK1 and ERK2 expression. Under these conditions, we observed a down- and up-regulation of ERK1 and ERK2 on culture with IL-12 + anti-IL-4 antibody and IL-4 + anti-IL-12 antibody, respectively (Fig. 1B). The addition of an antibody against the opposing cytokine further augmented the effect of the T-helper cell-inducing cytokine. Next, we examined the expression of ERK1 and ERK2 during T-helper cell differentiation. We observed a down-regulation of ERK1 and ERK2 under Th1-skewing conditions and their modest up-regulation under Th2-skewing conditions on d 4 (Fig. 1C). Interestingly, the early decline in the ERK1 and ERK2 level in Th1 cells fully recovered by d 7. Their level continued to increase in Th2 cells. The regulation of expression of ERK1 and ERK2 by IL-4 and IL-12, and their dynamic changes during T-helper cell differentiation, add a new dimension to the role of these kinases in the foregoing processes.

Effect of ERK1-null mutation on airway inflammation and hyperreactivity in the mouse model of chronic and acute asthma

As mentioned previously, ERK2 KO is embryonically lethal. To examine the role of ERK1 in Th2 differentiation and inflammation *in vivo*, we studied ERK1^{-/-} mice. We studied two different models of asthma, chronic and acute. For the chronic model, the KO and littermate WT mice were intranasally exposed to DRA 2 $\times$ /wk for 5 wk, as reported previously (15), rested for 3 wk, and then examined for airway inflammation and hyperreactivity. The littermate control mice showed intense airway inflammation (Fig. 2A, D), epithelial hypertrophy (Fig. 2C), subepithelial collagen deposition (Fig. 2B, E) and airway hyperreactivity (Fig. 2F). The ERK1^{-/-} mice showed negligible airway inflammation, epithelial hypertrophy, and collagen deposition. Their airway response to methacholine was also inhibited severely. We checked for ERK2 phosphorylation in the lung tissue by Western blotting. We found significant phosphorylation of ERK2 in ERK1^{-/-} mice when compared to ERK1^{+/+} mice (Fig. 2G).

For the acute asthma model, we used a commonly applied protocol where mice were immunized twice subcutaneously with an allergen (ragweed or *Aspergillus* as indicated) in alum followed by intranasal exposure to the allergen 2 $\times$ /wk for 2 wk. The mice were sacrificed 3 d later. Similar to the chronic asthma model, we found a significant inhibition of airway hyperreactivity to methacholine in ERK1^{-/-} as compared to ERK1^{+/+} mice (Fig. 3A). Airway inflammation was likewise reduced in these mice (Fig. 3B). The

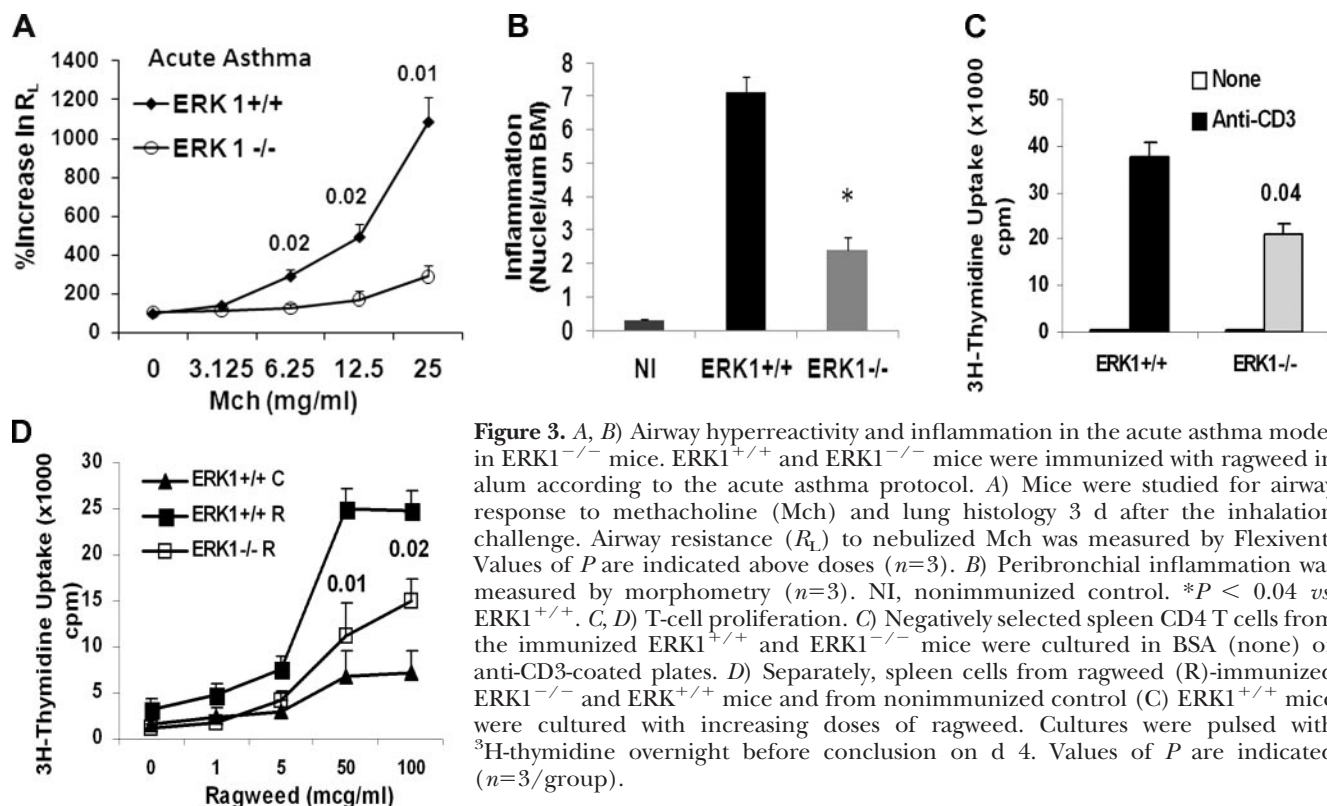


Figure 3. A, B) Airway hyperreactivity and inflammation in the acute asthma model in ERK1^{-/-} mice. ERK1^{+/+} and ERK1^{-/-} mice were immunized with ragweed in alum according to the acute asthma protocol. A) Mice were studied for airway response to methacholine (Mch) and lung histology 3 d after the inhalation challenge. Airway resistance (R_L) to nebulized Mch was measured by Flexivent. Values of P are indicated above doses ($n=3$). B) Peribronchial inflammation was measured by morphometry ($n=3$). NI, nonimmunized control. * $P < 0.04$ vs. ERK1^{+/+}. C, D) T-cell proliferation. C) Negatively selected spleen CD4 T cells from the immunized ERK1^{+/+} and ERK1^{-/-} mice were cultured in BSA (none) or anti-CD3-coated plates. D) Separately, spleen cells from ragweed (R)-immunized ERK1^{-/-} and ERK1^{+/+} mice and from nonimmunized control (C) ERK1^{+/+} mice were cultured with increasing doses of ragweed. Cultures were pulsed with ³H-thymidine overnight before conclusion on d 4. Values of P are indicated ($n=3$ /group).

results in aggregate suggested that the lack of asthma phenotype (acute and chronic) in $ERK^{-/-}$ mice was likely due to the inability to mount an effective Th2 inflammatory response.

T-cell proliferation in $ERK1^{-/-}$ mice

Thymocyte proliferation was reported previously to be reduced in $ERK1^{-/-}$ mice (8, 13). We confirmed this finding using splenic CD4 T cells. Anti-CD3-induced T-cell proliferation was significantly reduced (Fig. 3C). We also observed a significant reduction in allergen (ragweed)-stimulated spleen T-cell proliferation in KO mice from the acute asthma model (Fig. 3D).

Th2 immune response in $ERK1^{-/-}$ mice

$ERK1^{-/-}$ mice have been shown previously to have an augmented Th1 response (12, 13). We asked whether $ERK1^{-/-}$ mice had an abnormal T-helper cell differentiation response. Antigen-specific IgE production is a major outcome of a Th2 immune response. We checked for the level of total and allergen-specific IgE antibody. The serum level of total IgE from the chronic asthma model in $ERK1^{-/-}$ mice was similar to that in control mice (Fig. 4A). The level of *Aspergillus*-specific IgE antibody was <50% that of the control mice (Fig. 4B). We also checked for the presence of IgE antibody against an irrelevant antigen (ovalbumin). As expected, no ovalbumin-specific IgE was found in the serum.

Next, we examined *in vitro* differentiation of T-helper cells. We observed a significant reduction in the production of IL-4 and IL-5 but found no significant difference in the production of IL-17A and IFN- γ , which are the signature cytokines for Th17 and Th1, respectively (Fig. 4C–F). We also studied intracellular IL-4 production by flow cytometry. Most of the IL-4⁺ CD4 T cells were positive for CD44 in WT mice. The number of IL-4⁺ T cells was reduced by 50% in $ERK1^{-/-}$ mice (Fig. 4G). The reduced production of *Aspergillus*-specific IgE, and the cytokines IL-4 and IL-5 suggested an impaired Th2 response in $ERK1^{-/-}$ mice.

Eosinophil differentiation

One of the hallmarks of Th2 inflammation is eosinophilia. $ERK1/2$ plays an important role in eosinophil differentiation (22). For this reason, we studied eosinophilopoiesis *ex vivo* and *in vitro*. Eosinophils accounted for 1% of the total leukocytes in the bone marrow from $ERK1^{-/-}$ immunized mice, which was similar to that from $ERK1^{+/+}$ nonimmunized mice. In contrast, they constituted 4% of the total leukocytes in the bone marrow from immunized $ERK1^{+/+}$ mice. This finding suggested a possible problem in eosinophil differentiation in $ERK1^{-/-}$ mice. Next, we examined eosinophil differentiation *in vitro* from bone marrow cells in the presence of IL-3 and IL-5, as reported previously. The differentiation of MBP⁺ eosinophils from bone marrow cells was not impaired in KO mice (Fig. 5A). Thus, even

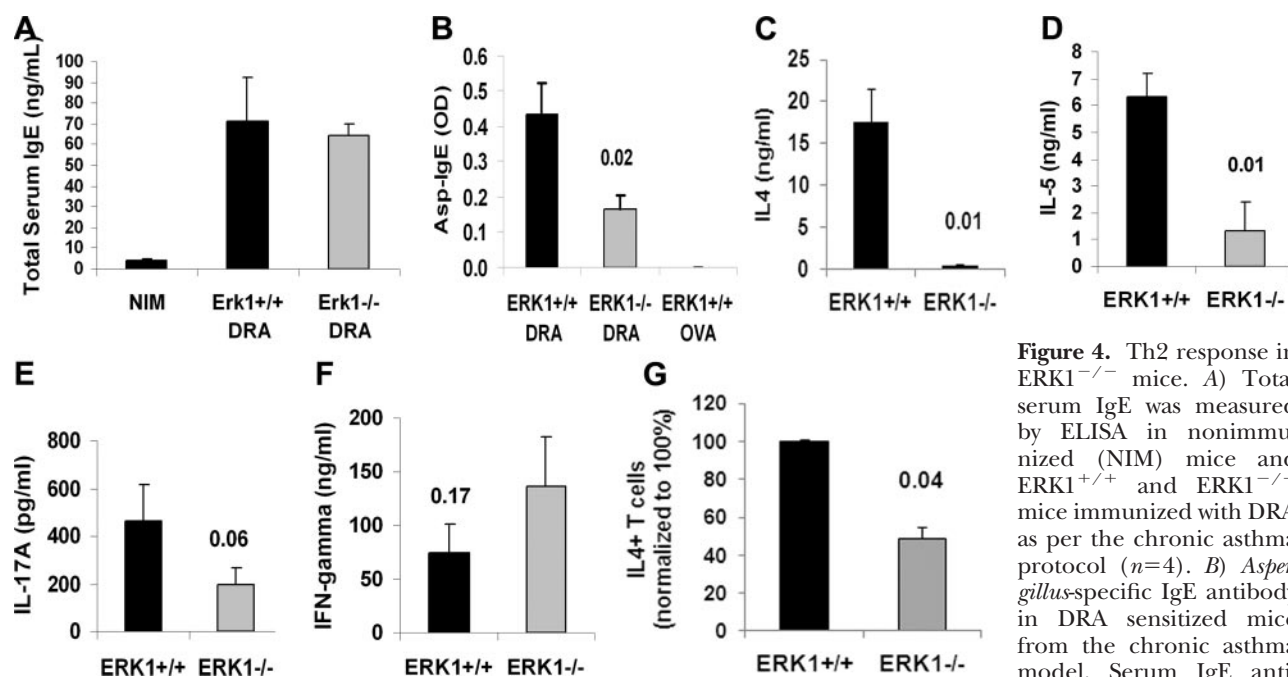


Figure 4. Th2 response in $ERK1^{-/-}$ mice. A) Total serum IgE was measured by ELISA in nonimmunized (NIM) mice and $ERK1^{+/+}$ and $ERK1^{-/-}$ mice immunized with DRA as per the chronic asthma protocol ($n=4$). B) *Aspergillus*-specific IgE antibody in DRA sensitized mice from the chronic asthma model. Serum IgE antibody against the immunizing allergen *Aspergillus* and an irrelevant antigen ovalbumin was measured by a modified ELISA ($n=6$). C–F) Spleen CD4 T cells from $ERK1^{+/+}$ and $ERK1^{-/-}$ mice were cultured *in vitro* under Th1-, Th2-, and Th17-inducing conditions. IL-4 (C), IL-5 (D), IL-17A (E), and IFN- γ (F) in the culture supernatant were measured by ELISA ($n=4$). G) Intracellular IL-4 was measured by flow cytometry in CD44⁺ T cells following gating for CD4 T cells that were cultured under Th2-inducing conditions. Number of IL4⁺ cells from $ERK1^{+/+}$ mice (which ranged from 4–15%) was normalized to 100%; data represent percentage reduction in $ERK1^{-/-}$ mice ($n=3$). Values of P are indicated above bars.

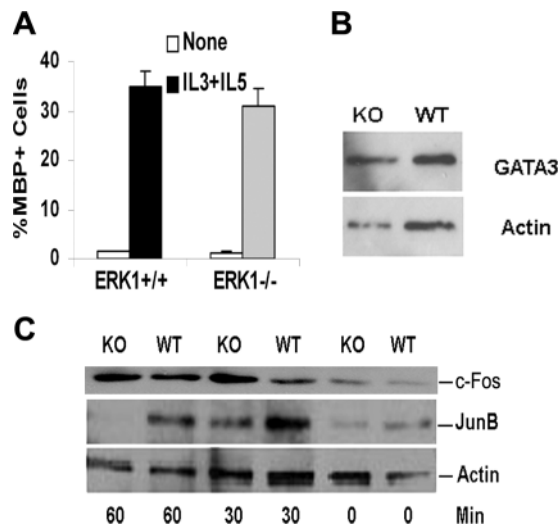


Figure 5. A) Eosinophil differentiation from bone marrow cells. Erythrocyte-depleted bone marrow cells from ERK1^{+/+} and ERK1^{-/-} mice were cultured in IL-3- and IL-5-containing medium for 9 d and then analyzed for MBP⁺ cells by flow cytometry ($n=3$). B) GATA3 expression. Spleen CD4 T cells were cultured under Th2-inducing conditions for 7 d, then GATA3 expression was compared by Western blotting. Membrane was re probed for actin ($n=3$). C) JunB and c-Fos expression in ERK1^{-/-} T cells. Spleen CD4 T cells from ERK1^{+/+} and ERK1^{-/-} mice were stimulated with anti-CD3 and anti-CD28 (2 μ g/ml) antibodies for the indicated period of time, and expression of JunB and c-Fos was examined by Western blotting. Jun-B membrane was re probed for actin ($n=3$).

though few eosinophils were observed in the lungs and bone marrow of sensitized and challenged ERK1^{-/-} mice, we found no deficiency in their expansionary response to IL-5 *in vitro*. Instead, the results pointed to defective Th2 differentiation, function, and/or survival.

JunB expression is reduced and short-lived in ERK1^{-/-} T cells

GATA3 is a critical regulator of Th2 differentiation. GATA3 expression was unaltered in ERK1^{-/-} Th2 cells following *in vitro* differentiation (Fig. 5B). The AP1 transcription factor also plays a critical role in T-helper cell differentiation (23). Previous studies have established an important role for JunB but not for c-Fos in Th2 differentiation (4, 24, 25). For this reason, we examined the expression of JunB in ERK1^{-/-} T cells. Stimulation with anti-CD3/CD28 antibodies rapidly induced JunB in CD4 T cells from ERK1^{+/+} mice (Fig. 5C). The amplitude (band intensity) of JunB induction and its duration were much reduced in CD4 T cells from ERK1^{-/-} mice. JunB expression was lost within 60 min after induction in ERK1 deficiency. We believe that this impaired JunB induction contributed to incomplete Th2 differentiation in ERK1^{-/-} mice.

Reduced CD44^{high} Th2 cell population in ERK1^{-/-} mice

Since we observed reduced proliferation in response to a recall antigen, we reasoned that the CD44⁺ CD4⁺ memory T-cell population might be affected in ERK1^{-/-} mice. We examined this population in spleen from immunized ERK1^{+/+} and ERK1^{-/-} mice. We observed an increased population of CD44^{high} CD4 T cells in ERK1^{+/+} immunized mice, which was significantly reduced in ERK1^{-/-} mice and was similar to that in control mice (Fig. 6A). ERK1^{-/-} mice have been shown previously to manifest exaggerated EAE (12, 13). Since CD44⁺ memory T cells are likely to be involved in EAE, we checked whether ERK1 differentially affected CD44 expression in Th1 and Th2 cells. To this goal, spleen CD4 T cells were induced *in vitro* to differentiate into Th1 and Th2 cells. Both Th1 and Th2 cells showed a spectrum of CD44 expression in ERK1^{+/+} and ERK1^{-/-} mice (Fig. 6B, top panel). Based on its bimodal distribution in Th1 cells, we arbitrarily divided CD44⁺ T cells into CD44^{high} and CD44^{low} populations. No difference was found in the CD44^{high} population between WT and ERK1-KO Th1 cells. Interestingly, the CD44^{high} population was reduced by 47% in Th2 cells from ERK1-KO mice when compared to ERK1-WT mice (Fig. 6B, bottom panel). Thus, ERK1^{-/-} deficiency seems to primarily affect the Th2 CD44^{high} but not the Th1 CD44^{high} population.

BIM and Mcl-1 expression is altered and CD4 T-cell survival is impaired in ERK1^{-/-} mice

T-cell apoptosis and survival are regulated by a number of molecules primarily belonging to the Bcl-2 family members. Among these molecules the proapoptotic molecule BIM (especially, BIM_{EL}; refs. 26, 27) and the prosurvival molecule Mcl-1 (28–30) are regulated by the ERK1/2 signaling pathway (31, 32). We examined the expression of these two molecules under basal conditions, in cultures without growth factors and after stimulation with anti-CD3 and -CD28 antibodies. We used spleen T cells from nonimmunized mice for these experiments. We observed a significantly higher expression of BIM in ERK1^{-/-} CD4 T cells on culture without growth factors (Fig. 7A). Mcl-1 expression was slightly higher at baseline in ERK1^{-/-} cells, which declined in the culture at a slightly faster rate as compared to the control cells (Fig. 7B). However, the differences were not significant. The ratio of BIM to Mcl-1 as a measure of proapoptotic and prosurvival factors clearly showed a stronger proapoptotic advantage in ERK1^{-/-} T cells (Fig. 7C). T-cell receptor (TCR) stimulation is known to induce Mcl-1 expression. Stimulation of T cells with anti-CD3/28 antibodies resulted in a significant increase in Mcl-1 expression in both study groups. However, the ERK1^{-/-} CD4 T

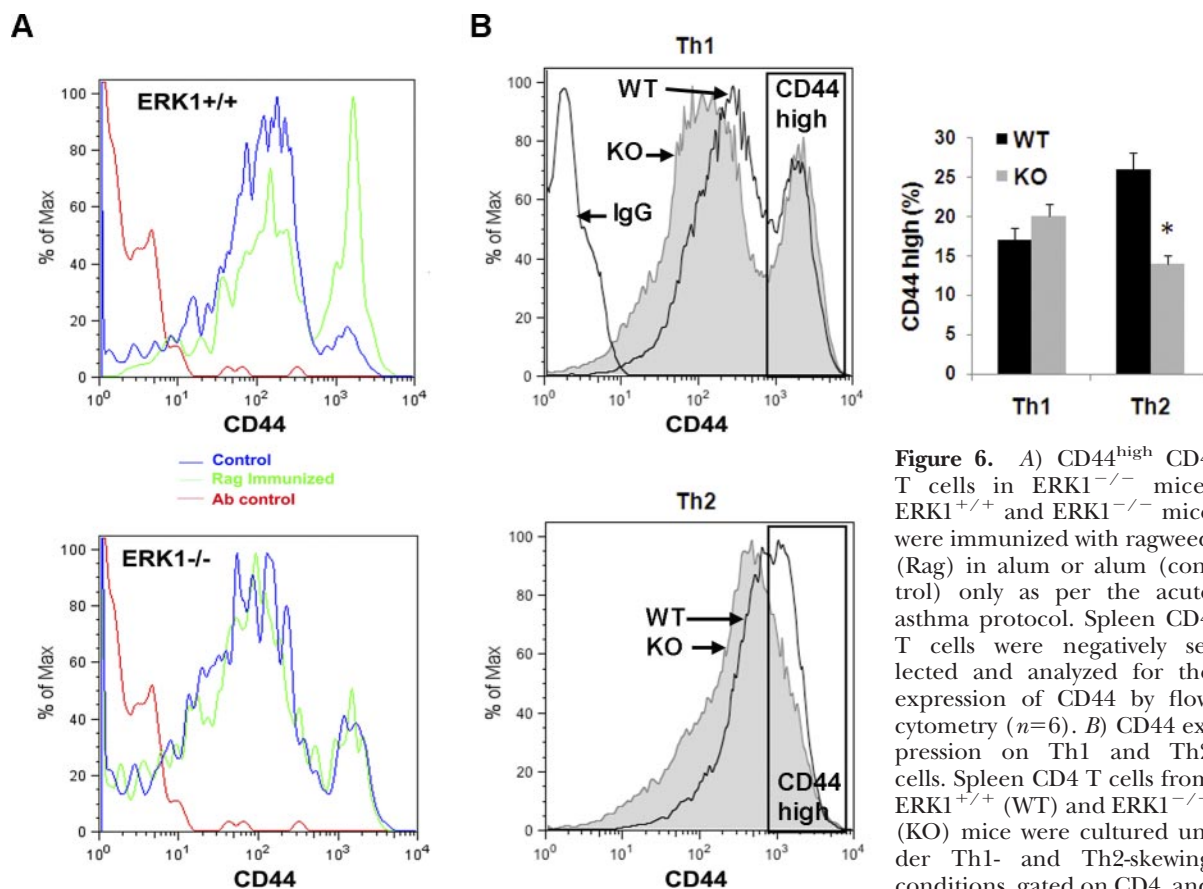


Figure 6. A) CD44^{high} CD4 T cells in ERK1^{-/-} mice. ERK1^{+/+} and ERK1^{-/-} mice were immunized with ragweed (Rag) in alum or alum (control) only as per the acute asthma protocol. Spleen CD4 T cells were negatively selected and analyzed for the expression of CD44 by flow cytometry ($n=6$). B) CD44 expression on Th1 and Th2 cells. Spleen CD4 T cells from ERK1^{+/+} (WT) and ERK1^{-/-} (KO) mice were cultured under Th1- and Th2-skewing conditions, gated on CD4, and analyzed for the expression

CD44 by flow cytometry ($n=4$). CD44^{high} population was arbitrarily defined (boxed area) based on its distribution in Th1 cells. Bar graph at right presents the percentage of CD44^{high} cells in Th1 and Th2 populations from ERK1 WT and KO mice. * $P = 0.04$; $n = 4$.

cells showed a significantly less increase in Mcl-1 expression (Fig. 7D).

Finally, we examined the biological relevance of altered BIM and Mcl-1 expression in ERK1^{-/-} CD4 T cells. We cultured spleen cells from *Aspergillus*-immunized mice (as per acute asthma protocol) in the presence of *Aspergillus* with and without recombinant FasL, and then examined live and apoptotic CD4 T cells on d 4. We observed a significant decline in survival in WT control cells cultured in the medium alone, which improved in the presence of the immunizing allergen (*Aspergillus*) but declined in the presence of FasL (Fig. 7E). The decline in survival was 2.3-fold higher in ERK1^{-/-} T cells in medium alone. The survival improved in the presence of the immunizing allergen, and the differences in survival between ERK1^{+/+} and ERK1^{-/-} cells were 1.7-fold. The difference further increased to 2.5-fold in the presence of FasL. These results in aggregate suggest that ERK1 is important for regulating specific survival factors in CD4 T cells. Since the CD44^{high} T-cell population is reduced in immunized ERK1^{-/-} mice, we induced Th2 differentiation *in vitro* and compared the survival of CD44^{high} and CD44^{low} population in a 4-d culture with the immunizing allergen. We observed a significantly higher loss of survival in

CD44^{high} but not in CD44^{low} Th2 cells in ERK1^{-/-} mice (Fig. 7F).

DISCUSSION

The role of the ERK1/2 signaling pathway in T-helper cell differentiation and the development of asthma has been investigated previously (2–4). Studies with a dominant negative Ras, an upstream activator of ERK1/2, have shown that the Ras-ERK1/2 pathway plays a critical role in Th2 differentiation (2) and the development of experimental asthma (33). ERK1/2 was shown to stabilize GATA-3 through inhibition of its ubiquitylation (34). Recently, ERK1 and ERK2 were shown to bind directly to the promoter of the IL-4 gene and facilitate the recruitment of the transcription factors (35). Pharmacologic inhibition of MEK1/2, the immediate upstream activator of ERK1/2, produced variable results on Th2 differentiation (2, 4). This finding is likely due to complexity of ERK1/2 function, which is determined by strength of activation, duration, subcellular localization, and interaction with other signaling molecules.

The level of ERK1/2 activation in cells is also determined by their expression level. Previous studies have

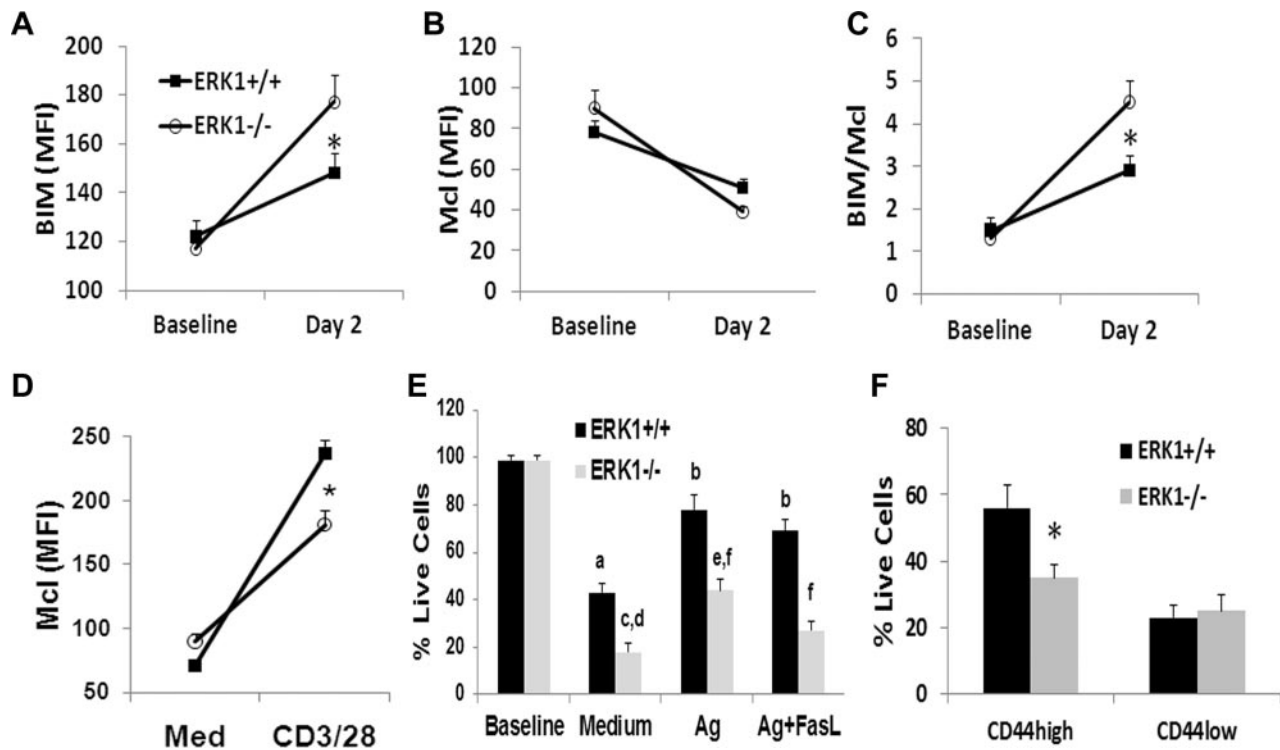


Figure 7. Expression of survival factors and T-cell survival in ERK1^{-/-} mice. A–C) Splenocyte cells from naive nonimmunized mice were cultured in medium with 10% FBS but without growth factors for 2 d. Expression of BIM (A) and Mcl-1 (B) on CD4 T cells was measured by flow cytometry at baseline and after culture, and ratio of BIM to Mcl-1 was plotted (C). * $P < 0.04$, $n = 4$. D) Induction of Mcl-1 in ERK1^{-/-} T cells. Spleen CD4 T cells from nonimmunized ERK1^{+/+} and ERK1^{-/-} mice were cultured with medium or anti-CD3/CD28 antibodies (2 μ g/ml) for 2 d; expression of Mcl-1 was measured by flow cytometry on d 2. * $P < 0.04$, $n = 3$. E) Survival of ERK1^{-/-} CD4 T cells. Splenocytes from *Aspergillus*-sensitized mice were cultured in medium plus 10% FBS alone (medium), *Aspergillus* (Ag; 3 μ g/ml), or *Aspergillus* plus recombinant FasL (10 μ g/ml) for 5 d. Live and dead cells were analyzed following staining for CD4, annexin V, and propidium iodide by flow cytometry. ^a $P = 0.01$ vs. baseline; ^b $P = 0.04$ vs. medium; ^c $P = 0.001$ vs. baseline; ^d $P = 0.04$ vs. ERK1^{+/+}; ^e $P = 0.04$ vs. medium; ^f $P < 0.04$ vs. ERK1^{+/+}. F) Th2 CD44⁺ cell survival. *Aspergillus*-sensitized spleen CD4 T cells were cultured under Th2-skewing conditions for 7 d and then stimulated with anti-CD3 antibodies for 2 d. Live and dead cells were analyzed by flow cytometry following staining with an anti-CD44 antibody and propidium iodide. CD44^{high} and CD44^{low} cell populations were defined as in Fig. 5. * $P = 0.04$, $n = 3$.

primarily focused on phosphorylation and enzymatic activation of ERK1/2 in T cells following cytokine stimulation. We now show that T-helper cell-inducing cytokines alter the expression level of ERK1 and ERK2. As a result, ERK1 and ERK2 undergo dynamic changes during T-helper cell differentiation. Cytokines are known to induce post-translational modifications of signaling molecules. Some of these modifications (ubiquitylation, SUMOylation) alter protein stability and thereby, change the expression level of the protein. STAT1 expression level changes in cells following their stimulation (36, 37). We speculate that a similar alteration contributes to the variable expression of ERK1 and ERK2 in T cells.

We have shown that ERK1 plays a nonredundant role in Th2 differentiation and the development of experimental asthma. In the absence of ERK1, the production of Th2 cytokines and allergen-specific IgE is reduced, and allergen-induced airway inflammation and other features of experimental asthma are severely inhibited. We have identified 3 mechanisms for this phenotype. First, JunB expression is reduced and short-lived due to ERK1 deficiency. JunB is known to play a critical role in

Th2 differentiation (24, 25). JunB directly binds to the IL-4 promoter and induces its transcription (24). JunB deficiency in T cells leads to impaired Th2 differentiation and asthma phenotype (25). Thus, the reduced expression of JunB in ERK1^{-/-} T cells is likely to impair Th2 differentiation. Second, ERK1-deficient Th2 but not Th1 cells are unable to express high levels of CD44. The high-level expression of CD44 is a hallmark of memory T cells (38). Thus, ERK1 deficiency leads to reduced Th2 memory formation. Finally, ERK1 deficiency predisposes CD4 T cells to heightened expression of BIM under growth factor-deprived conditions and reduced expression of Mcl-1 on stimulation. As a result, the cells undergo apoptosis at a faster rate on stimulation with the immunizing allergen and in the presence of FasL, a condition that is likely to be present during resolution of acute inflammation (39).

CD44 is a MEK-ERK1/2-regulated gene (40, 41). Inhibition of MEK1/2 blocks CD44 induction. Note that ERK2 deficiency leads to the reduced generation of antigen-specific CD44⁺ CD8 T cells (42). Although we did not study antigen-specific CD44⁺ T cells, we observed reduced CD44^{high} CD4 T cells in ERK1^{-/-}

immunized mice. ERK2, but not ERK1, was shown previously to regulate survival of activated CD8 T cells. Like ERK1^{-/-} CD4 T cells in our study, ERK2-deficient CD8 T cells express higher levels of BIM, which leads to their shortened survival (41). ERK1 and ERK2 are known to regulate BIM stability by phosphorylation, which leads to its degradation (31, 32). A cell-specific utilization of ERK1 and ERK2 is apparent. CD4 (especially Th2) and CD8 T cells preferentially utilize ERK1 and ERK2, respectively, for regulation of CD44 and BIM expression.

Although no reports focus on the effects of ERK1 deficiency on the manifestations of a Th2 disease, two groups investigated the effect of ERK1 deficiency on EAE (12, 13). Both studies reported heightened production of Th1 cytokines and exaggerated EAE in ERK1-deficient mice. Our IFN- γ data are in agreement with these studies. Agrawal *et al.* (13) also reported heightened IL-12 production by dendritic cells and increased serum level of IgG2b, which is consistent with the Th1 bias. The researcher also reported reduced T-cell proliferation, which is in agreement with the original report of Pagès *et al.* (8) and with our study but not with two other studies (11, 12). The researchers also reported reduced production of IL-5 in response to a recall antigen, which we confirmed in our model. Interestingly, anti-CD3/28 antibody-induced production of Th1 and Th2 cytokines was unchanged in the Agrawal study (13). Using the same KO strain and the same protocol of recall antigen stimulation, Nekrasova *et al.* (12) did not find any difference in T-cell proliferation and Th2 cytokine production. A tendency toward lower IL-4⁺ and IL-5⁺ T cells in the latter study should be noted, but differences did not reach significance due to the large data spread. Although the reason for these discrepancies is unknown, possible mechanisms include heterogeneity of the genetic background (the degree of backcrossing of sv129 to C57B/6) and differences in the experimental protocols.

The production of Th2 cytokines was reduced but not completely gone in the ERK1^{-/-} mice. This finding was reflected by a proportionate reduction in the levels of allergen-specific IgE antibody in these mice. However, the effect on the asthma phenotype was more remarkable. We speculate that this null phenotype results from a number of factors. The increased apoptosis of CD4 T cells and the reduced size of the CD44^{high} T-cell population are likely contributors. The ratio of activated ERK1/2 to activated p38 MAPK is important for determining the cytokine expression profile by dendritic cells (43, 44). Increased ERK1/2 favors heightened IL-6 and IL-10 and reduced IL-12 production, which results in a Th2 bias. Thus, ERK1 deficiency could alter this ratio in dendritic cells and affect differentiation. ERK1 is usually expressed at a lower concentration than ERK2 in many cell types; *e.g.*, lymphoid and myeloid cells. This finding is not the case in the lung tissue. The expression of ERK1 seems to be higher than that of ERK2 in the lung tissue (Fig. 1G). The absolute concentration of signaling molecules,

especially enzymes, plays a critical role in the level of their activation in the cell (45). Thus, ERK1 is likely to play an important role in many airway tissue cells. The complete inhibition of AEH in ERK1^{-/-} mice underscores this importance. Epithelial cells are known to elaborate important immunoregulatory molecules, such as thymic stromal lymphopoietin (TSLP; refs. 46, 47), and IL-25 (48). Thus, ERK1 deficiency in epithelial cells may affect the production of these important Th2-inducing cytokines and secondarily affect the Th2 phenotype. A recent report showed that ERK1/2 regulated airway smooth muscle tone by inhibiting β -adrenoreceptor-mediated relaxations, rather than an initiation of contraction (49). Thus, an absence of ERK1 in airway smooth muscle may have resulted in heightened relaxation and contributed to the lack of airway hyper-reactivity.

We have also observed a dichotomous effect on serum total IgE *vs.* allergen-specific IgE. ERK1-null mutation did not affect serum total IgE despite inhibiting Th2 differentiation. Although Th2 cytokines are the major regulators of IgE production, they are not the only ones. Many non-Th2 genes regulate serum total IgE (50–53). We speculate that the production of allergen-specific IgE is more sensitive to a reduction in Th2 cytokines than total IgE.

In summary, we have shown that ERK1 is important for Th2 differentiation, down-regulation of BIM, and the survival of CD44^{high} Th2 cells. The foregoing processes are impaired in ERK1-deficient mice. As a result, the mice fail to develop experimental asthma. FJ

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