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Polar Opposites: Erk Direction of CD4 T Cell Subsets

Chiu-Fang Chang,* Warren N. D’Souza, † Irene L. Ch’en,* Gilles Pages,‡ Jacques Pouyssegur,‡ and Stephen M. Hedrick*

Effective immune responses depend upon appropriate T cell differentiation in accord with the nature of an infectious agent, and the contingency of differentiation depends minimally on TCR, coreceptor, and cytokine signals. In this reverse genetic study, we show that the MAPK Erk2 is not essential for T cell proliferation in the presence of optimum costimulation. Instead, it has opposite effects on T-bet and Gata3 expression and, hence, on Th1 and Th2 differentiation. Alternatively, in the presence of TGF-β, the Erk pathway suppresses a large program of gene expression, effectively limiting the differentiation of Foxp3+ regulatory T cells. In the latter case, the mechanisms involved include suppression of Gata3 and Foxp3, induction of Tbx21, phosphorylation of Smad2,3, and possibly suppression of Socs2, a positive inducer of Stat5 signaling. Consequently, loss of Erk2 severely impeded Th1 differentiation while enhancing the development of Foxp3+ induced T regulatory cells. Selected profiles of gene expression under multiple conditions of T cell activation illustrate the opposing consequences of Erk pathway signaling. The Journal of Immunology, 2012, 189: 721–731.

The two Erk isoforms appear to act additively in cell cycle control (4); a loss of Erk1 (Mpk3) function reveals few phenotypic changes, whereas even a hemizygous loss of Erk2 (Mpk1) causes embryonic lethality in some strains of mice (5–8). The origin of Erk2 loss-of-function lethality was due, in part, to failure of the polar trophectoderm cells to proliferate (6). In several other physiological models there are differential requirements for Erk1 and Erk2 that do not correlate with dramatic differences in the relative amounts of Erk1 and Erk2 expressed (9).

The strength of signal through the TCR influences the outcome of differentiation; a strong and prolonged Erk signal gave rise to Th1 cells, whereas weak Ag stimulation or an attenuation of Erk gave rise to IL-2–dependent Stat5 phosphorylation, Gata3 expression, and IL-4 production (10, 11). Alternatively, an inhibitor of the Erk kinases, Mek1,2, was found to enhance Foxp3 expression and regulatory T cell (Treg) differentiation (12, 13). In this study, we used genetic ablation of Erk1 or Erk2 to study the T cell-intrinsic role of this pathway in proliferation, expansion, and differentiation of four types of CD4 effector T cells. We find that Erk2 is central to the contingencies governing the differentiation of Th1 cells and iTregs, and this is reflected in its effects on the subset-characteristic programs of gene expression. In particular, we show that a loss of Erk2 caused a large-scale increase in gene expression, specifically under conditions of TGF-β signaling.

Materials and Methods
Mice and viral infection
CreER12, Erk1<sup>−/−</sup>, Erk2<sup>−/−</sup> allele mice were described previously (8, 14, 15). Deletion ofloxP-flanked alleles in Erk2<sup>−/−</sup> CreER12 mice was induced by i.p. injection of 2 mg tamoxifen every day (Sigma) for six consecutive days, and mice were analyzed 2 d later. Where indicated, mice were infected i.p. with 2 × 10<sup>6</sup> PFU lymphohytic choriomeningitis virus (LCMV Armstrong). Bone marrow chimeras were made by reconstitution of lethally irradiated (10 Gy) Rag1<sup>−/−</sup> mice with T cell-depleted bone marrow cells from wild-type (WT; CD45.1+) or Erk2<sup>−/−</sup> CreER12 (CD45.2+) mice separately or mixed at a 1:1 ratio. Mice were analyzed 8 wk after reconstitution. For Treg induction, Erk2<sup>−/−</sup> CreER12<sup>−/−</sup> mice were crossed with Smarta transgenic mice (TCR transgenes specific for HA<sup>B</sup> bound with LCMV glycopetide 61–80) (16). Animal work was performed according to University of California, San Diego (UCSD) guidelines.

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Cell culture
CD4 T cells (purity > 90%) from lymph nodes and spleens were MACS purified (Miltenyi Biotec) by either positive selection for CD4 or by depletion using biotin-labeled B220, CD8, CD25, CD69, DX5, and MHC class II with streptavidin microbeads. T cell proliferation was stimulated with plate-bound anti-CD3 (5 ng/ml), with or without anti-CD28 added to the culture (1 μg/ml), as described (17). Where indicated, IL-2 (10 U/ml), PMA (20 ng/ml), and ionomycin (100 ng/ml) were added. For T cell differentiation, cells were stimulated with anti-CD3 and anti-CD28 plus IL-12 (10 ng/ml), IL-2 (50 U/ml), and anti-IL-4 (for Th1); IL-4 (10 ng/ml), IL-2, and anti–IFN-γ (for Th2); and IL-6, TGF-β, anti–IFN-γ, and anti–IL-4 (for Th17). To generate iTregs, CD4 cells were stimulated with anti-CD3, anti-CD28, TGF-β, and IL-2 (10 U/ml) in the presence or absence of anti-IL-4 and anti–IFN-γ. Cytokines were measured in the supernatant by ELISA (eBioscience).

Flow cytometry
Lymphocytes were explanted and stained with Abs specific for CD62L, CD44, CD4, and CD8. For the intracellular staining of Foxp3, cells were fixed and permeabilized with a Foxp3 buffer set (eBioscience). For Erk2 or phospho-Smad2,3 staining, cells were fixed with 2% paraformaldehyde and permeabilized with Perm III buffer (BD Biosciences). Cells were stained with Erk2- (Santa Cruz) or phospho-Smad2,3-specific Abs (Cell Signaling Technology), followed by goat anti-rabbit IgG-PE (Southern Biotech). For intracellular cytokines, cells were fixed and permeabilized with the BD Cytofix/Cytoperm Solution Kit (BD Biosciences) and stained for IFN-γ, IL-4, or IL-17 (eBioscience). H2-A b tetramers loaded with LCMV gp66–74 (dLck-Cre T cells) and RMA-S (WT or Erk2-deficient CD4 T cells) were visualized with streptavidin microbeads. T cell proliferation was stimulated with plate-bound anti-CD3 (5 ng/ml), with or without anti-CD28 added to the culture (1 μg/ml), as described (17). Where indicated, IL-2 (10 U/ml), PMA (20 ng/ml), and ionomycin (100 ng/ml) were added. For T cell differentiation, cells were stimulated with anti-CD3 and anti-CD28 plus IL-12 (10 ng/ml), IL-2 (50 U/ml), and anti-IL-4 (for Th1); IL-4 (10 ng/ml), IL-2, and anti–IFN-γ (for Th2); and IL-6, TGF-β, anti–IFN-γ, and anti–IL-4 (for Th17). To generate iTregs, CD4 cells were stimulated with anti-CD3, anti-CD28, TGF-β, and IL-2 (10 U/ml) in the presence or absence of anti-IL-4 and anti–IFN-γ. Cytokines were measured in the supernatant by ELISA (eBioscience).

Statistics
Prism 4.0c software (GraphPad software; San Diego, CA) was used for Student t test analyses. The p values are indicated in the figure legends.

Results
Erk2-deficient CD4 T cells from Erk2fl/fl CreER T2 mice
We previously analyzed the effects of Erk2 deletion on the function of CD8+ T cells using a distal Lck promoter-Cre transgene (dLck-Cre); however, this transgene caused deletion in only 80% of CD4 T cells (22). To analyze the role of Erk2 signaling in peripheral CD4 T cells, we crossed Erk2fl/fl mice to CreER T2 mice, and deletion of theloxP-flanked exons of Erk2 was induced by tamoxifen (Fig. 1A) (15). By PCR, Western blot, and flow cytometry, the deletion of Erk2 was uniform within the population and virtually complete. Tamoxifen-treated Erk2fl/fl CreER T2 mice are referred to as Erk2−/−. Erk2 deletion led to reduced thymic cellularity, preferentially affecting the CD4+CD8+ population (8); however, Erk2−/− mice displayed similar proportions and numbers of CD4 and CD8 T cells within the secondary lymphoid tissues (Supplemental Fig. 1A). Similarly, naive and effector/memory cell populations based on CD44 and CD62L expression were also unchanged (Supplemental Fig. 1B).

Costimulation can replace the requirement for Erk2 in T cell proliferation
Consistent with our analysis of CD8 T cells (22), Erk2−/− CD4 T cells, stimulated with anti-CD3 in the absence of added costimulation, accumulated at the undivided stage (Fig. 1B) and displayed incomplete induction of CD25 and CD44 (Fig. 1C). Higher concentrations of anti-CD3 did not induce proliferation (data not shown). However, the defect in Erk2−/− CD4 T cell proliferation was rescued by the addition of a CD28-mediated costimulatory signal (Fig. 1B, 1C). In fact, Erk2−/− T cells appeared to undergo more rounds of division compared with WT T cells; one explanation is suggested by the Erk2 dependence of the cyclin-dependent kinase inhibitor, p21Cip1/Waf1, as shown below. Contrary to our expectations, similar results were obtained using T cells deleted for both Erk1 and Erk2 (data not shown). These results imply that a signaling pathway downstream of the costimulatory receptor CD28 can replace a requirement for Erk activation in TCR-mediated cell cycle progression.

Similar to CD8 T cells, proliferation and survival were only partially rescued by the addition of IL-2 (Supplemental Fig. 1C) (22). However, Erk2−/− CD4 T cells proliferated to the same extent as did WT T cells upon PMA and ionomycin stimulation (Supplemental Fig. 1D). Finally, in marked contrast to the results with Erk2−/− T cells, there was no effect of Erk1 deletion on CD4+ T cell proliferation in response to TCR-mediated stimulation, with or without costimulation (Supplemental Fig. 2A).

Erk is required for Th1, but not for Th2 or Th17, development in vitro
Because T cells can proliferate in the absence of Erk2, we were able to examine the role of Erk1 or Erk2 in Th1, Th2, or Th17 differentiation. There were no differences observed for WT and Erk1−/− cells when activated under the different Th (Th1, Th2, Th17) conditions (Supplemental Fig. 2B–D) (23). In contrast, Erk2−/− CD4 T cells displayed normal proliferation but a survival defect when activated under Th1- and Th2-polarization conditions (Fig. 2A). This survival defect may be the result of increased levels of the proapoptotic protein Bim that were observed in Erk2−/− cells relative to WT cells (Fig. 2B).

We further examined whether the loss of Erk2 affected the differentiation of Th1, Th2, and Th17 cells, as measured by the intracellular production of IFN-γ, IL-4, or IL-17 (Fig. 2C). The proportion of IFN-γ-producing Erk2-deficient CD4 T cells and the amount of IFN-γ levels were reduced compared with WT cells, and there was a 4-fold decrease in IFN-γ levels measured in the
FIGURE 1. Erk2−/− T cells require costimulation for cell cycle progression. (A) Purified CD4 T cells from lymph nodes and spleen were analyzed for the presence of Erk2 by PCR, Western blotting, and flow cytometry. (B) Purified WT or Erk2−/− CD4 T cells were stimulated in vitro. CFSE profiles were analyzed for CD4+ T cells. (C) T cells (WT, Erk2−/−) were analyzed for CD25 and CD44 expression on day 3 following the stimulation. Similar results were found on day 4. The data are representative of three or more independent experiments.

culture supernatant (Fig. 2D). Despite this defect, T-bet and p-Stat5 were induced in Erk2−/− T cells to an extent equivalent to WT T cells (Fig. 2G). In addition, under Th1 conditions, Erk2−/− CD4 T cells exhibited higher Gata-3 expression, consistent with increased IL-4-independent Th2 differentiation found under conditions of Erk attenuation (10, 11). In contrast, the amount of p-Stat5 was reduced (Fig. 2G). Under optimal, polarizing Th2 conditions, there was a trend toward increased Th2 differentiation, but the difference did not reach significance given the number of trials (Fig. 2C, 2E). There was no difference in Th17 differentiation in the absence of Erk2 (Fig. 2C, 2F).

Impaired Th1-driven viral response in Erk2-deficient mice

To examine differentiation of Th1 cells in vivo, we infected WT, Erk1−/−, or Erk2−/− mice with LCMV Armstrong and assessed the number of IFN-γ–producing T cells at day 8 postinfection. Erk1−/− and WT mice displayed similar CD4 responses, whereas there was a substantial reduction in the proportion and the absolute cell number of IFN-γ–producing cells in Erk2−/− mice (data not shown). To determine whether this effect was T cell intrinsic, Rag1−/− mice were reconstituted with bone marrow cells from WT or Erk2ft CreERT2 mice or an equal mixture of both. Eight weeks postreconstitution, the mice were treated with tamoxifen and challenged with LCMV. The number of Ag-specific CD4 T cells, as measured by MHC class II gp66–77 tetramers or antiviral IFN-γ–producing TH1 cells, was dramatically reduced in the mice that were reconstituted with Erk2ft CreERT2 cells (Fig. 3). Even within the mixed bone marrow chimeras, the population of Ag-specific Erk2−/− T cells was reduced compared with WT T cells in the same animals. The number of tetramer+ cells was approximately equivalent to the number of IFN-γ+ T cells; thus, the absence of Erk2 did not redirect differentiation, but it either diminished Th1 differentiation or survival.

Increased iTreg differentiation in absence of Erk2

The role of Erk in the differentiation of iTregs in culture was examined. Erk2−/−, but not Erk1−/−, T cells generated increased proportions and numbers of CD25+Foxp3+ iTregs (Fig. 4A, 4B, data not shown). iTregs were also induced in vivo by transferring naive Smarta CD4 T cells (depleted of CD4+CD25+ cells) and immunizing mice with LCMV gp61–80. Under these conditions, there was an increased proportion of Erk2−/− Smarta T cells that converted to Foxp3+ cells (Fig. 4C). In addition, there was an increase in the proportion of Foxp3+ natural Tregs present in lymphoid organs of Erk2ft Cd4Cre mice (data not shown).

To determine whether Erk2−/− Tregs were functional, we used cell culture and in vivo measures of Treg activity. As shown, CD4+ CD25+ cells from Erk2−/− mice were at least as effective as WT Tregs in their ability to inhibit T cell proliferation (Fig. 4D). Erk2−/− Tregs also suppressed weight loss in an inflammatory bowel disease model to the same extent as did WT Tregs (Fig. 4E) (24). We can conclude that Erk2−/− T cells more readily differentiate into iTregs, and Erk2−/− natural Tregs exhibit at least equivalent function compared with those from WT mice.

Erk2 dependence of Dnmt expression and Smad signaling

The regulation of Foxp3 depends importantly on methylation, such that, in the absence of Dnmt1, Foxp3 is efficiently expressed in activated CD4 and CD8 T cells (25–27). Thus, we considered the possibility that enhanced iTreg induction in Erk2−/− T cells was the result of reduced Dnmt1 expression. Analysis by qPCR indicated that Dnmt1 expression is progressively induced with time, and the induction is partially Erk2 dependent (Fig. 5A) at 2 d in culture. As shown below, Foxp3 is induced by 1 d, and its expression is enhanced in Erk2−/− cells. Thus, simple regulation of Dnmt1 mRNA does not appear to explain the TGF-β–induced Foxp3 induction.

Another potential mode of regulation is direct inhibition of TGF-β signaling through the inhibitory phosphorylation of Smad2.
and Smad3 at their linker regions, as shown by the analysis of Mekk2,3-deficient T cells (28). Because Mekk2,3 are upstream of all of the MAPKs, the possibility exists that Erk constitutes the primary mediator of this effect. Smads are activated by TGF-β–mediated phosphorylation at the C-terminal SXS motif; this, in turn, causes nuclear localization and downstream gene activation.

As a means of detecting activated Smad2 and Smad3, we probed the amount of SXS phosphorylation of Smad2/3 both by intracellular fluorescence staining and immunoblotting. The results showed an increased induction of pSmad2 in Erk2-deficient cells compared with WT cells by 30 min, and this difference was enhanced at 2–3 d after stimulation (Fig. 5B, 5C). We conclude that

**FIGURE 2.** Erk2−/− CD4 T cells display impaired survival and polarization to the Th1 subset. (A) CD4 T cells were cultured under different conditions for 3 d. CFSE profiles were analyzed as in Fig. 1 (representative of three experiments). (B) Purified CD4 T cells from WT and Erk2−/− mice were cultured for Th1 polarization. Cell lysates were analyzed by Western blotting, and the amounts of BimEL and BimL/S were normalized to ZAP70 (representative of three separate experiments). (C) Cells were cultured in polarizing conditions, and CD4 T cells are shown (representative of three experiments). (D) The percentage of IFN-γ+ T cells and the amount of IFN-γ from cultures of restimulated Th1 cells (mean ± SEM from three experiments). Percentages of IL-4–producing (E) and IL-17–producing (F) cells (mean ± SEM from three experiments). (G) T cells were analyzed for T-bet, Gata-3, p-Stat1, and p-Stat5 at day 3. Isotype (mouse IgG1) control was used for T-bet and Gata-3 staining; goat anti-rabbit PE was used for the p-Stat–staining control. Representative of three experiments. *p < 0.05, **p < 0.001.
inactivation or removal of Erk2 promotes greater Smad signaling, which, in turn, enhances Treg development.

iTreg gene expression is suppressed by Erk2

To further understand the role of Erk2 in T cell differentiation, we performed microarray analyses of WT and Erk2–/– T cells in eight conditions. Basic analyses revealed that the data set reflects Erk2-dependent, T cell-specific mRNA expression under multiple stimulatory and polarizing conditions (Supplemental Fig. 3A). To identify a set of genes dependent on Erk2 for expression, we averaged the ratios (WT/Erk2–/–) across all eight conditions. There were 49 Erk2-dependent probes (mean ratio ≥ 1.5), and the highest 14 are shown as a correlated heat map (Supplemental Fig. 3B). These include 3/3 Mapk1 (Erk2); 2/2 Dusp6, an Erk dual-
stimulated under iTreg conditions for 30 min. Cell lysates were analyzed by Western blotting at the indicated time points. ZAP70 was used as loading.

transcript under 1-d iTreg conditions identified a small number of probes for which Erk2 was suppressed by Erk2, 26 dependent on Erk2) for Th0 and 77 probes suppressed by Erk2, 35 dependent on Erk2) for Th1. In contrast, the same comparison under 1-d iTreg conditions identified 42 suppressed by Erk2, 35 dependent on Erk2) for Th1. In contrast, the same comparison under 1-d iTreg conditions identified 42 suppressed by Erk2 (mean ratio 1.5 or 0.67) (Supplemental Fig. 3C). These include Ccr8; Ebi2, an oxytostrepspecific nuclear receptor controlling T and B cell migration (30); Gata3; and Klf2, a Foxo1 target controlling T cell homing (31).

We conclude from these analyses that the data set reflects Erk2-dependent T cell-specific mRNA expression under multiple stimulatory conditions.

Scatter plots comparing Erk2−/− with WT values under 1-d Th0 or Th1 conditions identified a small number of probes for which Erk2−/− was >1.5 or <0.67 compared with WT: 71 probes (45 suppressed by Erk2, 26 dependent on Erk2) for Th0 and 77 probes (42 suppressed by Erk2, 35 dependent on Erk2) for Th1. In contrast, the same comparison under 1-d iTreg conditions identified 643 probes, a >8-fold increase over the number identified under Th0 or Th1 conditions (544 suppressed by Erk2, 99 dependent on Erk2) (Fig. 6A). Using Venn diagrams, we graphed the number of overlapping or unique probes that were suppressed by or dependent on Erk2 under each of the polarizing conditions. The Erk2-suppressed changes in gene expression specific to iTregs (503 probes) constituted, by far, the largest category (Fig. 6B).

This large-scale increase in gene expression at 1 d in Erk2−/− T cells could be consistent with a hypomethylated genome as a result of reduced Dnmt1; however, the qPCR data showed no Erk2-dependent difference in Dnmt1 expression at this time point (Fig. 5A). The array data showed that, of the Dnmt isoforms assessed, only Dnmt1 and Dnmt3b were expressed over background, and neither was affected by the addition of TGF-β (Fig. 6C): equivalent induction was seen at 1 d under Th0, Th1, and iTreg conditions of stimulation. Moreover, the effects of Erk2 deletion on Dnmt1 and Dnmt3b expression were small compared with the magnitude of induction. We conclude that Erk2 signaling constitutes an important suppressor of gene expression in the presence of TGF-β, although the extent to which this results from Dnmt1 regulation is unknown (discussed below).

Thus, the iTreg dependence on Erk2 provided a tool to more finely characterize a program of iTreg-specific genes. We identified those genes that were induced in 1-d iTreg conditions, but not under Th0 or Th1 conditions, and were suppressed by Erk2 (Fig. 6D). This algorithm identified 45 unique probes, including all 3 probes specific for Foxp3 and both probes specific for the Treg-specific neuropilin-1 (Nrp1). Gpr83, Ecm1, Cmtm7, Nkg7, Socs2, and Glrx were previously found to be iTreg specific (32); of these, we also identified Gpr83 and Nkg7. Socs2 was strongly (>10-fold) induced in Th0, Th1, and iTreg conditions (see below) and, thus, was not iTreg specific (Fig. 7D). Ecm1 was induced at 3 h but not at 1 d. Cmtm7 and Glrx were relatively unchanged across all conditions (data not shown).

Other genes identified in this subset that may be involved in immune function included Dscr1l2, which inhibits calcineurin-dependent transcriptional responses; Ctsw (cathepsin W), which functions in T cell cytolytic activity; Faim3, a caspase-8 inhibitor; Rcan3, which binds and inhibits calcineurin A; Tnfrsf1b (Tnfr1); and Tnfrsf9 (4-1BB) (Fig. 6D). We also analyzed gene expression at 3 h postactivation, with or without the addition of TGF-β. The rationale was that gene expression directly affected by TGF-β signaling would be induced early, yet none of the probes specific to iTreg induction at 1 d were already induced at 3 h in the presence of TGF-β. All of the probes induced at 3 h with TGF-β were also induced in its absence under Th0 conditions (Fig. 6D). Likewise, we examined whether stimulation with TGF-β for 1 or 3 h after 1 d of Th0 activation would induce a subset of the signature genes, and we identified five of the genes listed in Fig. 6D. These included Ccr8, Ctsw, Dscr1l2, Emp1, and Edod1. Foxp3 and Nrp1 were not induced under these conditions. We conclude that the iTreg program of gene expression becomes elaborated in naive T cells between 3 h and 1 d post-TGF-β stimulation.

A separate analysis of the array data based on an unbiased hierarchical clustering of the entire data set (Pearson correlation, average linkage) was carried out, and a heat map of a cluster containing Foxp3 was selected (Supplemental Fig. 4A). Although there is a large overlap in the genes identified using the algorithm.

**FIGURE 5.** Erk2 regulates Dnmt1 and phosphorylation of Smad2. (A) WT or Erk2−/− cells from individual mice were stimulated under iTreg conditions for 0–5 d. The mRNA samples were analyzed by real-time quantitative PCR for Dnmt1 in duplicate and normalized to the Ppia gene (± SEM) (WT, n = 3; Erk2−/−, n = 3). The data are representative of two separate experiments. (B) WT or Erk2−/− naive CD4 T cells were stimulated under iTreg conditions and analyzed for pSmad2,3 expression by flow cytometry (representative of three experiments). (C) Purified CD4 T cells from WT and Erk2−/− mice were stimulated under iTreg conditions for 30 min. Cell lysates were analyzed by Western blotting at the indicated time points. ZAP70 was used as loading control. Plots show the phospho-Smad2 density normalized to Smad2 or ZAP70. Relative expression level was normalized by WT day 0 expression, as 1.
described above (e.g., Nrp1), unique genes were also identified. Notably, this list includes Ccr6, encoding a chemokine receptor that plays a role in inflammatory bowel disease (33), and three transcription factors not previously known to play a role in Treg differentiation: Cux1, Stat5, and Zfhx3 (discussed below).

**Gene expression associated with Th0, Th1, and Th2 polarization**

For each of the T cell subsets (Th1, Th2, Th17, and iTreg), there is a transcription factor, which, if not a lineage-specification factor, is characteristic and essential for function. As such, we charted the profiles of T-bet (Tbx21), Gata3, Rorγ (Rorc), and Foxp3 (Fig. 7A, Supplemental Fig. 4B) over the conditions tested. Remarkably, the profiles for Tbx21 (T-bet) and Gata3 were mirror images of one another, both for conditions of induction and Erk2 dependence. For all eight conditions, Tbx21 was dependent on Erk2, and Gata3 was suppressed by Erk2. This graphically illustrates the polarity of these two T cell subsets and reinforces the Erk2 dependence of Th1 differentiation contrasted with enhanced Gata-3 expression and Th2 differentiation found under conditions of Erk2 attenuation (Figs. 2C–G, 3A, 3B) (11). Equally striking was the profile of Foxp3. It was only induced under iTreg conditions for 1 d; consistent with increased induction of iTregs from Erk2-deficient precursors, its expression was further enhanced 2-fold by the de-
letion of Erk2 (Fig. 7A, Supplemental Fig. 4A). In addition, under iTreg conditions, Erk2 deletion attenuated Tbx21 and enhanced Gata3, gene expression predicted to further promote iTreg differentiation (34, 35). Rorc \((Rorc)\) expression was not detected.

This analysis was extended by an examination of the signature cytokines for the different T cell subsets (Fig. 7B). IFN-\(\gamma\) was only induced under Th1 conditions, but it was not appreciably dependent on Erk2. Because the mean fluorescence intensity of intracellular IFN-\(\gamma\) was higher in WT versus Erk2-deficient T cells (Fig. 2C), there may be a posttranscriptional Erk2-dependent enhancement. IL-4 was induced at 3 h under Th0 conditions, but it was close to baseline levels by 1 d. TGF-\(\beta\), sometimes characteristic of iTregs, was induced at 3 h but not at 24 h, and IL-10 was not induced under any conditions. These data reveal that there is not a strict correlation between the lineage-characteristic transcription factors and subset-specific cytokines; this underscores the notion that T cell differentiation is not governed by the expression of a single, signature transcription factor (36, 37).

In addition, we analyzed those genes that are dependent on Erk2 and induced under different conditions of T cell activation. Twenty-one unique probes were induced under Th0 conditions at 1 d and were dependent on Erk2 (Fig. 7C). This set contained Egr2, Ccl1.
dependent on cytokines and costimulation, strong activation can favor a Th1 response, whereas weaker activation favors either a Th2 response or iTreg differentiation (36, 41). In this study, we show that this strength of signaling contingency is mediated, in part, through Erk2. Although we found no phenotypic effect of Erk1 deletion, the effects of Erk2 deletion mimicked a weak signal, inhibiting Th1 differentiation while promoting iTreg differentiation. In addition, gene-expression studies clearly illustrated the polarity of Th1 versus Th2 differentiation based on the presence or absence of Erk2. The origin of Erk1 and Erk2 differences is unknown, but their differential association with upstream kinase-signaling complexes or localization within the cell may provide important information with respect to the signaling important for T cell differentiation.

Under conditions supporting Th1 differentiation both in culture and in response to LCMV infection, the loss of Erk2 resulted in a decreased accumulation and percentage of IFN-γ+ cells. This is consistent with a study showing that impaired H-ras and K-ras resulted in decreased IFN-γ expression (42). Reduced Th1 survival in the absence of Erk2 correlated with the increased expression of all three forms of Bim, including the highly apoptogenic Bim, form (43). From the array data, this is due, at least in part, to differences in RNA expression, as we showed for CD8 T cells (22). In addition, Bcl-2 expression was reduced upon T cell activation, whereas Bcl-XL was increased (44), and these changes were dampened in the absence of Erk2. The Bcl2a1 paralogs, like Bim, were strongly induced at 3 h, but their expression decreased at 1 d postactivation. Bcl2a1d has a role in early thymocyte survival, and it is a direct target of NF-κB (45). Its expression pattern and promoter sequences suggest that it is also a direct target of Egr-2; however, the role of Bcl2a1d in mature T cell activation is unknown. The cell survival defect in Th1 cells was not universal, because there was no Erk2-associated difference in survival or differentiation of IL-17+ cells.

Under optimal conditions supporting Th2 differentiation, there was also reduced survival in the absence of Erk2, and a trend toward an increased percentage of IL-4+ cells. Previous work showed that Th2 differentiation in the absence of added IL-4 is enhanced by the attenuation of Erk (10, 11, 46, 47). The dichotomy of Th1 versus Th2 differentiation was clearly illustrated by the array-expression patterns of Bcl2a1 and Gata3, which were almost perfect mirror images of one another with respect to Erk2 dependence and eight different conditions of activation. The Foxp3 locus is regulated by methylation, and a possibility is that partial demethylation, accompanying TGF-β signaling and a decrease in Dnmt1 activity, is sufficient for iTreg differentiation (25–27, 48, 49). Furthermore, pharmacological inhibition of Erk promoted Foxp3 expression and suppressor function in naive T cells, and this was also correlated with a diminished expression of Dnmt1, at least after 4 d of culture (12). Consistent with this, under iTreg conditions for 1 d, the array value changes associated with a loss of Erk2 were much more numerous than were those found for Th0 or Th1 conditions. Furthermore, the loss-of-Erk2 changes were overwhelmingly skewed toward increased expression. The implication is that there is suppression of gene expression, including Foxp3, by TGF-β that is dependent on Erk2. This would be consistent with diminished expression of the maintenance methyltransferase, Dnmt1, or possibly the de novo methyltransferase, Dnmt3b. However, neither Dnmt1 nor Dnmt3b mRNA was suppressed by TGF-β at 1 d, a time when Foxp3 has already been induced. In addition, array values and qPCR indicated that Erk2 deletion had a minimal effect on Dnmt1 mRNA expression at 1 d compared with the magnitude of induction, although the small decrease in Dnmt1 in Erk2−/− T cells was seen in all of the array conditions that included TGF-β. At later time points, we detected significant Erk2-dependent differences, but it is unknown whether the small Erk2-dependent differences seen at 1 d are of biological significance. We conclude that these results are not consistent with a simple model of iTreg differentiation.
based entirely on the amount of Dnmt1 mRNA expressed; but we note that the regulation of Dnmt1 activity derives from a large allosterically controlled protein complex that assembles on chromatin in a cell cycle-dependent manner (50). Independent of mRNA amount, Dnmt1 activity may be regulated in an Erk- and TGF-β-dependent manner that is not yet understood. Future studies will characterize the entire methylome of WT and Erk2−/− T cells under alternate conditions of activation and differentiation.

The set of genes that was specific to iTreg conditions and further induced in the absence of Erk2 included a number known to be part of the Treg signature, including Foxp3 and Nrp1. Other genes identified in this set may suggest mechanisms of T cell differentiation, but none encodes a DNA-binding transcription factor. A separate unbiased hierarchical cluster analysis was performed, and the clustering including Foxp3 identified an overlapping set of genes that also included three candidate transcription factors. One, Cux1, is a TGF-β–regulated homeobox gene that gives rise to many proteins with differing functions (51). One of the Cux1 knockout mouse strains had multiple defects in T and B cell development; thus, we speculate that it controls part of the iTreg program (52).

Another mechanism of Erk2 suppression involves TGF-β signaling, a central facet of iTreg differentiation. There was an increased amount of activated p-Smad2,3 in Erk2-deficient T cells, suggesting that Erk2 attenuates the TGF-β–mediated activation of Smads. A similar result was found for Mekk2- and Mekk3-deficient T cells, which show impaired phosphorylation of the Smad2,3 linker region, enhanced Smad transcription activity, and impaired IFN-γ production (28). In addition, Mekk3-deficient T cells were impaired in their Erk activation and were defective in their Th1 responses (61). An implication from these studies and the present work is that Mekk2,3, upstream of all of the MAPKs, are specifically acting through Erk phosphorylation to affect TGF-β signaling. A further mechanism involves Gata3, shown to be essential for Treg function (34), and in the absence of Erk2, there is a substantial increase in the expression of Gata3 under all conditions. Finally, Socs2 is a positive regulator of Stat5, signaling, and Erk2 suppresses its expression under Th0 and Th1 conditions. It is possible that, under conditions of limiting TGF-β, the signals such as those transmitted through the IL-2R are dampened by the activation of Erk2 (39). In sum, the strength of Erk2 signaling arbitrates a contingency of T cell differentiation through its role as a highly connected node in signal transduction.

An unexpected result was that the most important role for Erk2 in T cells is survival and differentiation of effector T cells, separate from its role in cell proliferation. Erk is well established as an essential signal transducer in cell cycle entry and progression in all cells previously examined and under all stimulatory conditions tested (62); yet, with the addition of costimulation through CD28, we found that T cells could still initiate cell cycle entry and complete division with a loss of Erk2 or even a complete loss of Erk1 and Erk2 (data not shown). This is not congruous with studies using pharmacological inhibitors of Mek1,2, suggesting that such inhibitors have off-target effects (63). Consistent with increased division in Erk2-deficient T cells, p21Cip1/Waf1 is strongly dependent on Erk2 for expression, making it an important pathway for negative-feedback control of T cell proliferation. This may explain, in part, the role of p21Cip1/Waf1 in tolerance and suppression of autoimmunity (64). The mechanism underlying the bypass of Erk in T cell cycle progression is a topic of current study.

Because finely tuned Erk signaling is essential for appropriate T cell differentiation, it is perhaps not surprising that defects in Erk expression are associated with autoimmunity. Studies showed that loss of Erk activation correlated with diminished Dnmt1 and DNA hypomethylation in patients with systemic lupus erythematosus (65, 66). From the data presented in this article, we predict increased numbers of Tregs in such patients; in one study, newly diagnosed and untreated systemic lupus erythematosus patients had increased frequencies of CD4+FOXP3+ T cells, which correlated positively with disease severity (67). It is unclear why an increased proportion of Tregs should correlate positively with autoimmune disease, but this is most likely correlating and not causative. Rather, changes in Erk activation underlie aberrant differentiation of T effector cells, leading to a loss of tolerance. Overall, Erk signaling is clearly pivotal in T cell differentiation and important for the regulation of T cell homeostasis.

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References


