UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of Erk MAPK pathway in CD4 T cell proliferation and differentiation

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biology

by

Chiung-Fang Chang

Committee in charge:

Professor Stephen M. Hedrick, Chair
Professor Ananda W. Goldrath
Professor Cornelis Murre
Professor David Traver
Professor Carl F. Ware
Professor Maurizio Zanetti

2011

The Dissertation of Chiung-Fang Chang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego
2011

DEDICATION

To my parents, for their encouragement and support

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ACKNOWLEDGEMENTS

I would like to thank Dr. Stephen Hedrick for letting me joining the lab and providing mentoring on the projects. Dr. Hedrick always encouraged us to participate in immunology class and conferences. Also, I would like to thank my committee members Dr. Cornelis Murre, Ananda Goldrath, David Traver, Maurio Zanetti for their suggestions over years. It always made me nervous to prepare each committee meeting but it always turned out to have lots of suggestion through past few years.

I would like to thank Dr. Warren D'souza. He taught me when I was a rotation student and even after I joined the lab. Warren is a great mentor and he taught me from basic mouse work to experiment design. He always provides his suggestions and I learned a lot from him in these two years. I really appreciated all his effort and patience for training me. Even after he left the lab, we still have some discussion about the Erk project and others. Our lab manger Carol, she is always very helpful and like to share her magical PCR buffer and food for the lab. It is great to have great PCR buffer when I need to do hundreds of genotyping every month. She always takes good care of everyone in the lab and answers every question whenever you have any confusion. Irene always interacts with everyone and likes to share her experiments with us. She is also in charge of most things, including finding the best vendors for our regents or

equipment. I would especially thank her for helping proofreading my manuscript and pushing me to write my manuscript and thesis. Laura, she joined the lab right after mine. I would like to thank her for sharing the techniques for preparing antibodies from cell lines. She always likes to share her life experience and make our bench bay more alive. There are so many great and smart post-docs in our lab during past few years. Manqing always have some new idea and I really appreciated his suggestion whenever I have a question for the project. Maureen, she likes to share her experiment and her suggestion for autoimmune disease models really help me save time to try several conditions. Also, I would like to thank Lewis, Anna, and Norberto to take good care of our mice. I would like to thank Andrea to provide her help for many things. It is nice to join in the lab and get help from all nice lab mates. I would like to thank all rest lab previous and current members Bryce, Erica, Kevin, Jennifer, Hannah, Ian, Rodrigo and Dan. With all your kindness help, it makes possible for me to make progress on the projects.

The text of Chapter 2 & 3 & 4, in part, has been prepared for manuscripts and gotten closed to submit for publication. I was the primary author and with co-authors Warren N. D'souza, Irene L. Ch'en, Saswata Talukdar, Stephen M. Hedrick, performed, directed and supervised the research, which forms the basis of these chapters.

VITA

EDUCATION:

2005-2011	Ph.D. Biology Division of Biological Sciences University of California, San Diego
2001-2003	M.S. Biochemical Sciences National Taiwan University, Taiwan
1997-2001	B.S. Agricultural Chemistry National Taiwan University, Taiwan

HONORS:

2010	Best Poster Presentation Award (36th La Jolla immunology conference)
2005-2008	Graduate student fellowship (Taiwan) Studying abroad on Government funds of Ministry of Education
2001	Research Award for the outstanding poster (39th annual meeting of the agricultural chemical society of Taiwan)
2000-2001	Undergraduate Research fellowship (National Science Council)
1997-1998	President's Award (Agricultural Chemistry, National Taiwan University)

RESEARCH AND WORK EXPERIENCE:

Graduate student (Ph.D. University of California, San Diego, USA)
Mentor: Professor Stephen M. Hedrick
I. The role of Erk in CD4 T cell proliferation and differentiation
II. The Erk2 MAPK regulates CD8 T cell proliferation and survival

Research Assistant (Institute of Biological Chemistry, Academia Sinica, Taiwan)
Mentor: Professor Chun-hung Lin
I. Study for the α-L-Fucodisase from Thermotoga maritima

II. Chemical modification and substrate protection of sialic acid synthase in E. coli K1

Graduate student (Master, Institute of Biochemical Science, National Taiwan University)
Mentor: Professor Chun-Hung Lin
Site-directed mutagenesis of sialic acid synthase in E. coli K1.

Undergraduate Independent Research (Agricultural Chemistry department, National Taiwan University)
Mentor: Professor Hui-Fen Wu
Substrate recognition of the Escherichia coli ClpYQ (HslUV) protease implicated by in vivo protein-protein interaction in the yeast two-hybrid system

Lecture and Laboratory Teaching Assistant (Biochemistry Lab, National Taiwan University)
Teaching Assistant (Recombinant DNA Laboratory, University of California, San Diego)

PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

The role of Erk MAPK pathway in CD4 T cell proliferation and differentiation

by

Chiung-Fang Chang

Doctor of Philosophy in Biology

University of California, San Diego, 2011

Professor Stephen M. Hedrick, Chair

The Erk (Extracellular signal-regulated protein kinase) signaling pathway is thought to be critical for T cell survival, proliferation and differentiation. However, the role of Erk in Th1 and Th2 differentiation is controversial. Most of the previously published studies used a MEK inhibitor to examine the function of Erk in CD4 T helper cell and induced regulatory T cell differentiation; yet the role of Erk2 and its distinction from Erk1 in CD4 T effectors and regulatory cells remains unclear. In this study, we used germline Erk1 knockout mice and tamoxifen-regulated conditional Erk2 knockout mice to identify the role of these two major Erk isoforms within mature CD4 T cells.

In vitro studies have revealed that Erk1 is dispensable for most aspects of CD4 T cell activation, while Erk2 performs several non-redundant functions. In the scope of this research project, we observed that Erk2 supported early

CD4 T cell proliferation, as well as the differentiation of Th1, but not Th2 or Th17 cells. In addition, virus-specific Th1 responses in the absence of Erk2 were greatly impaired, Furthermore, the reduction in Erk2 signaling enhanced induced regulatory T cell (iTreg) function in vitro and in vivo. Both $Erk1^{-/-}$ and $Erk2^{-/-}$ nature regulatory T cells can suppress CD4 T cell proliferation similar to wild type. In addition, when compared to wild type T cells, we observed down-regulated DNA methyltransferase (DNMT), and increased phospho-Smad2/3 in $Erk2^{-/-}$ CD4 T cells cultured under iTreg conditions. We hypothesized that this decrease in DNMT and increase in TGFβ signing is induced by an increase in Foxp3 expression. In summary, this study demonstrates that Erk2 plays an important role in CD4 T cell proliferation and differentiation in vitro and in vivo.

Chapter. 1
General introduction

MAPK (mitogen activated protein kinase) pathway

MAPK (mitogen activated protein kinase) pathways are highly conserved from lower to higher organisms and they are involved in cell differentiation, proliferation, survival, learning and memory, and the elaboration of cell-type specific functions (Chang et al., 2003; Meloche and Pouyssegur, 2007; Morozov et al., 2003; Rozengurt, 2007; Villegas et al., 2010). In mammals, at least four distinct groups of MAPKs have been recognized: extracellular signal-regulated kinases 1 and 2 (Erk1/2 or p44/p42), c-Jun amino-terminal kinases or stress-activated protein kinases (JNK/SAPK) 1-3, p38MAPKα, β, γ, δ and Erk5. These serine/threonine kinases are regulated by phosphorylation of organized specific modules. Upon stimulation, three modules are activated in series: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK (Chang and Karin, 2001; Chen et al., 2001).

MAP-kinase signaling pathways are not redundant and each pathway may play role in different T cell types and differentiated stages. Taking Erk signaling as an example, the Ras-Raf-Mek-Erk signaling cascade is activated upon the stimulation of T cells via T cell receptors (TCRs). Upon phosphorylation, Erk molecules can translocate into the nucleus and thereby activate transcription factors, such as Elk, Egr, Fos and possibly many others. These transcription factors are involved in cell development, proliferation, apoptosis and survival (Chang and Karin, 2001). Erk interacts with many

substrates and regulators in the cytoplasm, to modulate signal transduction, and in the nucleus to program gene expression (von Kriegsheim et al., 2009; Yoon and Seger, 2006). It constitutes a highly connected node in the physiology of cell signaling. This pathway plays a role in early thymocyte development by several studies such as dominant-negative and constitutively activated mutants of Ras (Yamashita et al., 1999). Erk kinases directly activate several transcription factors such as Elk, c-fos1 and thereby initiate gene expression required for activated T cells (Dong et al., 2002; Rincon, 2001).

ERK1/2 and their substrates

ERK1 (Mapk3) and ERK2 (Mapk1) are ubiquitously expressed in different tissues, but the relative abundance is variable. ERK is involved in cell proliferation and differentiation. Several researchers have used MEK inhibitors (PD98059, UO126) or germline Erk1 knockout mice to study the role of Erk signaling. An early study indicated that Erk1 is required for thymocyte development (Pages et al., 1999); however more recent studies showed that a deficiency in Erk1 does not affect T cell development (Fischer et al., 2005; Nekrasova et al., 2005), nor in vitro T cell activation. This implied that Erk1 deficiency could be compensated by Erk2. In constrast, a homozygous or hemizygous deletion of the Erk2 locus leads to embryonic lethality at embryonic day E6.5 (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003). This suggested that inactivation of Erk2 couldn't be rescued by Erk1 in early embryo

development. Although these two isoforms share 84% amino acid identity in their sequence and have similar specificities, they appear to play distinct roles in embryonic development.

Fischer et. al. showed that Erk pathway is required for β-selection and positive selection by using conditional Erk2 knockout mice (Fischer et al., 2005). The Erk kinases in this pathway can go into nucleus from cytosol and thereby activate their responsible transcription factors, such as Elk, Egr1, etc. Also, the mechanisms to regulate T cell development, proliferation and survival have been an important issue. The difference could derive from disparate functions, or more simply, it could be the result of different amounts of expression. This issue has been difficult to formally resolve. A recent study shows ERK2 is not only a kinase but also can be a transcriptional factor that can directly regulate expression of interferon gamma (IFNγ)-induced genes (Hu et al., 2009). This study suggest a new role or function of ERK2 protein.

Erk often plays a profound role in cellular differentiation and function, but a difficulty arises in separating this from the role of Erk in basic cellular processes such as cell cycle progression and survival. Many studies used noncompetitive inhibitors of Erk's proximal upstream kinase, Mek1, 2 to study the role of Erk, but these studies have inherent complications. The effectiveness of these inhibitors depends on the strength of the activating signal, they may engender off-target effects, and they do not distinguish Erk1 and Erk2 signaling

(Alessi et al., 1995; Duncia et al., 1998). In T cells, inhibitors were found to block proliferation in a manner that was dependent on the strength or mode of stimulation (DeSilva et al., 1998; Dumont et al., 1998; Sharp et al., 1997), and thus the requirement for Erk in T cell activation and proliferation is not entirely understood.

Various substrates of Erk were found in several cell types. Based on the location of Erk substrates, there were three types: membrane proteins (CD120a, Syk), nuclear substrates (Elk-1, NF-AT, c-fos, c-myc) and cytoskeleton proteins (neurofilament, paxillin). Of interest with respect to immune function activity of Erk for Bcl2 family members, Bim belongs to BH3-only Bcl-2 family member, and it is found in three isoforms of Bim (Bim_{EL}, Bim_{L}, Bim_{s}). Bim is an essential initiator of PCD (programmed cell death) and stress-induced apoptosis. Previous research showed that it is required for apoptosis of thymocytes in the process of negative selection (Owaki et al., 2006). TCR signals regulate the apoptotic activity of Bim by phosphorylation and then subsequent changes in binding to Bclxl in immature thymocytes. In addition to central tolerance, Bim is essential for apoptosis associated with peripheral tolerance (in peripheral lymph organs) (Bouillet et al., 2002). Recently, naïve and memory T cell homeostasis was shown to be controlled by a balance of Bim/Bcl-2 as demonstrated by using $Bim^{+/-}Bcl2^{-/-}$ mice (Wojciechowski et al., 2007). It was also shown that Erk1/2 could phosphorylate Bim_{EL} in fibroblast

cells as well as in macrophages. In macrophages, Bim phosphorylation led to its proteasomal degradation (Hacker et al., 2006). In fibroblast cells, phosphorylated Bim_{EL} at Ser65 will dissociate from Mcl-1 and Bclxl and thereby increase the turnover of Bim_{EL} Those results provided new mechanism for the role of Bim in cell death and its regulation by the ERK survival pathway (Ewings et al., 2007).

$p27^{kip1}$ controls cell cycle progression by binding to and inactivating cyclin-cyclin depentent kinase (CDK) complexes. It was found as a CDK inhibitory activity p27 can be phosphorylated by ERK in mesangial cells in vitro (Wolf et al., 2003). Thymic hyperplasia occurred in $p27^{kip}$ deficient mice because T lymphocyte proliferation was enhanced. In the spleen, a $p27^{kip1}$ deficiency increased proliferation of hematopiotic progenitor cells occurred in absence of $p27^{kip1}$ (Fero et al., 1996). In CD4 T cells, $p27^{kip1}$ plays a complex role in the regulation of cell division and effector function (Rowell et al., 2005). $p27^{kip1}$ promotes early G1 to S phase in activated CD4 T cells and then it functions as a negatively regulates the T cell clonal expansion following the peak of response.

T cell activation and differentiation

T cells are developed from thymus (primary organ) and they can travel through blood and reach to secondary lymphoid organ such as spleen and lymph nodes. When infection occurs, antigen-presenting cells such as B cells,

dendritic cells can present antigen to T cells. T cells can be activated through T cell receptor signaling and thereby activate several signaling cascade, such as ERK signaling, JNK signaling, NFκB and NFAT (nuclear factors of activated T cells) pathways etc (Fields et al., 1996). T cell receptor (TCR) is a CD3 complex including several subunits (alpha, beta, delta). T cell receptor signaling (signaling 1) is major signaling to activate T cells. Besides, T cells also require costimulatory signaling through costimulatory molecule such as CD28 or CTLA4. This costimulatory signaling can either activate or inactivate T cells. Furthermore, cytokine receptor such as IL-2R, IL-4 or IL-17 on T cells can receive the cytokine from itself or antigen presenting cells to further differentiate into different T cell subsets (Fields et al., 1996). CD8 T cells are also activated through TCR signaling and costimulatory signaling. They can be further developed as effector T cells and memory T cells. In CD8 T cells, D'souza et al. showed Erk2 is required for TCR signaling alone. Erk2 is essential for later survival upon optimal stimulation TCR and co-stimulatory signaling as well as the responses for LCMV infection. Increased apoptotic protein Bim expression and decreased anti-apoptotic protein Bcl2 and Bclxl expression were found in $Erk2^{-/-}$ CD8 cells. $Erk2^{-/-}$ $Bim^{-/-}$ (double knockout) can compensate the survival defect from $Erk2^{-/-}$(D'Souza et al., 2008).

Immune responses

A typical immune response includes proliferation, contraction and death. It starts with pathogen infection and it will initial immune responses through antigen presenting cells. The stages of specific T-cell immunity include proliferative expansion (expansion phase), acquisition of effector function (contraction phase) and memory (memory phase). After infection, both CD4 T cells and CD8 T cells go through these three phases. However, CD4 T cells have 20-fold lower peak expansion than CD8 T cell did (Brooks et al., 2005; De Boer et al., 2003), and they exhibit a longer contraction phase than CD8 T cells. Surprisingly, the level of $CD4^+$ memory cell gradually declined whereas $CD8^+$ T memory cells were stable (Sun et al., 2004). However, CD4 T cell response is critical to sustain CD8 T-cell function to prevent persistent infection and provide signals to promote the development of CD8 T cell memory.

Autoimmune disease, where the immune system attacks self-antigen, is a failure of tolerance. Autoimmune disease can be divided into two categories: organic specific (such as multiple sclerosis, diabetes) and systemic (such as system lupus erythematosus, SLE). Multiple sclerosis was thought to be due to Th1 responses, which have excessive secretion of INFγ and TNFα. EAE (experimental autoimmune encephalomyelitis) is an experimental model of human disease multiple sclerosis. It has been traditionally associated with dysregulated Th1 responses although IFNγ deficient mice still develop EAE

(Kalman et al., 1995; Krakowski and Owens, 1996). Alternatively, Th17 has been shown to mediate the inflammation associated with EAE (Kroenke and Segal, 2007). Besides, Erk1 deficient mice were more susceptible to EAE disease although mice have normal CD4 effector function (Agrawal et al., 2006).

CD4 T cell differentiation

T helpers

Within CD4 T lymphocytes, there are three major types of T helper (Th) cells: Th1, Th2 and Th17 (Murphy and Reiner, 2002). Th1 cells mediate effective immunity against intracellular pathogens (bacteria or virus), and they are characterized by the production of IFNγ. Th2 cells provide protection against certain extracellular pathogens (helminths) and they are characterized by the production of IL-4, IL-5, and IL-10. Th17 cells produce IL-17A and IL-17F, and provide a defense against extracellular bacteria by mediating inflammation (Weaver et al., 2006).

Th1 cells are elicited by intracellular pathogens (Mycobacterium, Salmonella, etc.) and are characterized by their production of IFNγ and lymphotoxin-α(Lt-α). The cytokines produced by Th1 cells are hallmarks of many organ specific autoimmune diseases, such as rheumatoid arthritis, experimental autoimmune encephalomyelitis (EAE), insulin-dependent diabetes mellitus etc. T-bet is a major transcriptional factor for Th1 differentiation, and it

directly activates IFNγ and silences IL-4 (Zhuang et al., 2009). However it is also widely expressed in other T subsets, such as Treg, CD8 T cells. Previous studies showed that T-bet deficient mice have more susceptibility to virus or bacteria infections (Sullivan et al., 2005; Szabo et al., 2002). In addition, they are more resistant to autoimmune diseases (Bettelli et al., 2004; Lazarevic and Glimcher, 2011; Neurath et al., 2002). Erk1 deficient mice exhibit normal T helper differentiation while dendritic cells (DCs) from Erk1 knockout mice tend to make more IL-12 and thereby induce more Th1 differentiation (Agrawal et al., 2006). Interestingly, Erk1 knockout mice are more susceptible to experimental autoimmune encephalomyelitis (EAE) (Agrawal et al., 2006). However, inhibition of Erk signaling (PD98059, MEK inhibitor) can attenuate EAE through suppression of IL-23 and IL-1 driven IL-17 production (Brereton et al., 2009). It remains unclear what role of Erk2 plays in T helper cell differentiations.

On the other hand, Th2 cells provide protection against certain extracellular pathogens (bacteria, parasites) and produce different cytokines (IL-4, IL-5, IL-9, IL-10, IL-13 and so on). Those cytokines produced from Th2 cells help B cells to proliferate and differentiate into antibody-secreting plasma cells. The balance between Th1 and Th2 is critical for a T-dependent immune response. Autoimmunity may result in mice, which have CD4 cells tend to differentiate Th1 cells, whereas immuno-deviation of Th2 development will lead

to allergy and asthma (Murphy and Reiner, 2002; Reinhardt et al., 2006). It has been shown that Ras-Erk signaling may regulate GATA3 stability and Jak1/Stat6 activity for Th2 differentiation (So et al., 2007; Yamashita et al., 2005). However, inhibiting Erk activation can induce early IL-4 expression as well as alter AP-1 DNA activity (Jorritsma et al., 2003). Moreover, in long term Th2 culture, there is no effect on IL-4 expression.

The role of Erk in Th1 and Th2 differentiation is controversial. Jorritsma et.al showed that inhibition of Erk activation using PD98059 resulted in increased IL-4 production by CD4 T cells (Jorritsma et al., 2003; Yamashita et al., 2005). However, Erk1-deficient CD4 T cells showed normal proliferation and cytokine production (including IL-4 and IFNγ), but were slightly more susceptible to EAE on a 129 Sv, but not B6 background (Nekrasova et al., 2005). In addition, Agrawal et al. demonstrated Erk1$^{-/-}$ mice exhibit increased Th1 cell polarization in vivo (not in vitro) and were more susceptible to EAE (Agrawal et al., 2006). Moreover, IL-27 induces Th1 differentiation via two distinct pathways and one of them is ICAM-1/LFA-1/ERK1/2-dependent pathway (Owaki et al., 2006). Based on those researches, it suggested ERK play a role in Th1/Th2 differentiation but it still remains unclear that different roles of Erk1 and Erk2 in CD4 T cell differentiation and their proliferation.

In addition, a new lineage was discovered to produce the cytokine IL-17, and they are named Th17. These cells are notable in providing defense against

extracellular bacteria and in mediating inflammation (Harrington et al., 2006; Stockinger and Veldhoen, 2007; Weaver et al., 2006; Weaver et al., 2007). Also, it was shown that certain autoimmune disease (such as rheumatoid and experimental autoimmune encephalomyelitis) maybe caused from the improper regulation of IL-17 and other cytokines associated with Th17 cells. IL-6 and TGF-β were found to promote the differentiation of Th17 (Mangan et al., 2006) and IL-23 is essential for Th17 to produce IL-17 (Thakker et al., 2007). IL-2 inhibits the differentiation of Th17 cells in vitro and an IL-2 deficiency is associated with enhanced IL-17 production (Mangan et al., 2006). Th17 cells are found related to autoimmune disease because of the balance between Th17 and regulatory T cells. Erk1 deficient cells can have normal T helper function, whereas inhibition of Erk signaling (PD98059, MEK inhibitor) can attenuate EAE through suppression of IL-23 and IL-1 driven IL-17 production (Brereton et al., 2009). Besides, treatment of a MEK inhibitor in mice induced colitis (Tan and Lam, 2010).

Regulatory T cell

Regulatory T cells can originate in the thymus (nTreg cells) or they can be induced from mature, naïve CD4 T cells (iTreg). Treg cells are identified by the expression of the Foxp3 transcription factor and their ability to suppress T cell expansion. Foxp3, though not a lineage commitment factor, is essential for the function of Treg cells and the maintenance of immune system quiescence.

Within the Foxp3 locus, there exist a promoter and enhancers (CN1, 2, 3) with Smad3, and Stat5 binding sites, and this correlates with the requirements for TGFβ and IL-2 (or other γ-receptor cytokines) in the development of T reg cells (Feuerer et al., 2009; Shen et al., 2009).

Regulatory T cells express Foxp3 and control the immune responses by their suppressive activity. T cell receptor, costimulatory and cytokine mediated signaling are required for Foxp3 expression. TGFβ signaling activates Smad2/3 and it is required for Th17 and regulatory T cell differentiation. However, the higher TGFβ signaling lead to differentiate Treg but low TGFβ signaling with IL-6 signaling results in Th17. $Smad2^{-/-}$ mice are embryonic lethal (Nomura and Li, 1998) and Smad3 deficient mice display strong inflammatory diseases (Yang et al., 1999). Smad2 and Smad3 are redundant required for the TGFβ-induced Treg differentiation (Takimoto et al., 2010).

A role for Erk signaling in the regulation of T reg cell differentiation was shown with the use of MEK inhibitor (UO126) (Luo et al., 2008). TGFβ signaling led to reduced Erk phosphorylation and preferential iTreg differentiation correlating with the down-regulated expression of DNA methyltransferase (DNMT) (Luo et al., 2008). Furthermore, the eliminiation of N-Ras or K-Ras signaling via siRNA resulted in an increase of Foxp3 mRNA and Foxp3 protein (Mor et al., 2008). A recent study showed decreased phosphorylation of

Smad2/3 linker region and increased Smad2/3 activity for enhancing iTreg differentiation in MEKK2/3 CD4 T cells (Chang et al., 2011).

Autoimmune diseases and CD4 T cells

There are two major mechanisms to prevent autoimmunity. One is through central tolerance, which this process occurs in the thymus and eliminates auto-reactive lymphocytes. A second is via peripheral tolerance which controls immune responses through regulatory T cells (Nurieva et al., 2011). EAE (Experimental autoimmune encephalomyelitis) is induced by the injection of Myelin basic protein (MBP) or Myelin oligodendrocyte glycoprotein (MOG) resulting in encephalitogenic CD4 T cells. Adding normal CD4 T cells with diverse TCR repertoires can prevent the disease (Apostolou and von Boehmer, 2004; Paust and Cantor, 2005). In addition, it was shown that Erk1 deficient mice have more severe EAE disease than WT mice on 129 background, but not B6 (Agrawal et al., 2006). Therefore, Erk signaling may be involved for T helper differentiation for autoimmunity.

Inflammatory bowel disease (IBD) is used as a model to examine the function of regulatory T cells. Colitis can be induced by the transfer of naïve CD4 T cells. The disease onset is about 4-6 weeks and it enhanced Th1 and Th17 responses in the colons. Co-transfer of Treg can reduce the severity of the disease apparently because Treg cells can inhibit Th1 and Th17 cell proliferation and differentiation. With MEK inhibitor study (UO126), impaired Erk

signaling enhanced Th17 responses, which in turn increased the severity of disease (Tan and Lam, 2010).

Erk aberrant phosphorylation has been found in several diseases such as cancer, rheumatoid arthritis (RA), EAE, colitis, SLE (system lupus erythematous) and infectious diseases. Early system lupus erythematous (SLE) is an autoimmune disease which is characterized by general decreases of DNA methylation and related with defect in Erk signaling (Gorelik and Richardson, 2009; Javierre and Richardson, 2011).

Summary

MAPK kinases are involved in many aspects of immune responses: innate immunity, adaptive immunity, cell proliferation and differentiation. It has been showed that Erk signaling is involved in T cell immune response and development (Pearson et al., 2001). If an improper balance of polarized T helper cells occurs, there can occur autoimmune diseases or allergies. By using conditional or inducible knockout mice, we are able to investigate the role of Erk (one of MAPK kinase) in CD4 T cell responses (activation, proliferation, survival and death) and their differentiation. Better knowledge of the mechanism of ERK signaling may help in the design of therapeutic drugs to selectively modulate the balance of T cell subsets and thus treat some immune disorders or autoimmune diseases.

In this study, we used genetic ablation to study the different functions of Erk (Erk1 and Erk2) in T helper and iTreg differentiation. Our data suggest that the loss of Erk2 causes a defect in T cell proliferation that can be overcome with co-stimulation. In addition, we also found an Erk2-associated defect in Th1 survival and differentiation. Most prominently, we observed an increased propensity for induced regulatory T cell differentiation in Erk2-deficient CD4 T cells in vitro and in vivo.

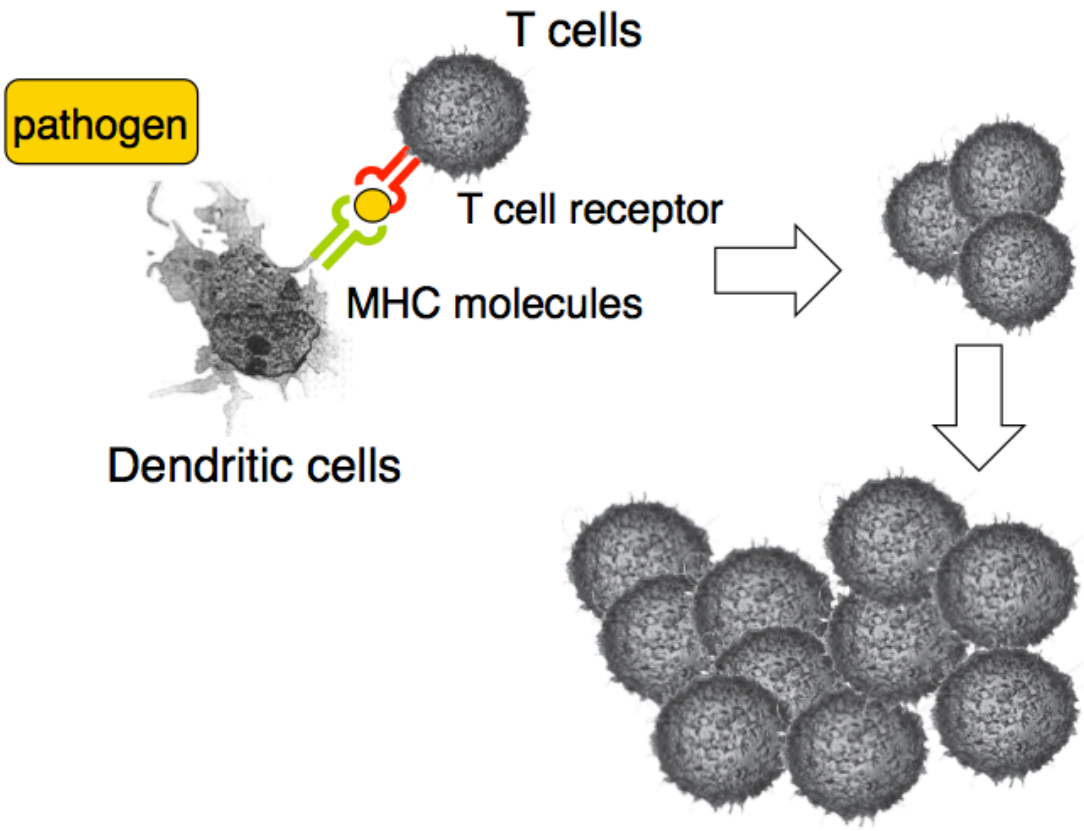

Figure 1-1. T cells activation and proliferation. Pathogens were up taken by dendritic cells (DCs) and DCs present the pathogen peptide on the MHC molecules, which are recognized by T cell receptors. Signaling through T cell receptors activate T cells and thereby leads to cell proliferation.

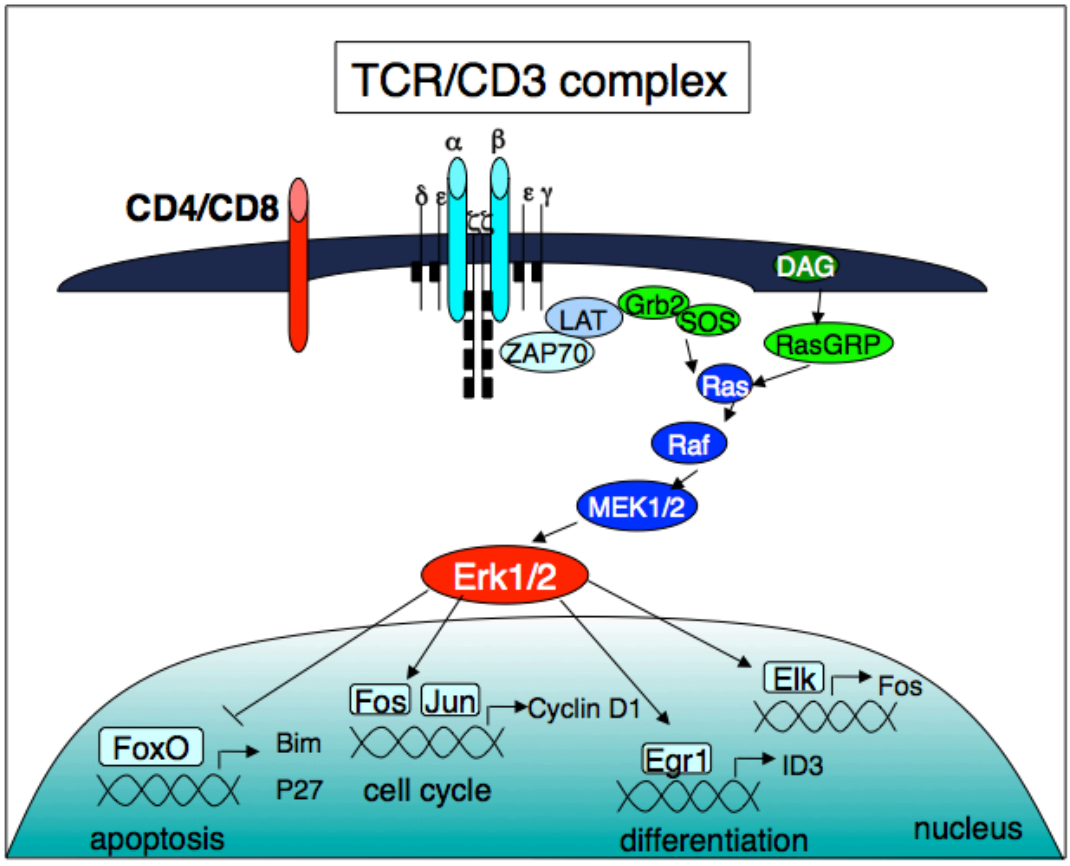

Figure 1-2. T cell receptor signaling and Erk target genes. Erk is downstream of MAPK signaling and it can translocate into nucleus and interact with several substrates. Erk can activate Fos, Egr1, and Elk and turn on gene expression for cell cycle and cell differentiation. On the other hand, Erk can inactivate FoxO and prevent apoptosis.

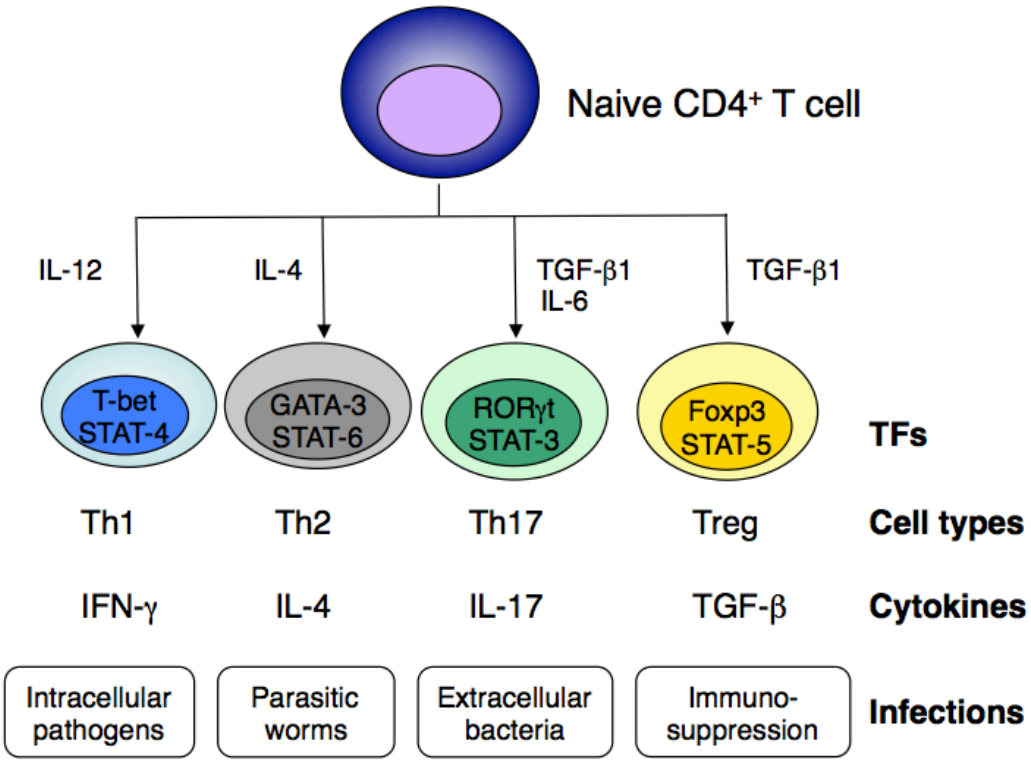

Figure1-3. Differentiation of T helper cells and regulatory T cells. Naïve CD4 T cells can differentiate into Th1, Th2, Th17 and regulatory T cells (Treg). Important transcription factors (TFs), cytokines and type of infections are listed in different CD4 T cell subsets. For example, Th1 cells require IL-12 signaling to activate Stat4 signaling and further un-regulate Th1 key transcription factors T-bet. When there is an intracellular pathogen infection such as virus infection, naïve CD4 T cells can differentiate into IFNγ-producing Th1 cells.

Chapter. 2

Phenotype of $Erk2^{f/f}$ $CreER^{T2}$ mice and CD4 T cell activation

Abstract

We crossed Erk2$^{f/f}$ mice with ERCre mice. The deletion of Erk2 can be achieved by administering tamoxifen to Erk2$^{f/f}$ ERCre mice. Following tamoxifen treatment, Erk2$^{f/f}$ERCre mice appear normal in lymph node and spleen cellularity in T cell subsets. However, we did see a dramatic decrease cellularity in thymus.

Proliferation of Erk1 deficient CD4 T cells appears normal; however, Erk2 deficient CD4 T cells accumulated as undivided cells upon TCR signaling alone. This impaired proliferation was rescued by stimulation of TCR signaling along with costimulatory signaling (anti-CD28). This implies a role for Erk2 in cell division in absence of costimulatory signaling.

Introduction

Erk often plays a profound role in cellular differentiation and function, but a difficulty arises in separating this from the role of Erk in basic cellular processes such as cell cycle progression and survival. Many studies used noncompetitive inhibitors of Erk's proximal upstream kinase, MEK1, 2 to study the role of Erk, but these studies have inherent complications. The effectiveness of these inhibitors depends on the strength of the activating signal, they may engender off-target effects, and they do not distinguish Erk1 and Erk2 signaling (Alessi et al., 1995; Duncia et al., 1998). In T cells, inhibitors were found to

block proliferation in a manner that was dependent on the strength or mode of the stimulation (DeSilva et al., 1998; Dumont et al., 1998; Sharp et al., 1997), and reasons for the requirement for Erk in T cell activation and proliferation is not entirely understood.

Other studies have taken advantage of targeted deletion of Erk1 and Erk2. Although initial studies showed a role for Erk1 in T cell development and activation (Pages et al., 1999), other reports found no phenotypic effect of Erk1 deletion (D'Souza et al., 2008; Fischer et al., 2005; Nekrasova et al., 2005). In contrast, a loss of Erk2 strongly inhibited T cell development and this was compounded by the additional loss of Erk1 (Fischer et al., 2005). Similarly, mature CD8 T cells require Erk2 for proliferation and survival (D'Souza et al., 2008).

T cells can be fully activated through T cell receptor signaling (Signal1), costimulatory signaling (signal 2) and cytokine signaling (Dave, 2011; Riha and Rudd, 2010; Zhu and Paul, 2010). Signal 1 is generated by interaction of an antigenic peptide with TCR-CD3 complex. This complex will recruit several kinases and thereby activate MAPK kinase pathways and many others. Erk is the downstream of TCR signaling and belongs to one of the MAPK kinase pathways. Costimulatory signaling is provided by the interaction between CD28 on the T cells and members of the B7 family on the antigen presenting cells (APCs) (Riha and Rudd, 2010). These interactions result in activation of PI3K

signaling and NF-KB signaling. CD28 cytoplasmic tail (tyrosine residues) can act as a regulator and substrate for different tyrosine kinases such as EMT and LCK (King et al., 1997). In addition, CTLA4 can be the ligated by B7-1,2. CTLA-4 has similar structure as CD28 although it acts antagonistically (Rudd, 2008). CD28 delivers a positive costimulatory signal to T cells whereas CTLA-4 delivers an inhibitory signal. T cells cannot usually be activated with costimulatory signaling alone but it requires both signal 1 and 2. Cytokine signaling can further drive CD4 T cell differentiation into different T helper cell types or regulatory T cells. IL-2 is a major cytokine, involved in the process and supports of T cell survival and proliferation (Malek and Castro, 2010). IL-2 can bind to IL-2 receptor (includes 3 subunits IL-2α, IL-2β, IL-2γ) and then activate Stat5. The dimer of phos-stat5 can activate their responsive genes such as Foxp3 (Cheng et al., 2011).

Here we used loxP-Cre system (Hernandez et al., 2006) to target the Erk2 locus. The Cre system we used is $CreER^{T2}$ (Figure 2-1), which is a mutant form of estrogen receptor attached to Cre recombinase driven by Rosa26 promoter (Guo et al., 2007). When mice are treated with tamoxifen, it can be metabolized to 4-hydroxy tamoxifen. 4-Hydroxyl tamoxifen will help mutant estrogen receptor release from heat shock protein and thereby mutant estrogen receptor with recombinase will translocate into nucleus. Therefore, Cre recombinase can target on the floxed allele. The floxed allele will be deleted after tamoxifen treatment. The advantage of this system is to keep normal cell

differentiation and then acutely delete the target protein. However, tamoxifen may affect thymus development depending on the dose of tamoxifen (Guo et al., 2007; Higashi et al., 2009).

We also crossed $Erk2^{f/f}$ ERCre mice with Smarta mice. Smarta mouse is TCR transgenic mouse, in which T cells are specific for LCMV glycoprotein peptide GP61-80 (Oxenius et al., 1998). CD4 T cells were isolated from Smarta wild type and Erk2 deficient mice and activated with GP peptide in presence or absence of irradiated APC. In addition, there are multiple pathways to lead autoimmunity including CD4 T cells, IL-7 and lymphopenia via Smarta CD4 transgenic system (Calzascia et al., 2008).

Here we analyzed the T cells (CD4 and CD8) present in Erk2-ERCre mice in thymi, lymph nodes and spleens after tamoxifen treatment (induce Erk2 deletion). Also, we examined the role of Erk in CD4 activation and proliferation upon different amount TCR signaling and costimulatory signaling. Besides, we also use TCR transgenic Smarta cells stimulated with specific peptide in presence very low amount and regular amount antigen presenting cells (as providing costimuatory signaling) in order to examine the role of Erk signaling under specific peptide stimulation.

Results

An examination of Erk2$^{f/f}$CD4Cre mice demonstrates that Erk2 regulates several stages of T cell development (Fischer et al., 2005). These mice with a T cell specific deletion of Erk have more mature T cells in the lymph nodes and spleens. In addition, Erk2$^{f/f}$dlck-iCre mice still express a measurable amount of Erk2 in CD4 T cells despite deletion driven by a distal Lck Cre (D'Souza et al., 2008). To analyze the role of Erk2 signaling in peripheral CD4 T cells, we crossed Erk2$^{f/f}$ mice to CreER^{T2} mice that express Cre recombinase under a mutated estrogen receptor (Figure 2-1). Mice were treated with 2mg of tamoxifen for six days and rested for two days before analysis. The extent of Erk2 deletion in purified CD4 T cells from lymph nodes and spleen was examined by PCR, western blot, microscopy and FACS staining. Based upon this data, it was clear that majority of Erk2 was effectively deleted (more than 10 fold decrease**)** (Figure 2-2). T cells from these mice will be abbreviated Erk2$^{-/-}$. We then examined CD4 and CD8 compartments in primary and secondary lymphoid tissues. The deletion of Erk2 resulted in a defect in thymus development as Erk2$^{-/-}$ thymi have reduced cellularity and percentage of CD8 and DP compared to wild type (Figure 2-3A). Despite the difference in T cell development, Erk2-deficient mice have similar proportions of CD4 and CD8 T cells in the secondary lymphoid tissues (Figure 2-3). Examination of the naïve and effective/memory cell population by the activation marker CD44 and CD62L

revealed a similar percentage of $CD44^{hi}$ cells in the Erk2 deficient and wild type mice (Figure 2-3B).

Erk2 is required for T cell proliferation through TCR signaling alone

Naïve CD4 T cell activation, proliferation and differentiation are dependent upon T cell antigen receptor (TCR) signaling (Dave, 2011). Erk is activated downstream of TCR signaling, and mediates a wide range of cellular activities. We examined the requirement for Erk in the activation of CD4 T cells upon stimulation of the TCR alone (anti-CD3) or TCR with costimulatory signals (anti-CD3 and anti-CD28). MACS-purified CD4 T cells from lymph nodes and spleens were labeled with CFSE for analysis of proliferation upon stimulation. We found that there was no difference in proliferation between WT and Erk1-deficient CD4 T cells following stimulation (Figure 2-4). This suggested that Erk1 is dispensable for CD4 T activation and proliferation. We then examined the role of Erk2 in CD4 T cell activation and proliferation. Interestingly, although the total cell number of wild type and $Erk2^{-/-}$ per well was similar under conditions of no stimulation, Erk2- deficient CD4 T cells accumulated at the undivided stage ($CFSE^{hi}$) when stimulated with anti-CD3 (2.5ng) alone (Figure 2-5A). This impaired proliferation was also observed at higher concentrations of anti-CD3 (250ng). We found that these $Erk2^{-/-}$ cells (anti-CD3 alone) display a lower expression of CD25 and CD44, suggesting that Erk2 plays an important role in driving cell proliferation when stimulated with TCR signaling alone (Fig 2-

5B). However, this defect in proliferation can be rescued by introducing a strong co-stimulatory signal (anti-CD28). We found that Erk2$^{-/-}$ CD4 T cells have a similar proliferation phenotype as wild type T cells following stimulation through the TCR in the presence of a co-stimulatory signal (Fig 2-5B). Although the number of divisions carried out by Erk2 deficient T cells appeared to be increased, this may be a result of enhanced metabolic activity of wild type T cells causing more rapid exhaustion of the culture medium. The implication is that a signaling pathway downstream of the co-stimulatroy receptor (Appleman et al., 2002). Interestingly, Erk2-deficient T cells appeared to display enhanced division under these conditions, although this may be a result of enhanced metabolic activity of wild type T cells causing a more rapid exhaustion of the culture medium.

We also observed the similar results using CD4^{+} T cells from Smarta TCR transgenic mice that express a transgenic TCR specific for the glycoprotein. Purified Erk2$^{-/-}$ Smarta cells activated with GP61-80 peptide in vitro displayed impaired proliferation and reduced expression of CD25 and CD44. However, with additional antigen presenting cells to provide costimulation rescued this defect (Figure 2-6), implying that CD28 signaling from APCs together with peptide induced TCR signaling can help Erk2 deficient T cells display normal proliferation. Finally, we were interested in determining how T cells would respond in the apparent absence of both Erk1 and Erk2. T

cells were analyzed from *Erk1*$^{-/-}$ *Erk2*$^{f/f}$ ERCre mice after tamoxifen treatment. As shown, there was one-division delay at 2 days compared to WT T cells that was no longer apparent at 3 days post activation. We did notice a reduced recovery T cells from Erk deficient T cells, especially after 3 days of culture (Figure 2-7).

As Erk has been shown to be essential for cell cycle entry (Pages et al., 1993), we considered the possibility that the responding T cells represented the expansion of a small population that had escaped deletion of the *Erk2* gene. To analyze this, T cells were examined for the presence of Erk directly ex-vivo and after 3 days of stimulation in culture. As shown, Erk was not detected either before or after stimulation (Figure 2-7A). In addition, the dividing cells are not a small subpopulation of the explanted T cells, but rather representative of the entire culture. Finally, there is no loss of T cells in the lymphoid organs following tamoxifen treatment indicating that there is no selective survival of T cells in vivo that retain Erk. These results imply that, unlike other cell types investigated, T cells can traverse the cell cycle without Erk1, 2 activity, or that a small amount of Erk still present 8 days after the initiation of tamoxifen treatment (Figure 2-2B), is sufficient for cell cycle initiation and continued progression—even after multiple division cycles. At a minimum, these results indicate that T cells are much less dependent on Erk for cell cycle progression than other cell types, at least when stimulated through CD3 and CD28.

Discussion

Erk is important to interact with several transcriptional factors to turn on gene expression upon TCR signaling. However, Erk1-deficient CD4 T cells have normal T cell activation and proliferation, which suggests Erk2, can compensate loss of Erk1. Erk2 is critical for cell proliferation with TCR signaling alone in both CD4 and CD8 T cells. Moreover, Erk2 is indispensable for it late survival under optimal stimulation in CD8 T cells (D'Souza et al., 2008). In Erk2 deficient CD4 T cells, they have normal proliferation upon optimal stimulation. Co-stimulatory signaling may help the proliferation in Erk2-deficient CD4 T cells but not for CD8 T cells. It suggests there is different requirement for CD4 and CD8 proliferation by Erk2. Pervious study also support that CD4 T cells have more preferential to CD28 signaling than CD8 T cells (Abe et al., 1995). Besides, Erk deficient ($Erk1^{-/-}2^{-/-}$) CD4 T cells had both proliferation and survival defect upon stimulation through TCR and co-stimulatory signaling (Figure 2-7). Some Erk deficient cells underwent proliferation and upregulated activated marker CD44 and CD25. One possibility is extreme lower amount of Erk2 can support those cells for activation and proliferation. The other possibility is other signaling such as Akt pathways, other MAPK pathways (p38, Jak) can help Erk deficient cells proliferate and activate. This result is different from what we saw in mouse fibroblast cells (MEFs), which we found Erk deficient cells cannot proliferate at all (Li et. al. unpublished data). It maybe due to different cell types and

stimulation pathways, which suggests Erk may play more significant role to drive cell proliferation in certain cell type.

Experimental procedures

Mice and regents

C57BL/6 mice were obtained from the Jackson Laboratory (Bar Habor, Maine). Erk1-deficient mice and the ERCre mice were kindly provided by Dr. Giles Pages (Nice, France) and Dr. Thomas Luwig (Rockerfeller University) (Feil et al., 1997; Hayashi and McMahon, 2002). We have previously reported on the generation of conditional $Erk2^{f/f}$ allele mice (Fischer et al., 2005). $Erk2^{f/f}$ mice were crossed to ERCre mice and deletion of the loxP-flanked alleles was induced by intraperitioneal (i.p.) injection of once daily doses of 2mg tamoxifen (Sigma) emulsified in 200ul of sunflower seed oil (Sigma) for 6 consecutive days. All mice were housed and bred in the University of California, San Diego and animal work was performed according to the institutional guidelines.

Cell culture

CD4 T cells from lymph nodes and spleens were purified by positive or negative selection using magnetic beads (Miltenyi Biotec). To analyze cell proliferation, purified CD4 T cells were labeled with CFSE at $10x10^6$ cells/ml. Cells were stimulated with anti-CD3 with or without anti-CD28 in complete RPMI

media for indicated periods of time and harvested at various time points. In some experiments, the cells were labeled with CFSE prior to culture.

Flow cytometry

Lymphocytes isolated from lymph nodes, spleens, thymus or peripheral blood were stained with the following surface makers: CD62L, CD44, CD4 and CD8. For the intracellular staining of Foxp3, cells were fixed and permeabilized with Foxp3 buffer set (eBioscience) and stained with anti-Foxp3 antibody. Samples were collected on a FACS Calibur or LSRII (BD Biosciences) and analyzed by FlowJo software (Tree Star).

Genomic PCR

Purified CD4 T cells from WT or $Erk2^{f/f}ERCre^{T2}$ were suspended in lysis buffer (Tris-EDTA, SDS, protease K). Genomic DNA was isolated by isopranol and ethanol precipitation and resuspended in 50mM Tris-buffer (pH8.0). Primers Erk2 1590, 1870 and lopSA were used to amplify the floxed fragment or deleted fragment.

SDS-PAGE and western blotting

Harvested T cells were subjected to lysis buffer (50mM HEPES, 150mM NaCl, 1% Triton, EDTA, 1.5mM $MgCl_2$, glycerol) with protease and phosphatase inhibitors (Calbiochem). Protein concentrations were determined by Bradford assay. Supernatants were run on 4-12% Bis-tris gel and transferred to PVDF

membrane. Blots were incubated with primary antibody at 4°C overnight and secondary HRP antibody (Vector Laboratories or SouthernBiotecha) for 1 hr. ECL were used to visualize protein expression. The primary antibodies were used as follows: anti-Erk1, 2 (santa cruz) and anti-ZAP70.

Microscopy

Purified WT and Erk2 deficient CD4 T cells were fixed in 4% (vol/vol) formaldehyde, then were made permeable with 0.02% (vol/vol) Triton X-100. Erk2 antibody (C14) was used as primary antibody and followed the secondary antibody with chrome. DAPI (4'-6-diamidino-2-phenylindole) was used to stain for the nucleus. All cells were visualized with a microscope (Axiovert 200M; Carl Zeiss MicroImaging) with a 63 times objective. Images were captured with an Axiocam monochrome digital camera and were analyzed with Axiovision software (Carl Zeiss MicroImaging).

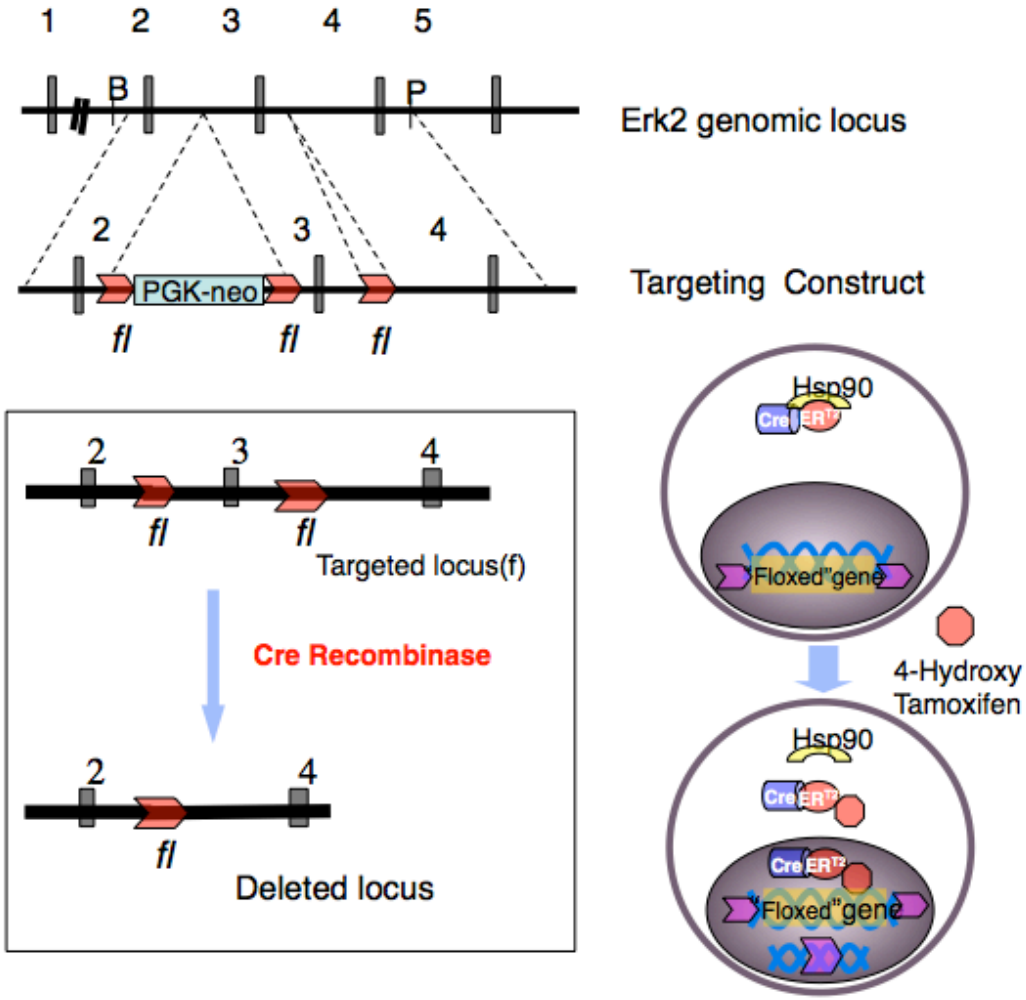

Figure 2-1. Erk2$^{f/f}$CreER^{T2} mice. The Erk2$^{f/f}$ mice were crossed to CreER^{T2} mice. The floxed allele was targeted upstream and downstream of exon 3 in Erk2 locus. CreER^{T2} mice carry mutant form estrogen receptor with Cre (recombinase). This complex (mutant estergon with Cre recombinase) will translocate in the nucleus upon 4-hydroxyltamoxifen treatments.

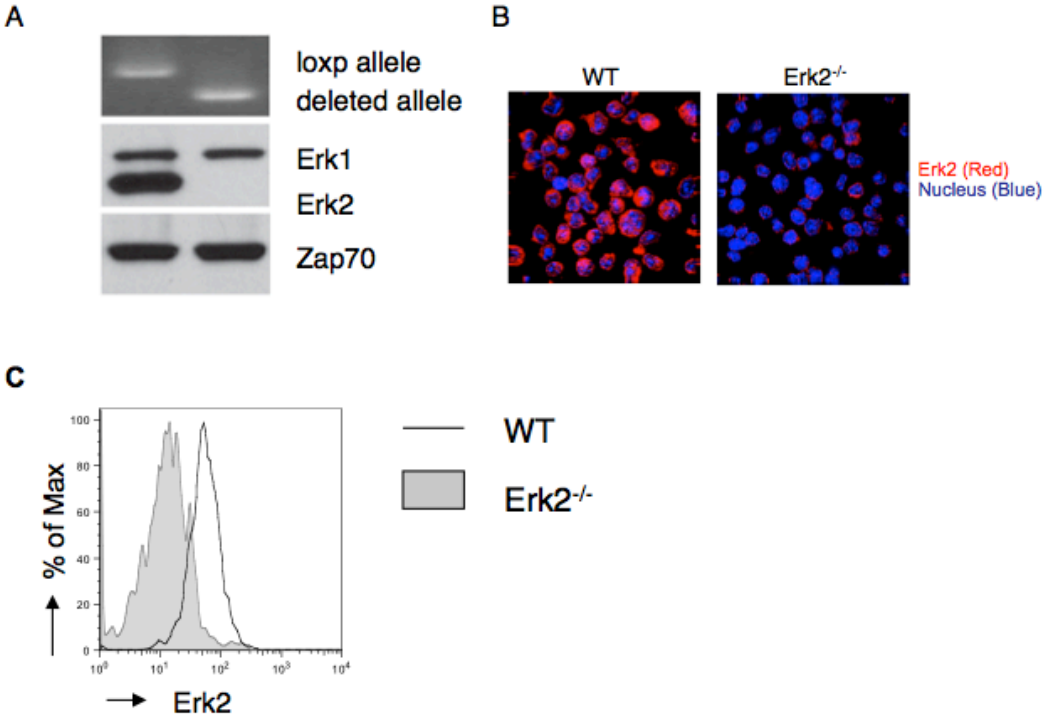

Figure 2-2. Deletion of Erk2 in Erk2$^{f/f}$ Cre-ER^{T2} mice following tamoxifen treatment. WT and Erk2f/f Cre-ERT2 (Erk2-/-) mice were treated with tamoxifen for 6 days. (A) Purified CD4 T cells from lymph nodes and spleen were analyzed for the presence of Erk2 by PCR, Western blotting (B) Nucleus-DAPI and Erk2 were stained fluorescence microscopy (C) Facs staining for Erk2.

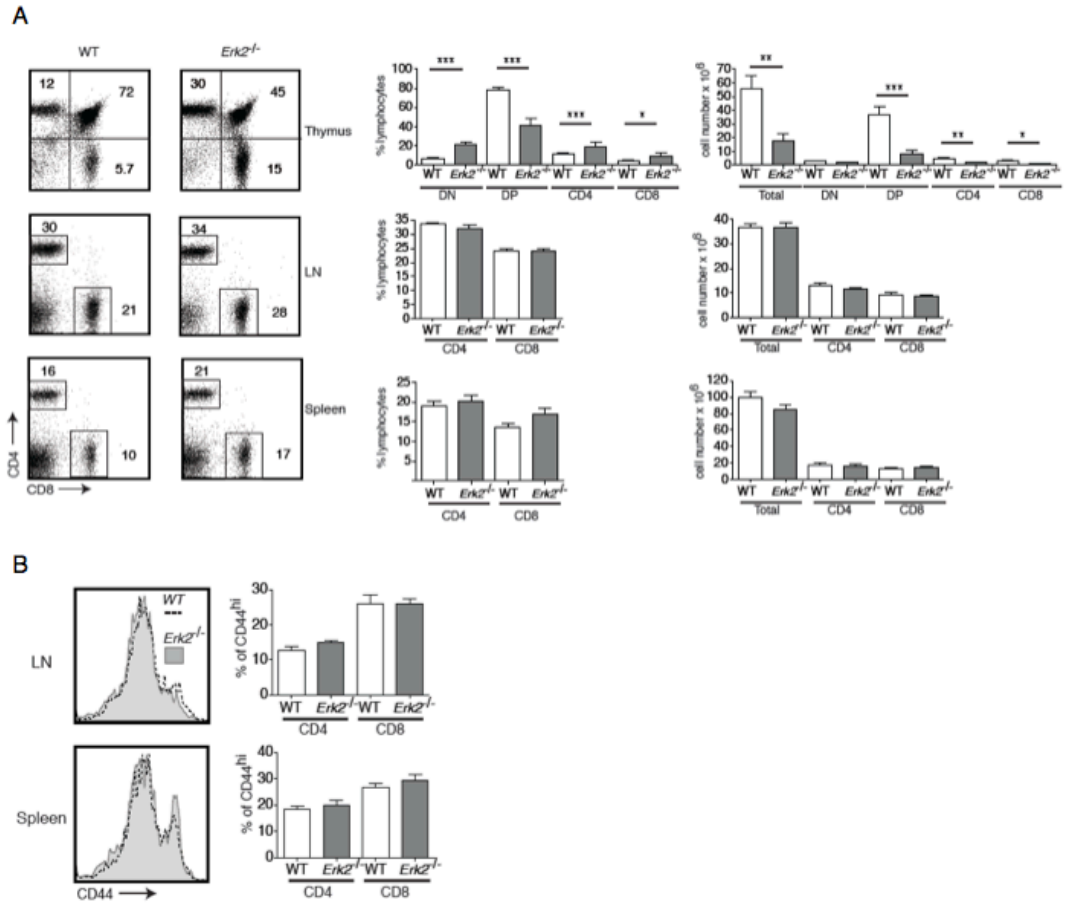

Figure 2-3. Secondary lymphoid tissues of Erk2$^{f/f}$ Cre-ER^{T2} mice appear normal but there is a defect in Erk2$^{f/f}$ Cre-ER^{T2} thymus. (A) WT and Erk2$^{f/f}$ Cre-ER^{T2} (Erk2$^{-/-}$) mice were treated with tamoxifen for 6 days. Thymus, lymph nodes and spleens of 8-12 week old WT mice (Erk2$^{f/f}$Cre^{-} or Erk2$^{+/+}$Cre^{+}) and Erk2$^{f/f}$Cre^{+} mice treated with tamoxifen (Erk2$^{-/-}$) were analyzed by flow cytometry (n=15 for each group). (B) Proportion of CD44^{hi} CD4 and CD8 T cells in the lymph nodes and spleens of WT and Erk2f/fCre+ mice treated with tamoxifen (Erk2$^{-/-}$)

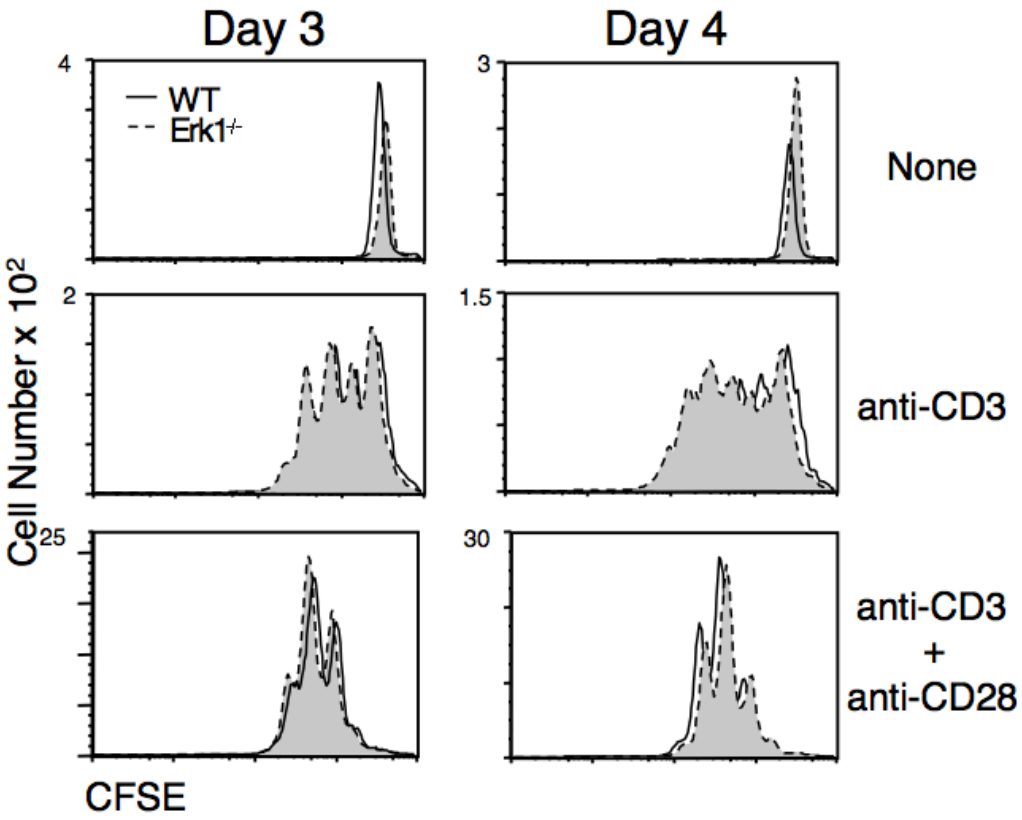

Figure 2-4. Erk1 deficient CD4 T cells exhibit normal proliferation under stimulation. Purified WT or Erk1$^{-/-}$ CD4 T cells were labeled with CFSE and stimulated in vitro with plate-bound anti-CD3 (2.5ng) +/- soluble anti-CD28. CFSE profiles are gated on CD4^{+} T cells. The actual number of cells run on the FACS in a fixed time-period is plotted.

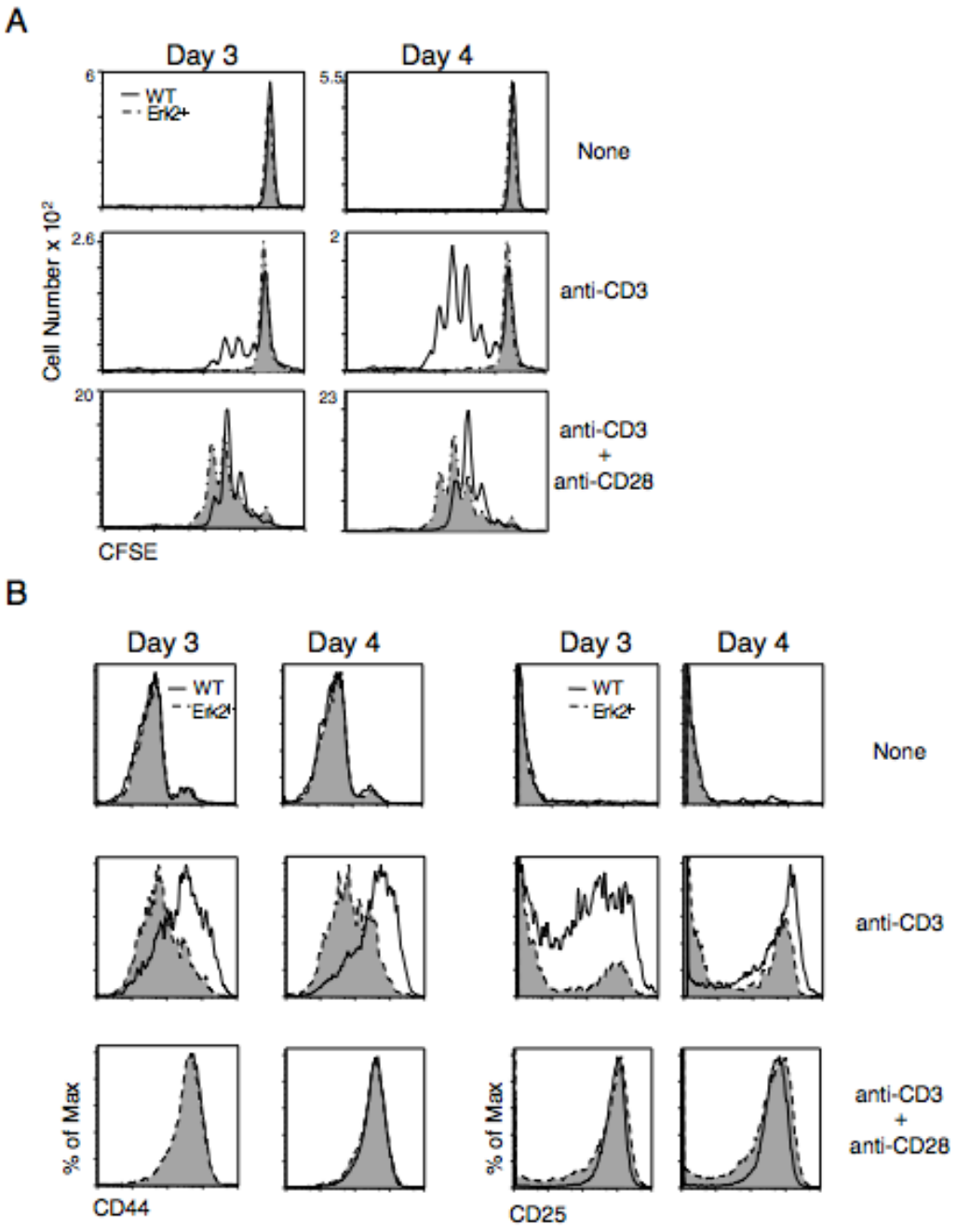

Figure 2-5. Erk2 deficient CD4 T cells have impaired proliferation with TCR signaling alone but exhibit normal proliferation under optimal stimulation in vitro. (A) Purified WT or Erk1$^{-/-}$ or Erk2$^{-/-}$ CD4 T cells were labeled with CFSE and stimulated in vitro with plate-bound anti-CD3 (2.5ng) +/- soluble anti-CD28. CFSE profiles are gated on CD4^{+} T cells. The actual number of cells run on the FACS in a fixed time-period is plotted. (B) Profiles of CD44 and CD25 were shown in FACS plots.

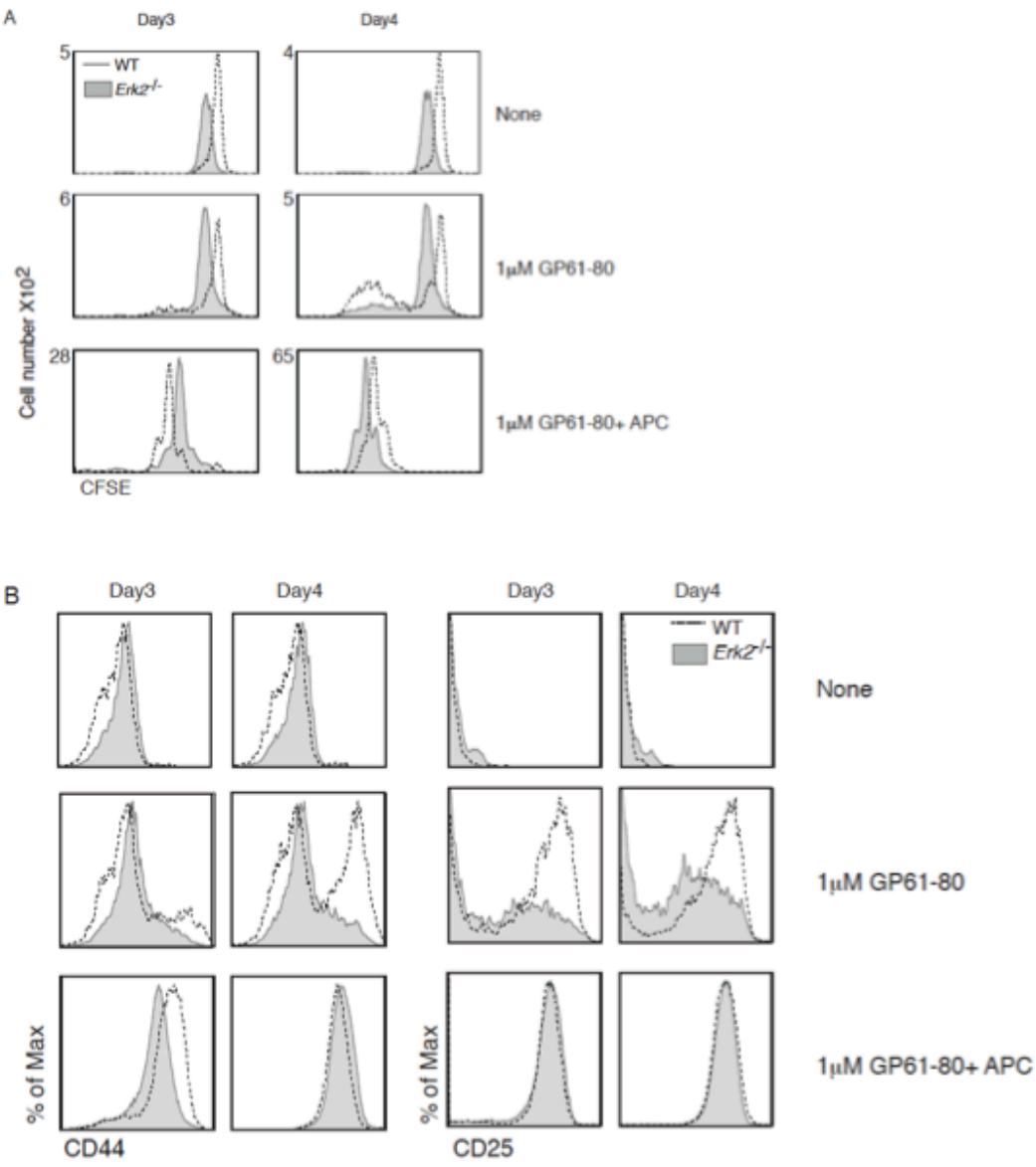

Figure 2-6. Erk2-deficient Smarta CD4 T cells have impaired proliferation with GP peptide alone but exhibit normal proliferation under optimal stimulation in vitro. (A) Purified WT or Erk2$^{-/-}$ Smarta CD4 T cells were stimulated in vitro with GP61-80 peptide (1uM) in presence or absence of mitomycin-treated antigen presenting cells (APC). CFSE profiles are gated on CD4^{+} T cells. (B) Cell surface marker CD25 and CD44 were analyzed under different conditions.

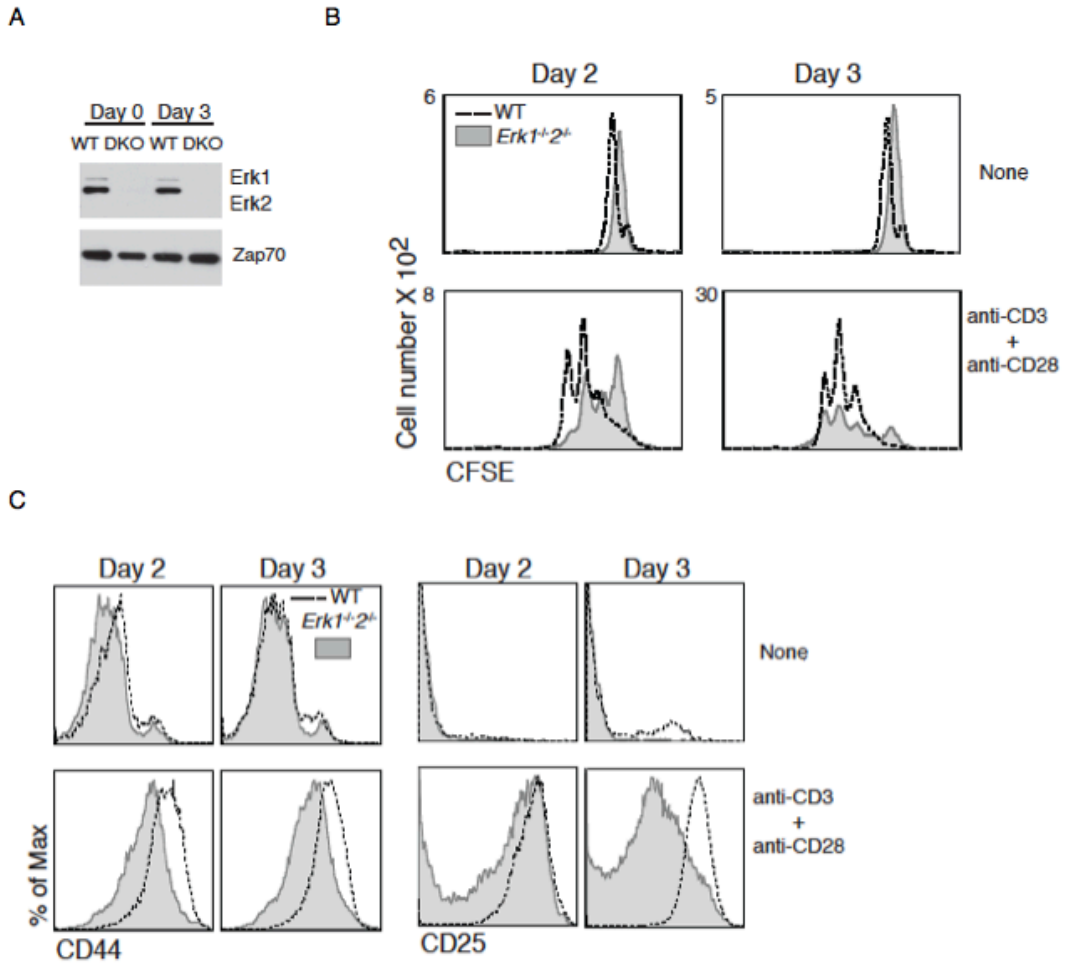

Figure 2-7. Erk deficient CD4 T cells have impaired survival and proliferation under optimal stimulation in vitro. (A) Purified CD4 T cells from WT and Erk1$^{-/-}$2$_{-/-}$ were analyzed for the presence of Erk2 by Western blotting. (B) WT or Erk1$^{-/-}$ or Erk2$^{-/-}$ CD4 T cells were labeled with CFSE and stimulated in vitro with plate-bound anti-CD3 (2.5ng) +/- soluble anti-CD28. CFSE profiles are gated on CD4^{+} T cells. The actual number of cells run on the FACS in a fixed time-period is plotted. (C) Profiles of CD44 and CD25 were shown in FACS plots.

Chapter. 3

The role of Erk in CD4 T helper proliferation and differentiation

Abstract

CD4 T cells can differentiate into different T helper lineages, such as Th1, Th2, Th17. Here we investigated the role of Erk in T helper proliferation and differentiation. We generated Th1 responses in mice by LCMV (Lymphocytic choriomeningitis virus) infection and found that Erk2 is important to regulate early Th1 proliferation. In addition, Erk2 is required for Th1 differentiation in vitro and in vivo. It is also true that Erk deficiency showed impaired Th1 differentiation when we examine Erk1$^{-/-}$2$^{-/-}$. Furthermore, Erk2$^{-/-}$T cells also have defect in cell survival due to increased expression of apoptotic protein Bim and decreased expression of anti-apoptotic protein Bcl2 and Bclxl.

Introduction

Within CD4 T lymphocytes, there are three major types of T helper (Th) cells: Th1, Th2 and Th17 (Murphy and Reiner, 2002). Th1 cells mediate effective immunity against intracellular pathogens (bacteria or virus), and they are characterized by the production of IFNγ. Th2 cells provide protection against certain extracellular pathogens (helminths) and they are characterized by the production of IL-4, IL-5, and IL-10. Th17 cells produce IL-17A and IL-17F, and provide a defense against extracellular bacteria and mediating inflammation (Weaver et al., 2006). Erk1 deficient mice exhibit normal T helper

differentiation while dendritic cells (DCs) from Erk1 knockout mice tend to make more IL-12 and thereby induce more Th1 differentiation (Agrawal et al., 2006). Interestingly, Erk1 knockout mice are more susceptible to experimental autoimmune encephalomyelitis (EAE), a murine autoimmune disease (Agrawal et al., 2006). However, inhibition of Erk (PD98059, MEK inhibitor) can attenuate EAE through suppression of IL-23 and IL-1 driven IL-17 production (Brereton et al., 2009). This discrepancy for the Erk1 and Erk2 in T helper cell differentiation is still not known.

Results

Erk2 is required for Th1, but not for Th2 or Th17 development in vitro

We next examined the effect of Erk1 or Erk2 on T cell proliferation and differentiation when activated under Th1, Th2 or Th17 polarizing conditions. There was no difference observed in the proliferation (Figure 3-1) and cytokine production of WT and Erk1 knockout cells when activated under the different T helper (Th1, Th2, Th17) conditions (Figure 3-2). Thus, Erk1 was not required for T helper differentiation in vitro, consistent with previous findings that Erk1-deficient CD4 T cells display normal T cell effector function (Nekrasova et al., 2005). We found that Erk2-deficient CD4 T cells displayed normal proliferation, but a survival defect when activated under Th1 and Th2 polarization conditions

(Figure 3-3). This survival defect may be the result of increased levels of the pro-apoptotic protein Bim and decreased levels of the pro-survival proteins Bcl-2 and Bcl-XL that were observed in Erk2$^{-/-}$ cells relative to wild-type (Figure 3-4). We further examined the effect the absence of Erk2 had on the differentiation of Th1, Th2 and Th17 cells as measured by the intracellular production of IFNγ, IL-4 or IL-17 (Figure 3-5). The proportion of IFNγ-producing Erk2-deficient CD4 T cells was greatly reduced when compared to wild type and this was confirmed by the 4-fold decrease in IFNγ levels that was observed in the culture supernatant measured by ELISA (Figure 3-5B and C). This is consistent with previous studies that used a MEK inhibitor (Badou et al., 2001). Despite this defect, Erk2 deficient T cells expressed a level of T-bet and phos-Stat1 equivalent to wild type (Figure 3-6A and C). However, Erk2 deficient CD4 T cells exhibited slightly more Gata-3 expression and generated an increased population of CD25^{+}Foxp3^{+} compared to wild type under Th1 skewing conditions (Figure 3-6B).

Previous studies have proposed a role for the Erk pathway in the induction of Th2 differentiation (Shinnakasu et al., 2008; Singh and Zhang, 2004; So et al., 2007; Yamashita et al., 1999; Yamashita et al., 2005), whereas other studies have shown that strong TCR-mediated signaling only gives rise to Th2 differentiation under conditions of Erk attenuation (Yamane et al., 2005). As such, we sought to test Th2 polarization of Erk2$^{-/-}$ T cells in vitro. In contrast,

there was no significant difference between WT and Erk2 deficient IL-4 or IL-17-producing cells. Thus, Erk2 does not appear to play a role in the differentiation of Th2 and Th17 cells at least in the presence of excess IL-4 or TGFβ and IL-6 (Fig 3-5A and B). Erk deficient CD4 T cells had defect in proliferation and survival under different T helper conditions (Figure 3-7). It was also true in $Erk1^{-/-}2^{-/-}$ CD4 T cells were defective in Th1 differentiation (Figure 3-8). Therefore, Erk2 has an indispensable role in Th1 cell differentiation and proliferation.

Impaired Th1-driven viral response in Erk2-deficient mice

To determine whether Erk2 was equally important for the differentiation of Th1 cells in vivo, we immunized mice WT, $Erk1^{-/-}$ or $Erk2^{-/-}$ mice with LCMV Armstrong and assessed the number of IFNγ producing T cell at the proliferative peak of responses (day 8 post-infection). Erk1-deficient mice displayed similar CD4 responses as wild type mice (Figure 3-9). However, the CD4 T responses in Erk2-deficient mice were 10 fold lower than those observed within the WT controls (Figure 3-9). There also was a dramatic reduction in the proportion and the absolute cell number of IFNγ producing cells in absence of Erk2. To determine whether the defect in Th1 cell expansion was T cell intrinsic, $Rag^{-/-}$ mice were reconstituted with bone marrow cells from wild type, or $Erk2^{f/f}$ ERcre mice or with an equal mixture of both. Eight weeks post-reconstitution, the mice were treated with tamoxifen and then challenged with LCMV. The levels of anti-virual IFNγ producing Th1 cells were dramatically reduced in the mice that were

reconstituted with Erk2$^{f/f}$ ERcre (Figure 3-10). Importantly, even within the mixed bone marrow chimeras, the Erk2 deficient cells again displayed reduced accumulation of antigen-specific IFNγ producing Th1 cells, confirming that there was a T cell-intrinsic requirement for the Erk2 signaling in Th1 cell differentiation. The results obtained using MHC-Class II tetramers to track LCMV-specific CD4 T cells mirrored those observed using intracellular IFNγ staining (Figure 3-10). Therefore, Erk2 plays an important role in CD4 Th1 cell survival and differentiation in vivo.

Discussion

In T helper polarization, Erk2 is required for early proliferation in Th1 and Th2, but not Th17. Erk2$^{-/-}$ CD4 T cells still a normal level of T-bet and GATA-3 as lineage factor for Th1 and Th2. In Th1 conditions, both the survival and differentiation is defect in absence of Erk2. It seems that Erk2-deficient CD4 T cells make less IL-2 and fewer cells survive. Also, Erk2-deficient CD4 T cells induce more Bim expression and thereby lead to cell death. Moreover, Erk2$^{-/-}$ CD4 T cells showed diminished activation because of lower expression of CD25 and CD44. Therefore, Erk$^{-/-}$ Th1 cells made less IFNγ when cells were restimulated through TCR signaling. Following LCMV infection, fewer IFNγ and tetramer positive cells were detected in Erk2-deficient cells. The tetramer

positive cells were able to make IFNγ in absence of Erk2. These results were consistent with what we saw in Erk2 deficient CD8 responses for LCMV (D'Souza et al., 2008). Therefore, Erk2 is required for cell survival in Th1 responses.

Experimental procedures

T helper differentiations in vitro

CD4 T cells from lymph nodes and spleens were purified by positive or negative selection using magnetic beads (Miltenyi Biotec). To analyze cell proliferation, purified CD4 T cells were labeled with CFSE at $10x10^6$ cells/ml. Cells were stimulated with anti-CD3 or anti-CD3 and anti-CD28 in complete RPMI media for indicated periods of time and harvested at various time points. For T helper differentiations, cells were stimulated with anti-CD3 and anti-CD28. Under Th1 conditions, IL-12 (10ng/ml) and anti-IL-4 were added to the culture. Under Th2 conditions, IL-4 (10ng/ml) and anti-IFNγ were added to the culture. Under Th17 conditions, IL-6, TGFβ, anti-IFNγ and anti-IL-4 were added to the culture.

LCMV responses

For bone marrow chimeras experiments, T cell-depleted bone marrow cells from B6 (CD45.1+) and $Erk2^{f/f}$ $CreER^{T2}$ (CD45.2+) mice were mixed at a 1:1 ratio and injected intravenously into lethally irradiated $Rag1^{-/-}$ mice. Mice were infected with LCMV until 8 weeks after reconstitution.

Flow cytometry

Lymphocytes were isolated from lymph nodes, spleens, thymus or peripheral blood. Cells were stained with the following surface makers: CD62L, CD44, CD4 and CD8. For the intracellular staining of Foxp3, cells were fixed and permeabilized with Foxp3 buffer set (eBioscience) and stained with anti-Foxp3 antibody. MHC ClassII GP66-77 Tetramers were provided by NIH tetramer facility. Samples were collected on a FACS Calibur or LSRII (BD Biosciences) and analyzed by FlowJo software (Tree Star).

Cytokine production

Cells were restimulated, harvested at the indicated time points, and centrifuged. The supernatant was removed and analyzed for the production of cytokines with ELISA kits (eBioscience) or Th1/2/17 CBA assay kits (BD sciences).

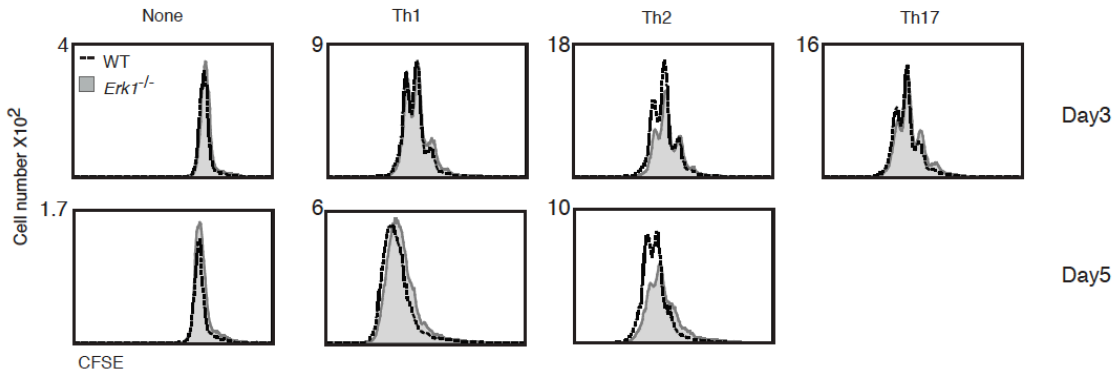

Figure 3-1. Normal proliferation of Erk1 deficient CD4 T cells under different T helper skewing conditions. Purified WT or Erk1$^{-/-}$ CD4 T cells were labeled with CFSE and stimulated in vitro with plate-bound anti-CD3, soluble anti-CD28 and different cytokines for 3 or 5 days. CFSE profiles are gated on CD4^{+} T cells. The actual number of cells run on the FACS in a fixed time-period is plotted.

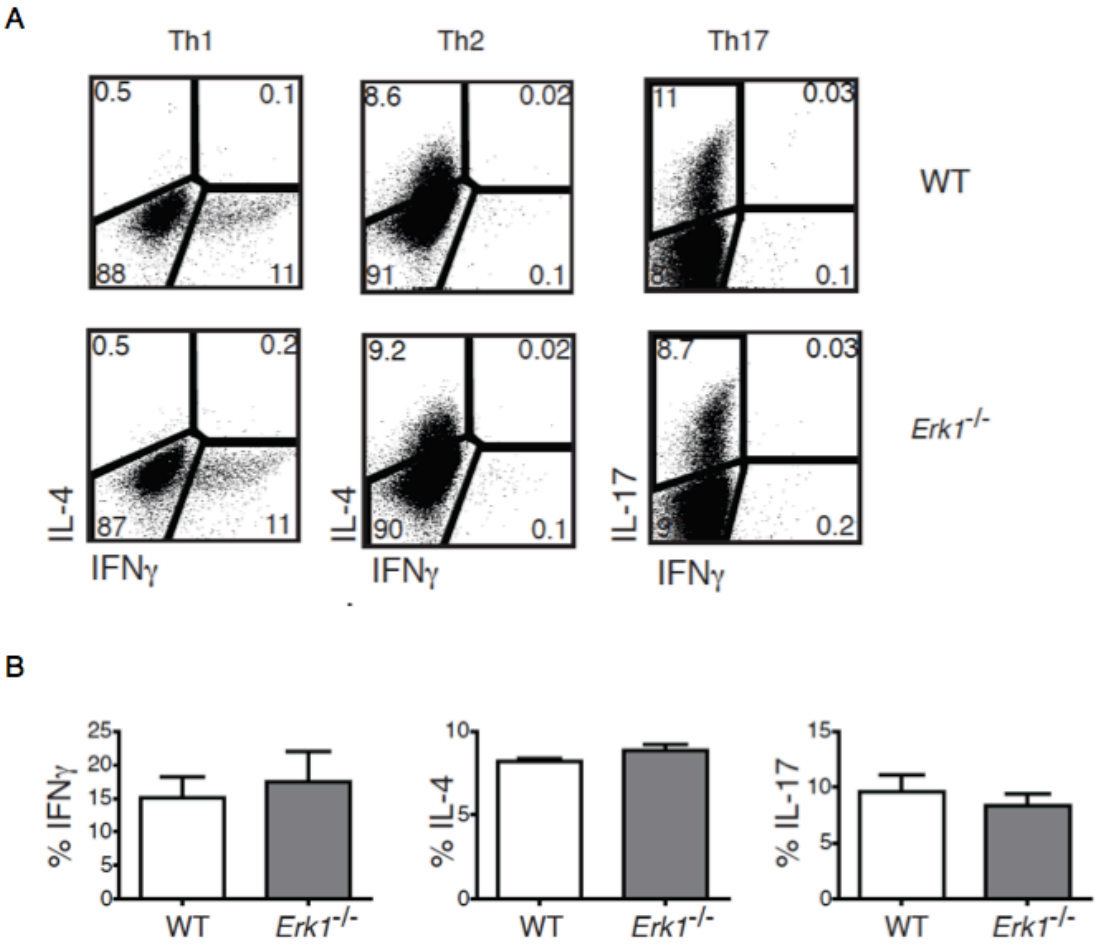

Figure 3-2. Erk1 deficient CD4 T cells exhibit normal CD4 differentiations. (A) On day 5, Th1 or Th2 cells were washed and restimulated for an additional 2 days with anti-CD3. Monensin was added during the last 5 hours of culture. For Th17 cells, cells were treated for 5 hours with PMA and ionomycin in the presence of monensin on day3. (B) Plots are gated on CD4 T cells and the numbers indicated are percent of CD4 T cells Percentage of IL-4, IL-17 from 3 independent experiments is shown.

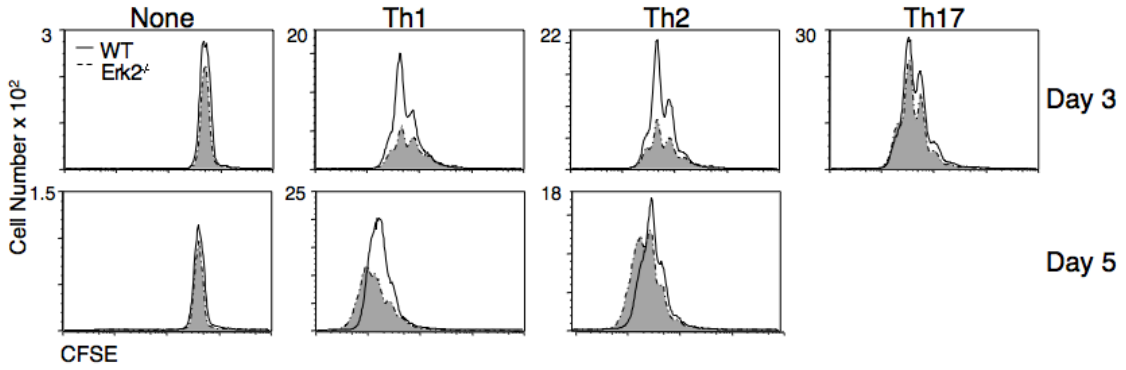

Figure 3-3. Erk2$^{-/-}$ CD4 T cells display impaired survival to the Th1 subset. CD4 T cells were purified, labeled with CFSE and cultured in media alone or under different T helper conditions for 3 or 5 days. CFSE profiles are analyzed in the same way as Figure 3-1 (Wild type is in white and Erk2$^{-/-}$ is in gray).

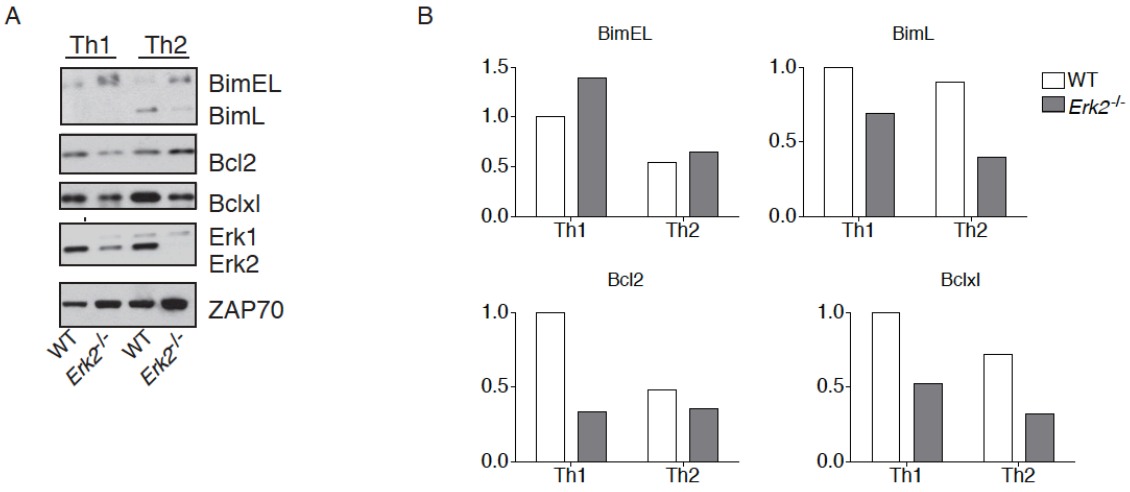

Figure 3-4. Increased Bim level in Erk2$^{-/-}$ CD4 T cells under Th1 polarization. (A)(B) Purified CD4 T cells from WT and Erk2$^{-/-}$ mice were stimulated under Th1 or Th2 condition for 5days and restimulated live cells for 2 days. Day7 Th1 or Th2 cell lysates were analyzed by western blotting. ZAP70 was used as loading control. Plots were BimEL, BimL, Bcl2 and Bclxl level normalized to ZAP70.

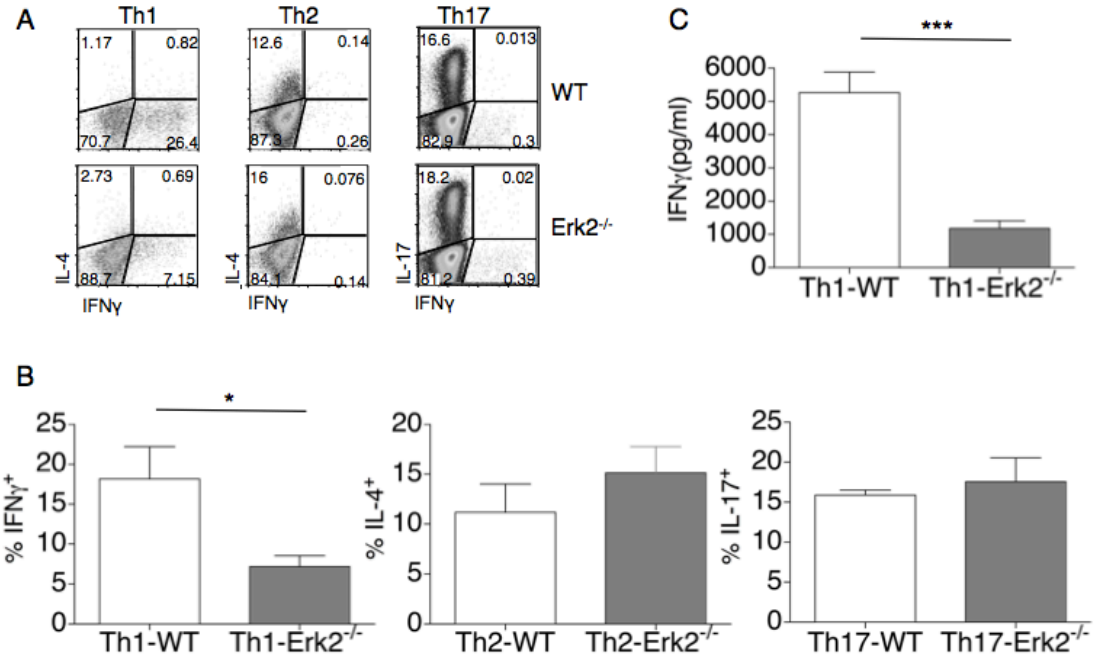

Figure 3-5. Erk2$^{-/-}$ CD4 T cells exhibit impaired polarization to the Th1 subset. (A) On day 5, Th1 or Th2 cells were washed and restimulated for an additional 2 days with anti-CD3. Monensin was added during the last 5 hours of culture. For Th17 cells, cells were treated for 5 hours with PMA and ionomycin in the presence of monensin on day3. Plots are gated on CD4 T cells and the numbers indicated are percent of CD4 T cells. (B) Percentage of IFNγ, IL-4, IL-17 from 3 independent experiments. (C) The level of IFNγ in re-stimulated Th1 supernatants were analyzed by ELISA.

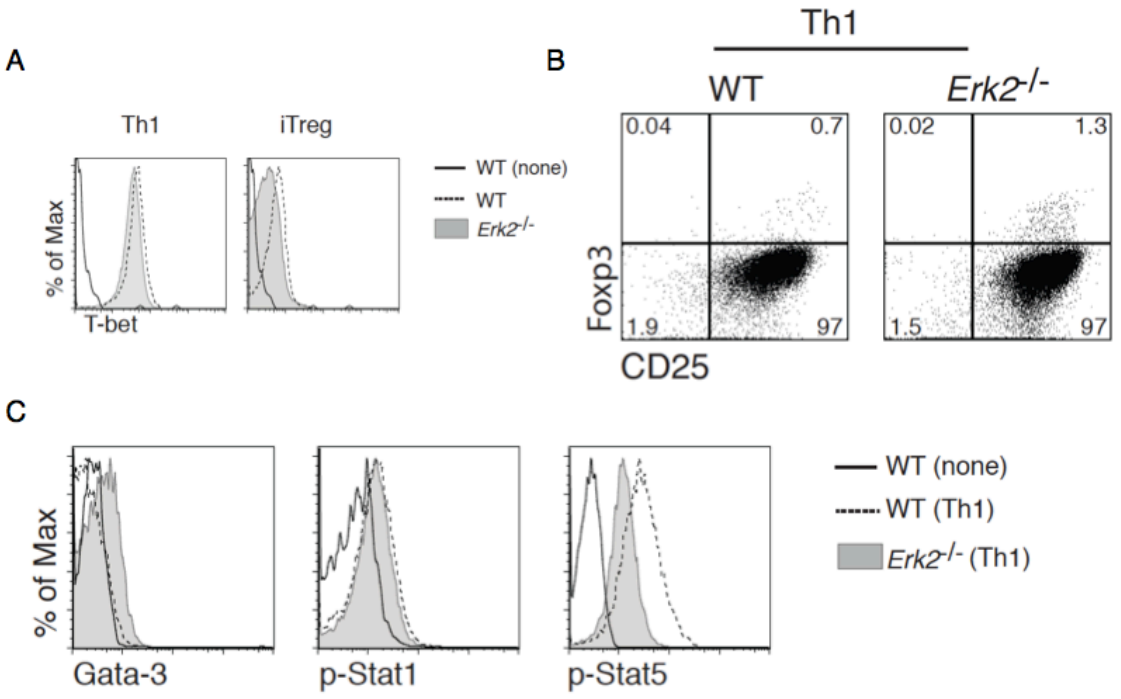

Figure 3-6. Erk2-deficient CD4 T cells express normal T-bet and phos-stat-1, but more Foxp3, Gata3 under Th1 skewing condition. (A) The cells (WT, Erk2$^{-/-}$) were analyzed for transcription factor T-bet at day 3 of Th1 and iTreg conditions. (B) Day 3 iTreg population (CD4+CD25+Foxp3+) were analyzed under Th1 and iTreg condition. (C) Expression of Gata-3, phos-Stat-1 and phos-Stat5 were shown by Facs analysis.

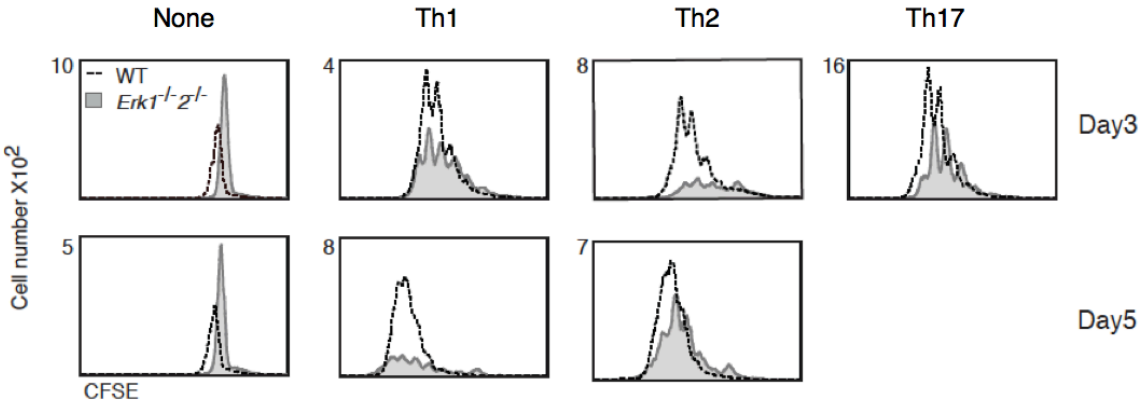

Figure 3-7. Erk1$^{-/-}$2$^{-/-}$ CD4 T cells display impaired survival to the Th1 subset. CD4 T cells were purified, labeled with CFSE and cultured in media alone or under different T helper conditions for 3 or 5 days. CFSE profiles are analyzed in the same way as Fig 3-1. Wild type is in white and Erk1$^{-/-}$2$^{-/-}$ is in gray.

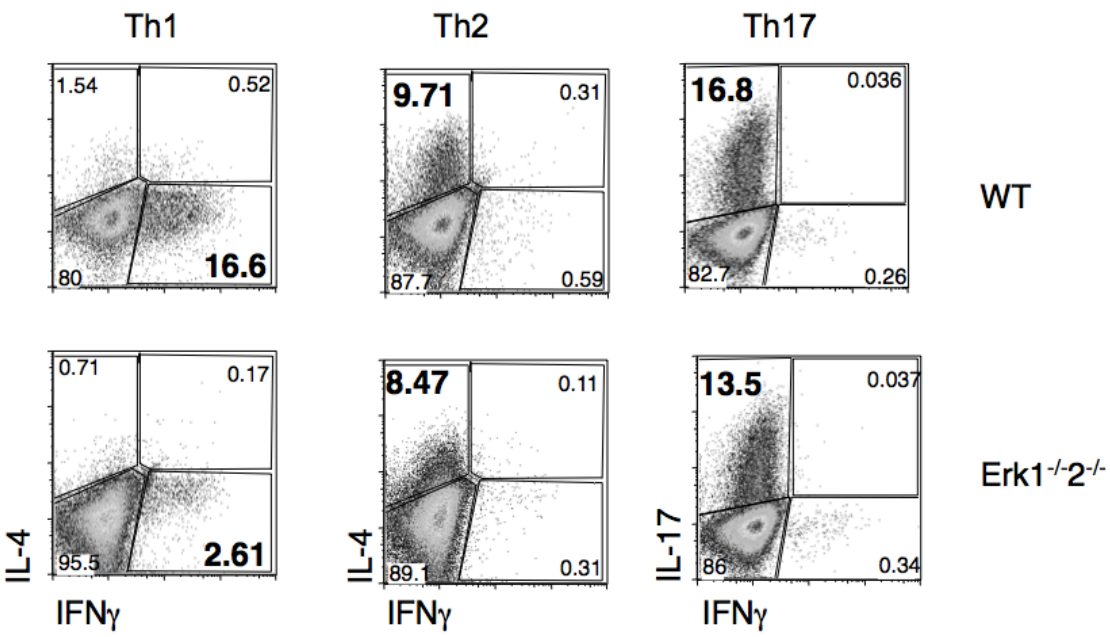

Figure 3-8. Erk1$^{-/-}$2$^{-/-}$ CD4 T cells impaired polarization to the Th1 subset. CD4 T cells were purified, labeled with CFSE and cultured in media alone or under different T helper conditions for 5 days. On day 5, Th1 or Th2 cells were washed and restimulated for an additional 2 days with anti-CD3. Monensin was added during the last 5 hours of culture. For Th17 cells, cells were treated for 5 hours with PMA and ionomycin in the presence of monensin on day3. Plots are gated on CD4 T cells and the numbers indicated are percent of CD4 T cells.

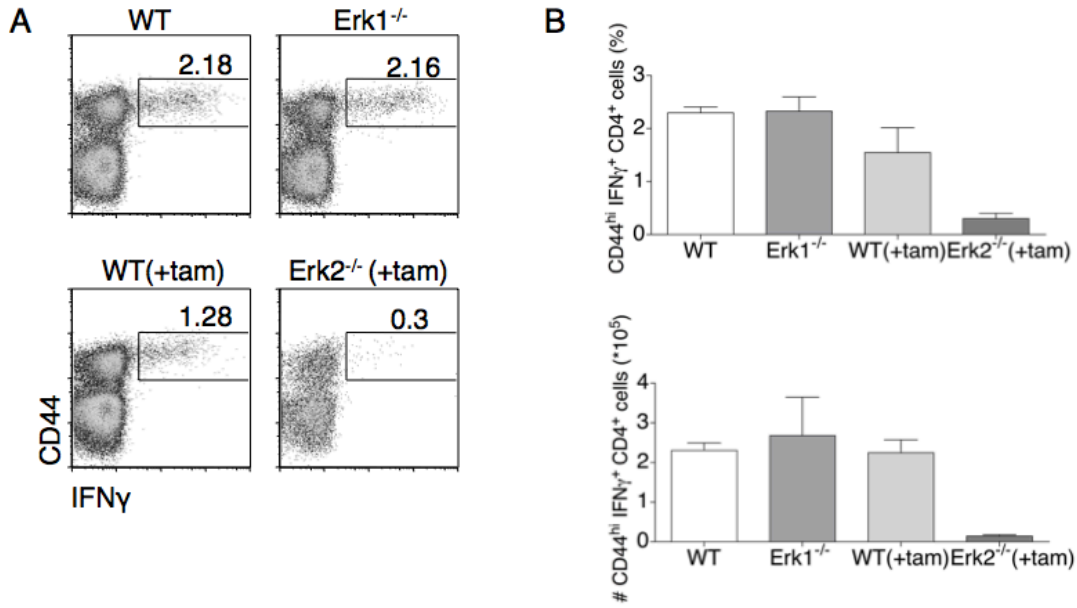

Figure 3-9. Erk2 deficient mice have the impaired anti-viral Th1 responses. (A)(B) WT, Erk1$^{-/-}$, and Erk2$^{-/-}$ mice were infected *i.p.* with 2 x 10^5 *pfu* LCMV and sacrificed at day 8 post-infection. Antigen-specific CD4 T cell responses within the spleen were assayed by intracellular cytokine staining following a 5 hour restimulation with the LCMV peptides GP61-80 and NP309-327. Representative FACS plots of Erk deficient and relevant control mice (gated on CD4$^+$ cells) are shown. Also indicated is the mean results +/- standard deviation of antigen-specific CD4 T cells present in spleen.

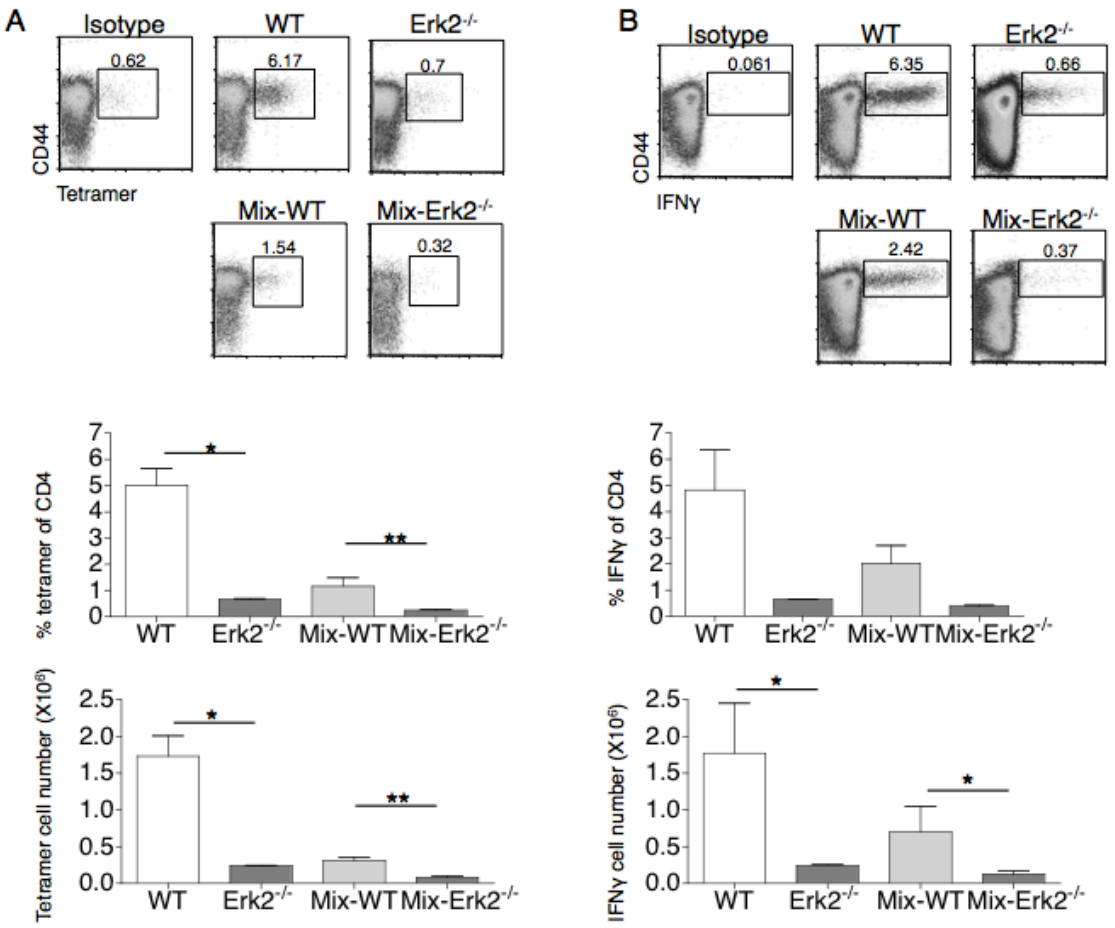

Figure 3-10. Impaired anti-viral Th1 responses in absence of Erk2. (A)(B) Rag1$^{-/-}$ mice were reconstituted with bone marrow from wild type, Erk2-deficient mice or both. Two months later, mice were treated with tamoxifen and then infected with LCMV. Antigen-specific CD4 T cell responses were analyzed in the same way as Figure 3-8 and also stained with tetramer GP-66-77. Representative FACS plots of Erk deficient and relevant control mice (gated on CD4^{+} cells) are shown. Also indicated is the mean result (n=3 per group) +/- standard deviation of antigen-specific CD4 T cells (percentage and cell number) present in spleen. (*, P<0.05; **, P<0.001.) Representative data were shown from one of three independent experiments.

Chapter. 4

The role of Erk in induced regulatory T proliferation and differentiation

Abstract

Here we examine the role of Erk in induced regulatory T cell differentiation. Both Erk1$^{-/-}$ and Erk2$^{-/-}$ natural regulatory T cells can suppress CD4 T cell proliferation similar to wild type in vitro. We also examine the function of Erk2-/- regulatory T cells by inflammatory bowel disease and found that Erk2-/- regulatory T cells have similar suppressive activity as wild type regulatory T cells. Impaired Erk2 signaling enhanced induced Treg (iTreg) differentiation in vitro and in vivo. In addition, we observed a down-regulated DNA methyltransferase (DNMT) in Erk2$^{-/-}$ CD4 T cells when compared to wild type. Also, an increased phos-Smad2 and TGFβ receptor II were found in Erk2$^{-/-}$ CD4 T cells under induced regulatory T cell condition. The decrease of DNMT in and increase TGFβ signaling in Erk2$^{-/-}$CD4 T cells possibly induced an increase of Foxp3 expression.

Introduction

Naïve CD4 T cells can differentiate into regulatory T cells ($CD4^+CD25^+Foxp3^+$). Regulatory T cells express Foxp3 as an important transcription factor and function to suppress the immune responses (Feuerer et al., 2009). There are two types of Treg cells: natural Treg (nTreg) produced

in the thymus, and induced Treg (iTreg) differentiated from naïve CD4 cells in secondary lymphoid organs. Both of them have been shown with suppressive activity, which may come from cell-cell contact inhibition as well as cytokine production such as TGFβ and IL-10 (Ke et al., 2011). In addition, Foxp3 expression requires T cell receptor, costimulatory and cytokine mediated signaling (Shen et al., 2009). The *Foxp3* locus contains a promoter and at least three enhancers (CN1, 2,3) that include Smad3, and Stat5 binding sites, and these sites presumably respond to TGFβ and IL-2 (or other γ-receptor cytokines), respectively (Chen et al., 2011; Zheng et al., 2010). TGFβ signaling leads activated Smad2 and Smad3 through the phosphorylation at C-terminal conserved residues (Abdollah et al., 1997; Souchelnytskyi et al., 1997). Furthermore, it has been showed that Smad3 and NFAT would bind to *Foxp3* locus and enhance its expression (Tone et al., 2008). IL-2 is a cytokine required for T cell survival and IL-2 signaling can activate Stat5 protein and thereby target in Foxp3 locus. It was known that IL-2 knockout mice develop autoimmunity because of a decreased number of regulatory T cells (Furtado et al., 2002). Furthermore, DNMT1 (DNA methyltransferase 1) and DNMT 3b are associated with Foxp3 locus and regulate the methylation of CpG islands to repress Foxp3 expression (Lal and Bromberg, 2009). In summary, regulatory T cells play an important role to prevent autoimmunity and are regulated.

Studies have shown that TGFβ signaling during T cell activation leads to lower phosphorylation of Erk and enhanced differentiation to iTreg cells (Luo et al., 2008). The preference for iTreg differentiation was also promoted by inhibition of MEK (using UO126)-the upstream activator of Erk1, 2. Either of these treatments caused a loss of DNMT expression (Luo et al., 2008). In accord with these results there was found to be an increase in *Foxp3* mRNA achieved using siRNA knockdown of either N-Ras or K-Ras, the upstream effectors of the Erk signaling pathway (Mor et al., 2008). A role for Erk signaling in the regulation of T reg cell differentiation was shown with the use of MEK inhibitor (UO126). TGFβ signaling led to reduced Erk phosphorylation and preferential iTreg differentiation correlating with the down-regulated expression of DNA methyltransferase (DNMT) (Luo et al., 2008). A recent study showed decreased phosphorylation of Smad2/3 linker region and increased Smad2/3 transcriptional activity may lead to enhanced iTreg differentiation in MEKK2/3 deficient CD4 T cells (Chang et al., 2011). However, the role of Erk1 and Erk2 in regulatory T cell differentiation and function is still not well understood. Here we used Erk knockout mice to address the questions in vitro as well as in vivo.

Results

Increased induced Treg differentiation in absence of Erk2

We observed an increased percentage of nature regulatory T cells (nTregs, $CD25^{+}Foxp3^{+}$) in $Erk2^{f/f}CD4Cre$ mice (Figure 4-1). Also, the acute deletion of Erk2 in $Erk2^{f/f}CreER^{T2}$ mice after tamoxfien treatment results in a slightly increased percentage of nTreg (Figure 4-2). To examine a role for Erk in induced regulatory T cells, naïve CD4 T cells were stimulated under iTreg conditions (anti-CD3, CD28, TGFβ, IL-2) for 5 days. The population of iTreg was analyzed for CD25 and Foxp3. Erk2 deficient CD4 T cells displayed similar proliferation as wild type (Figure 4-3A), but Erk2-deficient cells displayed an increased percentage of $CD25^{+}Foxp3^{+}$ (iTreg) in vitro (Figure 4-3B). However, there was no difference between wild type and $Erk1^{-/-}$ (Figure 4-4). Moreover, $Erk1^{-/-}Erk2^{-/-}$ CD4 T cells also showed increased in the percentages of iTreg (Figure 4-4). We also generated antigen-specific induced regulatory T cells in vivo by transferring Smarta CD4 T cells (depleted of $CD4^{+}CD25^{+}$ cells) and immunizing mice with LCMV Glycoprotein peptide 61-80. This cell population retained 1% $CD25^{-}Foxp3^{+}$ cells prior to transfer to recipient mice. The results showed a significant increase in the percentage of antigen-specific T cells that converted to a Foxp3 phenotype comparing $Erk2^{-/-}$ vs. wild-type T cells (Figure 4-5). These data are consistent with a role for Erk2 in repressing Foxp3 expression in vitro and in vivo.

Furthermore, the function of regulatory T cells was analyzed by two suppression assays. Compared to wild type, $Erk2^{-/-}$ Tregs exhibited an

enhanced suppressive function in Erk2 deficient regulatory T cells (Figure 4-6). Thus, Erk2 plays an inhibitory role for regulating induced Treg differentiation. To analyze the function of Erk2 deficient regulatory T cells, we used a model of inflammatory bowel disease generated by transferring naïve CD4 T cells in presence or absence of regulatory T cells (Figure 4-6B). The results showed that Erk2 deficient nature T cells can suppress the disease and mouse increase weight. It suggested that Erk2 deficient nTregs have similar ability to suppress T cell proliferation in vitro and in vivo.

Erk2 regulate Foxp3 expression

Studies showed that DNA methyltransferase (DNMT) was down regulated after UO126 treatment under induced Treg condition (Luo et al., 2008). We would thus predict that there would be decreased expression of DNMT in Erk2-deficient CD4 T cells upon iTreg differentiation. CD4 T cells were stimulated for different time points, RNA was isolated and DNMT level was analyzed by QPCR. The results showed that DNMT is down regulated in Erk2-deficient CD4 T cells compared to wild type (Figure 4-7A). Erk is also involved in the inhibitory phosphorylation of Smad protein in other cell types (Liu et al., 2011). In addition, Erk may also directly phoshorylate the linker region of Smad2 and Smad3. However, the phosohorylation of Smad2/3 iat the C-terminal is required for their transcriptional activity. Therefore, we examined the cell lysates for the phosphorylation of Smad (C-terminal) in order to see how

Erk regulate Smad signaling and their activity. We normalized expression phos-smad2 by Smad2 itself or loading control ZAP70. As shown, there was increased level of phos-Smad2 in Erk2-deficient cell compared to wild type in 30 minutes (Figure 4-7B). Therefore, Erk2-deficient cells may increase Foxp3 expression through downregulation of DNMT and increase TGFβ signaling.

Discussion

Foxp3 expression were regulated by TCR signaling, costimulatory signaling and cytokine-mediated signaling. Several transcription factors (Stat5, Smad3, AP-1, NFAT) binds to promoter or enhancer to increase Foxp3 expression. We see an increased differentiation of Foxp3 in absence of Erk2 but no difference in Erk1-deficient cells. TGFβ signaling downregulates the phosphorylation of Erk in naïve T cells. There is an increase differentiation of induced Treg with MEK inhibitor (UO126) because of down regulating the DNA methyltransferase (Luo et al., 2008). Our results agree with this study. Besides, we also found out Erk2 deficiency may enhance TGFβ signaling by increasing phos-Smad2 (Figure 4-7) and TGFβ receptor II (data not shown). Besides, there is higher percentage of regulatory T cells ($CD25^{+}Foxp3^{+}$) in $Erk2f^{f/f}CD4Cre$ mice. Also, it is true that Erk2 deficiency enhanced peptide induced regulatory T cells in vivo. Furthermore, we also examined the function activity of Erk2 deficient T

regulatory T cells. It showed that Erk2 deficient regulatory T cells have similar activity as wild type regulatory T cells (Figure 4-6). It indicates that T regulatory cells can have normal suppressive function in absence of Erk2.

In summary, Erk2 does repress Treg differentiation both in vitro and vivo. However, Erk1$^{-/-}$ cells are all normal in CD4 T cell proliferation and differentiation. Erk is known to drive cell proliferation and differentiation. Also, Erk has several substrates to further regulate cell responses (Chen et al., 2001). Our results provide the better way to understand how Erk regulate CD4 T iTreg differentiation and distinguish the difference between Erk1 and Erk2. CD4 CD4 T cell differentiation is important to further investigation for improving or inhibiting immune responses. Treg cells are important to keep balance for immune response between different T helper cells Th1, Th2 and Th17. Also, form human lupus studies (need refs), it showed the patients have increased population of CD25-Foxp3+ and Erk may be involved in this process (Gorelik and Richardson, 2009, 2010). Therefore, it would be interesting to find out novel therapies by enhancing Treg differentiation or inhibiting T cell responses in order to cure the disease.

Experimental procedures

Differentiation of induced Treg (iTreg) in vitro

To generate iTregs, CD4 cells were stimulated with anti-CD3, anti-CD28, TGFβ, IL-2 (10U/per well) in the presence or absence of anti-IL-4 and anti-IFNγ. In some cultures, cells were labeled with CFSE for analyzing cell proliferation.

Regulatory T cell assay (Suppression assay)

Naïve and regulatory T cells were purified from lymph nodes and spleens with $CD4^+CD25^+$ Regulatory T cell isolation kit (Miltenyi Biotech). Naïve T cells ($5* 10^4$) were stimulated with anti-CD3 (1ug/ml) and mitomycin C-treated splenocytes ($2*10^5$). Different amount of regulatory T cells were added to the culture and incubated at 37 degree for 3 days. [H^3] thymidine was added for the last 8 hr of the culture.

Generated induced regulatory T cells in vivo

Naïve Smarta CD4 T cells (CD45.2/2) were transferred into B6 mice (CD45.1/1). Next day the recipient mice were immunized with 10ug glycoprotein peptide (GP61-80) or PBS (as control). Five days later, lymph nodes and spleens were harvested and analyzed Foxp3 expression by FACS.

Inflammatory bowel disease

Naïve CD4 T cells ($CD25^-$ $CD45RB^{hi}$) and regulatory T cells ($CD25^+CD45RB^{low}$) were sorted by BD Aria. Cells (Naïve T cells, Naïve T cells with wild-type or $Erk2^{-/-}$ regulatory T cells) were transferred into $Rag^{-/-}$. We

measured the weight of mice weekly and scarified mice when losing 20% of starting weight. Colon sections were stained with H & E.

SDS-PAGE and Western blotting

Harvested CD4 T cells were resuspended in cell lysis buffer (50mM HEPES, 150mM NaCl, 1% Triton, EDTA, 1.5mM $MgCl_2$, glycerol) with protease and phosphatase inhibitors (Calbiochem). Protein concentrations were determined by Bradford assay. Supernatants were run on 4-12% Bis-tris gels and transferred to PVDF membranes. Blots were incubated with primary antibody at 4°C overnight and secondary HRP antibody (Vector Laboratories or SouthernBiotech) at room temperature for 1 hr. ECL was used to visualize protein expression. The primary antibodies are used as follows: anti-Erk1, 2 (Santa Cruz), anti-phos-Smad2 (Cell Signaling), anti-Smad2/3 (Cell Signaling), anti-ZAP70 (BD Biosciences).

RNA isolation and quantitative real-time PCR

Total RNA was isolated by Trizol (Invitrogen), treated with DNase using the DNA-free kit (Ambion), followed with cDNA synthesis by the SuperScript III reverse transcriptase and random hexamers (Invitrogen). The cDNA was amplified and detected using the Power SYBR Green mix (Applied Biosystems) and ROX reference dye (Stratagene) on an Mx3005P machine (Stratagene). Samples were normalized to GAPDH or Cph (cyclophilinA). Relative expression

was calculated as the difference (ΔCt). Primers are used as followed: *Dnmt*: fw 5'-gga agg cta cct ggc taa agt caa g-3', rev 5'-act gaa agg gtg tca ctg tcc gac-3'; *Cph*: fwd-cac cgt gtt ctt cga cat c, rev 5'-att ctg tga aag gag gaa cc-3'; *Gapdh*: fwd –cca gta tga ctc cac tca cg-3', rev 5'-gac tcc acg aca tact ca gc.

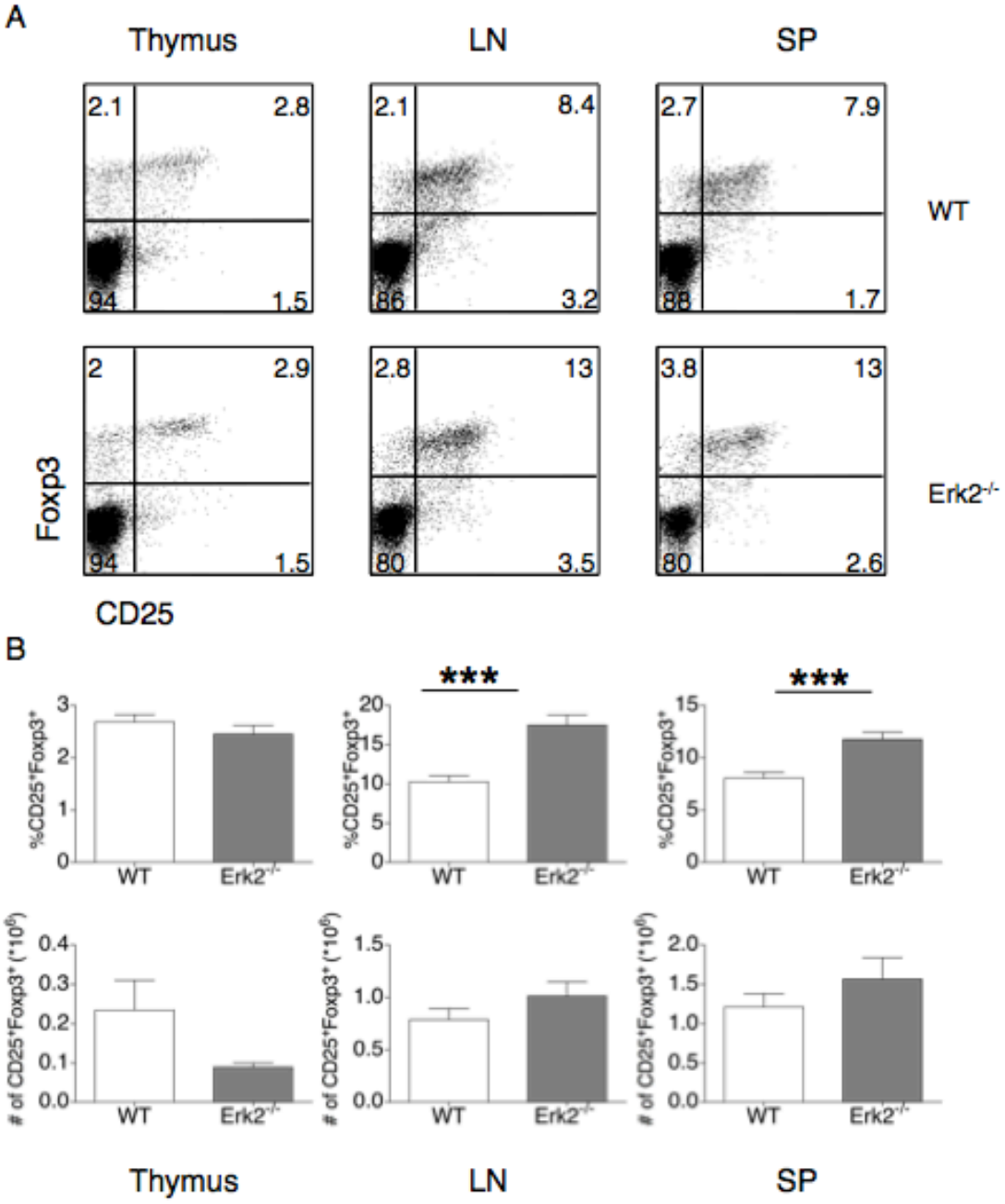

Figure 4-1. Slightly increased percentages and cell number in Erk2$^{f/f}$CD4Cre lymph nodes and spleens. (A) Representative FACS plots were shown from wild type and Erk2$^{f/f}$CD4Cre mice (6-12 weeks). (B) Percentage and cell number of nature Treg (CD4+CD25+Foxp3+) in thymii, lymph nodes and spleens were analyzed. (n=12 for each group)

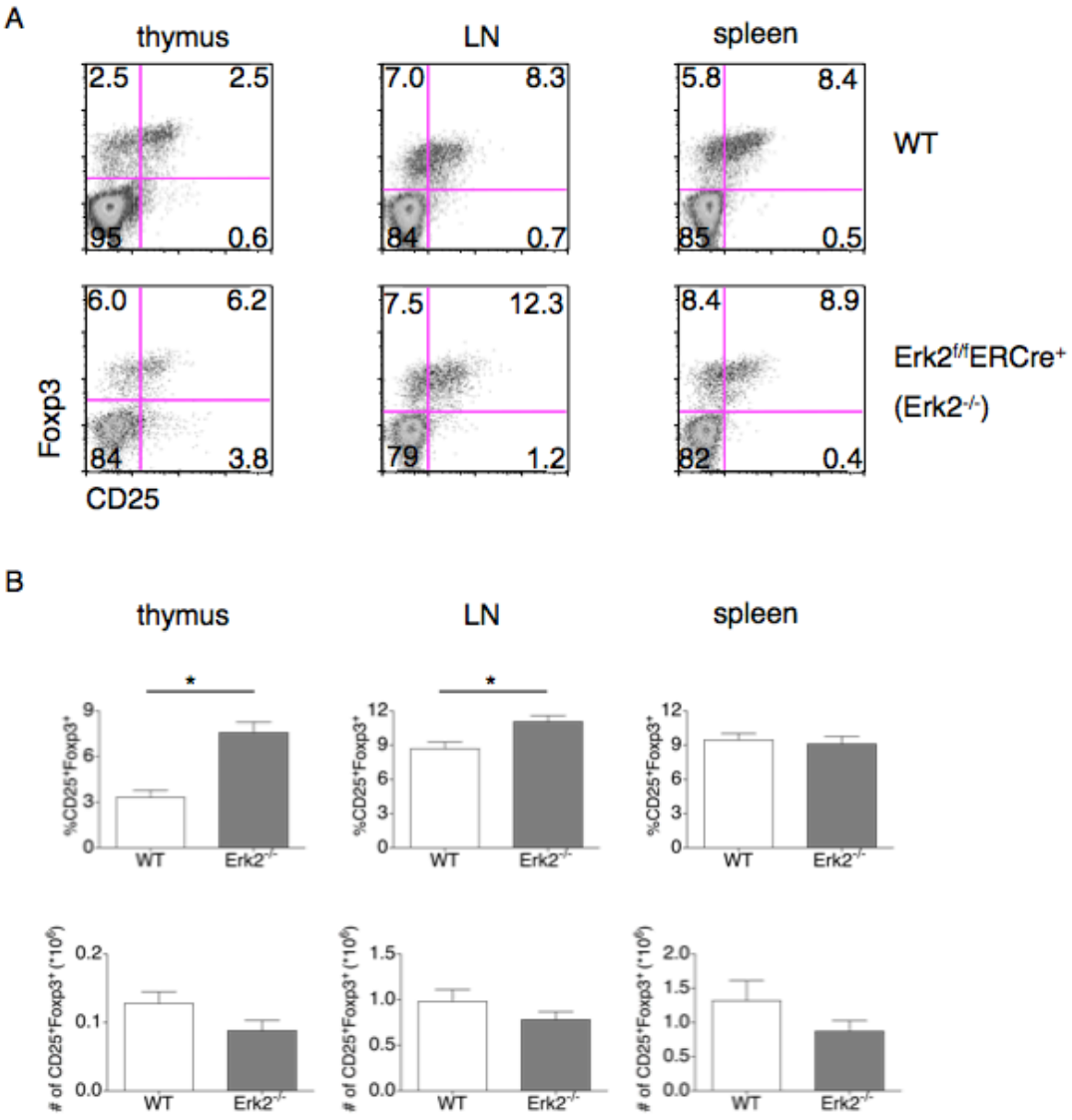

Figure 4-2. Slightly increased nTreg percentages and normal nTreg cell number in Erk2$^{f/f}$ERCre mice. (A) Representative FACS plots were shown from wild type and Erk2$^{f/f}$ERCre mice (6-12 weeks). (B) Percentage and cell number of nature Treg (CD4^{+}CD25^{+}Foxp3^{+}) in thymii, lymph nodes and spleens were analyzed. (n=10-20 for each group)

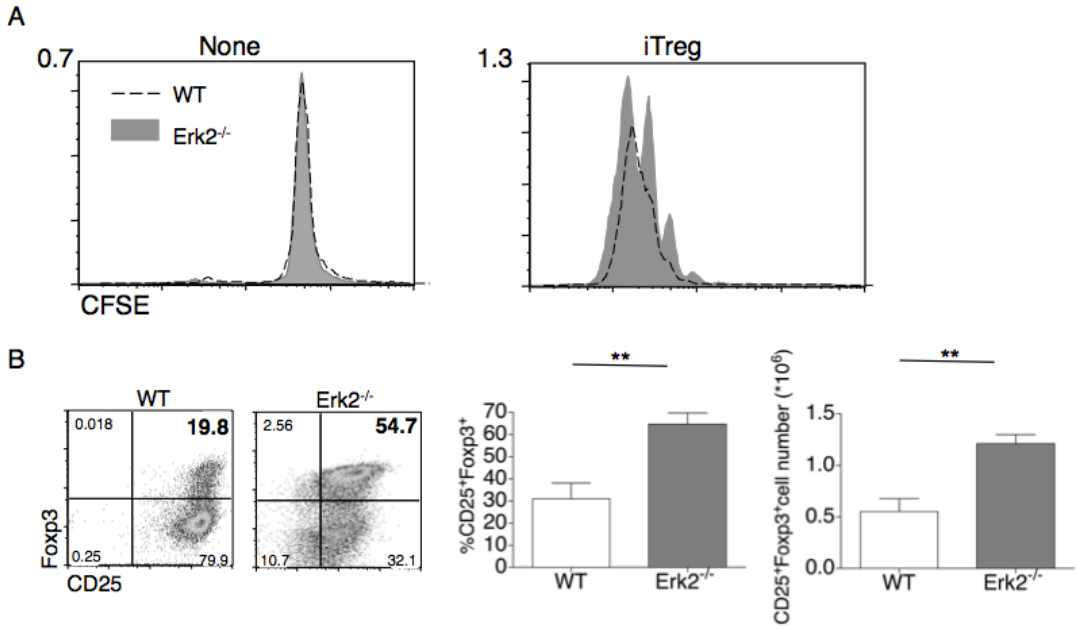

Figure 4-3. Enhanced differentiation of induced Treg in absence of Erk2. (A) CD4 T cells were purified, labeled with CFSE and cultured in media alone or under iTreg condition (anti-CD3, anti-CD28, TGFβ, IL-2) for 5 days. CFSE profiles are analyzed. Wild type is in white and Erk2$^{-/-}$ is in gray. (B) Purified CD4 T cells from WT and Erk2$^{-/-}$ mice were stimulated under iTreg conditions for 5 days. Cells were stained with CD4, CD25 and Foxp3, followed by flow cytometry analyses. The results shown are representative of four separate experiments.

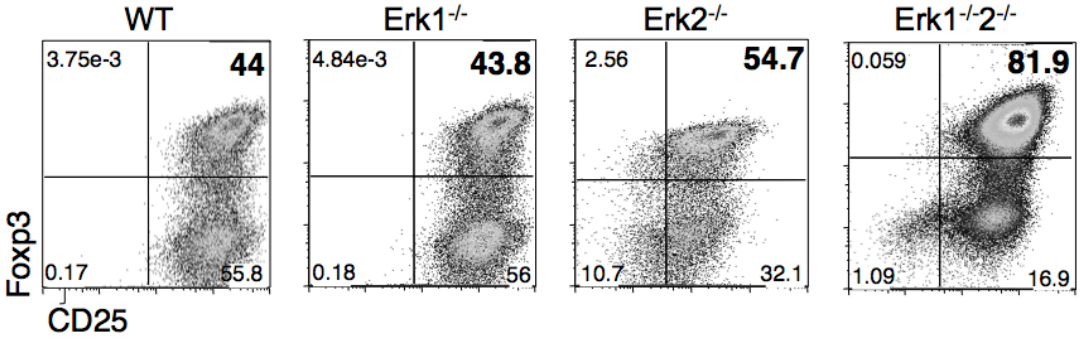

Figure 4-4. Enhanced differentiation of induced Treg in absence of total Erk (Erk1 and Erk2) and Erk2, but not Erk1 alone. Purified CD4 T cells from WT, Erk1$^{-/-}$ and Erk1$^{-/-}$2$^{-/-}$ were stimulated under iTreg condition for 5 days. Cells were stained with CD4, CD25 and Foxp3, followed by flow cytometry analyses.

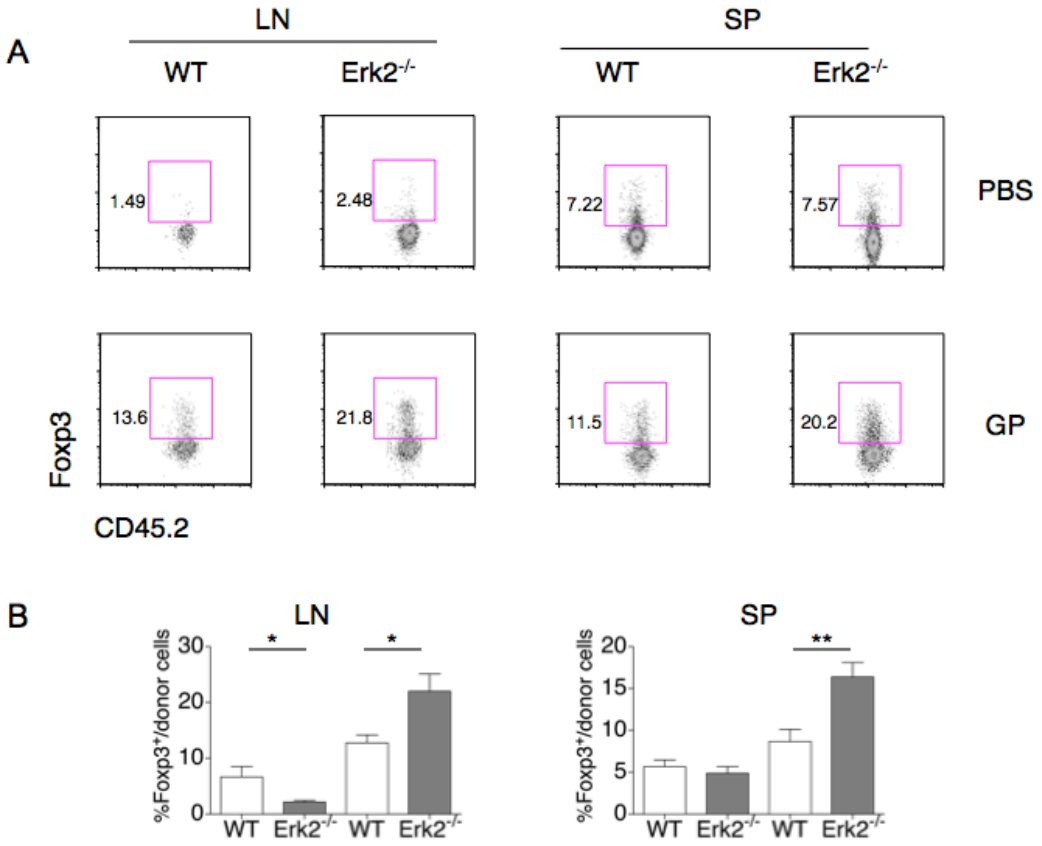

Figure 4-5. In vivo enhanced differentiation of induced Treg without Erk2. Naïve Smarta T cells from WT and Erk2$^{-/-}$ mice were adoptively transferred to different cogenic marker mice (CD45.1/1). Next day the recipient mice were immunized with 50ug GP61-80 peptide and 5 days later lymph nodes and spleens were harvested. FACS plots of Erk deficient and relevant control mice (gated on CD4+ cells) are shown. Peptide-induced regulatory populations are CD45.2^{+}Foxp3^{+}. Mean percentages of Foxp3^{+} (iTreg) in donor Smarta T cells were shown with standard derivation. Data were from two independent experiments (PBS treated group n=8, GP peptide treated group, n=10). *, P<0.05; **, P<0.001.

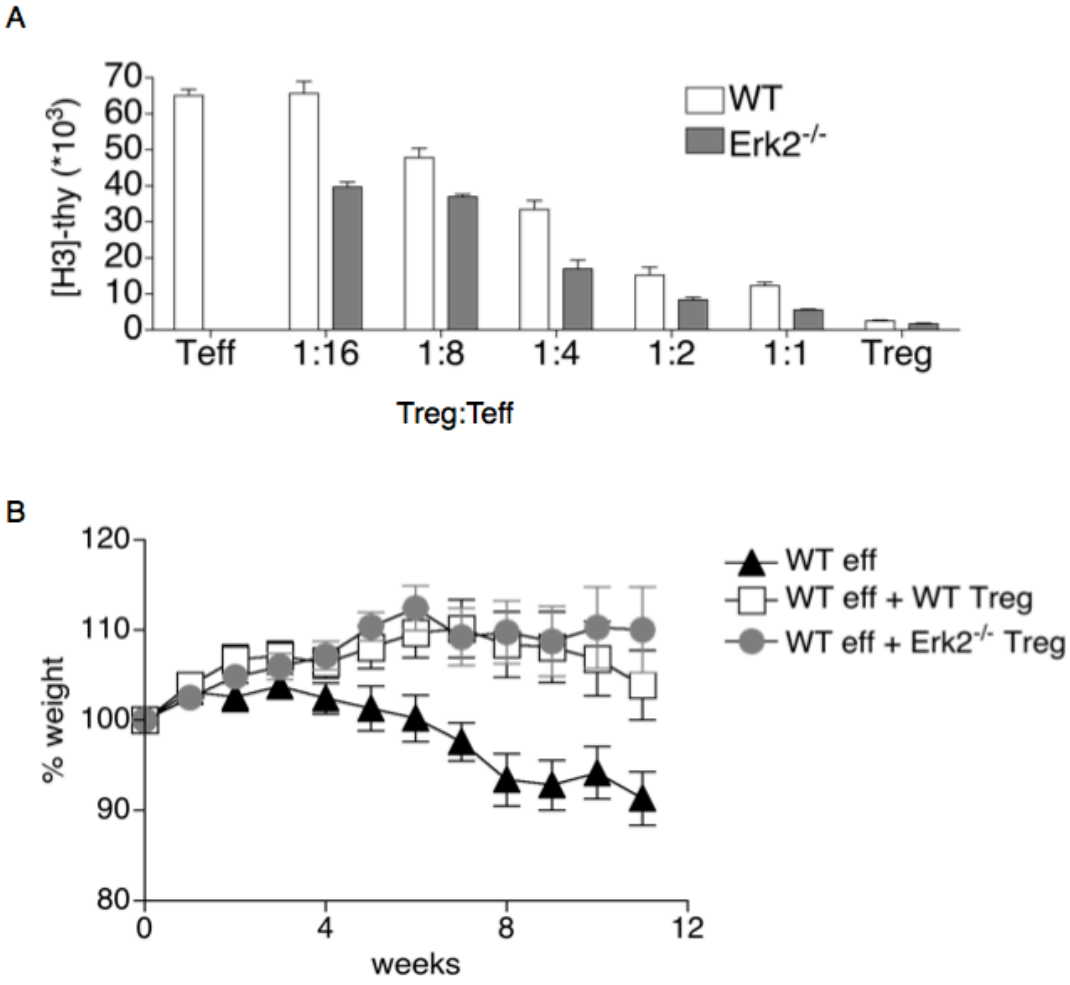

Figure 4-6. Normal function of Treg in absence of Erk2. (A) WT Teff ($CD4^+CD25^-$) were co-cultured with WT or Erk2 deficient Treg ($CD4^+CD25^+$) and stimulated with anti-CD3 and mitomycin-treated APC. Suppression of proliferation was measured by [^{3}H] thymidine uptake. (B) Naïve WT CD4 T cells in presence or absence of wild-type or Erk2 deficient nTreg were transferred into $Rag^{-/-}$ mice. Mice were weighed every week and the percentage of initial weight was calculated as indicated.

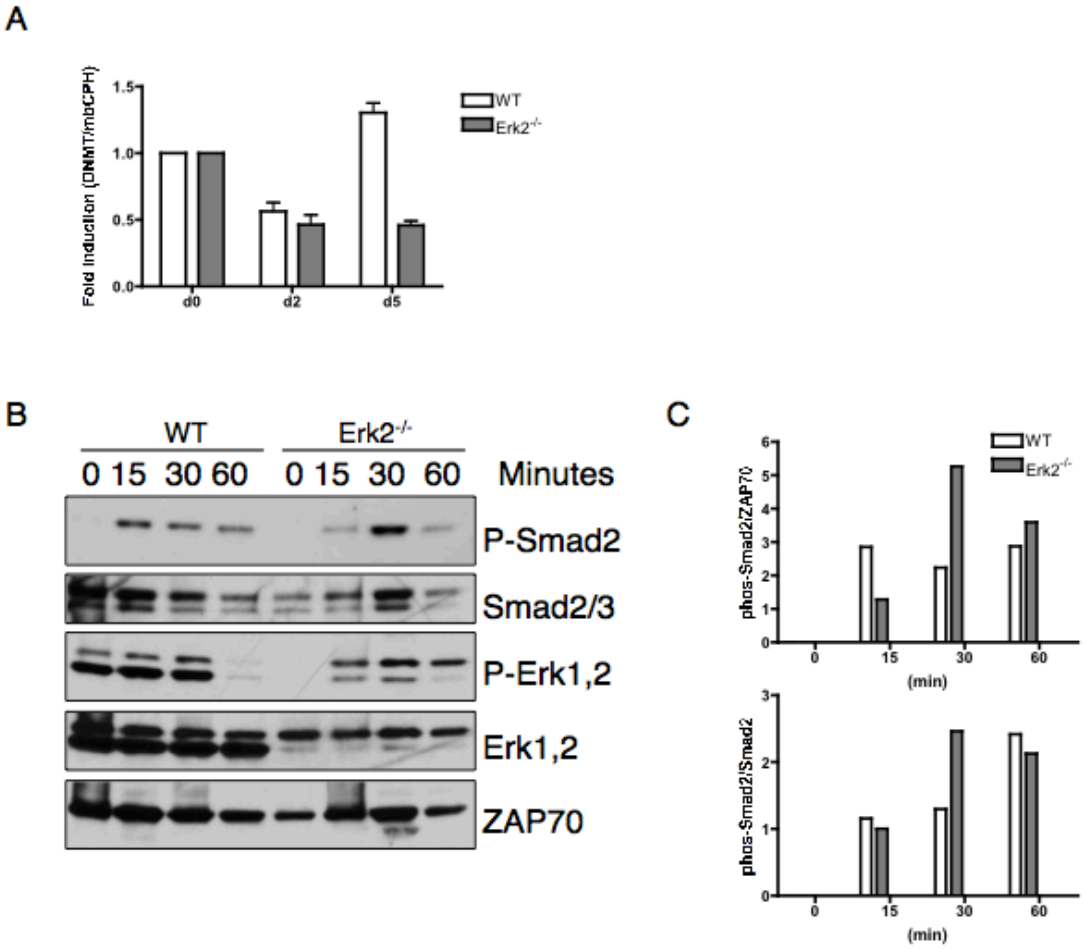

Figure 4-7. Erk2 regulate DNMT and phos-Smad under iTreg condition. (A) RNA was isolated from unstimulated WT or Erk2$^{-/-}$ cells that were stimulated under iTreg condition for 3 or 5 days. The mRNA level of DNMT1 (DNA methyltransferase I) was measured by real-time quantitative PCR and then normalized to a house keeping gene.(B)(C) Purified CD4 T cells from WT and Erk2$^{-/-}$ mice were stimulated under iTreg condition for short period of time. Cell lysates were analyzed by western blotting for indicated time points. ZAP70 was used as loading control. Plots were phos-Smad2 level normalized to ZAP70 or Smad.

Chapter. 5

Microarray analysis for the role of Erk2 in Th1 and iTreg

Abstract

From previous chapter III and IV, we showed Erk2 promoted Th1 differentiation but negatively regulated iTreg differentiation. Here, we performed microarray analysis for further examine the role of Erk2 in Th1 and induced Treg differentiation. In addition, we could find out the molecules are Erk2-dependent or independent for the mechanism in regulating T helper and regulatory T cells. We also analysis several signature genes in T helper cells and regulatory T cells. We found out that the decreased expression of Fos and stat-4 gene and increased expression of gata-3 genes and Foxp3-regulated genes (Ecm1, Pdlim4) in Erk2 deficient CD4 T cells under Th1 skewing. Besides, down-regulated of DNMT and up-regulated of Foxp3, Foxp3-induced genes or some Treg signature genes were found in Erk2 deficient CD4 T cells. This indicated Erk2 deficient CD4 T cells alter plasticity of CD4 T cell differentiation.

Introduction

Microarray analysis is a great tool to analysis large-scale of gene expression. Scatter plots and heatmaps are used to see profile of gene up- or down-regulated. There are several websites and software such as genepattern (Golub et al., 1999; Reich et al., 2006), D-chip (Li and Wong, 2001), cytoscape

(Smoot et al., 2011) to provide tools for analysis microarray data. Furthermore, filtered or selected genes (up or down-regulated genes) can be visualized by heatmap and further clustered by their profile of expression. In addition, these interested genes can be analyzed by their function and their protein-protein interaction as well as their roles in the cell signaling pathways.

We found out Erk2 promoted Th1 differentiation but negatively regulated iTreg differentiation. To further investigate the role of Erk2 in Th1 and iTreg, we performed microarray analysis for comparing wild type and Erk2 deficient cells under different conditions. We did short time point 3 hours to look at early transcription effects and also have stimulated for CD4 T cells for one day under Th0 (anti-CD3, anti-CD28, IL-2), Th1 (Th0+IL-12+anti-IL-4) and iTreg (Th0, TGFβ) conditions as well as have activated cells for a day and then add TGFβ for 1 or 3 hours to understand TGFβ signaling effects. (Fig 5-1) The purpose of this experiment is to screen potential Erk2-dependent substrates or regulated protein for Th1 and iTreg differentiation.

Results

RNA was isolated from different conditions from wild type and Erk2 deficient CD4 T cells in Figure 5-1. Microarray experiments were generated in UC San Diego core facility by using illumina mouse microarray kit. Normalized

expression data were obtained and analyzed by gene pattern website. Here we are going to discuss the results based on the points as below.

1. Up- and down- regulated genes in each condition

2. Find out common or unique genes in 3hr or day 1

3. T helper and Treg signature genes

4. Genes are involved for T helper and Treg differentiation

Day 0 (Fig 5-2, Tab 5-1)

Purified naive CD4 T cells ($CD4^+CD25^-$) were collected for d0 mRNA and further analyzed for gene expression. There are several upregulated genes (18 genes, KO/WT expression >1.5) in $Erk2^{-/-}$ CD4 T cells compared to wild type such as Hba-a1, Mid1, Erdr1, Dusp6, Vim, Ccr6, Rgs1, Cdkn1a and Lif etc. Hba-a1 (CD31) is hemoglobin alpha adult chain1, adult, expressed highly in most tissues and responsible for oxygen transport. However, there is one study showed that Hba-a1 is one of Erk–dependent substrates (Salzmann et al., 2006). Rgs1 are considered as signature nature regulatory T genes. Rgs1 blocked Erk2 phosphorylation (Cho et al., 2003) in human cells.

There are 11 downregulated genes (KO/WT expression <0.6) such as MAPK1 (Erk2), Socs3, Eno3 and Gdpd3 etc. As we expected, Erk2 (MAPK1) is downregulated in Erk2 deficient CD4 T cells. Socs3 belongs to suppressors of

cytokine signaling (SOCS) family and regulate various cytokine responses via inhibiting Stat3 signaling and IL-17 production (Zhang et al., 2006). In human T cells, it showed that JNK and MEK inhibitors suppressed peptide-induced expression of Socs3 (Kanda et al., 2011). Eno3 stands for beta Enolase and it is a glycolytic enzyme in glycolysis pathway. There are Eno1, 2, 3. Previous studies showed Erk signaling regulates ATP level and thereby induces Eno1 (alpha-Enloase) expression (Mizukami et al., 2004).

3hr Th0 & iTreg

We compared all the scatter plots from day0, 3hr and day 1 of Th0 and iTreg. There are more down-regulated genes in 3hr than day 1 when compared wild type and Erk2 deficient CD4 T cells (Figure 5-3). It may suggest that early cell proliferation may require the initial TCR signaling through Erk signaling and turn on more gene expression. However, some genes can still be activated through Erk1 signaling (weak signaling) after one-day stimulation. In table 5-2 and 5-3, we showed the list of up- and down-regulated genes in Erk2 deficient CD4 T cell after 3hr activation. Besides, there are few common genes showed up- or down regulated in Erk2 deficient CD4 cells in most conditions. It suggested Erk2 regulates their expression profiles directly or indirectly. On the other hand, there are some unique genes, which are specific for certain

conditions. Here we showed the number of genes is common or unique for different conditions after activation (Figure 5-5).

Using a VennDiagram, we show the number of genes in Erk2 deficient CD4 T cells that are uniquely up or down regulated under different conditions (Figure 5-5). There are some common up-regulated genes such as Gdpd3, Phlda and Eno3 and down-regulated genes such as Hba-a1, Mid1, Lta, Lyz, Cdkn1a and Ccl3. Some of them were shown in naïve Erk2 deficient CD4 T cells. It seems that the numbers of the unique up- or down-regulated gene expression in Erk2 deficient CD4 T cells are higher under iTreg condition than Th0 (Figure 5-5A). It suggests that TGFβ signaling may lead to more change of gene expression in absence of Erk. We also found out those up-regulated genes such as Usp18, Pdlim4, Emp1 can be Foxp3 target genes (Table 5-2 and 5-3).

Day1 Th0 & Th1 & iTreg

To explore the mechanism by which Erk2 affects Th1 and iTreg differentiation, we performed microarray analysis for WT and $Erk2^{-/-}$ (KO) post-activation under different conditions: Th0 (anti-CD3, anti-CD28, IL-2), Th1 (Th0+IL-12+anti-IL-4) and iTreg (Th0+TGFβ) conditions. Figure 5-4 depicts the number of up-regulated genes (expression >1.5 fold or more) and down-

regulated genes (expression <0.6 fold or less) under the indicated condition. We found that the greatest number of changes in gene expression in Erk2 deficient T cells under iTreg conditions (Figure 5-4).

Day 1 Th1 &Th0 (Table 5-3, 5-4)

It is known that Egr1-mediated signaling in activated T cells regulates T-bet expression (Shin et al., 2009). Egr1 is one of downstream target of Erk signaling, which is activated upon T cell receptor signaling. As we expected, Egr1 level is downregulated in Erk2 deficient CD4 T cells in all activated conditions we have. Also, Egr1 and Egr2 but not Egr3 mRNAs are expressed in activated Th1 cells (Dzialo-Hatton et al., 2001). Egr2 are regulated by NFAT from NFAT knockout mice study (Rengarajan et al., 2000). In addition, we also found out Egr2 level is down-regulated in Erk2 deficient cells since Egr2 is also one of Erk target substrates. We also analyze genes regulate Th1, Th2, Th17 and iTreg such as their important transcription factors. When we compare wild type and Erk2 knockout cells, we did see lower expression of Fos, stat4 but higher expression of GATA3, IL-4 and Foxp3 in $Erk2^{-/-}$ CD4 T cells under Th1 condition. However, $Erk2^{-/-}$ can express similar level of Tbx21 (T-bet) and IFNγ. It suggests that GATA3 and Foxp3 can further inhibit Th1 differentiation in $Erk2^{-/-}$ CD4 T cells.

There are down-regulated genes such as Egr1, Dusp4, Egr2, Rgs16, Cdkn1a, Csda, Egln3 in Erk2 deficient CD4 T cells. Dusp4 (MKP-2) is also found be down-regulated d1 Th1 and d1 iTreg condition. Dusp4 (MKP-2) can be induced by growth factors, harmone or stress agents and can dephosphorylate Erk and Jnk. Dusp4 knockout mice display enhanced phosphorylation of Erk and showed decreased responses Th1 and up-regulated Th2 responses because of more cytokines production (IL-6, IL-12, TNFα) from MKP-2 deficient macrophage (Al-Mutairi et al., 2010). Cdkn1a ($p21^{Cip1}$) is upregulated in Th1 cells and inhibit cell proliferation. It has been showed that inhibition of MEK (PD98059) in Th2 cell lines lead to decrease p21Cip expression (Chen et al., 1999) as well as lower expression was found in Erk deficient MEFs (Lee et. al, unpublished data). Also, Egr1 has been shown as a transcription factor binding to p21 promoter and regulate p21 expression (Ragione et al., 2003). Both expression of Cdkn1a and Egr1 are down regulated in Erk2 deficient d1 Th1 cells, and it suggests that decreased Egr1 level may lead to down-regulate of p21.

Day1 iTreg

There are 544 up-regulated genes and 99 down-regulated genes when we compare wild type and Erk2 deficient CD4 T cells under iTreg condition

(Figure 5-4). For nTreg signature genes (Feuerer et al., 2009), it was found that Tnfrsf18 (GITR), CTLA4, Foxp3, Klrg1, Nrp1 and Gpr83 are expressed highly in Erk2 deficient T cells after 1day of activation compared to wild type (Figure 5-6). However, only a small subset of underrexpressd genes Enc1, Arhgap29, Itgb3, Ptger2 and Myo10 were downregulated in Erk2 deficient cells. Besides, it was found out that some known iTreg signature genes such as Sell (L-selection), Plm1, Lgals1 and Ecm1 express higher in Erk2 deficient cells after 1day activation. Also, in absence of TGFβ, Erk2$^{-/-}$ cells already express higher mRNA level of Ctla4, Foxp3 and Swap70 (Figure 5-6). There are several transcription factors involved for Foxp3 expressionIrf4 is negative regulator for Foxp3 expression and we did lower expression of Irf4 in Erk2$^{-/-}$ T cells after 1day culture. Besides, T-bet expression is lower in Erk2 deficient cells.

Common or unique genes in d1

There are few genes are common in other conditions such Th0 or Th1 or Th0 and Th1. There are lots of unique expression genes for Erk2 deficient CD4 T cells for iTreg skewing.

In Th0 conditions, Erk2$^{-/-}$ cells already express higher mRNA levels of Ctla4, Foxp3 and Tnfrsf18 compared to wild type (Table 5-4). Under Th1 skewing conditions, we observed Foxp3 target genes Emp1, Pdlim4, Socs2 were upregulated. Also, under Th1 conditions, there is a similar expression of

Tbx21 (T-bet) but higher expression of Gata3 in Erk2 deficient CD4 T cells compared to wild type (Figure 5-5B). As expected, Erk target genes such as Egr1, Egr2 and Egr4 were downregulated in Erk2 deficient CD4 T cells. Additionally, Foxp3 negatively regulated targets such as Dusp6 and Jak2 were downregulated in Erk2$^{-/-}$ cells, (Figure 5-5B). Under iTreg conditions, there were 544 upregulated genes and as we expected we saw higher expression in Foxp3 as well as several Foxp3 target genes such as Ecm1, Il7r, Pdlim4 and the TGFβ-induced genes Socs3 (Table 5-6). We also observed downregulated Th1 genes such as Tbx21 and Il12rb1 (Figure 5-6).

Day 1 Common genes in all three conditions (Th0, Th1, iTreg)

Up-regulated genes: Ptprk, Nkg7, Vim, Nsg2, S100a8, Gata3, Eno3, Gdpd3, Pdlim4, Adh1 and Myo6

Down-regulated genes: Rgs16, Exoc2, Mid1, Pdgfb, MAPK1, Dusp6, Erdr1, Gpr68, Ccl1, Dusp6, Egr2

Gata-3 is known as a major transcription factor for Th2. Some Foxp3-regulated genes are found such as Nkg7, Vim, S100a8 and Pdlim4. Eno3 and Gdpd3 also express differentially from d0 WT and Erk2 deficient CD4 T cells. Ptprk were upregulated in Erk2 deficient cells (at least Th0, Th1, iTreg). Ptprk is a protein tyrosine phosphatase receptor K and it was found as TGFβ

responsive genes in some cell lines, such as HL cells and epithelial cells. When lymphoma cells were infected with Epstin-Barr virus, Ptprk and Smad2 were both down regulated. It suggests virus infection can disrupt TGFbeta signaling Smad2 and its target genes Ptprk (Flavell et al., 2008). Also, inhibition of Ptprk suppresses Erk phosphorylation in a T cell lymphoma and activated thymocytes. This indicates Ptprk controls CD4 T cell development through positively regulating Erk signaling (Erdenebayar et al., 2009). In epithelial cells, Ptprk was upregulated by TGF β signaling and depends on different amount of HER signaling will lead to cell proliferation through Erk signaling or cell migration through Src (Wang et al., 2005). From above studies suggest Erk signaling is related with Ptprk, which is regulated by TGFβ signaling. Impaired Erk2 signaling lead to upregulated Ptprk, and we would saw an increased TGFβ signaling as shown in Figure 4- 7 (increase Smad phosphorylation).

Day 1 Unique genes for Th0

Up-regulated genes: Swap70, Map3k8, Dennd5a, Cd86, Tmem158, Lif, Efna5, Evi2a, Raver2, Foxk1, Lad1, Rapsn, Faim3, Entpd8, Igfbp7, Tmem50a,Ctla4

Down-regulated genes: Nfatc1, Pou2f2, Lyz

Day 1 Unique genes for Th1

Up-regulated genes: Bcl3, Socs2, Ets1, Pigt, Eid2, Tubb2b

Down-regulated genes: Plek, Sperpina3g, Cxcl9, Snx5, Cd83, Il3, Arl5b, Tnfsf11, Rgs1, Rel and Jak2

Day1 Unique genes for iTreg

Up-regulated genes: 503 genes, such as Bcl2, Bcl2l11, Cd27, Coro1a, Eomes, Ets1, Foxp3, Il10ra, Il10rb, Il7r, Tnfrsf18, Tnfrsf9, etc.

Down-regulated genes: total 77 genes, such as Adh4, Anxa3, Ccl3, Ccl4, Csda, Il12rb1, Il1a, Lta, Tbx21, etc.

Day1 plus 1hr or 3hr TGFβ

We also analyzed the up- and down regulated gene expression in Erk2 deficient in comparison of wild-type cells after one-day activation and then treat with TGFβ for few hours. We put the list of up- and down- regulated genes in Table 5-7 and 5-8. It seems that there are less up-regulated genes and more down regulated when we compare day1 Th0 and day1 Th0 plus TGFβ (Figure 5-4). It suggests that TGFβ signaling and lost Erk2 signaling may lead to increase the number down-regulated genes. However, the number of up-regulated genes is similar in presence or absence of TGFβ signaling. Erk2 deficient cells express more gata-3 in day1 plus 1hr or 3hr conditions (TGFβ)

than wild type. GATA-3 is known as important transcription factors for Th2 differentiation. However, it was also known that GATA3 expression is higher in regulatory T cells than in naïve T cells (Sugimoto et al., 2006). This suggests that Erk2 negatively regulates Foxp3 and Gata3 expression, and lead to preferential differentiation into iTregs but not Th1s.

T helper and Treg signature genes

We also examine the T helper and Treg signature genes from Wei et.al research on the specificity and plasticity CD4 T cells as below (Wei et al., 2009) (Figure 5-7). Some of signature genes may turn on at late stage of CD4 T helper differentiation because they analyzed the highly differentiated CD4 T cells more than 5-days culture.

Th1: Eomes, Il12rb2, Ifnγ, Tbx21, Il18r1, Asb2, Fasl, Il18rap

Eomes, Tbx21, Il18r1, Fasl, Il12rb2, IFNγ, Il18rap are list as Th1 signature genes. It is known T-bet (Tbx21) as important transcription factor for Th1 differentiation. Also, T-bet regulated Th1 responses through direct negatively regulate Gata3 function (Usui et al., 2006). We did see (Figure 5-7) Tbx21 is downregulated in 3hr Th0, 3hr and d1 iTreg, but not in d1 Th0 and d1 Th1 conditions. In Th1 skewing condition, Erk2 deficient T cells can still express normal T-bet as shown (Figure 3-6). However, T-bet is downregulated in both 3hr and d1 iTreg showing that Erk deficient CD4 cells prefer to differentiae into

iTreg than Th1 under iTreg. There is a downregulated of IFNg in 3hr iTreg, and this may be correlated to lower expression of Tbx21. Il18r1 is upregulated in d1 iTreg and d1 plus 3hr TGFβ. Fasl is down regulated in 3hr Th0 and iTreg (Fang et al., 2010). The sensitivity of T cells subsets to Fas mediated apoptosis is: Th1> Th17> Th2). ASKB2 stands for ankyrin repeat-containing protein with a suppressor of cytokine signaling box-2 gene and it was identified as a retinoic acid-response gene and. In myeloid leukemia cells, the expression of ASB2 inhibits growth and promotes differentiation (Heuze et al., 2005).

Moreover, Il12rb1 is down regulated in d1 iTreg and 3hr Th0, Impaired Il12rb1 lead to decrease IL-17 differentiation in human (de Beaucoudrey et al., 2008). Erk- and p38MAPK-activation modulate IFNγ expression of T-bet and this regulatory role of Erk1/2 on IFNγ released is impaired in asthma (Koch et al., 2007). There is no difference in expression level of Eomes, Asb2 when comparing wild-type and Erk2 deficient CD4 T cells.

Th2: Pparg, Il1rl1, Ccr1,Gata3, Il5, Il13, Il4, Areg, Il21, Il9r

Besides, Pparg, Il1rl1, Ccr1, Gata3, Il5, Il13, Il4, Areg, Il21, Il9r were identified as th2 signature genes (Wei et al., 2009).

There is an increased expression in Erk2 deficient d1 th0, iTreg, Th1 and d1 plus 1hr and 3hr TGFβ. Gata-3 expression is higher in Erk2 deficient CD4 compared to wild-type post one-day stimulation. However, Yamashita et. al

showed that Ras-Erk MAPK signaling regulate Th2 cytokines gene loci and stability of GATA-3 expression by studying MEK inhibitor PD98059 (Yamashita et al., 2005). In addition, Gfi1 (Growth factor independ 1) is identified as downstream target of Ras-Erk signaling to maintain the stability of Gata-3 (Shinnakasu et al., 2008). Both Gata-3 and stat-5 expression are required for th2 differentiation, and a low signal through TCR activation induces gata-3 expression (Paul and Zhu, 2010). In Erk2 deficient T cells, there is a weak signaling through Erk1 signaling, which lead to induce gata-3 expression.

Il4 gene encodes cytokine IL-4, which is signature cytokine for Th2 and its differentiation. Il4 is down regulated (0.7-fold) in Erk2 deficient cells when compared to wild type at d0. However, it is up regulated in d1 (1.7), d1 iTreg (1.7 fold), in Erk2 deficient cells when compared to wild type. Previous study showed cross-talk between Ras-Erk and IL-4/Stat4 are observed by using Jurkat T cells (So et al., 2007). Also, it showed differential activation of Erk1 and Erk2 upon IL-4 stimulation, and Erk2 is prominent whereas Erk1 shows less effect. IL-4 induced stat6 activity is suppressed and decreased interaction between Erk2 and stat6 with MEK inhibitor treatment. Ras-induced Stat6 activity and Il-4 expression via Erk dependent rather than PI3K pathways. Ras inhibits IFNg expression via PI3K-dependent pathways (Yamane et al., 2005). Studies showed the role of Erk is controversial. Some studies showed no effect on IL-4 expression (Cantrell, 1996), some showed increased (Dumont et al., 1998;

Jorritsma et al., 2003) and other showed decreased (Egerton et al., 1998; Yamashita et al., 1999; Yamashita et al., 2005).

There is no difference in Pparg, Il1r1, Ccr1, Il5, Il13 in different conditions. It may due to there be no condition for Th2 differentiation and those gene expressions are low. Pparg stands for peroxisome proliferator activated receptor gamma and it is required for CD4 T cell proliferation, survival (Natarajan et al., 2003). Pparg deficient cells enhance cell proliferation and increase IFNγ production upon IL-12 signaling. Pparg CD4 T cells exacerbate the inflammatory bowel disease, even mice loss expression of Pparg in CD4 T cells display a colitogenic phenotype (Hontecillas and Bassaganya-Riera, 2007).

Th17: Il17a, Rora, Il1rl1 (same as Th2), Il17re, Rorc

Th17 is characterized by expression IL-17A and its major transcription factor is Rora and Rorc. There is no difference in the level of Il17a, Rora, Rorc, and il1rl1 expression compared wild type and Erk2 deficient CD4 T cells under different conditions. It may be due to the expression Th17 signature genes are lower under no polarization (Th0), Th1 and iTreg and also time points (3hr and d1) we examined are too early. It requires both IL-6 and TGFβ signaling for Th17 polarization. TGFβ alone doesn't have dramatic effect on Th17 signature

genes when we compare wild type and Erk2 deficient CD4 T cells. It doesn't alter Th17 signature genes expression in absence of Erk2 signaling.

iTreg: Foxp3*, Ccr6, Il1rn, Il9, Myb, Tnfsf11*, Nr4a3*, Nr4a1*, Rel*, Tgif1*, Tgif2*, Cecr6*, Irf4, Ccl4, Pou2f2*, Irf8 (* common signature genes with nTreg)

When we compare with WT and Erk2 deficient cells, there is an increased expression in Foxp3, Ccr6, Cecr6 in Erk2-deficient CD4 T cells upon 3hr activation for iTreg condition; In contrast, there is decreased in Tnfsf11 (th1), Nr4a3 (3hr iTreg), Rel (d1 th1), Ccl4 (3hr Th0, 3hr iTreg, d1 iTreg), Pou2f2 (3hr iTreg, d1 Th0), Irf8 (3hr Th0, 3hr iTreg). There is no difference in Il1rn, Il9, Myb, Nr4a1, Tgif1, Tgif2 and Irf4.

Foxp3 is a major transcription factor for regulatory T cells. Mutant of Foxp3 leads to autoimmune disease in mice (scurfy mice) and in human (IPEX syndrome) (Sharma and Ju, 2010). In our studies, Erk2 deficient CD4 T cells enhance expression of Foxp3, and Erk2 inhibits TGFβ signaling through Smad-dependent signaling. From the microarray analysis, we did see increased expression of Foxp3 in d1 Th0, d1 iTreg, d1+1hr TGFβ. This is correlated increased expression of genes directly or indirectly regulated by Foxp3 such as Ecm1, Dusp6, CTLA4 and CD25(Hori and Sakaguchi, 2004). Although it is no different at early time 3hr post-activation, there is an increased expression of

Foxp3 at d1 Th0 and iTreg. It is also known the peak of expression Foxp3 mRNA is about 18 hr. Also, when we analysis Foxp3 protein expression by Facs, we start see a difference at day2 when compared WT and Erk2 deficient T cells.

CCr6 (C-C chemokine receptor type 6) and it is also known as CD196. This protein belongs to G-protein-coupled receptor superfamily and its ligand is CCL20. It is preferential expressed in immature dendritc cells and memory B and T cells (Liao et al., 1999). CCR6-deficient mice has less sensitive to intestinal pathology (Varona et al., 2003). Colorectal cancer cells showed their cell differentiation dependent on CCR6 expression via Erk, JNK, AKt signaling (Brand et al., 2006). Ccr6 is upregulated in d1 Th0, d1 Th1, d1+1hr TGFβ, d1+3hrTGFβ, but not in d1 iTreg. Ccr6 stands for cat eye syndrome region, candidate 6, and it is reported as Treg marker (antigen-specific, IL-10 producing regulatory T cells, which is involved for developing inflammatory bowel disease (Kitamura et al., 2010).

Genes involved for Th1 (IFNγ) or iTreg (Foxp3) and other transcription factors for Th2 and Th17

In previous paragraph, we already discuss about T helper and regulatory signature genes. Some of them are also as the major transcription factor such T-bet, Gata-3, Foxp3 for different CD4 T subsets. Here we put together with

genes involved for Th1 differentiation (IFNγ expression) and regulatory T cell differentiation (Foxp3 expression). We analyzed those genes in heatmap as in Figure 5-8. We found out that the decreased expression of Fos and stat-4 gene and increased expression of gata-3 genes and Foxp3-regulated genes (Ecm1, Pdlim4) in Erk2 deficient CD4 T cells under Th1 skewing. Besides, down-regulated of DNMT and up-regulated of Foxp3, Foxp3-induced genes or some Treg signature genes were found in Erk2 deficient CD4 T cells (Figure 5-8). Furthermore we also did some of genes for further QPCR experiment to confirm their gene expression in wild type and Erk2 deficient CD4 T cells. In addition, we performed the quantitative PCR to further confirm genes of interested under different conditions. It yielded similar results as our microarray data that showed upregulated CTLA4, Tnfrsf18 (GITR), Foxp3, GATA3 and downregulated Socs2 and Tbx21 (Figure 5-9).

Discussion

Here we did the microarray analysis for gene expression for wild type and Erk2 deficient CD4 T cell differentiation. We found out there are lots of up- and down- regulated genes, which may be direct or indirectly regulated by Erk2. As we would expect several Erk2 substrates such as Egr1, Egr2, Egr4 and Cdkn1a were down regulated. On the other hand, we found out several up-

regulated genes such as Usp18, Pdlim4, Ecm1, Emp1, which are known as Foxp3 targets (Zheng et al., 2007). It suggest in absence of Erk2 signaling maybe enhance TGFβ signaling and thereby enhance expression of Foxp3 and its target genes. Furthermore, many up- and down regulated genes in Erk2 deficient CD4 T cells are not really known about their functions in CD4 T cells. Besides, it is also no known that Erk2 is directly or indirectly regulate those genes. It would be interested to start with those common selected genes under different conditions as well as find out the unique selected genes for further investigation.

Experimental procedures

Cell culture

CD4 T cells from lymph nodes and spleen were purified by positive or negative selection using magnetic beads (Miltenyli Biotec). Purified CD4 T cells were stimulated with anti-CD3, anti-CD28 and IL-2 (Th0) and in presence of cytokine IL-12 and anti-IL-4 (Th1) and TGFβ (iTreg) harvested at different time points (3hr or 24hr). Also, CD4 T cells are activated (anti-CD3/CD28, IL-2) for a day and then add TGFβ for 1hr or 3hr. (Figure 5-1)

RNA isolation and quantitative real-time PCR

Total RNA was isolated by Trizol (Invitrogen), treated with DNase using the DNA-free kit (Ambion), followed with cDNA synthesis by the SuperScript III

reverse transcriptase and random hexamers (Invitrogen). The cDNA was amplified and detected using the Power SYBR Green mix (Applied Biosystems) and ROX reference dye (Stratagene) on an Mx3005P machine (Stratagene). Samples were normalized to GAPDH or Cph (cyclophilinA). Relative expression was calculated as the difference (△Ct). Primers are used as followed: *Dnmt*: fw 5'-gga agg cta cct ggc taa agt caa g-3', rev 5'-act gaa agg gtg tca ctg tcc gac-3' ; *Foxp3*: fw 5'-gaa aca gca cat tcc cag agt tc-3', rev 5'-atg gcc cag cgg atg ag-3'; *Tnfrsf18*: fw 5'-ttg cag atc ttg cac tga gg-3', rev 5'-ctg ctg cag cct gta tgc tc-3'; *Gata3*: fw 5'- gcg ggc tct atc aca aaa tga-3', rev 5'-gct ctc ctg gct gca gac agc-3'; *Ctla4*: fw 5'-cac tga agg ttg ggt cac ct-3', rev 5'-gcc ttc tag gac ttg gcc tt-3'; *Socs2*: fw 5'-aag aaa gtt cct tct gga gcc-3', rev 5'-cgc gag ctc agt caa aca g-3'; *Cph*: fwd-cac cgt gtt ctt cga cat c, rev 5'-att ctg tga aag gag gaa cc-3'; *Gapdh*: fwd –cca gta tga ctc cac tca cg-3', rev 5'-gac tcc acg aca tact ca gc.

Microarray analysis

Total RNA from wild type and Erk2 deficient CD4 T cells under different conditions were purified with an RNeasy kit (Qiagen). Samples (250ng RNA) were labeled, hybridized and amplified by biomedical genomics microarray core facility at UC San Diego using MouseRef-8 beadchip kit (illumina). Data were normalized by UC San Diego microarray core and further analyzed with the Genepattern software suite.

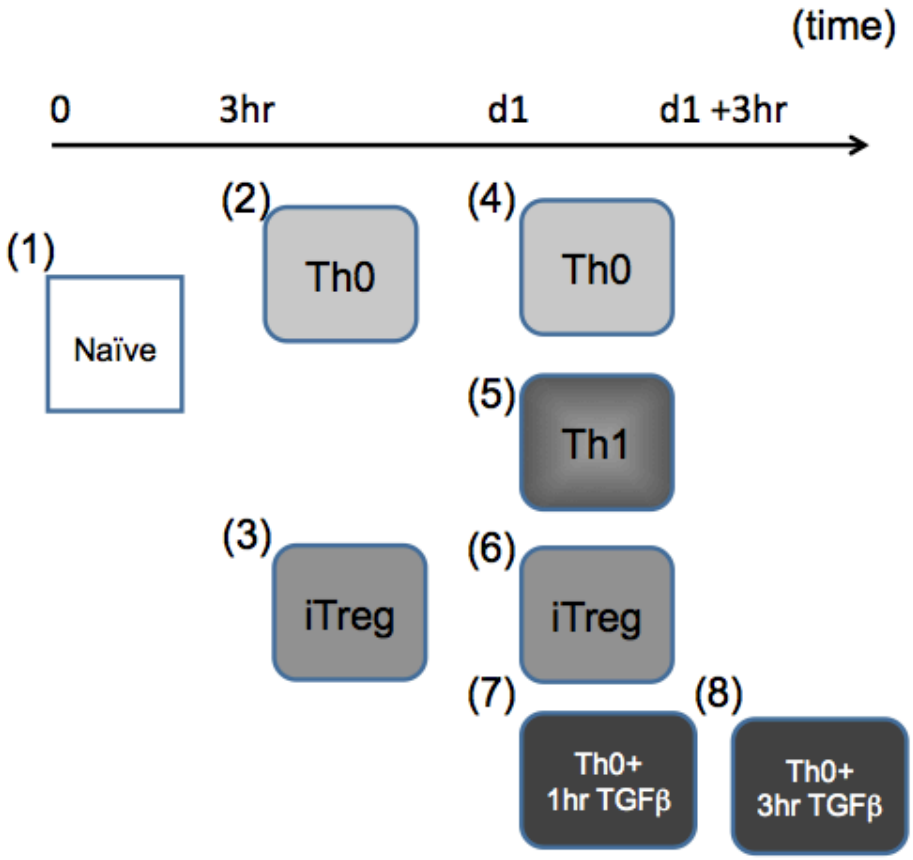

Figure 5-1. Experiment Design for microarray. Purified naive WT and Erk2$^{-/-}$ CD4 T cells are stimulated under different conditions as indicated. RNA were isolated for microarray analysis. Conditions: Th0 (anti-CD3/CD28, IL-2), Th1 (Th0, IL-12, anti-IL-12), iTreg (Th0, TGFβ).

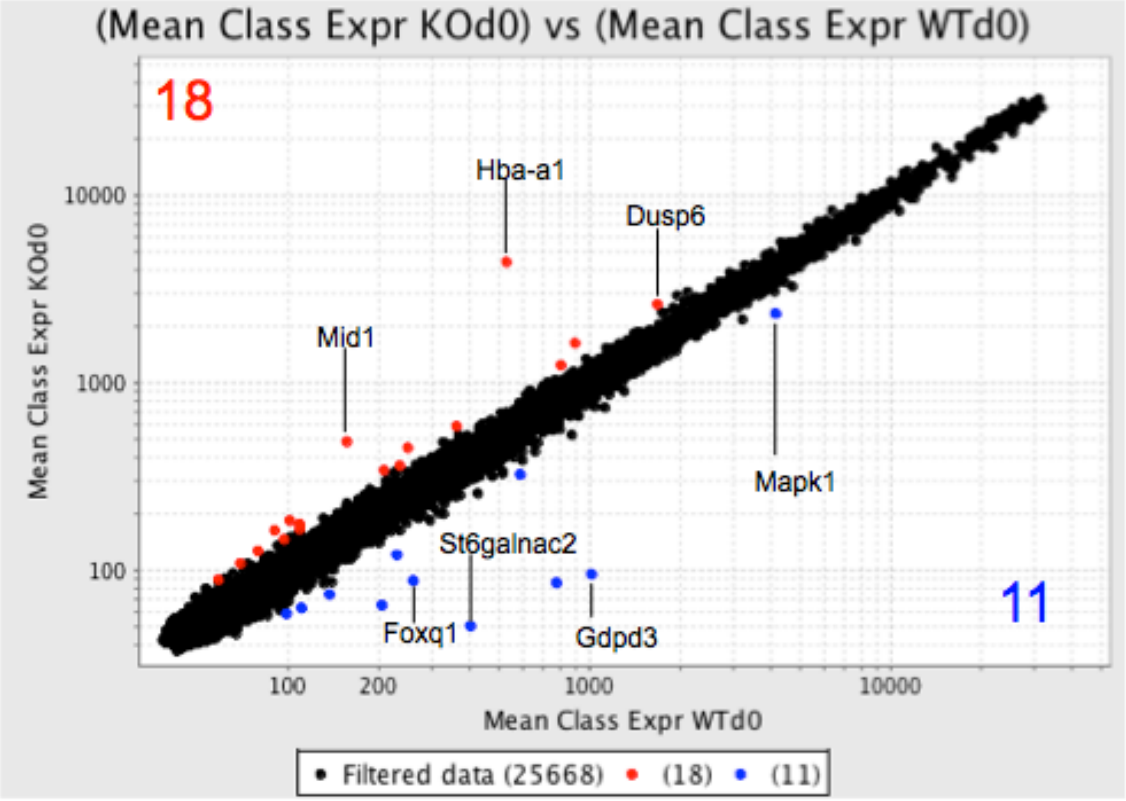

Figure 5-2. Day 0 scatter plot for the expression of WT and Erk2$^{-/-}$ CD4 T cells. It shows that the mean expression naïve wild type (WT) CD4 T cells in X-axis and mean Erk2$^{-/-}$ CD4 T cells in Y-axis. There are 18 up-regulated genes and 11 down-regulated genes when compare WT and Erk2 deficient CD4 T cells. Up-regulated genes (Fold change (Erk2$^{-/-}$/ WT) is over 1.5) are labeled in red and down-regulated (Fold change (Erk2$^{-/-}$/ WT) is less than 0.6) genes are labeled in blue.

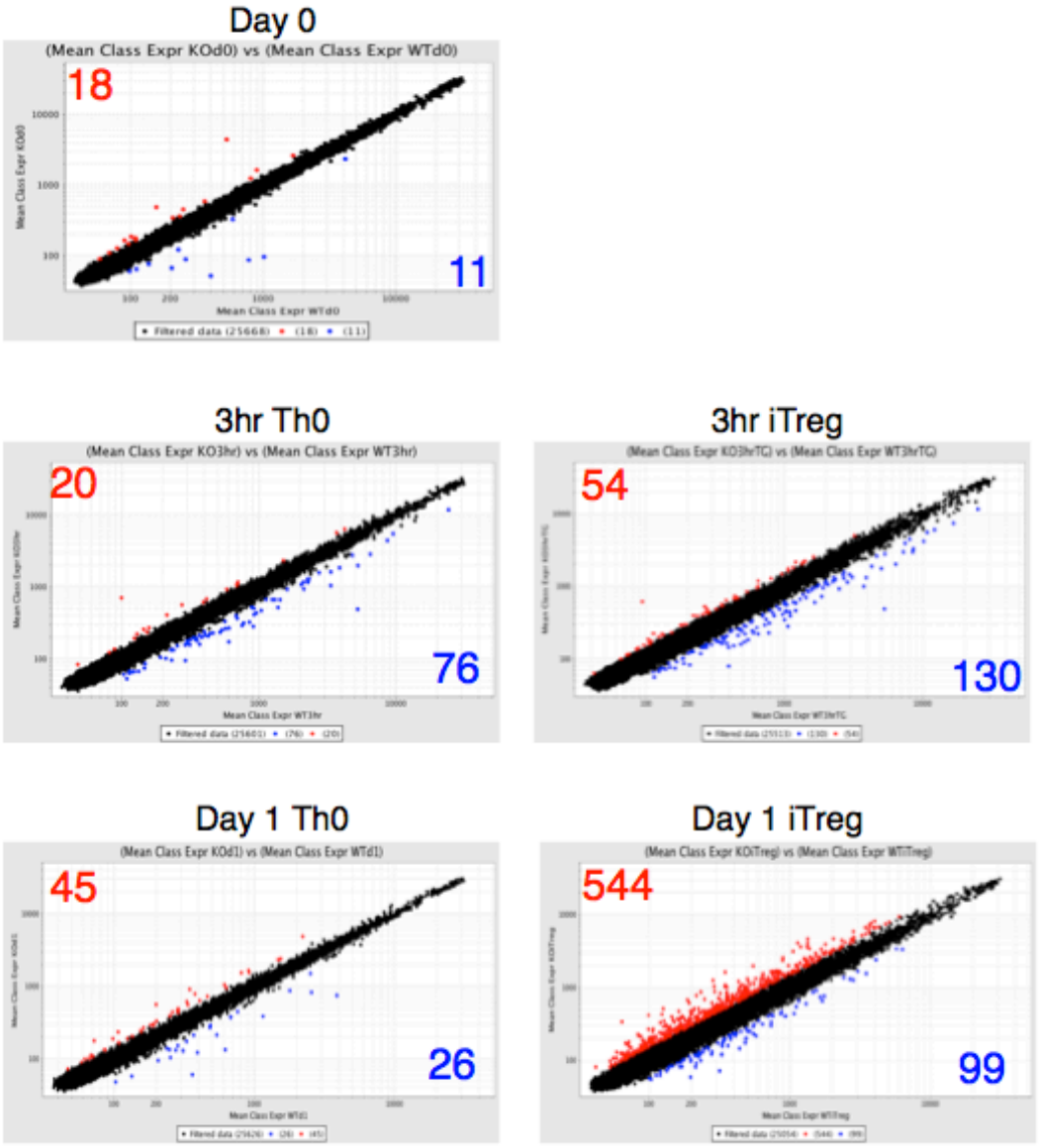

Figure 5-3. Scatters plots show that the mean expression of wild type (WT) CD4 T cells and Erk2$^{-/-}$ CD4 T cells under different conditions. The number of up-regulated genes (fold change > 1.5) is in red and number of down-regulated genes (fold change < 0.6) is in blue.

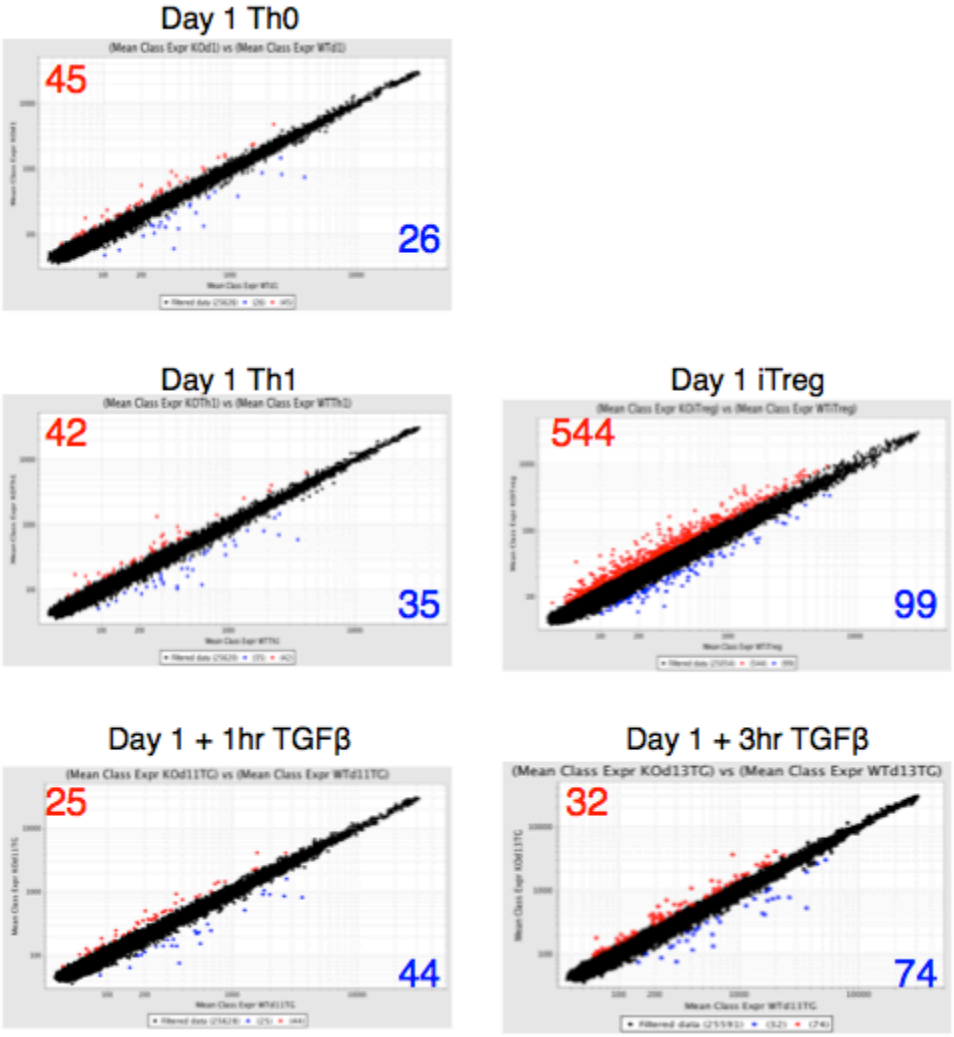

Figure 5-4. Scatters plots show that the mean expression wild type (WT) CD4 T cells and Erk2$^{-/-}$ CD4 T cells under different conditions (day 1). The number of up-regulated genes (fold change > 1.5) is in red and number of down-regulated genes (fold change < 0.6) is in blue.

Table 5-1. D0 up- and down-regulated genes in Erk2 deficient CD4 T cells.

(Fold change= Expression Erk2$^{-/-}$/ Expression WT)

Up	Fold change	Down	Fold change
Hba-a1	8.327	Gdpd3	0.094
Mid1	3.087	2300002D11Rik	0.110
Erdr1	1.819	St6galnac2	0.125
Chi3l3	1.817	2300002D11Rik	0.318
C530044N13Rik	1.809	Foxq1	0.337
Hbb-b1	1.802	2010001M09Rik	0.526
Lgals3	1.637	Marco	0.541
Litaf	1.617	Slpi	0.552
Scin	1.613	Mapk1	0.564
Mid	1.594	Sema3f	0.569
Chi3l3	1.586	Olfml3	0.596
Lcn2	1.563		
Dusp6	1.558		
Phlda	1.543		
Lyzs	1.537		
Alas2	1.514		
Camp	1.512		
Mmp28	1.504		

Table 5-2. 3hr Th0 top 25 up- and down-regulated genes in Erk2 deficient CD4 T cells.

(Fold change= Expression Erk2$^{-/-}$/ Expression WT)

Up	Fold change	Down	Fold change
Gdpd3	7.032	Hba-a1	0.092
Pdlim4	2.030	Mid1	0.284
Gtf2ird1	1.887	Dusp6	0.308
Lad1	1.766	Cxcl9	0.324
Accn1	1.735	Nr4a2	0.371
Slpi	1.696	Tnf	0.373
Phlda3	1.695	Lta	0.376
Mapre2	1.640	Lyz	0.384
B3galt6	1.625	Rgs16	0.402
D14Ertd449e	1.578	Cdkn1a	0.413
Usp18	1.561	A130090K04Rik	0.429
Prkcbp1	1.548	Hbb-b1	0.438
Bex4	1.548	1190002H23Rik	0.443
Ptpn6	1.538	Atf3	0.446
Lhfp	1.537	Ccl3	0.447
Chchd10	1.535	Hspa1a	0.449
1110038D17Rik	1.509	Chst2	0.456
Ebi2	1.505	Gfod1	0.463
Cntn3	1.505	S100a9	0.466
Ldoc1l	1.498	Sema7a	0.471
Usp2	1.493	Serpina3g	0.479
LOC56628	1.492	Lyz2	0.482
P2ry14	1.487	Nrn1	0.483
Nhedc2	1.485	Alas2	0.484
Eno3	1.473	Serpina3f	0.491

Table 5-3. 3hr iTreg top 25 up- and down-regulated genes in Erk2 deficient CD4 T cells.

(Fold change= Expression Erk2$^{-/-}$/ Expression WT)

Up	Fold change	Down	Fold change
Gdpd3	6.546	Hba-a1	0.092
5330431N19Rik	1.973	Cxcl9	0.201
Eno3	1.971	Dusp6	0.263
Mapre2	1.820	Mid1	0.293
Sac3d1	1.796	Tnf	0.314
Gtf2ird1	1.789	Sema7a	0.329
Klf7	1.761	Cxcl10	0.334
Pdlim4	1.761	Serpina3f	0.335
Dusp10	1.686	Serpina3g	0.336
Phlda3	1.681	Lyz	0.340
Lrmp	1.674	Lta	0.341
Mical1	1.646	Rgs16	0.351
Zfand2b	1.622	Gbp2	0.354
Aes	1.620	Actb	0.355
Dapl1	1.618	Nr4a2	0.359
Fbxo31	1.611	Areg	0.366
LOC100046930	1.609	Ccl3	0.379
Lhfp	1.606	Hbb-b1	0.381
Arhgap9	1.601	Irgm1	0.384
Pex7	1.601	Gfod1	0.387
Xylt2	1.599	1190002H23Rik	0.387
Adh1	1.588	Ifng	0.395
Ndufb4	1.585	Ccl4	0.396
Pik3r1	1.583	Cdkn1a	0.399
Emp1	1.582	Ccl1	0.399

Table 5-4. D1 Th0 top 25 up- and down-regulated genes in Erk2 deficient CD4 T cells.

(Fold change= Expression Erk2$^{-/-}$/ Expression WT)

Up	Fold change	Down	Fold change
Ptprk	2.820	Hba-a1	0.167
Eno3	2.622	Egr1	0.193
Gdpd3	2.450	Mapk1	0.215
Myo6	2.197	Egr2	0.334
S100a8	2.177	Dusp6	0.394
Ccr8	2.083	Pdgfb	0.414
Ccr6	1.918	Rgs16	0.415
Gata3	1.888	C530044N13Rik	0.420
Pdlim4	1.864	Ccl1	0.430
Ebi2	1.841	Gpr68	0.460
Il4	1.722	Egr4	0.466
St6gal1	1.708	Mid1	0.478
Nsg2	1.702	Cetn4	0.529
LOC100046930	1.681	Xcl1	0.533
Swap70	1.650	Pou2f2	0.538
Rapsn	1.642	Nrn1	0.548
Igfbp7	1.638	Erdr1	0.555
Efna5	1.638	Cdkn1a	0.556
Nkg7	1.627	Dusp4	0.572
Atp1b1	1.619	Lyz	0.586
S100a9	1.603	Egln3	0.592
Dennd5a	1.592	Exoc2	0.594
Tmem50a	1.575	Nfatc1	0.594
Trat1	1.570		
E430004N04Rik	1.568		

Table 5-5. D1 Th1 top 25 up- and down-regulated genes in Erk2 deficient CD4 T cells.

(Fold change= Expression Erk2$^{-/-}$/ Expression WT)

Up	Fold change	Down	Fold change
Myo6	4.936	Egr1	0.171
Gdpd3	2.824	Mapk1	0.193
Eno3	2.391	Pdgfb	0.263
Ptprk	2.361	Rgs16	0.343
Klf2	2.246	Nrn1	0.366
S100a8	2.202	Dusp6	0.393
Gata3	2.084	Il3	0.400
Sell	1.949	Mid1	0.421
Nkg7	1.932	Gpr68	0.422
Emp1	1.917	Plek	0.422
Socs2	1.868	Egr2	0.424
Ccr8	1.847	Dusp4	0.456
Pdlim4	1.807	C530044N13Rik	0.486
Ccr6	1.780	Slamf1	0.493
Npm3	1.768	Jak2	0.500
Ecm1	1.735	Ccl1	0.524
Slfn1	1.712	Cxcl9	0.525
Eid2	1.668	Bdh2	0.528
Ets1	1.661	Axl	0.529
Vim	1.642	Serpina3g	0.532
Gimap7	1.626	Cd83	0.544
Bcl3	1.625	Egr4	0.546
Cd2	1.622	Tnfsf11	0.556
LOC100046608	1.593	Exoc2	0.560
Tesc	1.580	Erdr1	0.575

Table 5-6. D1 iTreg top 25 up- and down-regulated genes in Erk2 deficient CD4 T cells.

(Fold change= Expression Erk2$^{-/-}$/ Expression WT)

Up	Fold change	Down	Fold change
Gdpd3	5.231	Hba-a1	0.227
Pdlim4	4.672	Il1a	0.299
Tsc22d3	4.391	Polr1a	0.333
Pou6f1	4.193	Csda	0.361
Ms4a6b	4.085	Il12rb1	0.368
Ecm1	3.735	Zbtb7a	0.370
Eno3	3.638	Anxa3	0.377
Il7r	3.624	Ccl1	0.379
Myo6	3.620	Egln3	0.388
Ifit3	3.590	Axl	0.408
Arhgef18	3.586	Chd4	0.410
Ifi27	3.476	Rgs16	0.413
Fam158a	3.464	Mapk1	0.421
Nsg2	3.203	Sesn2	0.425
Slfn1	3.091	Fam129b	0.430
Ypel3	3.062	Bdh2	0.434
Sell	3.040	Pdgfb	0.437
Ebi2	3.029	C530044N13Rik	0.438
Klf2	2.990	Cd200	0.441
Socs3	2.988	Tbx21	0.441
Samd9l	2.915	Lta	0.446
Unc84b	2.880	Exoc2	0.449
Mgst2	2.842	Erdr1	0.452
Ephx1	2.796	Dusp6	0.463
Gmfg	2.794	Mid1	0.472

Table 5-7. D1 plus1hr TGFβ top 25 up- and down-regulated genes in Erk2 deficient CD4 T cells.

(Fold change= Expression Erk2$^{-/-}$/ Expression WT)

Up	Fold change	Down	Fold change
Myo6	2.629	Hba-a1	0.200
Eno3	2.605	Egr1	0.227
Ptprk	2.499	Mapk1	0.276
Ccr6	2.107	Pdgfb	0.365
Gdpd3	2.065	Rgs16	0.410
Igfbp7	1.923	Ccl1	0.425
Pdlim4	1.895	Gpr68	0.426
Ebi2	1.892	Dusp6	0.426
Efna5	1.885	Mid1	0.432
Ccr8	1.863	Atxn1	0.485
Gata3	1.846	Nrn1	0.490
Lif	1.837	C530044N13Rik	0.501
Tsc22d3	1.814	Egr2	0.520
Crem	1.775	Serpina3f	0.533
Pim1	1.769	Pou2af1	0.546
Nkg7	1.735	Egr4	0.557
Vim	1.723	Lyz	0.562
Nsg2	1.717	Igf2bp3	0.566
Adh1	1.714	Akr1c18	0.572
Il6ra	1.679	Bdh2	0.582
LOC100046930	1.661	Rgs1	0.590
Dennd5a	1.637	Exoc2	0.597
Slfn1	1.635		
Il12a	1.627		
Il4	1.619		

Table 5-8. D1 plus 3hr TGFβ top 25 up- and down-regulated genes in Erk2 deficient CD4 T cells.

(Fold change= Expression Erk2$^{-/-}$/ Expression WT)

Up	Fold change	Down	Fold change
Myo6	4.164	Egr1	0.147
Pdlim4	2.866	Mapk1	0.221
Gdpd3	2.794	Hba-a1	0.252
Eno3	2.727	Pdgfb	0.256
Emp1	2.714	Rgs16	0.280
Ptprk	2.695	Dusp6	0.340
Klf2	2.425	Ccl1	0.386
Nsg2	2.233	Egr2	0.394
Igfbp7	2.217	Tnf	0.409
Vim	2.113	Bcl6	0.447
Socs2	2.050	Gpr68	0.463
Gata3	2.022	Mid1	0.477
Ifi27	2.003	C530044N13Rik	0.486
Ecm1	1.968	Slamf1	0.494
Ifit3	1.949	Axl	0.509
Ccr6	1.905	Hbb-b1	0.511
Tsc22d3	1.897	Jak2	0.512
Slfn1	1.890	Exoc2	0.536
Ebi2	1.873	Cd44	0.553
Nkg7	1.869	Serpina3g	0.558
Ccr8	1.847	Tnfsf11	0.564
Sell	1.827	Nrn1	0.567
Faim3	1.826	Dusp2	0.573
Aqp3	1.812	Tubb6	0.584
Usp18	1.795	Ptger2	0.586

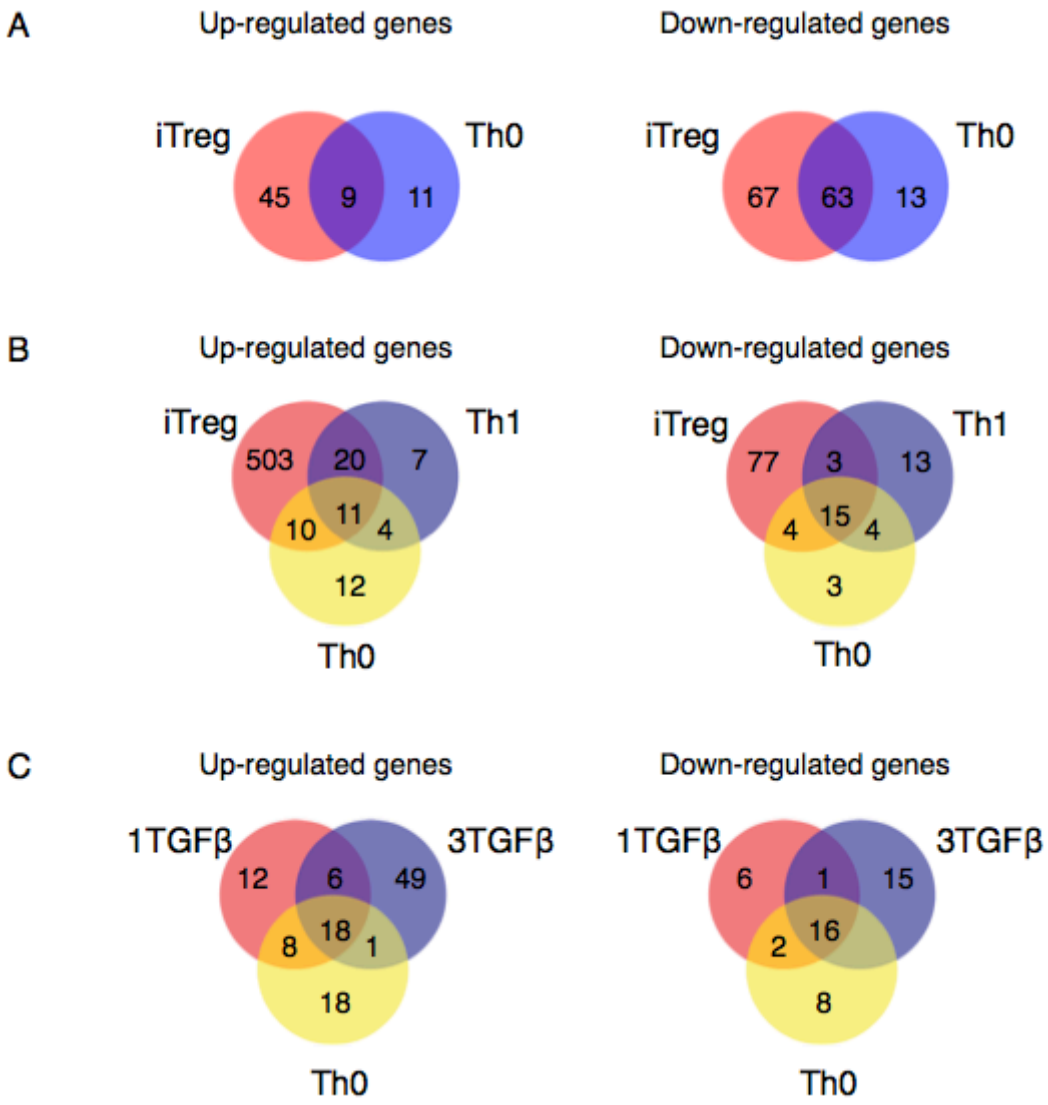

Figure 5-5. VennDiagrams show the number of up- or down-regulated genes in Erk2 deficient CD4 T cells under different conditions. (A) 3hr (Th0 and iTreg), (B) d1 (Th0, Th1 and iTreg) (C) d1 in absence or presence of TGFβ (Th0: no TGFβ, 1TGFβ: 1 hr TGFβ or 3TGFβ: 3 hr TGFβ)

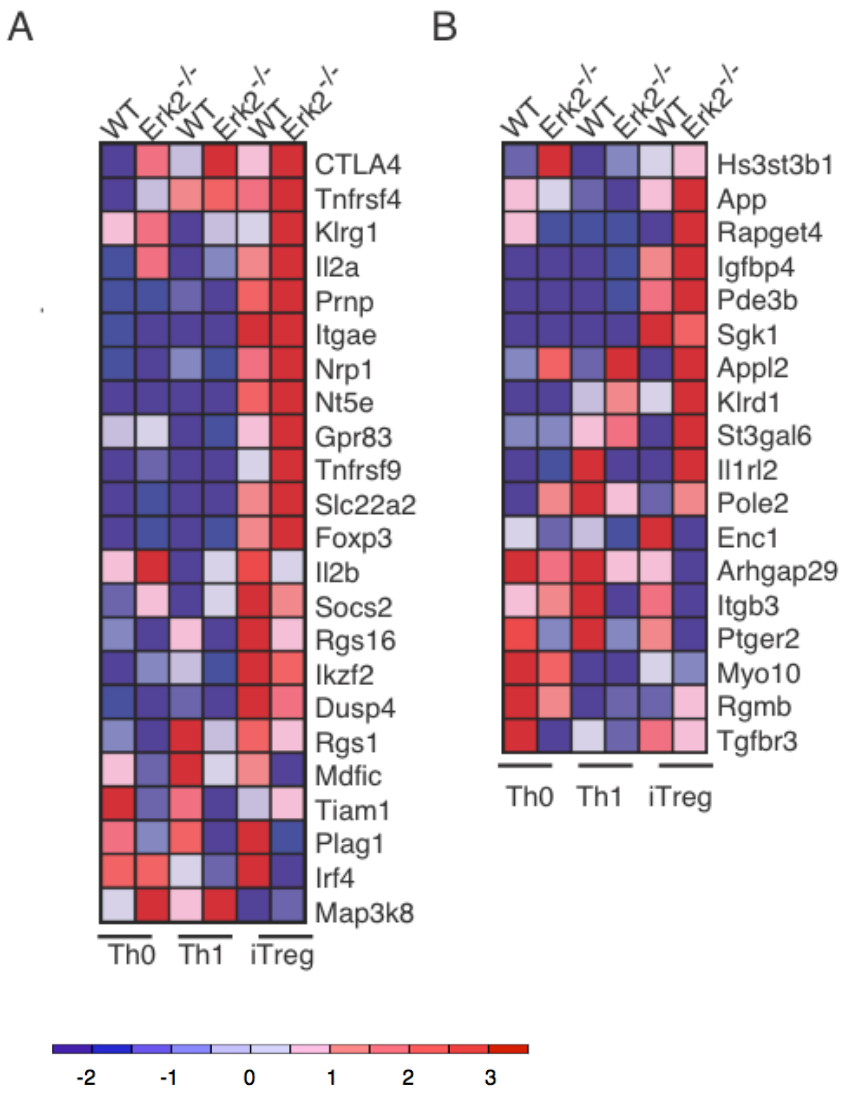

Figure 5-6. Up-regulation of Partial Treg signature genes in Erk2$^{-/-}$ CD4 T cells. (A) (B) Selected up-regulated or down-regulated n Treg signature genes are shown in heatmap. Higher expression genes were shown in red, otherwise were in blue.

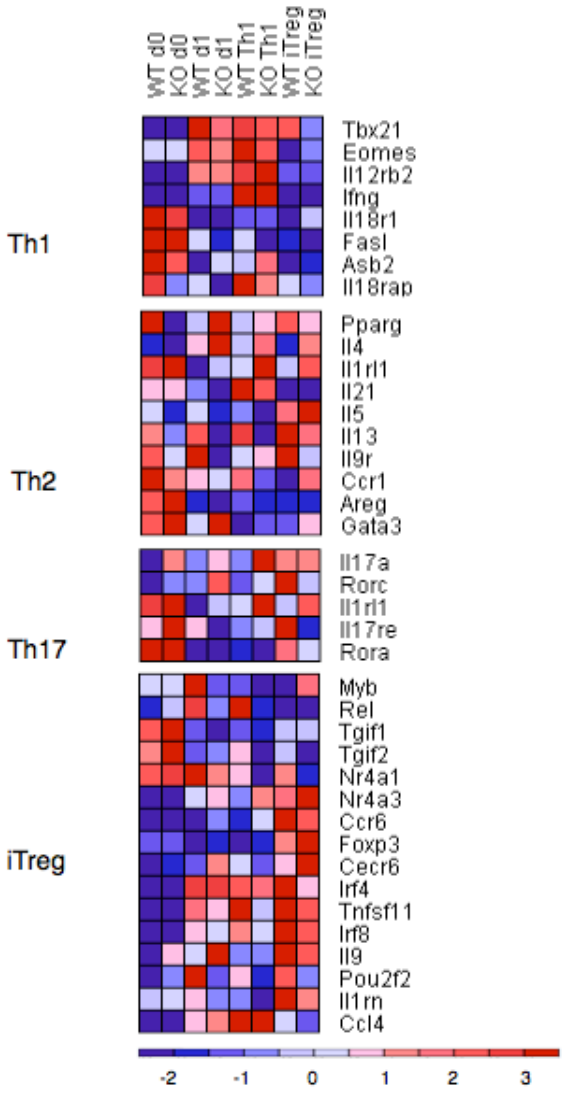

Figure 5-7. Heatmap of T helper and Treg signature genes are upregulated in day 1 Erk2$^{-/-}$ CD4 T cells. Higher expression genes were shown in red, otherwise were in blue.

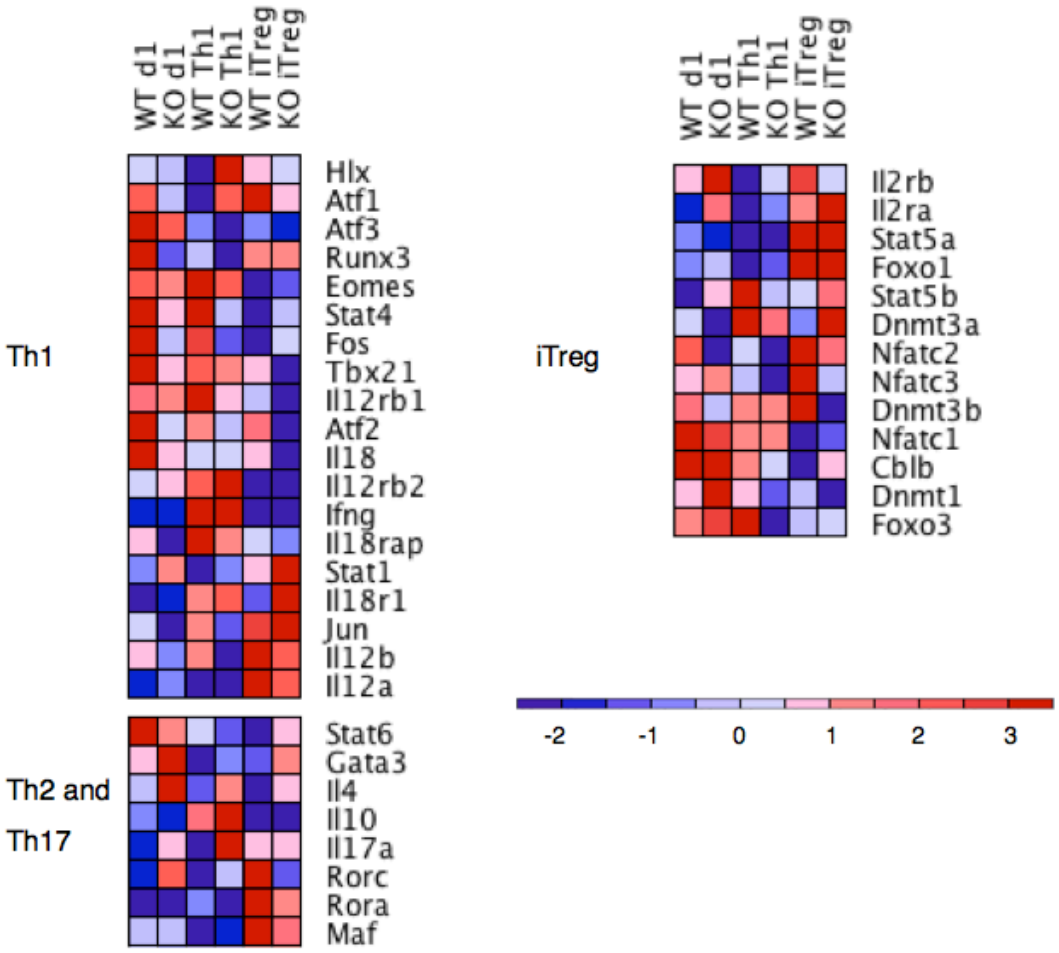

Figure 5-8. Heatmap of genes involved for T helper and Treg differentiation in day 1 wild type and Erk2$^{-/-}$ CD4 T cells. RNA was isolated from unstimulated WT or Erk2$^{-/-}$ cells (one representative data from 3 independent experiments) have been stimulated under indicated condition for 1 day. Higher expression genes were shown in red, otherwise were in blue.

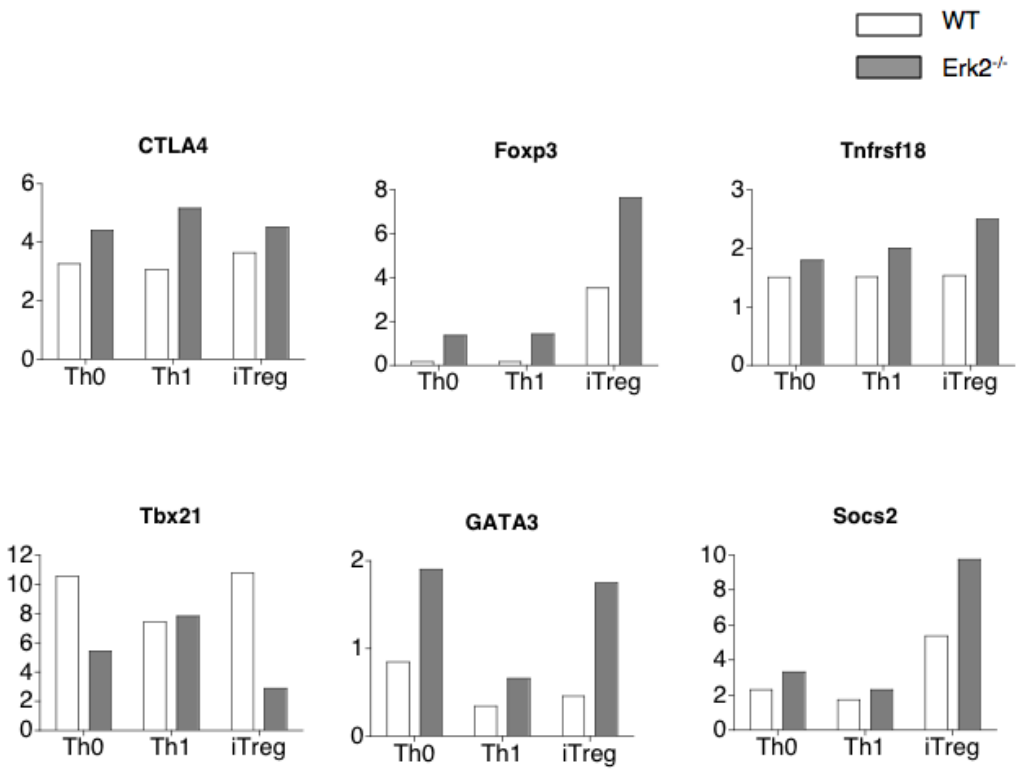

Figure 5-9. Increased iTreg related gene expression in day1 Erk2 deficient CD4 cells. RNA was isolated from unstimulated WT or Erk2$^{-/-}$ cells (one representative data from 3 independent experiments) have been stimulated under indicated condition for 1 day. The mRNA level of selected genes were measured by real-time quantitative PCR and then normalized to a house keeping gene. Relative gene expressions were showed and wild-type d0 expression level was used for normalization as 1.

Chapter. 6

General discussion

Upon TCR signaling, Erk is required for interacting with several transcriptional factors to turn on gene expression. Here we showed that Erk2 plays a role in promoting Th1 but inhibiting induced regulatory T cells. On the other hand, Erk1-deficient CD4 T cells have normal T cell activation and differentiation, which suggests Erk2 can compensate for the loss of Erk1. Erk2 is critical for cell proliferation with TCR signaling alone in both CD4 and CD8 T cells. Moreover, Erk2 is indispensable for the survival under optimal stimulation in CD8 T cells (D'Souza et al., 2008). Erk2 deficient CD4 T cells have normal proliferation upon optimal stimulation. Co-stimulatory signaling may help the proliferation of Erk2-deficient CD4 T cells but not CD8 T cells. This suggests there is a different requirement for Erk2 in CD4 and CD8 proliferation.

Additionally, Erk deficient ($Erk1^{-/-}2^{-/-}$) CD4 T cells had both proliferation and survival defects upon joint TCR and co-stimulatory signaling (Figure 2-6). Some Erk deficient cells underwent proliferation and upregulated the marker of activation, CD44 and CD25. It is also true that minority of Erk deficient CD8 T cells have ability to proliferation (D'souza, unpolished results). One possibility is that an extremely lower amount of Erk2 can support the activation and proliferation of those cells. The other possibility is that signaling through other pathways such as Akt or MAPK pathways (p38, Jak) can help Erk deficient cells proliferate and activate. This result differs from what we saw in mouse

fibroblast cells (MEFs), of which we found that Erk deficient cells cannot proliferate at all (Lee, et. al. unpublished data). This maybe due to different requirement for Erk signaling pathways in different cell types.

Our study also suggests that Erk2 is required for early proliferation in Th1, Th2 cells and has more important role for Th1 differentiation. $Erk2^{-/-}$ CD4 T cells still can express a normal level of T-bet and GATA-3, which are important lineage transcription factor for Th1 and Th2 (Figure 3-6). One possibility is that Erk2-deficient CD4 T cells produce IL-2 and thereby these cells have a survival defect at later stage. Another possibility is that Erk2-deficient CD4 T cells induce more Bim expression (Figure 3-4), leading to increased cell death. Also, $Erk2^{-/-}$ CD4 T cells were less activated with lower expression levels of CD25 and CD44. Therefore, those less activated $Erk2^{-/-}$ Th1 cells had less IFNγ production.

During LCMV infection, decreased amount and number of IFNγ and tetramer positive cells were detected in Erk2-deficient cells although those tetramer positive cells were able to make IFNγ in absence of Erk2. These results were consistent with what we saw during $Erk2^{-/-}$ CD8 responses to LCMV (D'Souza et al., 2008). Moreover, the Th1 responses of wild type from bone marrow chimera mice (Mix WT: $Erk2^{-/-}$=1:1) are about half lower than WT itself. There maybe two possibilities for seeing this decreased response: (1) half of the CD4 T cells population are $Erk2^{-/-}$, which results in decreased production

of IL-2 cytokine and less growth factor IL-2 in the surrounding environment, leading to less responses for wild type cells the ratio of WT and Erk2$^{-/-}$ is about 1:1, have less WT cells to further differentiate Th1 cells (Duncia et al.) (2) Although the percentage or cell number of Erk2$^{-/-}$ Treg cells are similar as wild type, but Erk2$^{-/-}$ Treg cells may inhibit the WT Th1 proliferation and differentiation. Therefore, Erk2 is required for cell proliferation and survival in Th1 responses in vitro and in vivo. Consistently, when we analyze the Erk double knockout CD4 T cells for T helper differentiation, there was a dramatic defect in Th1 differentiation (Figure 3-8).

Although dnRas showed increased percentage of IFNγ (Yamashita et al., 1999), the inhibitor study to block the Erk signaling pathway showed less Th1 differentiation (Brereton et al.). A recent study showed that impaired H-ras and K-ras resulted in decreased IFNγ expression (Iborra et al., 2011), and our result is consistent with the latter two studies. It may be due to purified CD4 T cells from mice of different genetic backgrounds and the strength of TCR signaling/co-stimulatory signaling and cytokine signals that were given for Th1 polarization.

From the microarray analysis, we did see lower expression of Fos and stat4 but higher expression of GATA3, IL-4 and Foxp3 in Erk2$^{-/-}$ CD4 T cells compared to wild type under Th1 conditions. However, Erk2$^{-/-}$ CD4 T cells can express similar levels of Tbx21 (T-bet) and IFNγ. Also, several known Foxp3

targets or regulatory T signature genes such as Emp1, Pdlim4, Vim, Ecm1, S100a8 and Socs2 have higher expression in Erk2$^{-/-}$ than WT under Th1 conditions. This suggests that GATA3 and Foxp3 may inhibit Th1 differentiation in Erk2$^{-/-}$ CD4 T cells.

The role of Erk in Th2 differentiation remains controversial. In our hands, we do see a slight increaseof both IL-4 producing cells and GATA-3 expression in Erk2$^{-/-}$ cells under Th2 conditions. However, there is no significant difference in Th2 differentiation between wild type and Erk2$^{-/-}$, even in Erk deficient (Erk1$^{-/-}$ 2$^{-/-}$) cells (Figure 3-8). It has been shown that Ras-Erk signaling may regulate GATA3 stability and Jak1/Stat6 activity for Th2 differentiation (So et al., 2007; Yamashita et al., 2005). However, inhibiting Erk activation can induce early IL-4 expression as well as alter AP-1 DNA activity. (Jorritsma et al., 2003) Moreover, in long term Th2 culture, there is no effect on IL-4 expression. Consistently, we also see no significant difference after culturing for 7-days. Interestingly, when Erk2$^{-/-}$ CD4 cells were stimulated with optimal CD3/CD28 in the absence of any Th1/Th2 skewing cytokine, those Erk2$^{-/-}$ CD4 T cells were producing higher levels of IL-4 compared to wild type. Our microarray data also showed that Gata-3 and IL-4 were upregulated in Erk2 deficient compared to wild type CD4 T cells, even without any cytokine for polarization (Table 5-4). It is consistent with early studies showing Gata3 expression was regulated by Erk-MAPK cascade (Yamane et al., 2005; Yamashita et al., 2005).

Additionally, Th17 cells require signaling through IL-6 and TGFβ. It was shown that using a MEK inhibitor leads to increase Th17 polarization because of less IL-2 production (Brereton et al.). A later study also suggested that enhanced Th17 responses in the absence of Erk leads to increased severity of IBD disease (Tan and Lam, 2010). We also observed slightly increased severity of IBD when we compared mice, which were injected with naïve WT or Erk2 deficient T cells. This difference maybe due to the fact that we only looked for the effect of Erk2 but not have both Erk1 and Erk2. Another possibility is that the inhibitor-treated mice may have other side effects to induce more severe disease. We did see less IL-2 production under different conditions (Th1, Th2, Th17) in $Erk2^{-/-}$ CD4 culture. However, in our study there is no significant increase in Th17 differentiation when comparing wild type and $Erk2^{-/-}$ T cells. Although there is a defeat for cell survival in Erk deficient cells ($Erk1^{-/-}2^{-/-}$), these cells can still make IL-17 producing cells. Erk deficient CD4 T cells have a lower proportion of IL-17 producing cells (Figure 3-5), possibly caused by fewer activated cells.

Foxp3 expression was regulated by TCR signaling, costimulatory signaling and cytokine-mediated signaling. Several transcription factors (Stat5, Smad3, AP-1, NFAT) binds to promoter or enhancer regions to increase Foxp3 expression (Yamashita et al., 1999). We see an increased differentiation of Foxp3 in the absence of Erk2 but no difference in Erk1-deficient cells. TGFβ

signaling down-regulates the phosphorylation of Erk in naïve T cells. There is an increased differentiation of induced Tregs with the addition of MEK inhibitor (UO126) as a result of down regulating the DNA methyltransferase (Luo et al., 2008). We found a similar result for DNMT1 as this study. However, we did not see a significant difference for DNMT3a and DNMT3b. Interestingly, we also found out Erk2 deficiency may enhance TGFβ signaling by increasing phos-Smad2/3 (Figure 4-7) and TGFβ receptor II (data not shown). A recent study of MEKK2 and MEKK3 deficient T cells revealed impaired phosphorylation of Smad2/3 linker region and enhance Smad transcription activity (Pfoertner et al., 2006).

Our ex vivo study of $Erk2^{f/f}$CD4Cre mice (Figure 4-1) and $Erk2^{f/f}$ERCre mice showed higher percentage of regulatory T cells ($CD25^{+}Foxp3^{+}$) (Figure 4-2). The in vivo study also demonstrated Erk2 deficiency enhanced peptide-induced regulatory T cells. From our microarray data, we observe that Foxp3 regulated genes and Treg signature genes are more highly expressed in $Erk2^{-/-}$ CD4 T cells (Figure 5-6). In the absence of TGFβ, $Erk2^{-/-}$ CD4 T cells express higher Foxp3, CTLA4 and Tnfrsf18 (GITR) mRNA level compared to wild type (Figure 5-9). This suggests Erk2 deficiency might lead to enhanced TGFβ signaling. GATA-3 is known as an important transcription factors for Th2 differentiation but it is also known to be more highly expressed in regulatory T cells than in naïve T cells (Chang et al., 2011). Irf4 is a negative regulator for

Foxp3 expression and we did lower expression of Irf4 in Erk2$^{-/-}$ T cells after 1day of culture. The Th1 major transcription factor, T-bet expression was expressed lower in Erk2 deficient cells under Th0 and iTreg conditions. Therefore, Erk2 negatively regulates Foxp3 expression.

In summary, Erk2 does positively regulate Th1 responses but represses Treg differentiation both in vitro and in vivo. However, Erk1$^{-/-}$ cells are all normal in CD4 T cell proliferation and differentiation. Erk has several target substrates to regulate cell responses (Shen et al., 2009). Our results provide the way to understand how Erk regulates CD4 T helper and iTreg differentiation. CD4 T helper cell differentiation is important for further investigation of improving or inhibiting immune responses. Treg cells are crucial for keeping balanced for immune responses between the different T helper cells subsets: Th1, Th2 and Th17. Also, human lupus studies showed that patients have an increased population of CD25^{-}Foxp3^{+} T cells and Erk may be involved in this process (Gorelik and Richardson, 2009, 2010). In addition, some increased Erk phosphorylation was found in autoimmune disease-Rhenumatoid arthritis (RA) patients in both naïve and memory T cells (Singh et al., 2009). Therefore, for the purposes of novel therapy, it would be interesting to explore enhancing Treg differentiation or inhibiting T cell responses as a mean to cure the disease.

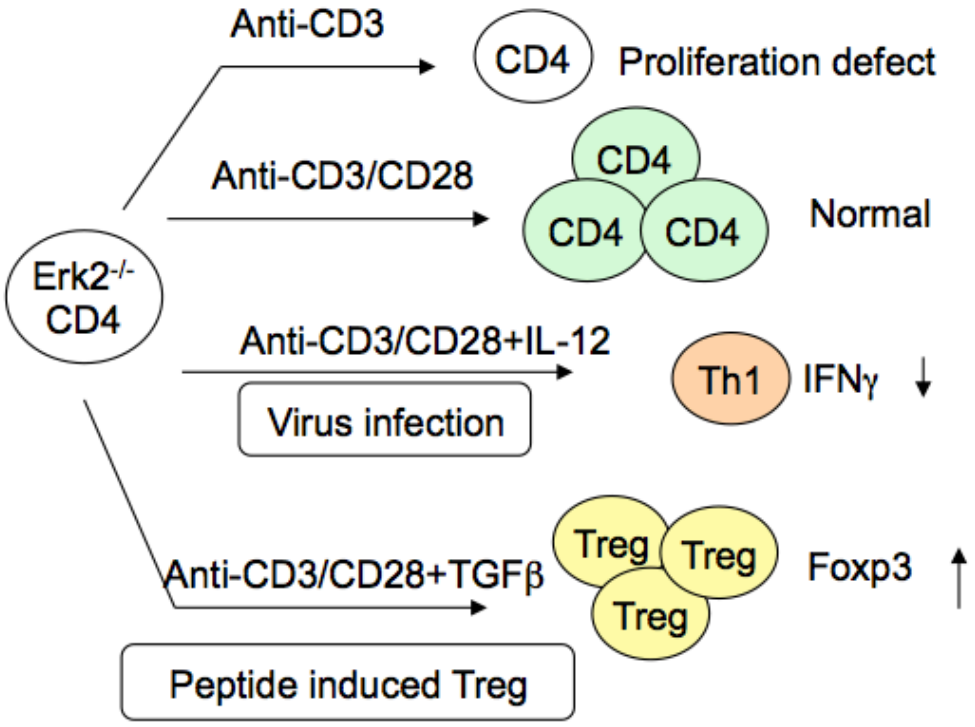

Figure 6-1. Summary diagram of Erk2 deficient CD4 T cell proliferation and differentiation. Erk2 positively regulates immune responses by promoting effector Th1 cell differentiation, and inhibiting the differentiation and function of Treg.

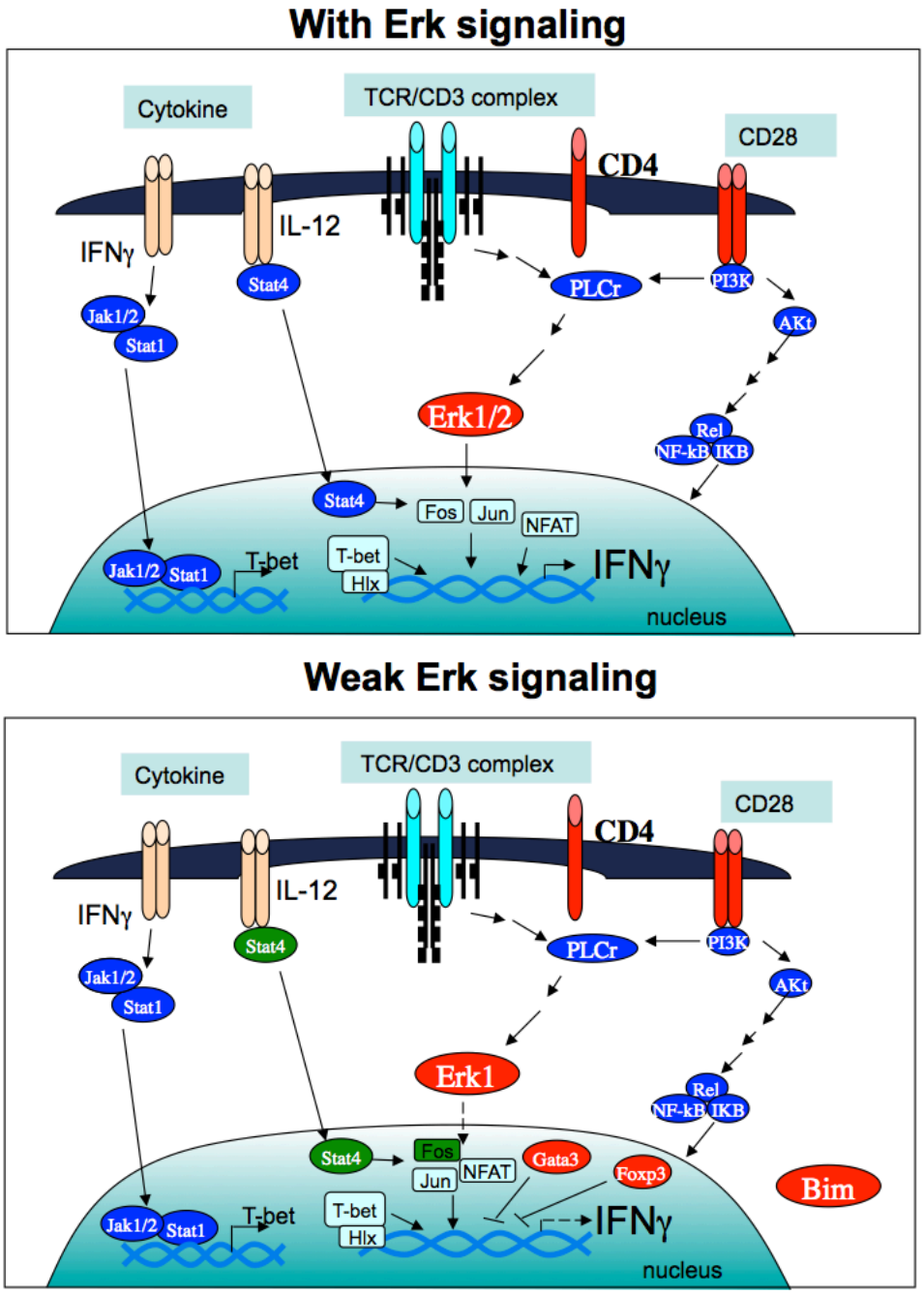

Figure 6-2. Graph of Th1 cells in presence or absence of Erk2 signaling. Deceased expression of Stat4 and Fos may lead to lower IL-12 signaling in Erk2 deficient CD4 T cells under Th1 condition. In addition, increased Bim level maybe related to Erk2 deficient Th1 CD4 survival.

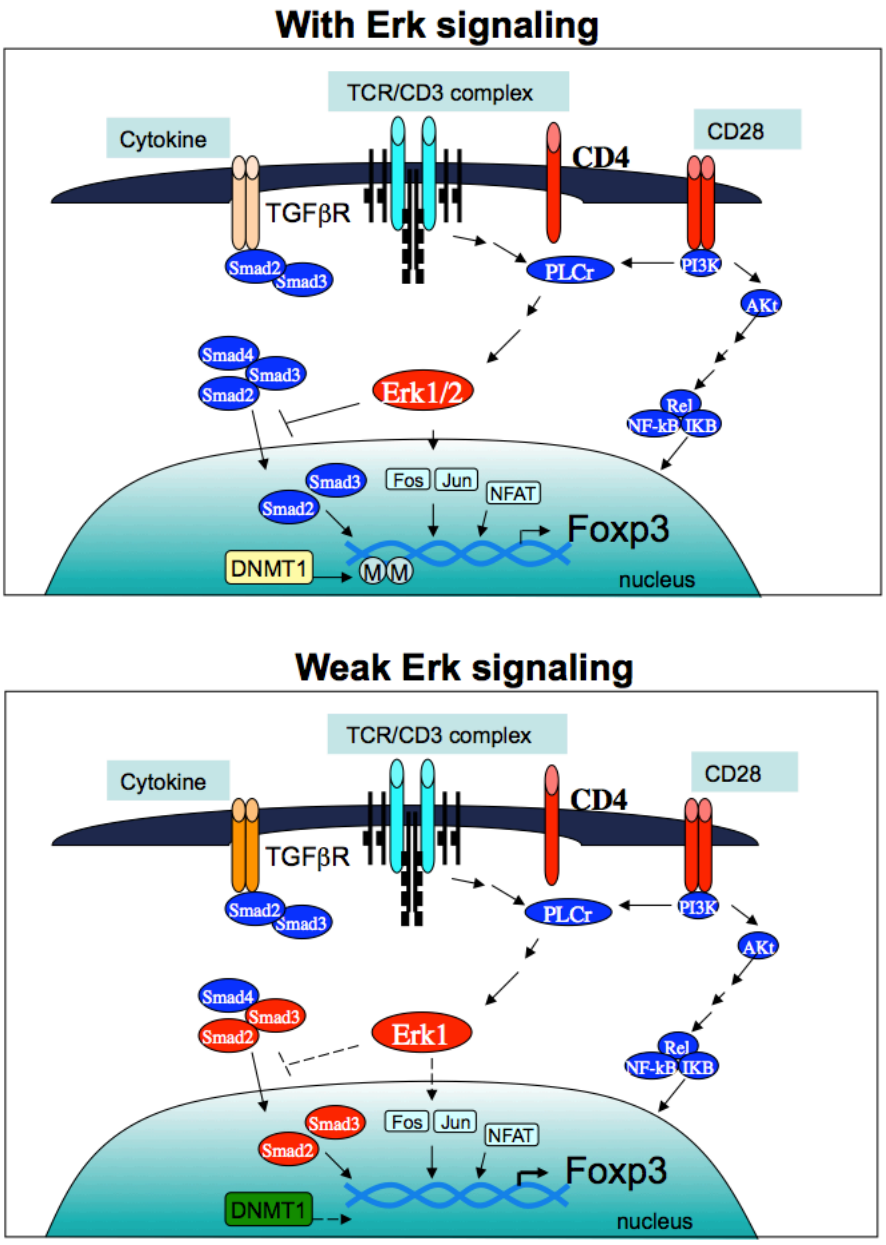

Figure 6-3. Graph of iTreg cells in presence or absence of Erk2 signaling. In Erk2 deficient CD4 T cells, there is only weak signaling through Erk1 and thereby partially loss inhibition of Smad signaling. In addition, DNMT level is down regulated in Erk2 deficient and thereby enhance the expression of Foxp3.

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