Early Growth Response Gene-2 Controls Inflammatory Responses of CD4+ T Cells by Negatively Regulating Th17 Differentiation.

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Declaration

I Meera Raymond confirm that I am author of this work and that where information has been obtained from other sources I have acknowledged this within the text or below.

All lymphoid organ extractions were performed at Brunel University by Dr Suling Li and her colleague's. The genotyping of Egr-2 conditional knock out mice was preformed by Dr Emma Ghaffari as well as the induction of EAE mog pepetide experiments. The analysis of the microarray data was carried out by Dr Alistair Symonds. EMSA experiments, Reporter gene Luciferase assay, Lentiviral transduction and mRNA silencing was performed with Dr Tizong Miao. Lentiviral vector constructs and Batf expression constructs were designed by Dr Tizong Miao. Clinical samples were obtained under the supervision of Dr Ute Meier and Alicia Rosello, From the MS Centre, Barts and Royal London Hospital, London.

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Abbreviations

AHR	Aryl Hydrocarbon Receptor
AICEs	AP-1 – IRF composite elements
AP-1	Activator protein 1
APC	Antigen Presenting Cell
ATF2	Activating Transcription Factor 2
BATF	Basic Leucine Transcription Factor, ATF like
bHLH	basic helix-loop-helix
Camp	Cyclic adenosine monophosphate
CIA	Collagen-Induced Arthritis
CLP	Common Lymphoid Progenitor
CSA	Cyclosporin A
DC	Dendritic cell
DN	Double Negative
dNTP's	Deoxynucleotide Triphosphates
DP	Double Positive
EAE	Experimental Autoimmune Encephalomyelitis
Egr	Early growth response
E2A	E-protein family of transcription factors
gd	gamma delta
HEK293	Human Embryonic Kidney 293 cells
ICOS	Inducible T cell COStimulator
ID	Inhibitor of DNA binding
IFN	Interferon

IFN-γ	Interferon gamma
IRF	Interferon Regulatory Factor
IRS2	Insulin Receptor Substrate 2
ISP	Immature Single Positive
Itec	Inducible T cell kinase
iTreg	Inducible T regulatory cell
IL	Interleukin
JNK	Jun N-terminal kinase
KO	Knockout
cKO	conditional Knockout
MAP	Mitogen activated protein
miRNAs	MicroRNAs
Mf	Macrophage
MHC	Major histocompatibility complex
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
NFAT	Nuclear factor of activated T cells
NK	Natural killer
n Treg	naturally occurring T regulatory cell
PPARg	Peroxisome proliferator-activated receptor g
PIO	Pioglitazone
PU.1	Purine rich nuclear protein
SP	Single Positive
SOCS	Suppressor of cytokine signalling

STAT	Signal transducer and activator of transcription
TBE	Tris base/Borate acid/EDTA
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
Th	T helper
TCR	T cell receptor
Tec	T cell-associated kinases
Tg	Transgenic
TNF	Tumour necrosis factor
TGF	Transforming Growth Factor

Abstract

$CD4^+$ T cells play a central role in controlling the adaptive immune response by secreting cytokines to control the responses of different lymphocytes. Naïve $CD4^+$ T cells differentiate into at least four subsets, Th1, Th2, Th17, and inducible regulatory T cells under antigen and corresponding cytokine microenvironment during an immune response. Therefore, each subset has unique functions for pathogen elimination. The differentiation of these subsets is induced in response to conditioned cytokine stimulation, which induces specific master regulator transcription factors. Multiple other transcription factors, both subset specific and shared, are also involved in promoting subset differentiation. Previously, our group has discovered that early growth response gene-2 (Egr-2) is important for the maintenance of T cell homeostasis and controls the development of autoimmune disease. However, the underlying mechanisms are unknown. In this study, using Egr-2 conditional knockout mice, a novel mechanism of Egr-2 in the control of Th17 differentiation has been discovered. Egr-2 is induced by TGFβ and IL-6 in naïve $CD4^+$ T cells. The induced Egr-2 negatively regulates the expression of IL-17, the signature cytokine for Th17. In contrast, Egr-2 has less effect on the expression of IL-2 or IFN-γ in effector T cells. In the absence of Egr-2, $CD4^+$ T cells produce high levels of Th17 cytokines. Deletion of Egr-2 in T cells renders mice susceptible to EAE induction; a model for Th17 mediated autoimmune diseases. T cells lacking Egr-2 show increased propensity for Th17, but not Th1 or Th2, differentiation in vitro. The key mechanism of Egr-2 in regulation of Th17 differentiation is to inhibit the function of Batf for transactivation of IL-17 expression and promotion of Th17 differentiation. Egr-2 interacts with Batf in $CD4^+$ T cells and suppresses its interaction with DNA sequences derived from the IL-17 promoter, while the activation of STAT3 and expression of RORγt is unchanged in Th17 cells in the absence of Egr-2. Thus, Egr-

2 plays an important role to intrinsically control Th17 differentiation. We also found that $CD4^+$ T cells from multiple sclerosis (MS) patients have reduced expression of Egr-2 and increased expression of IL-17 following stimulation with anti-CD3 in vitro. Collectively, our results demonstrate that Egr-2 is an intrinsic regulator that controls Th17 differentiation by inhibiting Batf activation which may be important for the control of inflammatory autoimmune diseases.

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

1.0 CD4+ T cells

The immune system consists of a sophisticated array of cells that have developed to recognize and eradicate a wide variety of microorganisms. It has been well established that to evade and overcome the innate immune response leads to the activation of adaptive immunity where the response is shaped depending on the pathogen eliciting the reaction (Janeway 1989). In adaptive immunity $CD4^+$ T cells (a major subset of T lymphocytes) play a central role in immune protection. T cells originate in the bone marrow, and go to the thymus where they mature and develop. They then exit to the peripheral immune system, and can be stimulated by antigen-presenting cells (APCs) that carry the major histocompatibility complex (MHC) presented with a peptide antigen. This is the stage that T cells will produce the cytokine IL-2 and express IL-2 receptors on the surface. Binding of IL-2 to its receptors, through the autocrine mechanism, induces clonal expansion of T lymphocytes. The activated T lymphocytes are subsequently differentiated to the effector cells for generating immune functions (see Fig 1-1, A-C).

In contrast to the MHC class I restricted $CD8^+$ T cells, $CD4^+$ T cells recognize antigen in context with MHC class II molecules (Janeway 1989). Importantly, $CD4^+$ T cells do not directly react on antigen-bearing target cells, but produce cytokines that regulate the function of different lymphocytes during immune responses (Tseng and Dustin 2002); so $CD4^+$ T cells are known as helper T cells (Th cells). $CD4^+$T cells are critical for host defense, but if they lose tolerance to self-antigens, they can also derive autoimmune diseases, asthma, and allergies (Stefanova, Dorfman et al. 2002). Based on the antigen and cytokine environment, $CD4^+$ T cells

differentiate into different subsets of T helper cells. The different Th subsets are defined by the signature cytokines produced in response to antigen stimulation (Jiang and Dong 2013). Th1 and Th2 cells are the classical Th subsets (Mosmann, Cherwinski et al. 2005). The mechanisms for Th1 and Th2 differentiation, the cytokines produced by Th1 and Th2 cells and the key transcription factors regulating the plasticity of these Th cells are well documented. See Fig 1.2 (O'Garra and Arai 2000). Further investigations in recent years demonstrate that the opportunities for helper diversity are far greater than just these two outcomes. The new diversity includes Th17, Th9, and Th22 cells; follicular helper T (T_{FH}) cells; and different types of regulatory T (Treg) cells. Figure 1-3 (Hirahara, Poholek et al. 2013). In addition, the emerging data point to the increased flexibility of these subsets. Understanding the molecular basis of this complexity is now a major focus in immunology, and the new insights will provide a better understanding of immune-mediated disease and new opportunities for therapy.

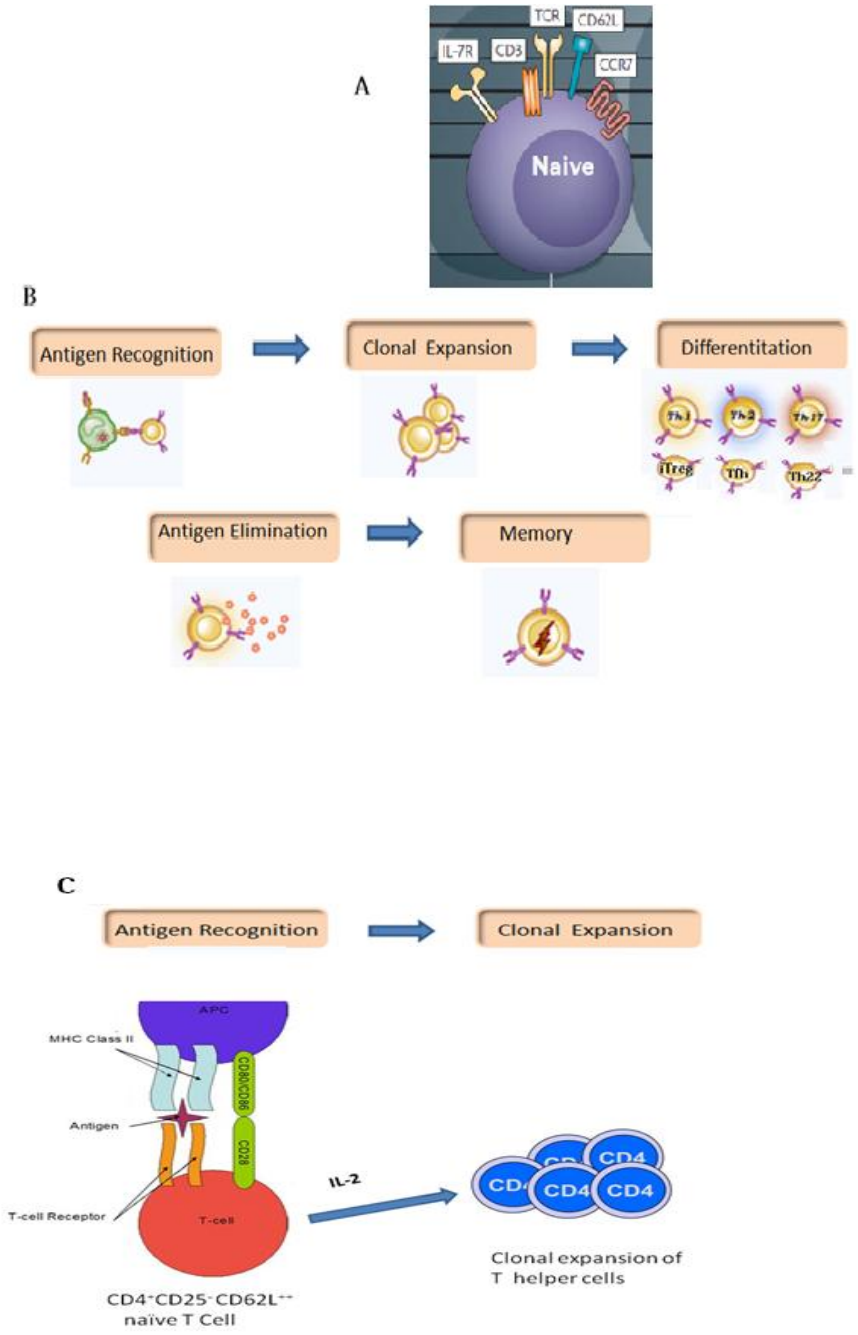


Figure 1-1. Schematic overview of naive T cells upon activation.

(A) Cell surface receptors expressed on naive $CD4^+$ T cells. (B) Flow chart showing upon encounter to antigen, $CD4^+$ T cells will clonally expand to T helper cells and differentiate to specific subsets of T helper cells. These effector $CD4^+$ T cells go on to eliminate foreign antigens by secreting cytokines and then stay as memory T cells already having previous exposure to the antigen. (C) Diagram showing APC carrying the MHC class II molecule presenting the antigen, binding to naive T cells along with the co stimulatory molecules activates the $CD4^+$ T cell to produce IL-2 and have Il-2 receptors on its surface which leads to clonal expansion of T helper cells.

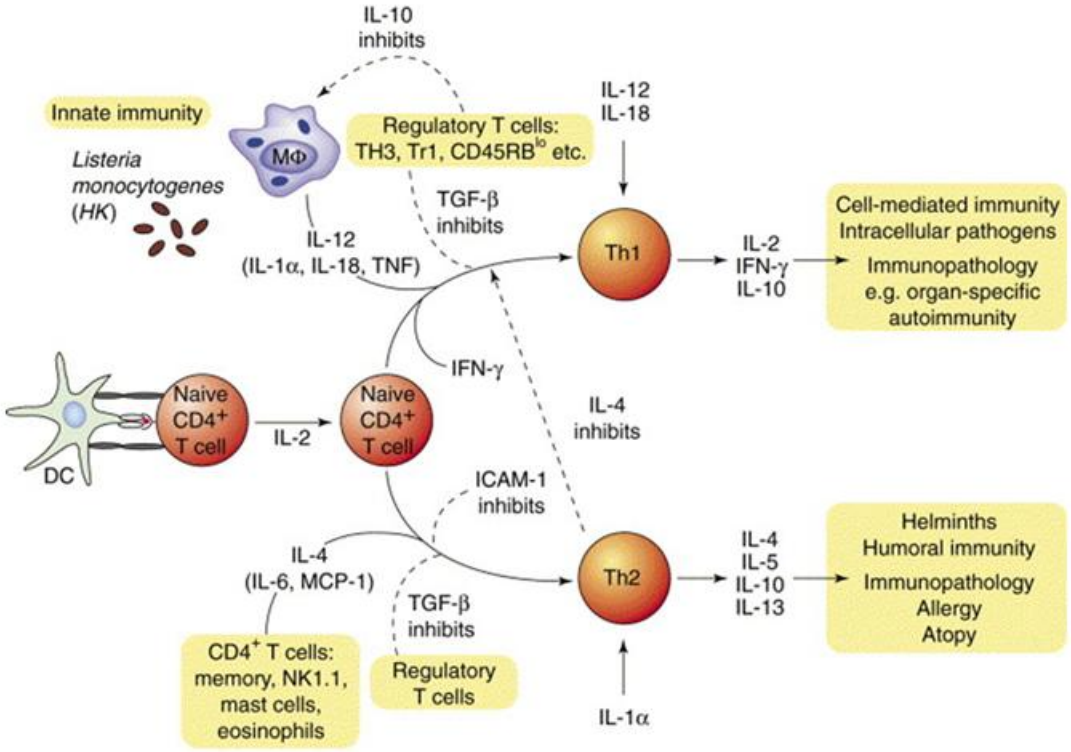


Figure 1-2. Differentiation and function of Th1 and Th2 cells. Adapted from *(O'Garra and Arai 2000).*

APCs, partly due to the cytokines that they produce, can induce the development of Th1 or Th2 cells. Naive $CD4^+$ T cells can develop into Th1 cells responsible for cell-mediated immunity in response to IL-12. Th1 development is dependent on IFN-γ, and maintenance of phenotype depends on stimulation in the presence of IL-12 and IL-18. Development of Th1 responses can be antagonized directly by IL-4 and indirectly by IL-10. Th2 cells, dependent on IL-4 to differentiate, and IL-1a for maintenance. Regulatory T cells have a suppressive function and this is part due to the action of transforming growth factor beta (TGF- β) and the ability to inhibit both Th1 and Th2 development.

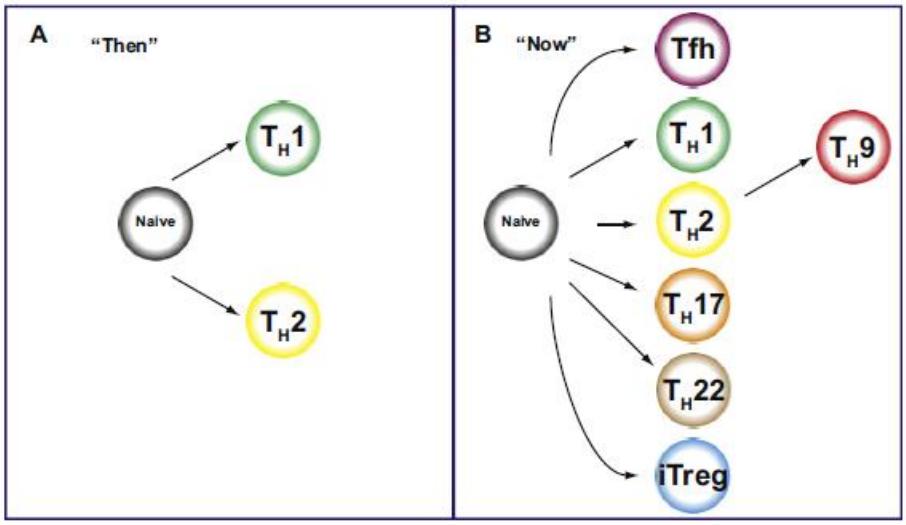


Figure 1-3. T helper subsets: What we knew before and what we know now. Taken from *(Hirahara, Poholek et al. 2013)*. More than two decades ago, helper T cells were viewed as having two major fates, Th1 and Th2 cells. We now recognize the new diversity of helper T cells including Th17, Th9, Th22, Tfh and different types of regulatory T cells.

1.1 Regulation of Th1 and Th2 cell differentiation

1.1.1 Cytokine induced development of Th1 and Th2 subsets

Early events in an immune response stimulate the production of cytokines, antigen-presenting cells (APCs) or a specific APC subset (e.g. distinct dendritic cell subsets), depending on cytokines that they produce, they can induce the development of Th1 or Th2 cells with discreet patterns of cytokine production.

Naive $CD4^+$ T cells can develop into Th1 cells by production of their hallmark pro-inflammatory cytokines interferon-γ (IFN-γ) as well as lymphotoxin, IL-2 and IL-10. They are responsible for directing cell-mediated immune responses, which leads to eradication of intracellular pathogens (Mosmann and Coffman 1989; Sher and Coffman 1992) including bacteria, viruses, parasites and yeasts. Maintaining the phenotype depends on stimulation in the presence of IL-12 and IL-18 (See figure 1-2). IL-12 is the dominant factor in directing the development of Th1 cells producing high levels of IFN-γ (Hsieh, Macatonia et al. 1993; Trinchieri and Scott 1995). IL-12 is a 75kDa heterodimer, produced by macrophages upon encounter with many microbial products. APCs are specialized in antigen capture, migration to secondary lymphoid organs, T cell priming (Steinman 1991) and can also produce IL-12 (Scheicher, Mehlig et al. 1995). A Th1 response is often accompanied by the production of complement-fixing antibodies of the IgG2a isotype, as well as the activation of natural killer (NK) cells and cytotoxic $CD8^+$ T cells expressing IFN-γ and perforin (Mosmann, Cherwinski et al. 1986; Sher and Coffman 1992;

Abbas, Murphy et al. 1996; Mosmann and Sad 1996). If uncontrolled, aberrant Th1 response can cause tissue damage or contribute to several inflammatory and autoimmune diseases such as multiple sclerosis (MS), type 1 diabetes and inflammatory bowel disease (Powrie and Coffman 1993; Liblau, Singer et al. 1995).

Human asthma, as well as animal models of allergic airway inflammation, revealed the importance of cytokines produced by Th2 cells, namely IL-4, IL-5, and IL-13 (Umetsu and DeKruyff 2006). Th2 cells activate mast cells and eosinophils and in addition can result in elevated levels of IgE and so have been implicated in atopy and allergic inflammation (Romagnani 1994). Th2 cells are dependent upon naïve $CD4^+$T cells exposure to IL-4 for differentiation at the initiation of an immune response (Swain, Weinberg et al. 1990). The effects of IL-4 in inducing Th2 development are dominant over Th1 polarizing cytokines (Hsieh, Heimberger et al. 1992; Seder and Paul 1994). So when IL-4 levels reach a certain threshold at the beginning of an immune response Th2 cells will differentiate, leading to increased levels of IL-4 progressively. This could explain why during an innate immune response where chronic stimulation takes place in the absence of inflammatory signals as well as the magnitude of an immune response could possibly drive Th2 responses (Abbas, Murphy et al. 1996). It was originally thought that IL-4 was only produced by differentiated T helper cells early in an immune response. There are now several candidates for IL-4 production occurring early in an immune response which could be possible for Th2 differentiation; these include MHC class II restricted $CD4^+$T cells (memory and naïve) (Hosken, Shibuya et al. 1995), the natural killer 1 ($NK1^+$) of $CD4^+$ and double negative (DN) T cells (reviewed in (Bendelac, Rivera et al. 1997), and non-T cell sources, such as mast cells, basophils and eosinophils (Paul, Seder et al. 1993). The production of B cell growth and differentiation factors by Th2 cells may explain why certain

immune responses are predominantly humoral, whereas delayed-type hypersensitivity is attributed to Th1 cells.

1.1.2 Th1 and Th2 paradigm

Cytokines from Th1 and Th2 can cross-regulate each other's function as well as development. The development of Th1 responses can be antagonized directly by IL-4 and indirectly by IL-10, which inhibits the production of inflammatory mediators such as IL-12 and IL-18 from macrophages and/or dendritic cells stimulated by the innate immune response (Moore, O'Garra et al. 1993). IFN-γ produced by Th1 cells inhibits the development of Th2 cells (Fitch, McKisic et al. 1993) as well as humoral responses.

Helper T cells and clones that produce both Th1 and Th2 type cytokines (termed Th0) have been described in human and mouse systems (Sher and Coffman 1992; Kelso 1995; Abbas, Murphy et al. 1996). Whether they are precursors for Th1 and Th2 subsets is still not clear; it is possible they are involved in eliminating many pathogens, where a balance of both regulated and an appropriate humoral response will eradicate the invading pathogen with little immunopathology. However in a chronic setting it could be pathogenic as the polarized Th1 and Th2 responses could have counter regulatory effects of cytokines from each subset.

In the past year, it has become clear that distinct subsets of regulatory T cells are responsible for regulating both Th1 and Th2 responses and preventing the development of immune pathologies. A common feature of many of these regulatory T cells is that their function is related to the action of transforming growth factor beta (TGF-β); this would be in keeping with the ability of TGF-β to inhibit both Th1 and Th2 development.

The attempt to link common autoimmune diseases with a simple Th1/Th2 paradigm has been problematic (Gor, Rose et al. 2003) even though there is evidence that excessive activation of Th1 cells contributes to organ-specific autoimmune diseases (Szabo, Sullivan et al. 2003). However, a number of lines of evidence suggest that autoimmune mechanisms cannot be reduced to the action of Th1 cells alone. In particular, the discovery of a new cytokine, IL-23, led to the recognition of a new subset of helper T cells and their importance in autoimmunity (Cua, Sherlock et al. 2003).

1.1.3 Costimulatory molecules and the control of Th1 and Th2 differentiation

The differentiation pathway for Th1 and Th2 cell development can be influenced by a number of factors, including the dose and form of antigen, the affinity of the peptide antigen T cell receptor (TCR) interaction (Constant and Bottomly 1997) and costimulatory interactions between cell-surface molecules. Appropriate Th lymphocyte stimulation requires two signals. The first response is by interaction of the T cell receptor (TCR) on the Th cell and the peptide-MHC complex on the APC. This signal is not sufficient to activate T cells due to the absence of a second signal from costimulatory molecules; TCR binding may result in anergy or cell death (Schwartz 1990). Adhesion and costimulatory molecules, which deliver the second signal to T cells, stabilize intercellular binding and influence Th1 and Th2 differentiation. The most important costimulatory molecule is CD28, which interacts with members of the B7 family. Other costimulatory interactions include ICAM-1 and LFA-1; CD40 and CD40L participate in the cross-talk between Th cells and APCs, they also influence the outcome of the response. These molecules modulate the duration and strength of Th cell activation and, similarly to that recognized for the antigen dose. Also the affinity of the peptide MHC complex to the TCR, have an effect on Th1 and Th2 cell differentiation (Gause et al. 1997;Ruedl et al. 2000). It has been

seen that ICAM-1 and LFA-1 interactions inhibit the expression of Th2 cytokines (Luksch, Winqvist et al. 1999). Although these factors might offer some explanation to the development of distinct Th cell subsets, cytokines are major inducers of Th cell differentiation (Paul and Seder 1994). In addition to the expression of distinct cytokine genes, the molecular basis for commitment to a Th1 or Th2 phenotype can probably be explained by multiple mechanisms, including exclusive cytokine receptor expression, differential cytokine signalling, differential expression of transcription factors and differential chromatin remodelling of Th1 and Th2 specific genes (Rincon and Flavell 1997; Agarwal and Rao 1998).

There are two main systems used for studying the factors driving Th1 and Th2 development. These are the polyclonal stimulation of T cells, triggering the TCR with antibody against CD3 together with the costimulator anti-CD28, and the stimulation of T cells derived from TCR-transgenic mice with specific antigen and APC. Using these systems, researchers have shown that cytokines such as IL-12 and IL-4 play a dominant role in inducing the differentiation of Th1 and Th2 cells, respectively (O'Garra, Steinman et al. 1997; Murphy, Ouyang et al. 2000). Naive T cells differentiate into homogeneous populations of committed Th1 or Th2 cells displaying an exclusive cytokine pattern. This is achieved mostly after repeated antigenic stimulation under the appropriate conditions. Th1 and Th2 populations are heterogeneous at an early stage in development, contain low frequencies of cytokine-producing cells and are reversible, suggesting that a number of molecular changes must occur in order to establish a committed profile of cytokine gene expression.

1.1.4 Signaling pathways for Th1 differentiation and commitment

The activation of the transcription factor STAT4 by IL-12 and through the IL-12 receptor (IL-12R) is crucial for Th1 responses. (Figure1-4, adapted from O'Garra and Arai 2000). The IL-12 receptor consists of two chains, IL-12Rb1 and IL-12Rb2, and mice deficient in IL-12 and STAT4 have markedly reduced, but not absent, Th1 responses (Magram, Connaughton et al. 1996; Thierfelder, van Deursen et al. 1996). It has been shown that STAT4 can bind to DNA-binding sites within the gene encoding IFN-γ, but differentiated T cells can produce IFN-γ in the absence of STAT4 (Ouyang, Jacobson et al. 1999). Also IFN-γ can activate STAT4 in human T cells but not in mice (Rogge, D'Ambrosio et al. 1998).

The receptor IL-12Rb2 is expressed in Th1 cells and is upregulated by IFN-γ, whereas Th2 cells lose the expression of this chain of the IL-12R by downregulation with IL-4, researchers have hypothesized this as a basis for Th1 commitment (Szabo, Dighe et al. 1997). Work by Nishikomori and his group and also Heath and his group have shown; ectopic expression of IL-12Rb2 in developing Th2 cells results in activation of STAT4 by IL-12 and to proliferate it does not lead to significant production of IFN-γ, suggesting that IL-12 driven STAT4 activation is not sufficient to maintain IFN-γ production by Th cells (Heath, Showe et al. 2000; Nishikomori, Ehrhardt et al. 2000).

IL-18 has recently been shown to synergize with IL-12 to stimulate the production of IFN-γ by Th1 cells and both chains of the IL-18 receptor are downregulated on Th2 cells but maintained on Th1 cells (Xu, Chan et al. 1998). IL-18 signals autophosphorylation of IRAK and activation of NF-kB52. Further, more mouse studies by Magram and Takeda have shown that IL-18 as well as IL-12-deficient mice show a defect in Th1 responses (Magram, Connaughton et al. 1996;

Takeda, Tsutsui et al. 1998). Mice deficient in IRF-1 have also shown a defect in development of Th1 cells and IFN-γ production, both at the level of IL-12 production by the APC and at the responsiveness of the Th1 cells to IL-12. This could be explained by studies showing that IRF-1 (Interferon regulatory factor 1) is a target gene of IL-12 (Coccia, Passini et al. 1999).

Exposure of cells to cytokines and environmental stresses activates the MAP (Mitogen activated protein) kinases c-Jun N-terminal kinase (JNK) and p38 pathways. Targets of these pathways include transcription factors such as c-Jun and ATF2 (Activating Transcription Factor 2). Activation of c-Jun and ATF2 plays an important role in activating cytokine genes in the innate and adaptive immune response.

In $Jnk2^{-/-}$ T cells, a large reduction in IFN-γ production was seen, which in part could be explained by a decreased expression of IL-12Rb2 and reduced STAT4 activation required for IFN-γ production (Yang, Conze et al. 1998). In another study, a dominant-negative inhibitory mutant of p38 selectively impaired Th1 responses and activation of the IFN-γ promoter (Rincon, Enslen et al. 1998).

Both the proximal and distal elements in transgenic reporter mice direct reporter expression in memory $CD4^{+}$ effector T cells. The consensus GATA motif is contained in the distal sites, also a potential regulatory motif found in the promoter regions of other cytokine genes, and factors binding to this site included GATA-3. GATA-3 is of interest as expression has since been shown to be Th2 specific and crucial for differentiation of Th2 cells (Lee, Takemoto et al. 2000).

NFAT and NF-kB have also been shown to bind to similar regions in the first intron of the IFN-γ gene (Sica, Dorman et al. 1997). A ubiquitous nuclear factor, Yin-Yang 1 (YY1), has been

shown to bind to two regions of the IFN-γ promoter and is proposed to act as a repressor of basal IFN-γ transcription (Ye, Cippitelli et al. 1996). Although these transcription factors have been proposed to regulate IFN-γ expression, future detailed functional analysis is needed to define their specific roles.

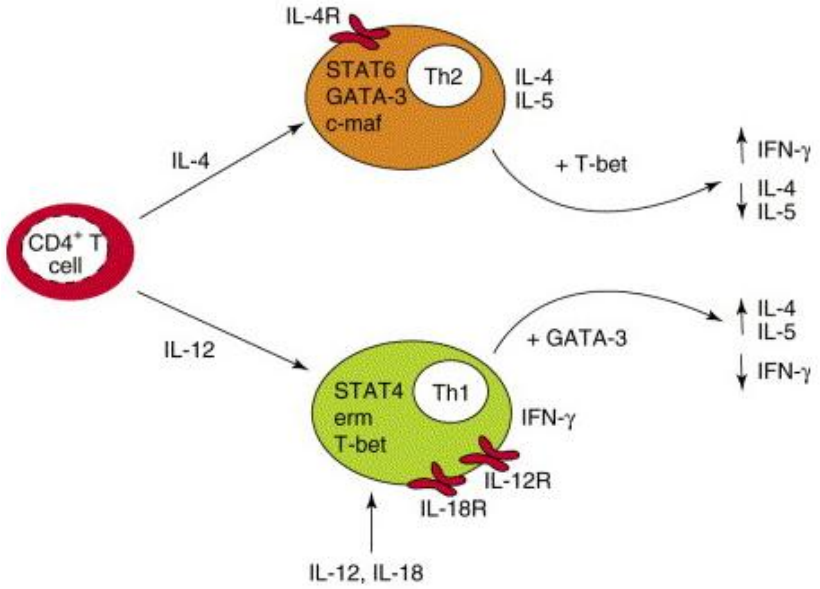


Figure 1-4. Regulation of Th1 and Th2 differentiation

Adapted from (O'Garra and Arai 2000) IL-12 and IL-4 direct the development of Th1 and Th2 subsets respectively, producing IFN-γ through STAT4 in Th1 cells or IL-4 through STAT6 dependent pathway in Th2 cells.

1.1.5 Transcription factors in Th1 development and commitment

Studies by Szabo, Glimcher and colleagues have found a cDNA encoding a protein they term T-bet (T-box gene expressed in T cells, since it is selectively expressed in thymocytes and Th1

cells) (Szabo, Kim et al. 2000). T-bet belongs to the T box family of transcription factors and play important roles in diverse developmental processes. T-bet was rapidly and selectively induced in Th1 but not Th2 cells. Co-transfection of the mouse thymoma (EL4) with a T-bet expression plasmid and an IFN-γ-luciferase reporter construct (containing the entire IFN-γ gene locus) results in significant T-bet-specific transactivation of the IFN-γ gene in both stimulated and unstimulated cells. Contrastingly, the activity of the IL-2 promoter is repressed by T-bet expression. This could explain previous observations that, although Th1 cells express IL-2, they downregulate its expression as they differentiate. Interestingly, T-bet neither inhibited nor enhanced IL-4 promoter transactivation.

After a week of culture the reversibility of Th1 and Th2 populations is mostly abrogated; however, stable commitment to a Th phenotype occurs only after repeated stimulation under polarizing conditions (Murphy, Shibuya et al. 1996). Interestingly, the introduction of T-bet into stably committed Th2 populations also dramatically increased the numbers of IFN-γ-producing cells and reduced the number of IL-4 and IL-5 producing cells, showing T-bet can convert Th2 into Th1 cells. However, ectopic expression of T-bet in a homogeneous Th2 clone only increased minimally the number of IFN-γ-producing cells. This might be because the chromatin configuration at the relevant loci is no longer accessible or because of the other factors necessary for inducing IFN-g gene expression is no longer present. However, T-bet expression in the Th2 clone still resulted in a significant reduction of IL-4 and IL-5-producing cells, showing that the ability of T-bet to suppress Th2 development extends even to long-term clones. So T-bet both initiates Th1 genetic programmes and represses the opposing Th2 programmes.

1.1.6 Signaling pathways for Th2 differentiation and commitment

Production of IL-4 early in an immune response directs the development of a Th2 response. Ligation of IL-4 with its receptor (IL-4R) activates at least two signaling pathways JAK–STAT and IRS2 (Insulin receptor substrate 2) pathways. Studies have showed that STAT6-deficient mice had defects in Th2 responses (Takeda, Tanaka et al. 1996), similar to the phenotype of IL-4-deficient mice, showing a crucial role of STAT6 in inducing Th2 differentiation. The STAT6-mediated signalling cascade was further studied by using a conditionally active form of STAT6 (STAT6:ER) which mimics IL-4-mediated signalling without ligation of the IL-4 receptor (Kurata, Lee et al. 1999). STAT6:ER, a fusion protein of STAT6 and the hormone-binding domain of the oestrogen receptor, is activated independently of IL-4 by an oestrogen analogue, 4-hydroxytamoxifen (4-HT). Work from Kurata and his group has shown that when STAT6:ER was transduced retro virally into naive T cells from mice with a transgenic T-cell receptor and driven under Th1 conditions. Activation of STAT6:ER induced the production of IL-4, IL-5 and IL-10 and repressed Th1 development and production of IFNγ although there was inhibition of the IL-12Rb2 receptor. Also a change in cytokine profile and the induction of mRNA for the Th2-specific transcription factors GATA-3 and c-maf was also observed upon the activation of STAT6:ER, suggesting that GATA-3 and c-Maf are downstream from STAT6 in the IL-4R mediated signalling cascade in Th2 development (Kurata, Lee et al. 1999). Studies by Ouyang et al, demonstrated that STAT6-independent GATA-3 autoactivation can direct IL-4-independent Th2 development and commitment (Ouyang, Lohning et al. 2000). In addition to these dominant pathways for Th2 cell differentiation, there might be crosstalk with other signaling pathways and so a requirement for other signaling molecules, such as the Tec (T cell-associated kinases) family kinase, which is required for the development of Th2 cells (Fowell, Shinkai et al. 1999).

1.1.7 Transcription factors for Th2 development and commitment

A number of transcription factors have shown to be implicated in Th2 cell differentiation. These include STAT6, NFATc/2, NF-IL-6, AP-1/Jun-β, c-Maf and GATA-3. Although AP-1 and NFAT proteins are important for inducing IL-4 and IL-5 transcription, they are present in both Th1 and Th2 subsets and so do not account for the Th2-restricted expression of IL-4. However, mice bearing targeted disruptions of NFATp have increased Th2 cytokines in the late phase of the immune response (Kiani, Viola et al. 1997). In addition, mice lacking both NFAT4/x and NFATp have extremely high levels of IL-4 and IgE (Ranger, Oukka et al. 1998), Showing that NFATp and NFAT4/x can exert a negative regulatory role on IL-4 expression *in vivo*, whereas NFATc positively transactivates the IL-4 promoter (Szabo, Glimcher et al. 1997).

GATA-3 and c-Maf are two Th2-specific transcription factors, c-Maf, an oncoprotein, is reported to activate the IL-4 promoter. c-Maf-deficient mice have been shown to be defective in IL-4 production but, interestingly other Th2-specific cytokines were unaffected (Kim, Ho et al. 1999). Ectopic expression of c-Maf did not induce the expression of IL-4 in developing Th1 cells.

Several investigators have demonstrated the importance of GATA-3 in Th2 cytokine gene expression and Th2 differentiation. Th1 cells from transgenic mice expressing GATA-3 have shown an increase in IL-4, IL-10 and IL-6 mRNA expression, although an effect on IFN-γ was not observed. Furthermore, expression of antisense GATA-3 in a Th2 clone significantly inhibited the expression of Th2 cytokines upon activation (Zheng and Flavell 1997). Retroviral infection of naive T cells with GATA-3 when cultured under Th1 conditions induced the production of Th2-cytokines and downregulated IFN-γ production through repression of IL-12 signaling (Ouyang, Ranganath et al. 1998). It also has been shown that expression of a dominant-

negative mutant of GATA-3 in transgenic mice inhibited the production of IL-4, IL-5 and IL-13 and inhibited airway inflammation in a murine model of asthma (Zhang, Yang et al. 1999). These observations indicate that GATA-3 might control the production of all Th2 specific cytokines and so maintains the Th2 phenotype.

It has been shown that the activation of STAT6:ER did not induce Th2-specific cytokines in committed Th1 cells (Kurata, Lee et al. 1999). Although activated STAT6:ER could induce Th2-specific cytokines and repress IFN-γ production in developing Th1 cells, this effect rapidly decreased when the activation of STAT6 was delayed. As all the Th1 cells within the population differentiated the lack of effect upon delayed activation of STAT6 also correlated with a decreased level of induction of the Th2-specific transcription factors GATA-3 and c-Maf. Experiments have shown that cAMP enhanced the GATA-3 effects to induce IL-4 and IL-5, although it had no effect on inducing these cytokines in Th1 cells in isolation (Lee, Takemoto et al. 2000).

It has been shown that cAMP (Cyclic adenosine monophosphate) levels are high in Th2 cells, and addition of cAMP as well as cAMP-elevating agents such as forskolin and PGE2 selectively upregulate Th2 cytokine production in Th2 clones, (reviewed by (Lee, Takemoto et al. 2000).

Studies of the gene promoter for IL-5 have established independently the important roles of GATA-3 and cAMP in specific expression of the IL-5 gene in a transformed thymoma cell line EL-4 as well as Th2 clones (Lee, O'Garra et al. 1998). A GATA-3-binding site was identified in the regulatory sequences that lie within 1 Kbp (kilo base pairs) upstream of the IL-5 gene; four *cis*-acting elements including the GATA-3 binding site have been mapped. By contrast, a role for GATA-3 in the activation of the IL-4 promoter has not been clear. Transfection and expression

of GATA-3 was sufficient for transcription of the IL-5 but not the IL-4 promoter in spite of the presence of putative GATA-3 sites in the IL-4 promoter (Zhang, Yang et al. 1998). An antigen-inducible GATA-3-binding enhancer was recently identified in the 39 flanking region of the IL-4 gene by DNase I hypersensitivity analysis (Agarwal, Avni et al. 2000). These findings suggest that GATA-3 controls IL-4 expression through distal enhancer elements outside the IL-4 promoter, possibly by mechanisms other than transactivation.

1.1.8 Th1 and Th2 cytokine gene regulation at the chromatin level

The regulatory elements in the Th2-specific gene cluster have been better defined than those in the Th1-specific gene cluster. Genes encoding IL-5, IL-13 and IL-4 are within the Th2 cytokine gene cluster in the long arm of human chromosome 5, q23–31, syntenic mouse chromosome 11, spreading over 160 Kbp. The deletion of this region, del (5q), is frequently observed in patients with a myelodysplastic syndrome. Several reports have indicated that differentiation to Th2 cells is linked with changes in chromatin structure in the IL-4 gene locus but not in the IFN-γ locus (Agarwal and Rao 1998). The DNA within transcriptionally inactive genes is found in regions of condensed chromatin, contains unmodified histones and is often densely methylated (methylation is an epigenetic mechanism for silencing genes). However, histone acetylation and DNA demethylation favour decondensation of chromatin, which can be demonstrated by the sensitivity of sites in the DNA to cleavage by the enzyme DNase I (DNase I hypersensitive sites), and provide increased access to factors such as transcription factors. Five clusters of DNase I hypersensitive sites (I–V) have been mapped within a 19-Kb fragment, flanked by the restriction sites for the enzyme BamH1, spanning the IL-4 gene in a Th2 clone, D10, and are accompanied by CpG demethylation. By contrast, a Th1 clone, D5, exhibited a relatively

‘closed’ chromatin structure, with only one hypersensitive site (IV)(Agarwal and Rao 1998). Similarly, differentiating Th2 cells rapidly acquired an accessible chromatin structure in the IL-13 genes as well. In addition to these hypersensitive sites observed in the IL-4 gene, three additional DNase I hypersensitive sites have been identified (HSS1, 2 and 3) in an intergenic region between the IL-4 and IL-13 genes separated by 15 Kb, both in a Th2 clone and in differentiated Th2 cells. The appearance of HSS1 and HSS2 is specific to Th2 cells but is constitutive and independent of stimulation (Takemoto, Koyano-Nakagawa et al. 1998).

Interestingly, the largest conserved noncoding sequence, CNS-1, was located in the intergenic region between IL-4 and IL-13, where the Th2-specific hypersensitive sites (HSS1 and HSS2) had been mapped previously. The biological properties of CNS-1 were characterized recently by establishing transgenic mice bearing a human YAC transgene (Loots, Locksley et al. 2000). Conditional deletion of the CNS-1 element in a 450-kb human transgene resulted in a decrease in the number of Th2 cells expressing human IL-4, IL-5 and IL-13. This indicates that CNS-1 might contain a crucial sequence controlling all cytokine genes within the IL-4 locus. Their results also suggested that GATA-3 functions as more than a sequence-specific transactivator and plays a role as a master switch in Th2 development.

Complementary changes in chromatin occur at the IFN-γ locus during Th1 commitment, but these are less well defined compared with those in Th2 cells. Specific DNase I hypersensitive sites were originally mapped in the IFN-γ gene. Further characterization of hypersensitive regions within the IFN-γ gene showed that Th1 cells developed these sites in the first and third introns during differentiation from a naive $CD4^+$ T cell. In addition, the locus is hypomethylated in Th1 but not Th2 cells (Agarwal and Rao 1998).

So, GATA-3 and T-bet are major inducers of Th1 and Th2 differentiation by controlling the production of their respective cytokines. In addition they are involved in the commitment and stable maintenance of the Th1 and Th2 phenotypes. GATA-3 might function not only in transactivation of genes but also by inducing epigenetic changes in the chromatin structure of the IL-4 locus.

There is a debate about whether the cytokines that induce Th1 or Th2 differentiation 'instruct' the developmental fate of naive Th cells or 'select' for cells which have already undergone a random process of gene activation and already produce a range of cytokines indicative of a Th1 or Th2 cell (reviewed in (Coffman and Reiner 1999)). As discussed above, stable cytokine expression is accompanied by demethylation and increased chromatin accessibility of the cytokine genes and, in addition, chromatin accessibility is acquired after repeated stimulation under Th1 or Th2 conditions, which correlates with a committed Th phenotype.

1.2 Regulation of T helper 17 differentiation

Over the last two decades, our understanding of helper/effector T cell differentiation has changed dramatically, with the addition of new T helper subsets that preferentially produce distinct cytokines. One includes cells that selectively produce IL-17A and IL-17F (Th17 cells). These cells are critical for host defence against extracellular pathogens (Ye, Rodriguez et al. 2001) and have been implicated in a number of autoimmune diseases (Annunziato, Cosmi et al. 2009; Kyttaris, Zhang et al. 2010; Steinman 2010). Th17 cells have also been shown to produce IL-9, IL-10, IL-21, and IL-22; however, subsets of cells that selectively produce these cytokines have also been identified (Nurieva, Chung et al. 2008; Veldhoen, Uyttenhove et al. 2008). The other

major lineage of $CD4^+$ T cells includes those that express the transcription factor Foxp3. These immunosuppressive, regulatory T (Treg) cells comprise both natural (n)Tregs and induced (i)Tregs. Th17 cells can mediate inflammatory responses and functionally oppose Treg cells.

1.2.1 T cell receptor (TCR) signaling and IL-17 production

Like Th1 and Th2 the obligate first step in the activation of $CD4^+$T cells is engagement of the T cell receptor: this is an important contributor to IL-17 regulation. For naive $CD4^+$ T cells, the strength of TCR signalling is an important driver of Th1 versus Th2 differentiation (Zhu and Paul 2008). Exactly how the signal strength influences IL-17 regulation is not completely understood. Recently it has been found that high dose antigen-loaded dendritic cells induce Th17 generation (Iezzi, Sonderegger et al. 2009). The Tec family tyrosine kinase inducible T cell kinase (Itk) is a tyrosine kinase required for full TCR-induced phospholipase C-g activation. $Itk^{-/-}$mice show decreased IL-17 production (Gomez-Rodriguez, Sahu et al. 2009). The same was seen with Raftlin, a protein located in lipid rafts, has been suggested to regulate the strength of TCR signaling. $Raftlin^{-/-}$T cells produce less IL-17 and $Raftlin^{-/-}$mice show reduced severity of experimental autoimmune encephalomyelitis (EAE) (Saeki, Fukuyama et al. 2009). The same has been found with an E3 ubiquitin ligase denoted GRAIL (gene related to anergy in lymphocytes) which regulates TCR-CD3 degradation and also influences IL-17 production (Nurieva, Zheng et al. 2010). These results are puzzling as these strong signals can induce T-bet expression and Th1 differentiation. In human $CD4^+$ T cells, it has been shown that weak TCR stimulation promotes Th17 responses (Purvis, Stoop et al. 2010). This could be due to upregulation of T-bet which attenuates IL-17 production.

Recent work has also shown that mammalian target of rapamycin (mTOR) complex 2 (mTORC2) impaired differentiation into Th1 and Th2 cells without significant effect on Th17 differentiation (Lee, Gudapati et al. 2010).

Signals from co-stimulatory molecules are also important for T helper cell specification. But it is still not clear for Th17 differentiation; both Inducible T cell COStimualtor (ICOS) and CD28 activation have been implicated in Th17 differentiation. $ICOS^{-/-}$ mice have exaggerated severity of Th17-mediated encephalomyelitis (Galicia, Kasran et al. 2009), while $ICOS^{-/-}CD4^{+}$ T cells show no significant increase of IL-17 production in vitro. More work is needed in this area.

1.2.2 Role of cytokines in Th17 differentiation

IL-23 is a heterodimeric cytokine composed of p19 and p40 subunits, with P40 subunit being shared with IL-12. IL-23 is a well-known inducer of IL-17. Studies have shown that gene targeting of p19 dramatically attenuated EAE, a disease that is dominated by Th cells that produce IL-17 (Cua, Sherlock et al. 2003). However, recently it has been shown that naive T cells do not express the IL-23R and that it needs to be induced (Zhou, Ivanov et al. 2007). This has led to the idea that IL-23 cannot be the sole inducer of Th17 differentiation. Cytokines such as IL-6 and IL-21 are implicated in the regulation and induction of IL-17 production. In vitro, IL-6 is a potent inducer of Th17 cells but only when combined with other cytokines including transforming growth factor β1 (TGF-b1). This led to the finding that both cytokines TGF-β1 and IL-6 are needed as the initial driver of Th17 specification (see Figure 1-5). However, $IL\text{-}6^{-/-}$ mice have reduced numbers of Th17 cells (Mangan, Harrington et al. 2006), suggesting that *in vivo* other cytokines can compensate for its actions. One candidate cytokine is IL-21, which is

produced by Th17 and follicular helper T cells (Tfh). IL-21 induces IL-17, itself and can also induce the expression of IL-23R (Veldhoen, Hocking et al. 2006; Korn, Bettelli et al. 2007). However, in disease models that depend on Th17 specification, IL-6 seems to be more relevant than IL-21. Studies looking at cytokine specific KO mice to IL-6 or IL-21 show them to be protective from EAE. Other studies have shown that the development of EAE was not impaired by the absence of IL-21 signals (Coquet, Chakravarti et al. 2008; Sonderegger, Kisielow et al. 2008). Suggesting that both IL-6 and IL-21 can promote Th17 differentiation, but neither appears to be very necessary *in vivo*.

The combination of TGF-β and IL-6 is a very effective for inducing naive T cells to become IL-17 producers. Consistent with this idea is that deleting TGF-β has also been reported to inhibit Th17 differentiation *in vivo*. In addition, transgenic overexpression of TGF-β in T cells was associated with increased susceptibility to the development of EAE in mice injected with MOG peptide (Bettelli, Carrier et al. 2006). It has been reported recently that the downstream signals of TGF-β and Smad2 are important for TGF-β mediated Th17 cell generation (Malhotra, Robertson et al. 2010; Martinez, Zhang et al. 2010). $Smad2^{-/-}$ mice showed less severe disease in an EAE model (Takimoto, Wakabayashi et al. 2010). It has also been shown that $CD4^{+}$ T cells that lack the molecular adaptor tumour necrosis factor receptor associated factor 6 (TRAF6) are more sensitive to TGF-β induced Smad2/3 activation and also exhibit a specific increase in Th17 differentiation (Cejas, Walsh et al. 2010).

However, findings have shown it is not that easy to input a role for TGF-β as an inductive factor in Th17 differentiation and it is complicated by the fact that mice lacking this cytokine or its receptor subunits develop a fatal systemic autoimmune disease. So the mechanism by which

TGF-β induces Th17 development still is not clear. An essential role of TGF-β in Th17 differentiation has recently been challenged by Das and Ren et al. They show that in mutant T cells lacking T-bet and STAT6, IL-6 alone was found to be sufficient to generate Th17 cells (Das, Ren et al. 2009). This suggests that TGF-β plays an indirect role in Th17 differentiation by suppressing the expression of T-bet and GATA3, and limiting differentiation to alternate fates.

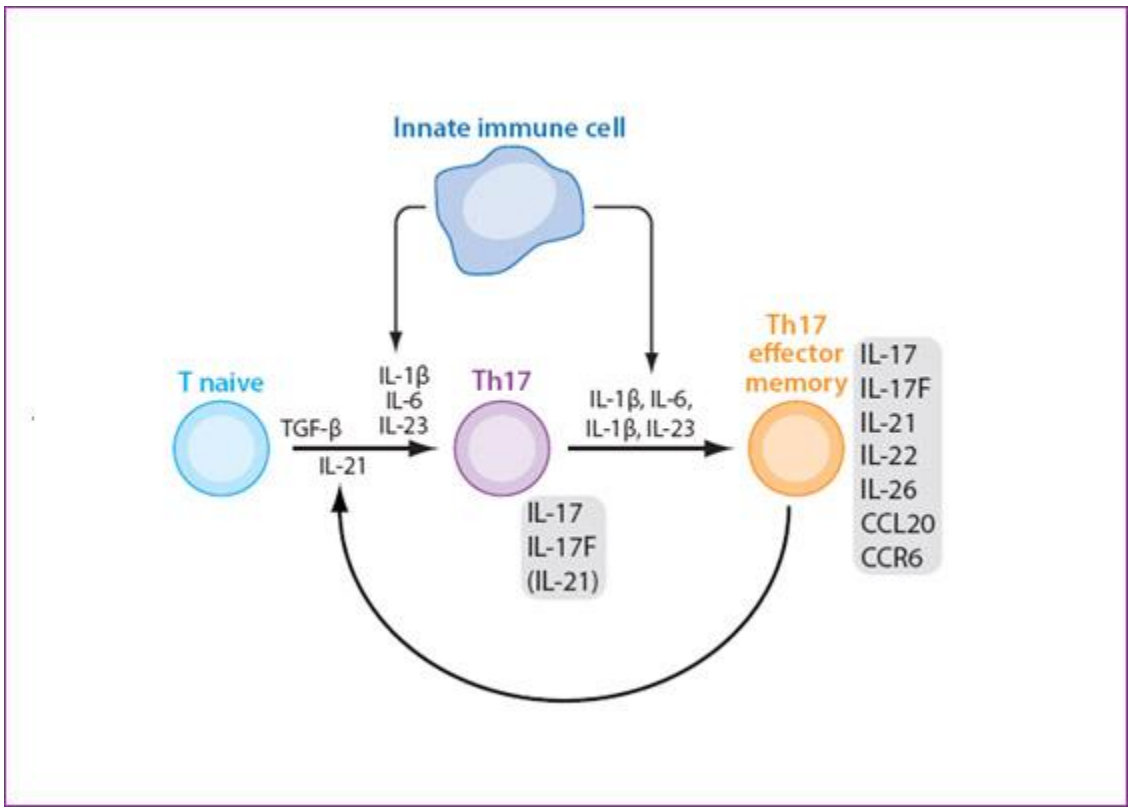


Figure 1-5 Cytokines involved with Th17 differentiation. *(Modified from Korn amd Bettelli 2009)*

Naïve T cells differentiate to Th17 cells in the presence of TGF-β and cytokines from innate immune cells (APCs) which lead to production of pro inflammatory cytokines IL-17A, IL-17F and also IL21 and IL-22.

1.2.3 Th17 and Treg paradigm

In addition to effector T cells there is another subset of novel $CD4^+$ T cells that regulate Th cells termed T regulatory cells (Tregs). They are essential for dominant immunological tolerance. Surprisingly, both Th17 and Treg cells can develop from naive $CD4^+$ T cell precursors under the influence of the same cytokine, transforming growth factor β1 (TGFβ1), (see figure 1-5). $FOXP3^+CD25^+CD4^+$ Treg cells have the ability to suppress the proliferation and function of other immune and non-immune cells including T effector cells (Teff cells), B cells, macrophages and dendritic cells (DCs). So far, at least two types of $FOXP3^+$ Treg cells, including naturally occurring Treg (n Treg) and inducible Treg (iTreg) have been identified.

Thymic-derived nTreg cells are constantly expressing FOXP3, and comprise nearly 10% of total peripheral $CD4^+$ T cells in mice and humans. nTreg cells function as immune suppressors and express high levels of FOXP3, which play a crucial role in the maintenance of self-tolerance and immune homeostasis. Genetic mutations of the forkhead box transcription factor FOXP3 leads to lethal X-linked syndrome "Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked Syndrome" (IPEX) in humans, and the scurfy (sf) phenotype in mice, which is usually accompanied by multiple immunopathologies including type I diabetes, inflammatory bowel disease as well as massive lymphocytic infiltrates.

iTregs are induced in the periphery by immune suppressive cytokines such as TGF-β after TCR stimulation (Chen, Jin et al. 2003). Other iTregs include Tr1 and Th3 cells. Tr1 cells often locate in the intestinal mucosa and so far no specific surface marker for Tr1 cells has been identified; Foxp3 gene expression cannot be constitutively induced in iTregs, and they highly express immune suppressive cytokines including IL-10 and TGF-β, showing that Tr1 cells are a subset of

Treg rather dissimilar from nTreg. Th3 cells are induced primarily from naive $CD4^+$ T cells and have been shown to have regulatory function in oral tolerance (Chen, Kuchroo et al. 1994). Th3 cells highly express TGF-β, and may also express Foxp3, so they cannot be identified as a distinct subpopulation of iTreg or as an activated nTreg cells. Both nTreg and iTreg cells have the ability to suppress the function of T effector cells and in maintaining host immune homeostasis. Recently, a $CD8^+$ regulatory T cell lineage was reported to be essential for self-tolerance. These $CD8^+$ Treg cells target Qa-1^+ follicular T helper cells and suppress lethal systemic-lupus-erythematosus-like autoimmune disease (Kim, Verbinnen et al. 2010).

The differentiation of Th17 and inducible Tregs (iTreg) from naive T cells depends on the level of TGF-β expression. See Figure 1- (Chen, Li et al 2010). A low level of TGF-β, IL23 or IL6 induces naive T cells to develop into Th17 cells (Zhou, Chong et al. 2009). High levels of TGF-β in the tissue microenvironment can lead to the development of iTreg (Zhou, Lopes et al. 2008). Loss of the balance between Th17 and Treg cells in favour of the former will break immune homeostasis in the host and lead to the development of autoimmune diseases. Moreover, $FOXP3^+$ Treg cells are not terminally differentiated cells, and can also redifferentiate into a variety of T effector cells including Th17 cells, Tfh cells, as well as autoaggressive and pathogenic memory T cells *in vivo* after losing FOXP3 expression under certain inflammatory conditions. Researchers have found that a significant number of $CD4^+FOXP3^+$ Treg cells in human peripheral blood and lymphoid tissue which express CCR6 (chemokine (C–C motif) receptor 6) can develop into Th17 cells with the capacity to produce IL-17 on mucosal sites during inflammation (Voo, Wang et al. 2009). Zhou and colleagues also found that a subset of Treg cells that lost Foxp3 expression and suppression function, named exTreg, could produce

IL2 and IFN-γ instead of CD25, GITR, and CTLA-4; consequently, these exTreg cells act as effector T cells rather than the conventional suppressors (Zhou, Bailey-Bucktrout et al. 2009).

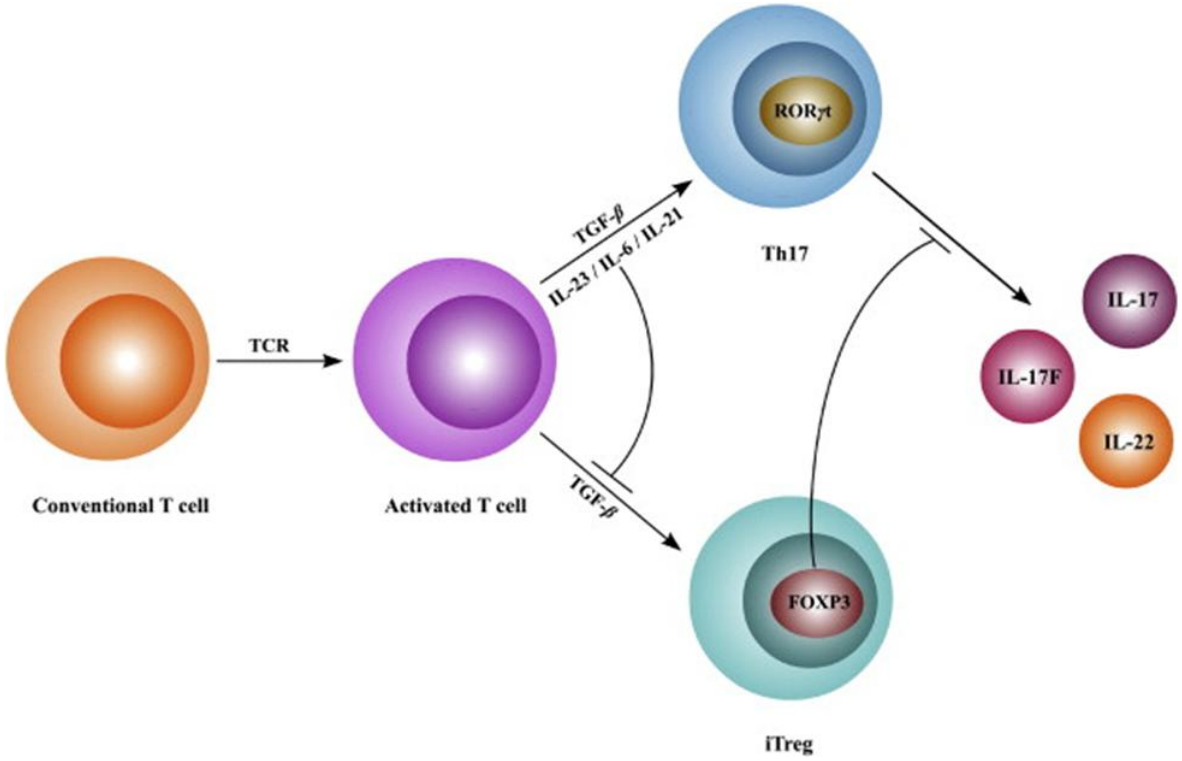


Figure 1-6. TGF-β mediated regulation of the differentiation and function of Th17 and iTreg cells

From Chen, Li et al. 2010. TGF-β orchestrates Th17 and Treg cell differentiation in a content and concentration dependent manner. Low concentration of TGF-β stimulation in cooperation with IL-6 and IL-21 favor the differentiation of Th17, whereas it is favourable towards the differentiation of Treg cells under high concentration of TGF-β that represses IL-23R expression.

1.2.4 IL-1 and Th17

The inflammatory cytokine, IL-1 has a new role with the recognition that it is a major promoter of Th17 development in both mouse and humans, synergizing with IL-6 and IL-23. Studies have shown that $IL1R^{-/-}CD4^{+}$ T cells are severely impaired to differentiate towards Th17 cells *in vivo* and $IL1R^{-/-}$ mice show reduced incidence of EAE (Ben-Sasson, Hu-Li et al. 2009). Also, gamma delta (gd) T cells activated by IL-1b and IL-23 have been found to produce IL-17, IL-21 and IL-22 (Sutton, Lalor et al. 2009). The IL-1R has homology to Toll-like receptors (TLR), and IL-1 engagement of its receptor results in the recruitment of the adapter MyD88 and IL-1 receptor-associated kinases (IRAK). A recent study has indicated that single Ig IL-1R-related molecule (SIGIRR), a negative regulator of IL-1R and TLR signalling, controls Th17 cell differentiation and proliferation. The study also showed that IL-1-induced Th17 cell expansion is dependent on mTOR (Gulen, Kang et al. 2010).

1.2.5 Regulation of IL-17 production by G-protein coupled receptors

Studies have shown that the addition of S1P (abundant lysophospholipid present in blood and lymph) to cultures of splenic $CD4^{+}$ T cells has increased IL-17 and reduced IFN-γ and IL-4 production (Liao, Huang et al. 2007). S1P signals via a G-protein coupled receptor (GPCR) known as S1PR1. S1P regulates T cell trafficking. Transgenic overexpression of an S1P receptor resulted in enhanced IL-17 production. Prostaglandins are another group of lipids that signal via GPCR. During inflammation, prostaglandin E2 (PGE2) is highly induced. Interestingly, culture of human peripheral blood mononuclear cells with PGE2 and IL-23 resulted in high levels of production of IL-17 and chemokine ligand CCL20, with less IFN-γ and IL-22 (Chizzolini,

Chicheportiche et al. 2008). PGE2 acts through prostaglandins EP2- and EP4-mediated signalling and cyclic AMP pathways to upregulate IL-23Rand IL-1R expression resulting in synergistic induction of Th17 cells with IL-23 and IL-1b (Boniface, Bak-Jensen et al. 2009). *In vivo* administration of PGE2 induced IL-23-dependent IL-17 production and exacerbated collagen-induced arthritis (CIA) in mice (Sheibanie, Khayrullina et al. 2007).

1.2.6 Regulation of Th17 differentiation by transcription factors

1.2.6.1 STAT3 in Th17 differentiation

Cytokines exert their effects through signal tranducers and activators of transcription (STATs).The major cytokines that induce IL-17, IL-23, IL-6 and IL-21 all activate STAT3. The critical role of STAT3 was established by the finding that deletion of STAT3 in T cells abrogated Th17 differentiation (Yang, Panopoulos et al. 2007). It has been found that retroviral overexpression of constitutively active STAT3 was sufficient to induce IL-17 production (Mathur, Chang et al. 2007). Importantly, conditional deficiency of STAT3 in $CD4^+$ T cells impairs IL-17 production *in vivo* and limits IL-17-associated pathology. Patients with Hyper IgE syndrome (HIES) (a primary immunodeficiency disorder, due to dominant negative mutations of STAT3) had severely impaired ability to produce Th17 cells (Holland, DeLeo et al. 2007; Renner, Rylaarsdam et al. 2008). This shows that there is a requirement for STAT3 in human IL-17 production. The impairment of STAT3 signalling and consequently attenuated Th17 generation may explain the patient's inability to clear bacterial and fungal infections.

STAT3 was initially found to directly bind to the IL-17 promoter by using chromatin immunoprecipitation (ChIP) assays. However, new technology, mostly ChIP and massive

parallel sequencing (ChIP-seq) provides the ability to define the function of transcription factors on a genome-wide scale (Park 2009).

The functional relevance of the binding can be found by genome-wide transcriptional profiling and by assessing the transcription factor-dependent changes in epigenetic modifications. This has been done for STAT3 in Th17 cells and it was found that STAT3 binds many promoters and enhancers of genes involved in Th17 development. STAT3 binds throughout the IL-17A and IL-17F locus. Interestingly, its binding is not restricted just within the promoter regions, STAT3 also binds the intergenic region (see figure 1-7). STAT3 binds and regulates IL21, IL21r and IL23r genes. Importantly, STAT3 directly controls expression of many of the other transcription factors that participate in Th17 differentiation including RORγt, IRF4, and Batf. Interestingly, STAT3 has also shown to play a role in proliferation and survival of Th17 cells (Durant, Watford et al. 2010) and is important for both the expression of IL-23R and the downstream signalling of this cytokine receptor complex.

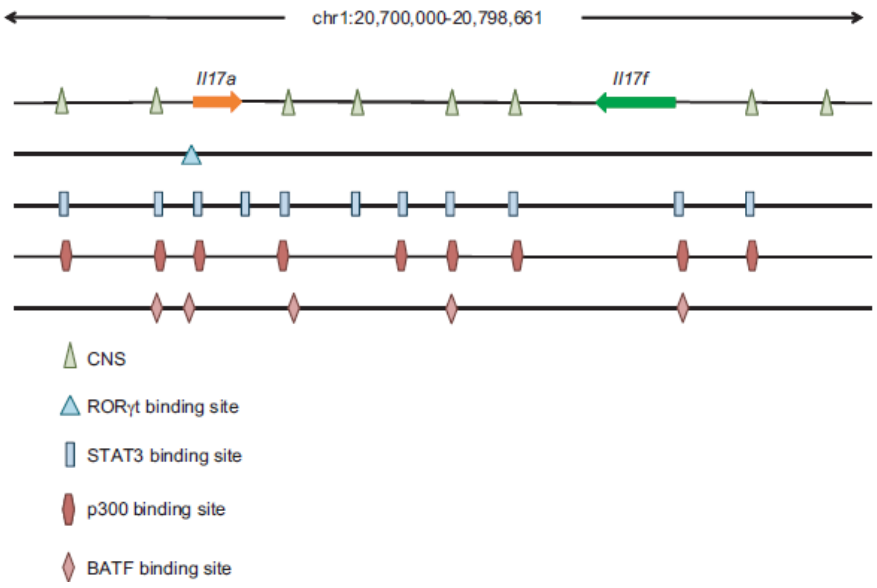


Figure 1-7. STAT3, BATF and RORγt binding sites in the IL17A/IL17F locus

From K. Hirahara et al, 2010. STAT3 have been found to bind strongly with multiple conserved non-coding sequences located in the intergenic region between IL17A and IL17F. Binding sites of BATF and RORγt have been reported by others. The significance of those individual binding sites is of interest for further investigations.

1.2.6.2 Retinoid receptors and Th17 cells

7Retinoid receptors are a family of nuclear receptors and are part of a larger family of steroid nuclear receptors that have direct transcription factor properties. Retinoid receptors can be divided into three families: classical retinoic acid receptors (RARα–γ), retinoid X receptors (RXRα–γ) and the retinoic acid orphan receptors (RORα–γ). RORγt is a splice variant of the Rorc gene that results from initiation by a distinct promoter within the full length of the Rorc gene. This results in products that differ in their amino terminus (Jetten 2009). Whereas RORγ is ubiquitously expressed, a transcript known as RORγt is exclusively expressed in lymphoid cells. RORγt is critical for T cell development and lymphoid organogenesis. This is evident by the fact

that Rorc$^{-/-}$mice exhibit reduced numbers of CD4^{+} single-positive and double positive thymocytes. Rorc$^{-/-}$ mice also lack lymphnodes, lymphoid tissue inducer cells and Peyer's patches. Rorc$^{-/-}$mice also have shown to have reduced Th17 differentiation and have reduced disease severity in the EAE model (Ivanov 2006). The opposite has been found with the overexpression of RORγt, promotes IL-17 expression. In this way RORγt acts similarly to transcription factors such as T-bet and GATA3 in Th1 and Th2 differentiation, respectively, and so has been proposed to be a master regulator for Th17 differentiation. A number of multiple RORγt binding sites are present in the IL-17 promoter, and by the use of ChIP, RORγt has been found to bind the IL-17 gene (Ichiyama, Yoshida et al. 2008). STAT3 binds the Rorc gene and studies have shown that STAT3-deficient T cells have poor expression of RORγt. However, overexpression of active STAT3 in Rorc$^{-/-}$cells resulted in poor IL-17 induction (Ivanov 2006), arguing that STAT3 is necessary but not sufficient for IL-17 expression. Rather, STAT3 and RORγt appear to act cooperatively on the IL-17 locus to drive production of this cytokine (Figure 1-7). RORα, is another related retinoic acid nuclear receptor, and is preferentially expressed in Th17 cells. In contrast to RORγt, deletion of RORα had little effect on IL-17 production. However, deficiency of both RORα and Rorc completely abolished IL-17 production and protected against EAE (Yang, Pappu et al. 2008). Interestingly co-expression of the RORα and Rorc enhanced the number of IL-17-producing cells.

1.2.6.3 IRF4 and Th17 differentiation

IRF4 is a member of the interferon regulatory factor (IRF) family of transcription factors and was originally thought to be a key inducer of GATA3 expression in Th2 differentiation. While, studies have shown IRF4-deficient T cells to impair IL-17 production in response to TGF-β and

IL-6. Recently, it has been shown that phosphorylation of IRF4 regulates IL-17 and IL-21 production (Biswas, Gupta et al. 2010). Also, IRF4-deficient mice are resistant to EAE. It has also been seen that IRF4-deficientT cells fail to upregulate RORγt expression and differentiate into Th17 cells. Overexpression of RORγt in such T cells could not restore IL-17 production. It has been suggested IRF4 might co-operate with STAT3 to induce RORγt expression, but IRF4 also interacts with NFATc1 and c2 (Hu, Jang et al. 2002). So in this way, it can affect other Th cell lineages. It is a rare example of a factor required for both Th17 and Th2 development, which is in contrast with the previously accepted links with Th17 and Th1 development via IL-23 (Harrington, Hatton et al. 2005) and Th17 and Treg development via TGF-β (Bettelli, Carrier et al. 2006).

1.2.6.4 Batf and Th17 differentiation

Basic leucine zipper transcription factor, ATF-like (Batf) is a member of the AP-1 transcription factor family. It contains a basic region and leucine zipper, lacks a transcriptional activation domain and so has been considered as an inhibitor of AP-1 transcriptional activity. Recently, it has been shown that Batf is essential for Th17 cell differentiation (Schraml, Hildner et al. 2009). Mice lacking Batf fail to induce RORγt, and fail to express Th17 specific cytokines. Batf not only controls Th17 development through regulating RORγt expression, but also directly controls Th17 specific gene targets, since reconstitution of *Batf* $^{-/-}$ T cells with RORγt fails to restore IL-17 expression. Observations consistent with this show that Batf directly binds to regulatory regions surrounding the IL-17 gene locus and the mechanism of gene regulation by Batf appears to arise from the formation of a heterodimer with Jun proteins that exerts transcriptionally unique actions on genes involved in the Th17 development. Batf binds to the promoters of IL-17, IL-

17F, IL-21 and IL-22 (figure 1-8) and the intergenic regions between IL-17 and IL-17f (figure 1-7). Studies by Schraml and his group have shown that immunization of *Batf* $^{-/-}$ mice with MOG peptide fails to induce EAE in contrast to wild-type mice, which is consistent with a requirement for Th17 development in EAE. This defect is due to a T cell intrinsic property of *Batf* $^{-/-}$T cells, since wild-type T cells transferred into *Batf* $^{-/-}$ mice restores the ability to manifest severe EAE after MOG immunization. However, Batf mRNA is not exclusively expressed in Th17 cells and it has been shown to be expressed in Th1 and Th2 cells and in activated B cells, with the development of Th1 and Th2 cells, mature B cells and all dendritic cell subsets being normal in *Batf* $^{-/-}$ mice. More recently, work by Murphy and his group has shown that Batf–Jun family protein complexes cooperate with IRF4 in binding to AP1–IRF composite elements (AICEs) in pre-activated $CD4^+$ T cells stimulated with IL-21 and in Th17 differentiated cells. Importantly, Batf binding was diminished in *IRF4*$^{-/-}$ T cells and IRF4 binding was diminished in *Batf*$^{-/-}$ T cells, consistent with functional cooperation between these factors. It was also shown that AP1 and IRF complexes cooperatively promote transcription of the IL-10 gene, which is expressed in Th17 cells and regulated by IL-21. These findings reveal that IRF4 can signal via AP1 motifs and so indicating new approaches for modulating IRF4-dependent transcription (Murphy, Tussiwand et al. 2013).

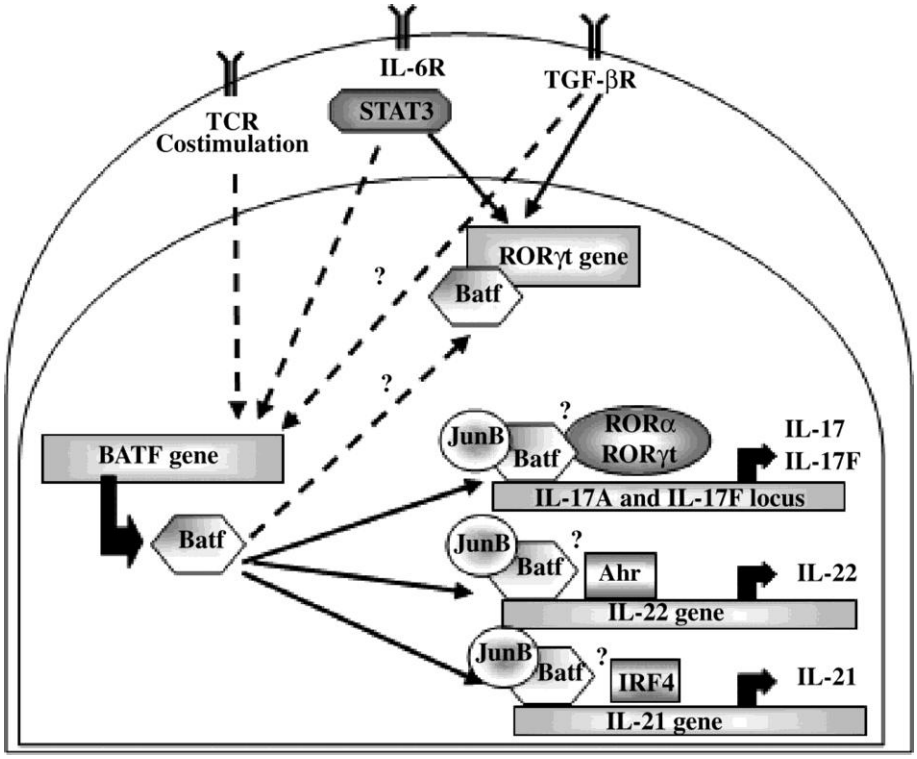


Figure 1-8 Batf regulates Th17 cell generation *(Martinez and Dong 2009).*

Batf is selectively required for Th17 cell induction. Whether T cell receptor (TCR) signaling or costimulatory molecules induce Batf expression is still unknown. Batf binds to IL-17A and IL-17F intergenic regions and IL-21 and IL-22 promoter regions, and induce Th17 cytokine production. Batf synergizes with RORγt in the induction of IL-17 expression. However, whether it also synergizes with RORα, Ahr and IRF4 in induction of IL-17, IL-22 and IL-21, respectively, needs to be further investigated. Moreover, Batf seems to be responsible for maintenance of RORγt expression, although the exact mechanism is unkown.

1.2.6.5 Runx transcription factors

Another crucial family of proteins that regulate $CD4^+$ T helper cell differentiation are Runx transcription factors. There are three mammalian Runx domain transcription factors: Runx1, Runx2, and Runx3. Runx1 is important for normal haematopoiesis, which includes thymic T cell development (Collins, Littman et al. 2009). Additionally, both Runx1 and Runx3 are reported to act as repressors of CD4 and Zbtb7b (the latter being encodes Th-POK, a transcription factor that

regulates $CD4^+$ differentiation), but at different stages of thymocyte development to affect the CD4/CD8 lineage choice (Setoguchi, Tachibana et al. 2008; Collins, Littman et al. 2009). In $CD8^+$ T cells Runx3 functions as a CD4 silencer and is involved in a feed-forward regulatory circuit in which T-bet induces Runx3 and then joins with Runx3 to direct activation of IFN-γ and silencing of IL-4 (Djuretic, Levanon et al. 2007). In Treg cells, Runx1 binds the N-terminus of Foxp3. This interaction is required for Foxp3 mediated suppression of IL-2 production and suppression activity of Treg cells. Recent work has shown that Runx1 also plays a role in Th17 differentiation (Zhang, Meng et al. 2008). Both Runx1 and RORγt bind to the IL-17 promoters and, by interaction with distinct functional partners (RORγt versus FOXP3), Runx1 may contribute to both Treg and Th17 differentiation.

1.2.6.6 Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AHR) is highly conserved in evolution and belongs to a bHLH-PAS family of transcription factors. AHR senses a wide range of small synthetic compounds and natural ligands. Importantly, environmental toxins such 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) bind AHR. Upon ligand binding in the cytosol, AHR undergoes conformational change, translocates to the nucleus and dissociates with its chaperone Hsp90 and p23. Th17 cells preferentially express AHR and tryptophan-derivedphotoproduct 6-formylindolo [3,2-b] carbazole (FICZ), promote expression of IL-17, IL-17F and IL-22. It has been shown that Th17 cells and also gd T cells express AHR (Martin, Hirota et al. 2009). Mice lacking AHR have reduced severity in EAE; however, AHR is important for IL-22 but not IL-17 production (Veldhoen, Hirota et al. 2008). TCDD is very immunosuppressive and activation of AHR by TCDD expands $CD25^+$ $FOXP3^+$Treg cells, which suppress EAE (Quintana, Basso et al. 2008).

Recent work has indicated that activation of AHR also induces $FOXP3^+$ Treg cells in humans (Gandhi, Kumar et al. 2010). How different AHR agonists seem to have distinct effects is puzzling; however, AHR expression in Th17 cells provides a link as to how environmental toxins might contribute to the pathogenesis of autoimmune diseases.

1.2.6.7 Nuclear factor of activated T cells and NF-kB family

As a result of TCR ligation there are elevated levels of intracellular $Ca2^+$, which leads to the dephosphorylation and activation of members of the nuclear factor of activated T cells (NFAT) transcription factor family. The proximal promoter of the human and mouse IL-17 genes contain multiple NFAT binding sites. Recently, it has been shown that $CD4^+$ T cells expressing hyperactivable NFAT1 produce more IL-17 (Ghosh, Koralov et al. 2010). Also, proteins that interfere with the DNA binding of NFAT can regulate IL-17 expression. It has been demonstrated that NR2F6, a nuclear zinc-finger orphan receptor, acts as a transcriptional repressor of Th17 cells, similar to zinc-finger proteins that repress GATA3 and Th2 cells (Hermann-Kleiter, Gruber et al. 2008). IL-1, a major promoter of Th17 development, activates NF-kB. A recent study has shown that the NF-kB family member IkBz regulates Th17 development by cooperating with ROR nuclear receptors (Okamoto, Iwai et al. 2010). Another member of the NF-kB family, c-Rel is also shown to play a critical role in the differentiation of Th17 cells (Chen, Hardy et al. 2010).

1.2.6.8 Negative regulation of Th17 differentiation

In addition to positive roles in regulating Th17 differentiation, cytokines that bind cytokine receptors can negatively regulate Th17 differentiation. STATs are not necessarily differentially

expressed among subsets. Cytokine receptor expression can be down modulated, but the action of STATs can be redundant. Consistent with work indicating that IFN-γ and IL-4 also antagonize Th2 and Th1 differentiation, respectively, IFN-γ and IL-4 also inhibit IL-17 production. IL-27 is an IL-12-related cytokine that consists of two subunits, p28 and EBI3 (Pflanz, Timans et al. 2002). Like the IL-6R, the IL-27R complex contains gp130, but in addition it also has a ligand-specific subunit named WSX-1. Even though there are similarities to IL-6, IL-27 has the opposite effect in regulating IL-17 expression; it is a critical negative regulator of Th17 differentiation (Batten, Li et al. 2006). Like IFN-γ, IL-27 activates STAT1 and the inhibitory effect of IL-27 on Th17 differentiation is abrogated in STAT1-deficient mice. Activated by IL-12, STAT4 is well known as a critical positive regulator of Th1 differentiation and IFN-γ production. As both IL-12 and IFN-γ suppress IL-17 production, it would be expected that STAT4 would negatively regulate IL- 17 expression. However, two studies have reported that IL-17 production is decreased in STAT4-deficient T cells, suggesting a positive role of STAT4 for IL-17 production (Hildner, Schirmacher et al. 2007; Mathur, Chang et al. 2007). The decreased IL-17 production in STAT4-deficient T cells might be related to the impaired IL-23 signalling, as IL-23 has been shown to activate STAT4 (Watford, Hissong et al. 2004). IL-2 is a well-known T cell growth factor *in vitro*, but at the same time the deficiency of IL-2 results in severe multi-organ autoimmune disease *in vivo* (Malek 2008). This is in part due to its role in promoting the differentiation of Tregs by STAT5, but recent work has shown that IL-2 also suppresses Th17 differentiation in a STAT5-dependent manner. Like $IL2^{-/-}$mice, STAT5-deficient mice suffer from inflammatory autoimmune disease that is associated with a loss of Treg cells and the simultaneous expansion of Th17 cells. STAT5a/b appear to be essential for FOXP3 expression and in constraining Th17 cells (Laurence, Tato et al. 2007; Yao, Kanno et al.

2007). However, the mechanisms underlying the blockade of Th17 differentiation by IL-2 via STAT5 are not clear. One possibility could be that it is mediated indirectly via induction of FOXP3, a factor known to bind and inhibit RORγt. Alternatively, STAT5a/b might act as a direct repressor to inhibit IL-17a expression. Negative regulation of Th17 by nuclear receptors is in contrast to RORγt and RORγ, other retinoic acid nuclear receptors have been suggested to inhibit Th17 differentiation. Several groups have shown that retinoic acid inhibits Th1, Th2 and Th17 differentiation in vitro. Retinoic acid down regulates expression of RORγt and enhances the expression of FOXP3 (Mucida, Park et al. 2007; Elias, Laurence et al. 2008)., and an RAR antagonist inhibited FOXP33 expression. FOXP3 binds to the Il17 promoter and so FOXP3 and RORγt, directly interact and modify each other's function.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of transcription factors. There have been a number of natural and synthetic PPARγ ligands have been described to exert anti-inflammatory effects on various levels. Upon ligand binding, PPARγ translocates into the nucleus and forms a heterodimer with the retinoid X receptor and then binds to PPARγ response elements located in the promoter region of target genes. Recently the relevance of PPARγ in Th17 differentiation has been demonstrated with the use of the synthetic ligand, pioglitazone (PIO) (Klotz, Burgdorf et al. 2009). Th17 differentiation and expression of Th17-assocated genes (Il23r, Il21, Il17 and Il17f) were dramatically inhibited by treatment of $CD4^+$ T cells with PIO, but this had no effect on Th1 differentiation. PIO ameliorated EAE clinical scores and mice had fewer IL-17 producing cells in the CNS. Consistent with this, the mice lacking PPARγ in $CD4^+$ T cells showed earlier onset and aggravated disease in the initial T cell dependent phase of EAE. However, there was no difference in the later effector phase of disease.

1.2.7 Socs3

Suppressor of cytokine signalling (Socs3) is a cytokine-inducible negative regulator of STAT3; Socs proteins are among the most prominent Stat-regulated genes providing a negative feedback to STAT signalling. It was first shown that T cell-specific deletion of Socs3 is associated with enhanced STAT3 phosphorylation and increased IL-17 levels (Chen, Laurence et al. 2006). Deletion of Socs3 in endothelial and hematopoietic cell compartment is associated with widespread autoimmune disease and also increased susceptibility to both CIA and EAE (Wong, Egan et al. 2006).

1.2.8 MicroRNA and negative regulation of IL-17

MicroRNAs (miRNAs) are a large family of post transcriptional regulators of gene expression that are approximately 21 nucleotides in length. Dicer is an RNaselll-like enzyme that is required for generating miRNAs. Specific deletion of Dicer in T cells resulted in impaired T cell development and aberrant IFN-γ production (Muljo, Ansel et al. 2005). Ets-1 is a transcription factor that associates with T-bet and binds the IFN-γ promoter. Ets-1 deficiency results in enhanced Th17 differentiation, along with increased levels of mRNA for IL-22 and IL-23R. Ets-1's actions are likely to be indirect, as no binding of Ets-1 to the promoter of IL17 was seen. Studies have shown that Ets-1 appears to negatively regulate Th17 differentiation through effects on IL-2. Specifically, Ets-1-deficient T cells were found to secrete less IL-2 and have impaired responsiveness to this cytokine. Ets-1 has been found to be a target of microRNA mir-326 and mir-326 has been reported to be upregulated in patients with multiple sclerosis and in murine

Th17 cells generated in vitro (Du, Liu et al. 2009). Overexpression of mir-326 enhances Th17 generation and disease severity in EAE whereas blocking mir-326 reduces Th17 generation and ameliorates disease severity of the EAE. This has been explained by regulating expression of Ets-1. Recently, miRNAs, mRNA, and ChIP-seq were performed to characterize the microRNome during lymphopoiesis within the context of the transcriptome and epigenome (Kuchen, Resch et al. 2010). The authors showed that H3K27me3 inhibited expression of induced miRNAs during lymphopoiesis. These studies reveal some of the epigenetic, transcriptional, and post transcriptional strategies that help orchestrate cellular abundance of miRNAs during lymphopoiesis.

1.2.9 Relationship of Th17 cells to other subsets: influence of epigenetic modification

Cellular differentiation is associated with heritable changes in chromatin in daughter cells that preserve gene expression. Epigenetic modifications such as DNA methylation, chromatin remodelling, histone modifications and incorporation of histone variants can all contribute to regulate gene expression. Cytokine genes in Th cell subsets are similarly regulated. The IL-17 gene is linked to the IL-17F gene on chromosome 1 (human histone marks are STAT3-dependent). Some of STAT3 binding sites are shared with p300 binding. Although the classic model of Th1 and Th2 differentiation suggests that these subsets behave like terminally differentiated cells, recent findings indicate more flexibility than first noted and recent findings provide mechanisms for flexibility in expression of key transcription factors. While the histone methylation at the proximal promoters of cytokines genes showed reciprocal permissive

(H3K4me3) and repressive (H3K27me3) marks in cell subsets that express the signature cytokines, the histone methylation patterns of the key transcriptional factors for lineage specification exhibit bivalent modifications of those genes are not expressed. For example, the promoters of Tbx21 and Gata3 are modified by both H3K4me3 and H3K27me3 in Th17 cells, suggesting that these genes are set up for expression. This is consistent with the reported transition of Th2 to produce IFN-γ following viral infection (Ghoreschi, Laurence et al. 2010).

1.2.10 Th17 cells in health and disease

The Th17 subset exhibits effector T cell function distinct from Th1 and Th2 cells and has been identified as an important mediator of autoimmune disorders (Steinman 2007). Th17 cells were characterized due to producing the cytokine IL-17. The IL-17 cytokine family contains six members, which include IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (i.e. IL-25) and IL-17F. IL-17 cytokines promote tissue inflammation via the induction of other pro-inflammatory cytokines and chemokines (Korn, Bettelli et al. 2009). Increased IL-17 levels have been detected in multiple autoimmune models in animals, as well as in human patients with various autoimmune disease syndromes, such as systemic lupus erythematosus, multiple sclerosis, inflammatory colitis, rheumatoid arthritis and psoriasis. In rheumatoid arthritis, Th17 cells may induce matrix metalloproteases and stimulate osteoclasts, which leads to the development of cartilage and bone destruction. IL-17 deficient mice or mice treated with an IL-17 receptor antagonist are resistant to the development of arthritis. Similarly, IL-17 deficient mice display delayed onset and reduced severity of experimental autoimmune encephalomyelitis (EAE). Evidence also showed that Th17 cells were involved in the pathogenesis of colitis and are capable of inducing EAE

when adoptively transferred into naive wild-type mice. In chronic inflammatory bowel disease, Th17 cells seem to be essential for inducing the breakdown of intestinal epithelial barriers. Once induced, Th17 cells drive immunopathology and tissue inflammation *in vivo*. These data all support the notion that IL-17-producing Th17 cells play an important role in the induction and propagation of autoimmune diseases.

The IL-23/Th17 axis has a strong impact on the pathogenicity of autoimmune diseases. IL-23 was shown to promote the production of IL-17 by activated T cells and IL-23 expanded T cells are able to transfer EAE and CIA (Langrish, Chen et al. 2005). Th17 cells cannot maintain their immune features without IL-23 *in vivo* and *in vitro*. IL-23 may not be involved in the initial steps of driving the differentiation of naive T cells into Th17 cells. Similar to IL-12 for Th1 cells, IL-23 plays a fundamental role in stabilizing the Th17 lineage and expanding Th17 responses. IL-23 also induces proinflammatory effector cytokines in Th17 cells. Therapeutic neutralization of IL-23 can prevent EAE relapses and decrease the expression level of IL-17 in the central nervous system (Gran, Zhang et al. 2002). Thus, IL-23 is essential for full differentiation and exhibiting of effector function by Th17 cells.

Th17 cells are now identified as potent inducers of autoimmunity, while Th1 cells have been well studied in induction and development of organ-specific autoimmune diseases. Th1 cells commonly found present along with Th17 cells in inflamed tissues, but the interaction between Th1 and Th17 cells during autoimmune disease is still unclear. Recent work indicated that Th17 cells might cross-regulate Th1 development in intestinal inflammation, in which IL-17 cytokine could mediate a protective effect (O'Connor, Kamanaka et al. 2009). These researchers demonstrate that IL-17 could suppress the expression of T-bet, osteopontin as well as the IL-

12β2 receptor to inhibit the development of Th1 cells. The mechanism underlying how IL-17 and Th17 cells cross-regulate other T cell subsets remains unclear.

1.3 Early growth response family

Early growth response (Egr) family comprises four members: Egr-1, Egr-2 (Krox-20), Egr-3 (Pilot) and Egr-4 (NGFI-C). The most closely related members are Egr-2 and Egr-3, followed by Egr-1. Egr-4 is much more distant. Several conserved sequences have been noted between all members: the zinc fingers and two basic regions flanking the zinc fingers are conserved between Egr-1, Egr-2 and Egr-3, the C-terminal one being also observed in Egr4; the Nab interaction domains present in Egr-1, Egr-2 and Egr-3 (Poirier, Cheval et al. 2008). They encode closely related transcription factors that contain three Cys2-Hys2 zinc fingers shared by the four members and can bind to the same GC-rich consensus DNA binding motif, the Egr response element (ERE). There is high homology between the three zinc finger sequences of the four members of the Egr family (Beckmann and Wilce 1997) suggesting that the proteins can bind to cis-regulatory regions of at least a subset of the same target genes (Chavrier, Vesque et al. 1990; Swirnoff and Milbrandt 1995). However, outside their common DNA-binding domain, the sequences of the different Egr members have less homology, suggesting they can be differentially regulated by selective signalling pathways and may therefore serve different functions. A few conserved domains have been identified and are depicted in figure 1-9. Of particular interest is the interaction domain for the Nab proteins, which is found in Egr-1, Egr-2 and Egr-3. Nab1 and Nab2 have initially been described as repressors of the transcriptional activity of Egr proteins (Russo, Sevetson et al. 1995; Svaren, Sevetson et al. 1996). However,

recent data suggest that they can also act as synergistic factors for Egr-2 function at least (Le, Nagarajan et al. 2005; Desmazieres, Decker et al. 2008).

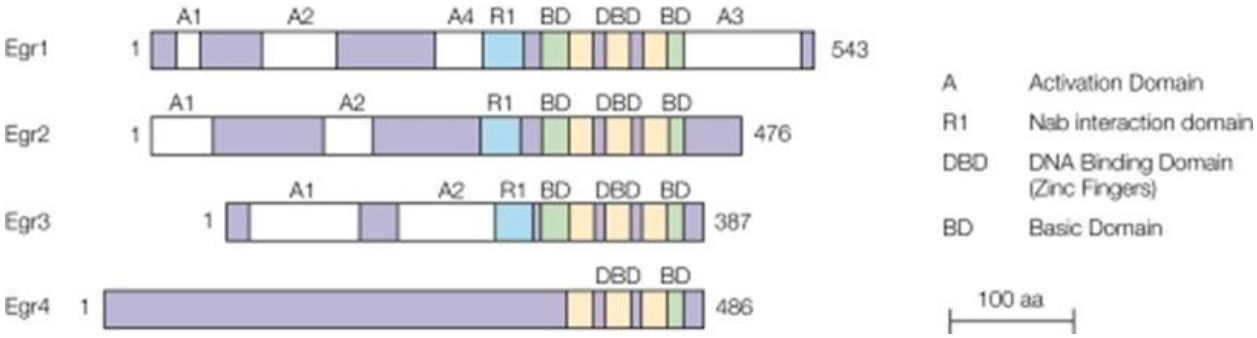


Figure 1-9.Schematic representation of the four members of the Egr family *(Beckmann and Wilce, 1997).*

DNA binding domain sequences showing the conserved regions of the Egr1-4 family domains. There is high homology between Egr-1, Egr-2 and Egr-3.

Egr molecules have mostly been investigated in brain. In brain, Egr family members show, in general, a similar regional profile of basal expression. mRNA and proteins are detected in several areas of the brain with different levels of expression (reviewed inBeckmann and Wilce 1997). In addition to their basal expression, the expression of Egr family members can be induced in different brain areas after a variety of neuronal stimulation paradigms such as the induction of LTP or maximal electroconvulsive shocks (MECS) inducing seizure activity, or in behavioural circumstances after exposure to novel environments of specific learning experiences.

1.3.1 Egr-2 in T cell development

During thymocyte development two control points govern the number and diversity of mature T cells. The first, β-selection, takes place in $CD4^{-}CD8^{-}$double-negative (DN) thymocytes (Michie and Zuniga-Pflucker 2002). The functional rearrangement of the β-chain of the TCR, and its association with the invariant pTα chain to form the preTCR, leads to a proliferative burst and differentiation into $CD4^{+}CD8^{+}$ double-positive (DP) thymocytes. During this transition, the TCR-α chain rearranges and associates with the β chain to form the mature αβTCR. The second control point, is a selection process that operates to ensure that only those cells bearing TCR with appropriate affinity for self-peptide-MHC survive. Most of the immature thymocytes have TCR with no or very low affinity for peptide-MHC, and so die by neglect. Thymocytes expressing TCR with very high affinity for peptide-MHC are deleted via negative selection. Those thymocytes whose TCR have intermediate affinity for peptide-MHC receive survival signals and develop into either $CD4^{+}$ single-positive (CD4SP) helper or $CD8^{+}$ single-positive (CD8SP) cytotoxic T cells. This process is termed positive selection (Starr, Jameson et al. 2003). Positive and negative selection are identified by the activation of distinct signalling pathways downstream of the TCR, with Erk1 and 2 essential for positive selection(Fischer, Katayama et al. 2005)and p38/Jnk and Erk5 mediating negative selection (reviewed in (Sohn, Thompson et al. 2007). Calcineurin signalling is also necessary for positive selection, activating its own downstream signalling cascade, and being required to establish the threshold for Erk activation during the selection process.

The early growth response (Egr) transcription factors Egr-1, Egr-2 and Egr-3 are key players throughout the development of T lymphocytes. All three are induced upon activation of the pre-

TCR (Carleton, Haks et al. 2002; Lefebvre, Haks et al. 2005) and overexpression can force progression through β-selection. Egr-1 and Egr-3 promote survival at β-selection (Carter, Lefebvre et al. 2007), and Egr-3 is also required for the post-β-selection proliferative burst to occur (Xi, Schwartz et al. 2006). These transcription factors are also induced rapidly following ligation of the αβTCR, both during thymocyte selection and in mature T cells responding to antigen-MHC, where Egr-1 has a role in upregulation of IL-2 transcription (Collins, Wolfraim et al. 2006), and Egr-2 and Egr-3 are required for induction of anergy (Harris, Bishop et al. 2004; Safford, Collins et al. 2005) and regulate expression of FasL (Rengarajan, Mittelstadt et al. 2000). Through its control of self-tolerance, Egr-2 has also been implicated in the development of late-onset autoimmune disease (Zhu, Symonds et al. 2008) and is required for the development and maturation of NKT cells (Lazarevic, Zullo et al. 2009).

Upregulation of Egr proteins during positive selection is dependent upon the Ras/MAPK pathway. Egr proteins are direct transcriptional targets of ternary complex factor Sap-1, which is itself a substrate of Erk and essential for positive selection. In addition, Egr-2 and Egr-3 are regulated by calcineurin signalling, likely via NFAT. Both Egr1 and Egr3 have roles in positive selection. Egr-1 overexpression enhances positive selection of cells with low affinity TCR. Conversely, Egr-1 deficient mice have impaired positive selection (Bettini, Xi et al. 2002); although the initial TCR signal is transduced, cells stall at the DP to SP transition, resulting in a numerical decrease in CD4 and CD8 SP. Animals deficient for both Egr-1 and Egr-3 have a similar but more marked selection phenotype, and CD8 differentiation is significantly impaired (Carter, Lefebvre et al. 2007). For both Egr-1 and Egr-3, the principal reason for the alterations in SP cell number is a change in the cell's susceptibility to apoptosis, at least partly through regulation of pro- and anti-apoptotic Bcl2 family members,(Bettini, Xi et al. 2002).

Egr-2 is similarly important in DP thymocytes. Recently, analysis of mice in which Egr-2 was deleted in DN thymocytes has shown that it is not required for negative selection, but that positive selection of both CD4 and CD8 lineages is impaired in the absence of Egr-2. This defect is at least partially due to increased apoptosis as it is rescued by overexpression of the survival factor Bcl-2; however, the mechanism by which Egr-2 might be regulating survival has not been established.

Egr-2 has been found to be important for NKT cell development and positive selection of thymocytes in separate studies (Lauritsen, Kurella et al. 2008; Lazarevic, Zullo et al. 2009; Lawson, Weston et al. 2010). We have now shown that together with Egr-1 and Egr-3, Egr-2 is expressed only in selected stages of T and B cell development and is repressed in ISP thymocytes and pro-B cells. Forced expression of Egr-2 severely impairs the development of DP cells in thymus and immature B cells in bone marrow, respectively. In contrast to the defects in precursor development in Egr-2 conditonal Transgenic mice (Egr-2 cTg) , the percentage of DP cells developed into SP and NKT cells and the percentage of immature B cells into mature B cells were increased, suggesting that Egr-2 has a general role in the regulation of progenitors and terminal maturation of lymphocytes, but at restricted stages.

Expression of Egr-1, -2 and -3 molecules can be induced in DN cells upon pre-TCR stimulation. The involvement of Egr proteins in β-selection is demonstrated by the fact that overexpression of Egr-1 or Egr-3 can overcome the deficiency in β-selection resulting from Rag deficiency and drives the development of cells to the ISP stage. However, the most significant and common phenotype displayed in transgenic models of Egr-1, -2 and -3 is the reduction of the DP population and the increase of ISP cells (Miyazaki 1997), which suggests that Egr molecules are important for the development of DN cells following β-selection and lack of Egr-2 expression

after β-selection is important for the differentiation of ISP cells to DP cells. Although Egr-1 or Egr-2 deficiency does not affect thymocyte development significantly, Egr-3 deficiency results in thymic atrophy due to impaired proliferation after β-selection, but the DN to DP transition is normal, indicating an important function of Egr molecules in supporting expansion and survival of thymocytes after β-selection. The defects in proliferation and survival of thymocytes were much more severe in mice lacking both Egr-1 and Egr-3 and observed in both DN and DP subsets suggesting a survival function of Egr molecules at early and late stages of thymocyte development (Carter, Lefebvre et al. 2007), which is directly supported by our findings that DN cells and percentage of DP differentiated to SP and NKT cells are increased in Egr-2 cTg mice. A response of transient Egr-3 expression to pre-TCR stimulation has been suggested to be important in order to allow later induction of Bcl-XL and RORγt since sustained expression of Egr-3 decreases the expression of Bcl-XL and RORγt (Xi, Schwartz et al. 2006): important factors for the survival of mature thymocytes during selection. Therefore, overlapping with Egr-1 and Egr-2, the regulated expression of Egr-3 in response to pre-TCR stimulation is important for the maturation of DP cells. The increased maturation of DP cells into SP and NKT cells in Egr-2 cTg mice indirectly supports the findings that Egr-2 is important for positive selection and the development of NKT cells.

Our results extend the function of Egr-2 to the development of B cells and indicate a similar mechanism for the modulation of Egr-2 expression and the function of Egr-2 in regulation of B cell progenitors and B cell maturation. The expression of Egr-1 can be induced in pre-, immature- and mature-B cells by stimulation with anti-IgM (Dinkel, Warnatz et al. 1998). However, the expression of Egr in B cell progenitors is unknown. We have now demonstrated that Egr-1, -2 and -3 are expressed in CLP and pre-pro-B cells and importantly, the expression is

repressed in pro-B cells, a similar expression pattern to that seen in DN and ISP thymocytes. In addition to the similar expression pattern, the pattern of defects is also similar between B and T cell development in Egr-2 cTg mice, as demonstrated by a severe reduction of pro-B cells and enhanced terminal B cell maturation, suggesting a common mechanism operated by Egr-2 in both lineages. Despite the severe defect of pro-B cells in Egr-2 cTg mice, the development of B cells in Egr-2 conditional knock out (cKO) mice is normal. This may be due to the weak activity of the CD2 promoter in B cells as shown previously or perhaps due to the redundant function of Egr-1 and Egr-3.

The normal expression of the major molecules involved in cell death and survival and the normal apoptosis of thymocytes in the thymus indicate that the abnormalities resulting from sustained Egr-2 expression are not due to increased apoptosis. Instead the impaired development of DP thymocytes and pro-B cells appears to be due to the blockade of the differentiation of early lineage-restricted cells into fully committed pre-B cells or DP thymocytes. One intriguing possibility is that the expression of Egr molecules may be inherited from hematopoietic stem cells and that Egr molecules may serve to support the early stages of lymphocyte development and are then downregulated before final commitment to a specific lineage.

It has been reported that Egr-1 induces expression of ID3 (Bain, Cravatt et al. 2001). ID3 is one of the ID proteins that modulate function of E2A proteins (Yashiro-Ohtani, He et al. 2009). The E2A transcription factors are essential for the commitment and differentiation of B and T lymphocytes. Deficiency in the E2A pathway results in a defective transition of DN to DP stage as seen in Egr-2 cTg mice (Bain, Cravatt et al. 2001). Although E-box ID pathways may be regulated by Egr-1, the expression of ID3 is unchanged in Egr-2 expressing DN and ISP cells suggesting that Egr-2 does not directly induce ID3 expression.

The involvement of Egr-2 in early development of T and B cells is supported by the impaired expression of lineage specific transcription factors Notch1 in thymocytes and Pax5 in pro-B cells in Egr-2 cTg mice. Activation of the Pax5 and Notch1 pathways is essential for the lineage commitment and maturation of B and T lymphocytes, respectively. Pax5 is exclusively expressed in the B lymphoid lineage from the committed pro-B cells to the mature B cell stage and controls the commitment of lymphoid progenitors to the B cell pathway. Notch signalling is important during the early development of thymocytes but the regulation of Notch expression is not clear (Rothenberg 2007). Notch1 is highly expressed at early stages of DN cells and the expression is reduced after β-selection and downregulated in DP cells (Yashiro-Ohtani, He et al. 2009). Although both Notch1 and Egr molecules are expressed in DN cells, only Notch1 is detected in ISP cells. Interestingly, sustained expression of Egr-2 does not affect Notch1 expression in DN cells, but reduces it in ISP cells, suggesting that regulation of Notch1 by Egr-2 is conditional upon other factors and may depend upon the stage of thymocyte development. Egr-2 has been found to play different roles in macrophage differentiation based on its association with PU (Laslo, Spooner et al. 2006). PU.1 is expressed in the early stages of T and B cell development; whether PU.1 regulates the expression and function of Egr-2 in suppression of the Notch and Pax5 signalling pathways at the early stages of DN or pro-B cell development, respectively, remains to be investigated. The enhanced maturation of T, NKT and B cells in Egr-2 cTg mice could not be explained by the reduced expression of Notch1 in ISP and Pax5 in pro-B cells. The completely opposite functions of Egr-2 induced at the early and late stages of lymphocyte development suggest that the function of Egr-2 may depend on the function of stage-specific transcription factors. The establishment of Egr-1, -2, -3 null and inducible Egr transgenic mice

will be essential to define the redundant and specific roles of these three Egr molecules at the early and late stages of lymphocyte development and to characterize stage-specific function.

1.3.2 Egr2 in regulation of immune tolerance

T cell anergy has been referred to as a tolerant state of T cells and characterized as impaired proliferation and IL-2 production following antigen stimulation. T cell anergy has been observed both *in vitro* and *in vivo*. The induction of anergy has also been observed with antigen stimulation in the absence of costimulatory signals (Anderson, Manzo et al. 2006). In addition to a nearly complete block in IL-2 production and proliferation, anergic T cells show reduced secretion of other cytokines such as interferon-γ (IFN-γ) and IL-3. The anergic state can be long-lived and stable for weeks, but in these model systems it is reversible by the addition of exogenous cytokines such as IL-2 or IL-15 (Beverly et al. 1992; Essery et al. 1988). This reversibility has motivated further investigation of anergy in the settings of cancer and chronic infection, in which restoration of correct T cell function is one of the main therapeutic goals. Thus, the understanding of tolerant mechanisms is important for immune intervention.

On the basis of the *in vitro* anergy model systems, the signal transduction events that drive the induction of anergy rather than full T-cell activation seem to involve the disproportionate over-activation of calcium/nuclear factor of activated T cells (NFAT) signalling compared with other biochemical signalling intermediates, such as the Ras/mitogen-activated protein (MAP) kinase pathway. The addition of calcium ionophores alone—such as ionomycin—is sufficient to induce anergy of T cells. Conversely, induction of anergy with CD3 monoclonal antibodies can be prevented by cyclosporin A, which blocks NFAT-dependent signalling. See figure 1-10 (Chai & Lechler, 1997). Together, these results suggest a model in which excessive

calcium/NFAT signalling results in transcriptional upregulation of negative regulatoryproteins that inhibit correct TCR/CD28 signalling.

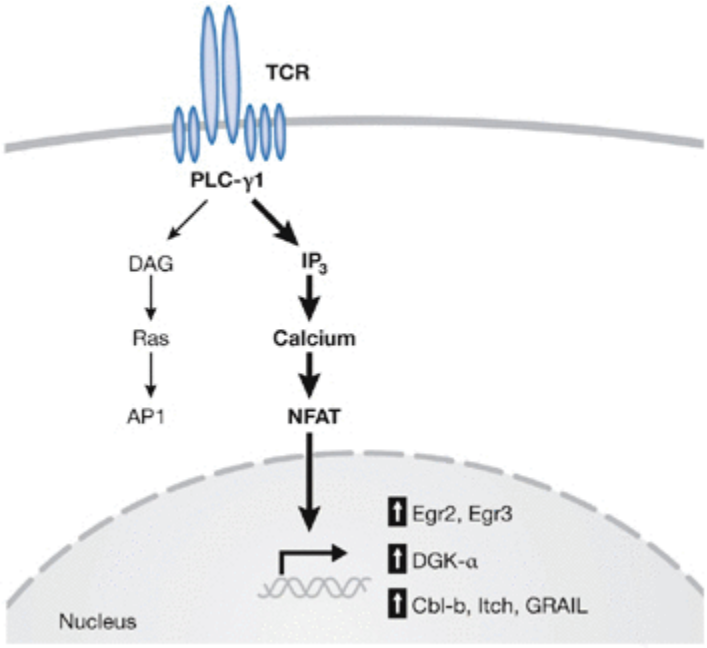


Figure 1-10.Signals leading to the induction of anergy *(Chai and Lechler, 1997).*

Excessive calcium/NFAT signalling leads to upregulated expression of negative regulatory factors that inhibit aspects of TCR/CD28-dependent signalling)

1.3.3 Potential role for Egr family transcription factors in the induction of T cell tolerance

Egr-2 and Egr-3 have been found to be induced in tolerant or anergic T cells by antigen receptor stimulation (Anderson, Manzo et al. 2006). The induction has been found to be dependent on NFAT activation (Hsiao, Liu et al. 2009). Overexpression of Egr-2 and Egr-3 reduced IL-2 production in T cells and has been shown to upregulate Casitas B-cell lymphoma-b (Cbl-b), one of the E3 ligase (Zheng, Zha et al. 2008). Conversely, Egr3-deficient T cells were relatively

resistant to anergy induction in an *in vivo* peptide-induced anergy model (Safford et al. 2005). As discussed below, Cbl-b has also been implicated as a contributor to the anergic state and is upregulated on Egr2/3 overexpressed T cells, perhaps suggesting that Egr proteins are regulating the transcription of several anergy-associated genes.

Consistent with a potential role for E3 ubiquitin ligases in the anergy phenomenon, T cells overexpressing Egr-2 or Egr-3 have a high basal level of Cbl-b. Conversely, no increase in Cbl-b expression was seen on anergy induction in Egr-3-deficient T cells (Safford et al. 2005). However, whether E3 ubiquitin ligases are involved in the induction compared with the maintenance of T-cell anergy is not yet clear, and how they relate to altered Ras activation in the anergic state has not been established.

The molecular alterations in anergic T cells have become clearer as a result of recently published experiments. A current working model suggests that excessive calcium/NFAT-based signalling leads to upregulated expression of Egr-2 and/or Egr-3, which in turn drives the expression of several negative regulators of TCR-based signalling. Upregulated E3 ubiquitin ligases might contribute to disrupted signalling through ubiquitination and either degradation or altered subcellular localization of other signalling intermediates. Further in-depth study of the regulation of T-cell anergy should lead to the development of pharmacological approaches either to promote the anergic state in settings of autoimmunity and transplantation, or to restore T-cell function in the settings of cancer and chronic infection.

1.3.4 Egr-2 regulates T cell homeostasis and controls the development of autoimmune diseases

Recently, we and others have found that Egr-2 is induced in tolerant T cells in response to antigen stimulation *in vivo* or TCR ligation *in vitro* (Harris, Bishop et al. 2004; Safford, Collins et al. 2005; Anderson, Manzo et al. 2006). Together with Egr-1 and Egr-3, Egr-2 is expressed in thymocytes and in mature T cells upon TCR stimulation. RNAi mediated knockdown of Egr-2 in an established T cell line rendered the cells less susceptible to anergy induction (Harris, Bishop et al. 2004), while overexpression of Egr-2 reduced T cell activation in vitro indicating that Egr-2 regulates genes involved in the suppression of T cell activation. However, the function of Egr-2 in T cells *in vivo* under homeostasis condition has not been studied as Egr-2 knockout mice die prenatally due to defects in hindbrain development (Topilko, Schneider-Maunoury et al. 1994).

To assess the function of Egr-2 in T cells *in vivo*, we have established Egr-2 conditional knockouts (Egr-2 cKO), in which the Egr-2 gene was deleted specifically in $CD2^{+}$ lymphocytes. The Egr-2-deficient T cells did not show altered primary activation, but were hyperproliferative in response to prolonged stimulation and exhibited a Th1 and Th17 bias leading to the development of a lupus-like syndrome in older mice. Defective expression of p21cip1 was detected in $CD44^{high}$ T cells from Egr-2 cKO mice and Egr-2 was found to interact directly with the promoter of p21cip1 *in vivo*. In addition, IFN-γ and IL-17 were highly induced in Egr-2 deficient T cells and accumulation of IFN-γ and IL-17-producing cells was associated with massive infiltration of T cells in multiple organs. Our results demonstrate that Egr-2 is important for controlling the self-tolerance of T cells and preventing autoimmunity through activation of negative regulators of cell proliferation and by controlling proinflammatory cytokine expression.

We and others have previously found that Egr-2 is induced in tolerant T cells following TCR stimulation (Harris, Bishop et al. 2004; Anderson, Manzo et al. 2006). We have now found that Egr-2 is normally expressed in $CD44^{high}$ T cells and that Egr-2 deficiency results in a massive accumulation of these cells. $CD44^{high}$ is a marker for effector or effector memory T cells (Surh, Boyman et al. 2006). The $CD44^{high}$ T cells in wild-type mice raised in a clean environment are presumably generated in response to commensal antigens, gut-flora or self-antigen. In contrast to the resting $CD44^{low}$ naive T cells, $CD44^{high}$ cells undergo slow and intermittent proliferation in response to homeostatic stimuli such as self-antigen and cytokines (Curtsinger, Lins et al. 1998), and also it has been found that $CD44^{high}$ memory T cells are intrinsically more sensitive than $CD44^{low}$ naive cells to stimulation through the TCR/CD8 complex.The expression of Egr-2 in $CD44^{high}$ cells may be induced from homeostatic responses to serve as a feedback mechanism to control the proliferation and activation of $CD44^{high}$ T cells. The development of systemic autoimmune responses in Egr-2 cKO mice demonstrates the importance of such controls.

Several findings in predisposed mice appear to support the notion that primary or secondary homeostatic lymphocyte perturbations, such as in MRL-Fas^{lpr}, and p21cip1 KO mice of C57BL/6 background, contribute the pathogenesis of spontaneous lupus (Balomenos, Martin-Caballero et al. 2000; Lawson, Baccala et al. 2004). These findings suggest that uncontrolled T cell proliferation can lead to T cell expansion and generation of effector cells to self-antigens resulting in the development of lupus-like systemic autoimmune diseases. Our results indicate that Egr-2 is an intrinsic regulator that not only controls proliferation, but also inflammation of effector T cells and inhibits the development of lupus-like autoimmune diseases. The accumulation of IFN-γ and IL-17 producing $CD4^+$ T cells in Egr-2 cKO mice suggests the possibility that the activated $CD4^+$ T cells are effector inflammatory T cells primed by self-

antigen and that the loss of tolerance in Egr-2 cKO mice leads to the expansion of these cells and autoimmune responses.

We found that the cell cycle inhibitor p21cip1 is a direct target gene of Egr-2. Egr-2 deficiency results in defective expression of p21cip1 in $CD44^{high}$ T cells. Most of the reports from $p21cip1^{-/-}$ mice have shown enhanced T cell activation, proliferation and autoimmune manifestations which are largely consistent with Egr-2 cKO mice. However, a study in the BXSB atypical lupus model showed that p21cip1 deletion increased apoptosis of activated T cells and decreased disease incidence in male mice suggesting that p21cip1 regulates proliferative responses by blocking apoptosis. However, the increased apoptosis in response to TCR stimulation suggests a differential effect of p21cip1 in the activation and proliferative responses of T cells. In a recent study in C57BL/6 mice, p21cip1 deletion only enhanced T cell proliferation at a late stage of stimulation consistent with our findings in Egr-2 cKO mice of C57BL/6 background. Taken together, these studies indicate that p21cip1 is involved in the regulation of effector T cell proliferation, but plays only a part of the tolerance program and its function in lupus development is dependent on the genetic pre-disposition. Indeed, in addition to the altered expression of p21cip1, enhanced differentiation of Th1 and Th17 cells in Egr-2 cKO mice was found. Although no studies have shown yet how Egr-2 regulates the expression of proinflammatory cytokines, the accumulation of Th1 and Th17 cells can directly result in infiltration of T cells into multiple organs and inflammatory autoimmune diseases (Porter and Clipstone 2002). These results have not been reported in p21cip1-deficient mice (Balomenos, Martin-Caballero et al. 2000).

The increased expression of IFN-γ and IL-17 in Egr-2 deficient T cells following TCR ligation suggests that Egr-2 may be involved in the control of inflammatory cytokines in effector T cells. Such control may not only be important to prevent inflammatory reactions of autoreactive T cells but also for the reduction of immunopathology during a productive immune response. Although both Th1 and Th17 are observed in most autoimmune models, the mechanisms mediating Th1 and Th17 differentiation *in vitro* are distinct (Porter and Clipstone 2002), while the *in vivo* mechanisms are still unknown. NFAT has been found to induce IFN-γ expression and activate the IL-17 promoter (Liu, Lin et al. 2004). Egr-2 is one of the NFAT target genes in T cells. Therefore, the expression of Egr-2 could act as negative feedback loop to control IFN-γ and IL-17 expression in response to TCR stimulation. Although we did not find altered activation of NFAT in Egr-2 deficient T cells from aged mice (data not shown), the possibility that Egr-2 physically interacts with NFAT and inhibits its activity has yet to be analysed. The reduced production of Th2 cytokines could either result from enhanced Th1 and Th17 differentiation or direct regulation by Egr-2; this remains for future investigation.

Both Egr-2 and Egr-3 are expressed in double negative thymocytes (DN) and defective expression of Egr-3 leads to the accumulation of DN and reduction of DP thymocytes in the thymus (Carter, Lefebvre et al. 2007), indicating that Egr-3 is involved in thymocyte development before thymocyte selection. However, we did not find a similar phenotype in the thymus of Egr-2 cKO mice suggesting that either Egr-2 function at this stage is redundant or that Cre mediated deletion was incomplete at this stage. T cell lines over-expressing Egr-2 or Egr-3 show an upregulation of Cbl-b and reduced production of IL-2 (Harris, Bishop et al. 2004; Safford, Collins et al. 2005). This finding led to the hypothesis that the mechanism for Egr-2 in maintaining T cell tolerance would be the downregulation of TCR signalling. However, we could

not detect differences between Egr-2-deficient and wild-type naive T cells in the major TCR signalling pathways, such as AP1, NFκB, NFAT and MAP kinase following TCR stimulation *in vitro* (data not shown). In addition, we did not observe hyperproliferation of naive Egr-2 cKO T cells in response to primary TCR stimulation. This normal response to TCR engagement could be due to functional compensation by Egr-3 (Safford, Collins et al. 2005). Nevertheless, the continuously proliferating T cell lines and pre-activated primary T cells used in these studies share some features of the hyperactive $CD44^{high}$ T cells in Egr-2 cKO mice. Therefore, the negative regulation of T cell activation in these reports is consistent with the hyperproliferation and activation of Egr-2-deficient T cells *in vivo*.

Egr-2 has been found to induce FasL expression in T cells (Mittelstadt and Ashwell 1999). Deficiency in Fas expression in MRL/lpr mice results in severe lupus-like disease due to the resistance of T cells to apoptosis (Lawson, Baccala et al. 2004). However, Egr-2 deficiency did not alter expression of FasL in T cells or apoptosis in either young or old mice and $CD3^{+}CD4^{-}CD8^{-}$ T cells were not detected in spleen of old Egr-2 cKO mice , suggesting distinct mechanisms for lupus-like disease in Egr-2 cKO and MRL/lpr mice. Recently, reduced expression of Egr-2 and Egr-3 has been found in T cells from BALB/c mice after induction of lupus like disease by an anti-DNA antibody, and the decreased expression was associated with increased IFN-γ secretion(Kang, Liu et al. 2007), suggesting that Egr molecules are involved in the maintenance of self-tolerance.

Although anti-nuclear antibodies are hallmarks of systemic lupus, the mechanisms responsible for the breakdown of self-tolerance are still unknown. One of the characteristics of the autoimmune disorder in lupus patients is Th1 mediated inflammation with a high level of IFN-γ production and increased serum IgG2a antibody, both of which we have observed in aged Egr-2

cKO mice (Zhu, Symonds et al. 2008). In addition, a massive increase of IL-17 production in activated T cells and an increased percentage of Th17 cells in Egr-2 cKO mice were observed. Th17 plays an important role in the development of lupus-like disease (Pernis 2009). These results suggest that autoimmune disorders can result from a loss of control of effector T cell expansion and inflammatory activation and that this control is mediated by genes regulated by Egr-2.

Peripheral T cells are tolerant to self antigens, which is achieved by limiting the expansion and activation of self-reactive T cells (Schwartz 2003). In addition to the function of regulatory T cells (Germain 2008), a number of intrinsic mechanisms have been discovered which either induce T cell anergy or limit T cell expansion (Lin and Mak 2007), such as the GRAIL E3 ligase which is induced in anergic T cells and inhibits IL-2 expression by targeting TCR signalling molecules. In addition, control of T cell growth and regulation of apoptosis are also important mechanisms to maintain self-tolerance. Egr-2 has been found to regulate expression of an E3 ligase (Safford, Collins et al. 2005) and we have shown that Egr-2 directly activates p21cip1 expression in $CD44^{high}$ T cells and is involved in the regulation of T cell homeostasis leading to the control of autoimmunity (Zhu, Symonds et al. 2008).

AIMS

Although Egr-2 plays an important role in the regulation of immune homeostasis, it function in regulation of effector T cells, especially in the differentiation of Th cells, is still unknown. This study aims to investigate the function of Egr2 in the control of Th17 differentiation and Th17 associated autoimmune diseases.

CHAPTER 2

MATERIALS AND METHODS

This study is supported by Arthritis Research UK

2 MATERIALS AND METHODS

2.0 Experimental Murine Models

For a better understanding of the Egr-2 gene intrinsically, we used mice as experimental models because they are small, prolific and we can manipulate their genetics in ways not possible with humans. The mouse strain we used were the C57BL/6 mice as a genetic background due to the availability of congenic strains, easy breeding, and robustness. All mice used in this study were maintained at Brunel University's Biological Service Unit and used according to established institutional guidelines under the authority of a UK Home Office project licence (Guidance on the Operation of Animals, Scientific Procedures Act 1986).

2.0.1 EGR-2 conditional knockout mice

Previous studies to look at the functions of physiological levels of Egr-2 in T cells *in vivo* have been proven difficult to study as Egr-2 knockout ($Egr2^{-/-}$) mice die postnatally due to defects in hindbrain development (Schneider-Maunoury, Topilko et al. 1993). Our group overcame this by establishing Egr-2 conditional knockouts (Egr-2 cKO) in which the Egr-2 gene was deleted specifically in $CD2^{+}$ lymphocytes using the cre loxP system.

We used the cre-loxP system as a transgenic tool to artificially control the expression of Egr-2 by deleting Egr-2 in $CD2^{+}$ lymphocytes. The human CD2 (hCD2) promoter, which is lymphocyte specific, drives expression of the Cre-recombinase. Therefore, the Egr-2 gene is selectively deleted in CD2 positive cells in Egr-2 cKO mice. This allows for lineage studies of lymphocytes

and allows us to further investigate the contribution of specific effector T cells to immune responses and immunological disorders (see Figure 2-1).

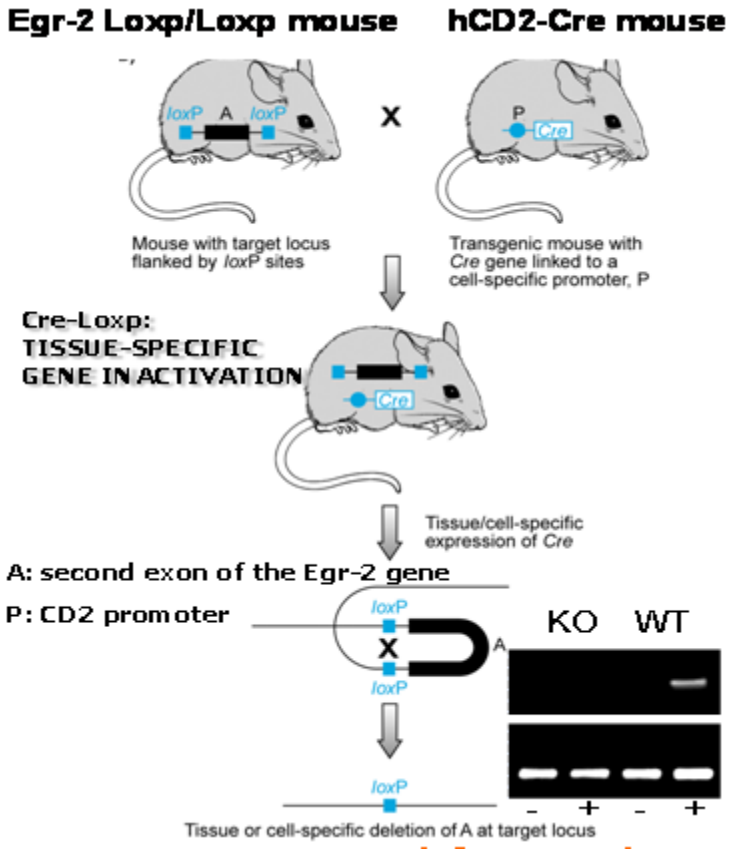


Figure 2-1.Cre-Loxp mouse model

Diagram showing the Egr-2 gene being deleted out and regulated by CD2 promoter of conditional EGR-2 KO mice.

The Egr-2 cKO model was generated by crossing of hCD2-Cre transgenic mice (a gift from Dr D. Kioussis, National Institute for Medical Research, London.) and Egr-2-floxed mice (Egr-2 $^{loxP/loxP}$) a gift from Dr P. Charnay, *Institut national de la santé et de la recherche médicale*, Paris

France. (Taillebourg et al. 2002; de Boer et al. 2003). In these mice, the Egr-2 expression is normal in the tissues in which the Cre is not expressed.

2.0.2 Genotyping

To identify the EGR-2 cKO mice used in this study, the mice were genotyped. Genomic DNA was extracted from mice tails using the REDExtract-N-Amp Tissue PCR Kit (Sigma) by manufacturer's instructions; this work was done at Brunel University by Emma Ghiraffe and Punam Bhullar. The mice tails were cut between 12-14 days of age as the tail tissue is soft and yield of DNA is at its highest.

The genomic DNA was then amplified up by Polymerase Chain Reaction (PCR).This technique allowed selective amplification of the Cre and LoxP locus using sequence specific primers to Cre and Lox-P transgene (see Figure 2-2). The advantage of this method is that it allows for exponential amplification of the variant DNA sequences using Taq (Thermus aquaticus) a thermostable DNA polymerase enzyme that is stable at very high temperatures required during PCR and is not denatured at the same temperature required for DNA denaturing and so allowing for many rounds of extension without adding more enzyme for each cycle. The Taq enzyme extends the primers by incorporating dNTPs complementary to the pre-existing strand and so producing a copy of the DNA.

Table 2-1. Primer sequences and PRC cycle run used for genotyping

Gene of interest	Primer sequence	PCR cycle
hCD2 EGR-2 KO LoxP	***Sense***5'-GGG AGC GAA GCT ACT CGG ATA CGG-3' ***Antisense***5'- GTG TCG CGC GTC AGC ATG CGT- 3'	95˚C for 5mins 94˚C for 40sec 65˚C for 40sec 30 cycles 72˚C for 40sec 72˚C for 5mins
hCD2 EGR-2 KO Cre	***Sense*** 5'-CCA ACA ACT ACC TGT TCT GCC G-3' ***Anti sense*** 5'-TCA TCC TTG GCA CCA TAG ATC AGG-3'	95˚C for 5min 94˚C for 40sec 56˚C for 40sec 30 cycles 72˚C for 40sec 72˚C for 5min

PCR was performed as per the manufacturer's protocol with a final primer concentration of 0.5µM. The DNA amplicons were then detected by gel electrophoresis using a 2% agarose gel (Figure 2-2). Mice positive for cre where either homozygote as in samples 1 and 5 that were present for LoxP and WT allel or heterozygote, those were positive for the LoxP allel. To confirm that Egr-2 $^{loxP/loxP}$ hCD2-Cre mice have Egr-2 deleted in the T cells, splenic CD4 T cells were isolated and stimulated with anti-CD3 and anti-CD28, Egr-2 mRNA was examined by Real Time PCR, while protein was analysed by Western Blot (Figure 2-2 B and C).

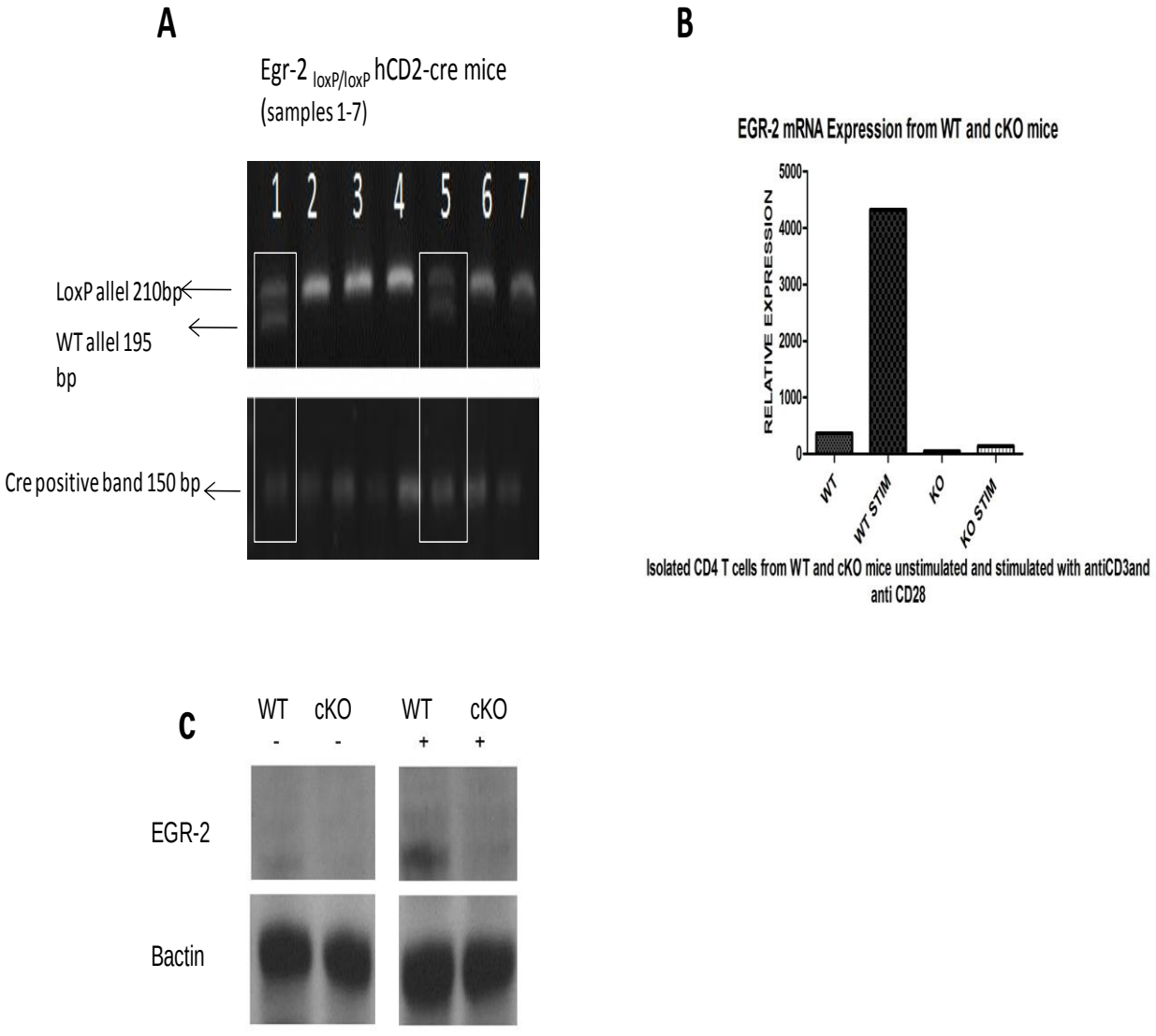


Figure 2-2.Genetic characterization of EGR-2 cKO mice

hCD-2-Cre mice were crossed with EGR-2 loxP/loxP mice to generate EGR-2$^{loxP/loxP}$ hCD2-Cre (EGR-2 cKO) mice.(A) Extraction of genomic DNA from tail samples of EGR-2 cKO mice and identified by PCR for Cre and LoxP. The white box highlights EGR-2 cKO heterozygous mice which were not used. Splenic CD4^{+} cells were isolated from EGR-2 cKO mice and C57/Blk6 WT mice and stimulated with anti-CD3 and anti-CD28 for 16 hours. mRNA expression of EGR-2

was quantified by Real Time QPCR (B) and western blot (C) Bactin was used as a loading control.

2.1 T cell isolation

To study the naive CD4 T cells of Egr-2 cKO mice and to compare them with WT mice we used the spleens and lymph nodes of each mouse (dissection preformed at Brunel University). We used young mice between 6 to 8 weeks to avoid bias that could be created in an Egr-2 deficient environment by the onset of inflammatory disease.

Total mice spleens and lymph nodes were first mashed to break up the tissue up using a cell strainer and syringe end, and then washed with Phosphate Buffer Saline (PBS). The cell suspension is centrifuged at 12,500 rpm for five minutes at room temperature (RT) and supernatant taken off. The erythrocytes are lysed from the cell pellet using 0.9% ammonium chloride lysis buffer at 37ºC for five minutes. The erythrocytes lysed due to the hypotonic solution in the lysis buffer. Next diluting with PBS stops the lysis and a further centrifugation at 12,500 rpm for five minutes at RT is needed.

To study the function of Egr-2 in lymphocytes we isolated naive $CD4^+$T cells and further purified them by CD62L. The cell adhesion molecule CD62L (L-selectin) is highly expressed on naive T cells and down-regulated upon activation. This makes sure that we start with a true naive $CD4^+$ T cell population and eradicates the possibility of already differentiated cells. We preformed Magnetic Activated Cell Sorting (MACS) using $CD4^+$ $CD62L^+$ T cell isolation beads

($CD4^+$ $CD62L^+$ T Cell Isolation Kit II mouse Miltenyl Biotec) according to manufacturer's instructions. Brief description: Total mice splenocytes of both WT and EGR-2 cKO mice were extracted as previously described in to a single cell suspension of 10^8 total cells respectively. Cells and solution buffers are kept cool to prevent capping of antibodies on the cell surface and non-specific cell labeling.

Non $CD4^+$ T cells were indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal anti-mouse antibodies against CD8a, CD45R, CD11b, CD25, TCRγ/δ and Ter-119 and incubated for 10 minutes at 4ºC. Anti biotin microbeads (isotype mouse IgG1) are added with buffer (PBS, 0.5% BSA and 2mM EDTA) and cells are further incubated for 15 minutes at 4ºC. Following centrifugation at 300xg for 10 minutes at 4ºC and resuspension in 500ul buffer. The cell suspension runs through a LS column, which magnetically attaches to a MACS separator; the unlabelled cells pass through and are collected. The total effluent contain unlabelled pre enriched $CD4^+$ T cell fraction. Next step cells are centrifuged 300 x g for 10 minutes and resuspended in 800ul of buffer. They are directly labelled with 200ul CD62L (L-selectin) Microbeads and incubated at 4ºC for 15minutes. Cells are then washed with 10 ml of buffer and centrifuged at 300 x g for 10 minutes. Following resuspension in 500ul of buffer the cells run through an MS column, magnetically attached to MACS separator. Cells are next isolated by positive selection from the pre-enriched $CD4^+$T cell fraction. The magnetically labelled $CD4^+CD62L^+$ T cells are retained on the MS column and eluted after removal of the column from the magnetic field (Figure 2-3). The isolated cells were 95% of $CD4^+CD62L^+$CD44lowCD25-.

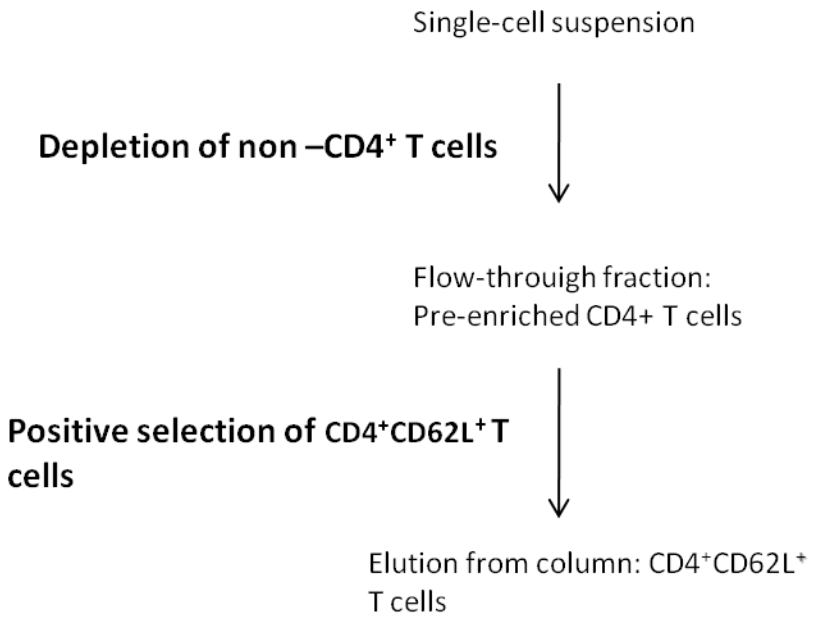


Figure 2-3. The principle of MACS Separation

2.2 Effector T helper differentiation studies

For differentiation studies of naive $CD4^+$ T cells to effector T cells, we had a starting population of naive 95% of $CD4^+CD62L^+$CD44lowCD25- for cells derived from both WT and EGR-2 cKO respectively. Co-stimulatory cytokines anti-CD3 5ug/ml and anti-CD28 2ug/ml were used to coat 48 well Nunc tissue culture treated plates and left overnight at 4ºC (we started with six well plates but found the cells grew better in a smaller surface area). Cells seeded at 0.25 $x10^6$ cells per well and cultured in 100ul Iscove's Modified Dulbecco's Media (IMDM) supplemented with300mg/ L-glutamine, 100U/ml penicillin, 100ug/ml streptomycin, $5x10^5$M β-

mercaptoethanol and 5% heat inactivated Fetal Calf Serum. Next step another 100ul of medium was added to all the wells together with various Th cytokine stimulation cocktails (see

Table 2-2**).** Anti IL-4 is added in Th1 conditions to stop differentiation of Th2, and anti IFN-γ in Th2 conditions to stop differentiation into Th1. Both cytokines anti IL-4 and anti IFN-γ is used to stop differentiation of Th1 and Th2 in Th17 conditions. To note: we first started using mouse IL-6 but found Human IL-6 to be more reactive. Cells are left for four days in culture at 37°C to proliferate and differentiate.

Table 2-2. Effector Th cytokine cocktail

Effector T cell	Cytokine cocktail added to 100µl of IMDM
Th0	No Cytokines added
Th1	10ng/ml IL12 10ng/ml anti IL4
Th2	10ng/ml IL4 10ng/ml anti IFNg
Th17	50ng/ml Human IL6 1ng/ml TGFB 10ng/ml IL1b 10ng/ml anti IFNg 10ng/ml anti IL4

2.3 Brdu Assay

To look into the proliferation of $CD4^+$ T cells in vivo, we used BrdU to detect its incorporation in to the cells. BrdU (Bromodeoxyuridine) is a thymidine analogue that is readily taken up by proliferating cells and incorporated into newly synthesized DNA by cells progressing through DNA synthesis (S) phase and cell cycle. Using an anti-BrdU antibody, S phase cells can then be detected and analysed by flow cytometer. BrdU (from Sigma) at 10µM was added to the cells for 4 hours. The cells are then harvested into microtubes and centrifuged twice . PE-Anxin V antibody is used at 2ul and incubated at 4° C for 30 min. Cells are then washed once with PBS and 200ul of fixation buffer (BD) is added and left on ice for 10 minutes . Cells are then centrifuged and supernatant removed. PBS is added at 1ml and washed twice. Cells are then permeambilised by adding 500ul of 0.5% Triton X-100 in PBS and incubated for 15 mines at room temperature followed by two wash steps with PBS. Resusupension of cells by adding 500ul of 1x DNase I buffer and 50ul DNase I solution. Cells are incubated at 37 C for 45 mins. This is followed by a further three wash steps with PBS. Anti – Brdu at 2ul is added and incubated for 20mins at room temperature. Cells are washed and 50ul of IL2 and incubated in the dark for 20 minutes at room temperature. Cells washed once and resuspended in 50ul RNaseA (100ug/ml) and 200ul PI (50ug/ml) and incubated for 10 minutes. Cells were then analysed on flow cytometer.

2.4 [$_3$H]TdR proliferation Assay to Methods

To analyse proliferation of T cells tritiated thymidine ([3H]TdR) incorporation was measured by help of Dr B. Zhu. This technique takes advantage of the fact that as cells proliferate they replicate their DNA. This technique uses the nucleotide thymidine (TdR) that is labelled with a radioactive isotope of hydrogen called tritium (3H). As the cells proliferate they take up this [3H]TdR and incorporate it into their DNA. Thustritium incorporation is proportional to DNA synthesis and hence to proliferation. To measure [3H]TdR incorporation, purified CD4+ T cells (5×104 cells/200μl) in 96-well plates were stimulated in triplicate for 72 hours. 1μCi of [3H]TdR was added to each well for the last 8 hours of culture, then the DNA was harvested onto glass fibre filters and unincorporated [3H]TdR was washed away. Tritium incorporation was measured by 54 scintillation counting; tritium emits radiation which excites scintillation fluid causing it to emit light which can then be detected.

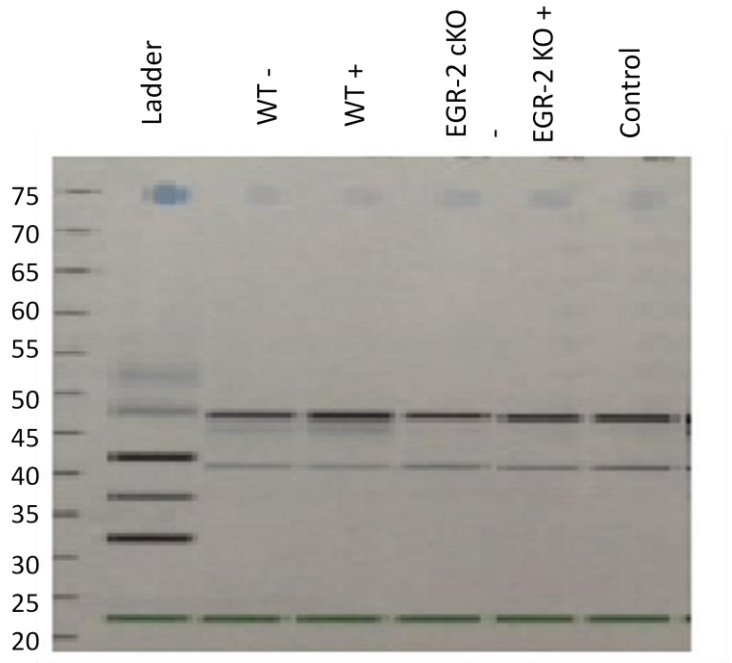
2.5 Extraction of RNA

Total RNA is extracted using the Trizol reagent (Invitrogen). This method separates nucleic acids from other cellular components. The protocol uses acidic conditions, which separates the RNA from the DNA and proteins following chloroform extraction. The solution also contains Guanidinium thiocyanate; a strong denaturing agent which denatures any proteins, such as RNAse, present in the sample (Chomczynski and Sacchi 2006). Cultured $CD4^+$ cells (under

various stimulating treatments) from WT and EGR-2 cKO mice were pelleted and then lysed in 1ml of Trizol per 10^7 cells and then incubated at room temperature for 10 minutes. 200µl of Chloroform is then added, the sample mixed vigorously, incubated at room temperature for five minutes and then centrifuged. After centrifugation the sample separated into a red organic phase and a clear aqueous phase separated by a white interphase. The RNA separated into the aqueous phase while proteins and DNA remained in the interphase. The aqueous phase is transferred to a new tube and the RNA was precipitated by the addition of isopropanol. Nucleic acids are not soluble in alcohol and so the RNA precipitates out of solution. In addition, in situations where the initial cell number was low, glycogen is added; glycogen is also not soluble in alcohol and co-precipitates with the RNA, aiding visualization of the RNA pellet. The RNA pellet is then incubated at −20°C for one hour and spun down. The RNA pellet is next washed with 70% ethanol and then resuspended in RNAse-free water.

2.5.1 RNA purity and integrity

High quality, intact RNA is essential for full length, high quality cDNA synthesis. To quantify the amount of RNA present, a nanodrop spectrophotometer is used to measure the absorbance at 260nm.



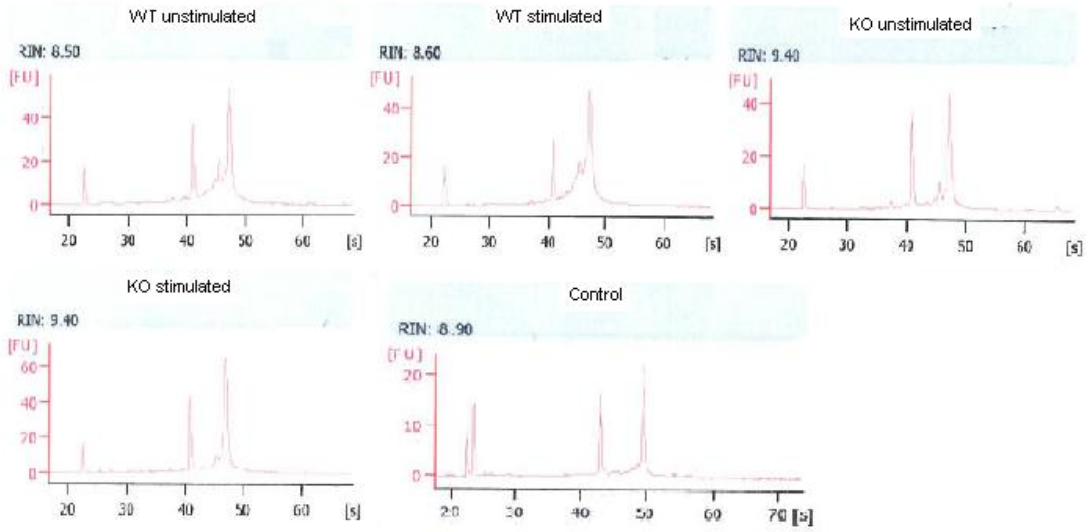


Figure 2-3.Gel and graphs showing integrity of RNA of 28s and 18s and rRNA bands

All samples had clear bands and RIN number of above 8.00

2.6 RNA to cDNA

RNA was converted to first strand cDNA (copy DNA) using a reverse transcriptase enzyme, superscript III from Invitrogen. The reverse transcriptase enzyme catalyses the formation of a complementary cDNA strand using the RNA strand as a template. Oligo-dT nucleotides will then anneal to the poly-A tails of mRNA and act as primers for reverse transcriptase. Using nuclease free thin walled PCR tubes 2µg of total RNA and 0.5µg of oligo-dT was added in to the tube along with sterile distilled water to give final volume of 13ul. dNTP's were also added 10µM of each dATP, dTTP, dCTP and dGTP. The samples were then heated to 65°C for five minutes to denature any secondary structures present in the RNA, helping the binding of the oligo-dT primers.

The samples were then placed on ice for 1 minute and 1µl of RNAse inhibitor, 1µl DTT (Dithiothreitol), 4µl 5x Reaction Buffer and 1µl Superscript III (200 units/µl) was added to each reaction (all from Invitrogen). The reactions were then heated to 37°C for 1 hour and then 70°C for 15 mins to inactivate the Superscript enzyme. The cDNA was then ready to be used as a template for Real Time Quantitative PCR.

2.7 Real Time Quantitative PCR

Real Time Quantitative Polymerase Chain Reaction (Real Time QPCR) uses the same principle as normal PCR which amplifies up large amounts of a target DNA sequence using specific primers and enzyme conditions. Real Time QPCR exceeds the limitation of PCR as the amplified DNA detects the reaction progresses in 'real time'. This is a new approach compared to standard

PCR, where the product of the reaction detects at its end-point on an agarose gel. The advantage of Real Time QPCR is that it allows quantification between samples against a reference gene. The reference genes are also known as housekeeping genes, they are constitutive genes that are required for the maintenance of basic cellular function, and isexpressed in all cells of an organism under normal and patho-physiological conditions. We tested a number of housekeeping genes on our samples for both mouse and human genes and we found the best housekeeping genes where there was no change in expression, and expression was high in mouse to be Beta actin (β-actin) and in humans to be B2M.

The method for the detection that we used was SYBR® Green I, a nonspecific asymmetrical cyanine dye (Zipper, Brunner et al. 2004) that intercalates with all double-stranded DNA (see Figure 2-4). The resulting DNA-dye-complex absorbs blue light (λmax = 497 nm) and emits green light (λmax = 520 nm). Detection is monitored by measuring the increase in fluorescence throughout the cycle. Real time PCR was performed with the QuantiTect SYBR Green PCR kit (Quiagen) as per the manufacturer's instructions using 1µl of relevant cDNA and a final concentration of 0.2µM of relevant sense and antisense primers (see Figure 2-5 and Table 2-3) in 1x SYBR Green master mix containing HotStarTaq DNA Polymerase (high specificity and sensitivity) and ROX dye which allows fluorescence normalization. Real Time QPCR performs a relative quantification based on the relative expression. The data was analysed using the Rotor-Gene Software. All samples were run in duplicate, and relative mRNA expression levels were obtained by normalizing against the level of β-actin for mice samples or B2M for human samples

from the same respective samples under the same program using$^{\Delta\Delta}$CT method (Livak and Schmittgen 2001): **relative expression=2^(CT$_{(\beta\text{-actin})}$– CT$_{(\text{target gene})}$)*10,000.**

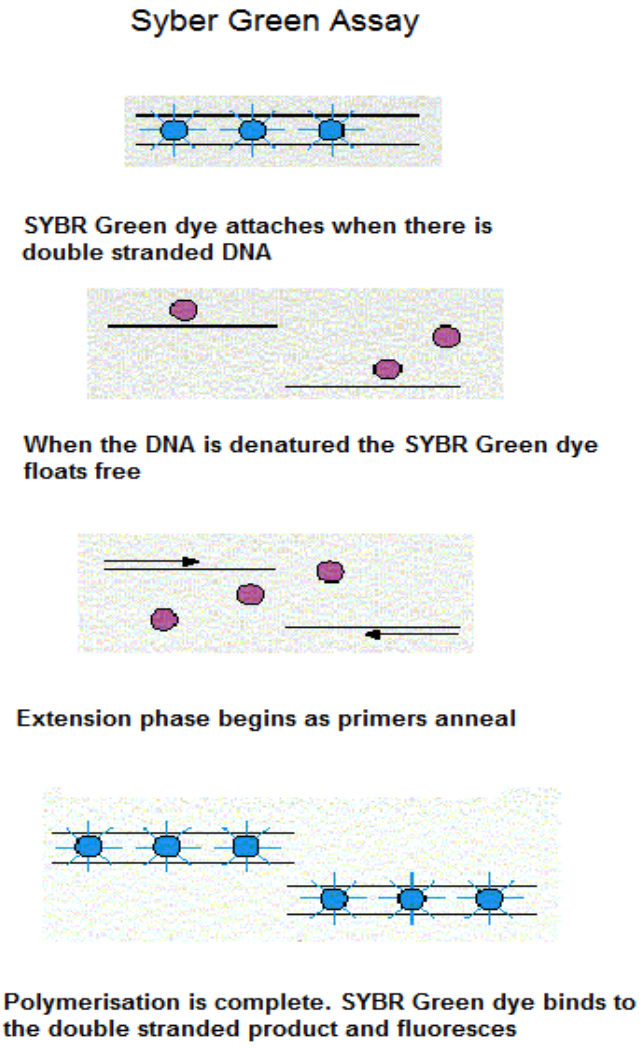


Figure 2-4.Diagram showing the binding of SYBR Green dye in Real Time QPCR

*Adapted from Applied Biosystems (**www.appliedbiosystems.com**)*

Real time PCR was performed on a Rotor-Gene system (Corbett Robotics) using the program listed below:

1. 95°C for 10min
2. 95°C for 20s
3. 58°C (varies depending on Tm) for 30s } 45 cycles
4. 72°C for 20s

Step 1: the initial activation step where HotStarTaq DNA Polymerase is activated by this heating step.

Step 2: in addition to denaturing the DNA, it also denatures the antibody that is bound to the DNA polymerase.

Step 3: Annealing, the temperature is always set 5-8C below the melting temperature of primers.

Step 4: Extensioncatalyse DNA synthesis during the elongation phase. During each elongation phase fluorescence data was collected. This cycle is repeated 45 times.

Figure 2-5. PCR cycle run 1

2.7.1 Primer design

To find the genes of interest to amplify up, we first looked up their nucleotide sequence using the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/) nucleotide database.

The sequence runs through the Primer-Blast software (www.ncbi.nlm.nih.gov/tools/primer-blast) where primers are picked to the specific PCR template, under certain critical criteria for optimal amplification with high yield. The primers were designed to be 20-25 nucleotides in length, long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature. They were also designed to have a 40-60% GC content to ensure stable binding of primer/template as G-C bonds contribute more to the stability (increased melting temperatures) of primer/template binding than do A-T bonds. The GC clamp was also set so there would be G or C base at the 3′ end of primers. This helps to promote correct binding at the 3′ end due to the stronger hydrogen bonding of G and C bases. However strings of G and C, more than 3 repeats in the first five bases of the 3' end were avoided. As they form internal, non-Watson-Crick base pairs that disrupt stable primer binding and form primer dimer (self dimers that are due to intermolecular interactions of the same primer and cross dimer that are intermolecular interactions between sense and antisense primers). We selected primers with a random base distribution and contained fewer than four complementary bases especially at the 3′ end as this can lead to the formation of product artifacts arising from amplified primer-dimers and primer-oligomers to form homo-dimers or hetero-dimers.

Table 2-3. Forward and Reverse mouse primers used for Real Time quantitative PCR

Primer	Sense	Antisense	Annealing Temperature
Egr-2	5'-CTTCAGCCGAAGTGACCACC-3'	5'-GCTCTTCCGTTCCTTCTGCC-3'	58°C
IL-2	5'-GCATGTTCTGGATTTGACTC-3'	5'-CAGTTGCTGACTCATCATCG-3'	58°C
IL-17A	5'-AGCGTGTCCAAACACTGAGG-3'	5'-CTATCAGGGTCTTCATTGCG-3'	59C
IL-17F	5'-AACCAGGGCATTTCTGTCCC-3'	5'-TTTCTTGCTGAATGGCGACG-3'	59C
IL-1a	5'-ATGTATGCCTACTCGTCGGG-3'	5'-ATGAGGTCGGTCTCACTACC-3'	57C
IL-1b	5'-CCTGTGTAATGAAAGACGGC-3'	5'-GGTGCTGATGTACCAGTTGG-3'	58C
β-actin	5'-AATCGTGCGTGACATCAAAG-3'	5'-ATGCCACAGGATTCCATACC-3'.	59C
Nfkbiz (L S)	5'-TCCAGAATGTCCCAGTCTCC-3'	5'-GAGTCTCAGTTTGGGGTGGA-3'	60C
Nfkbiz (L D)	5'-CGACACCGGCTACCTGTC-3'	5'-CACCAAGGTTTACCAGATCTTG-3'	60C
Nfkbiz (L)	5'-CGCTCAACCTGGCTTACTTC-3'	5'-GGGCTCAACTTGAGGGCG-3'	60C
IL-21	5'-ATCCTGAACTTCTATCAGCTCCAC-3'	5'-GCATTTAGCTATGTGCTTCTGTTTC-3'	58C
Rorc s	5'-AGGGGATTCAACATCAGTGC-3'	5'-AGGGGATTCAACATCAGTGC-3'	59C
IL-22	5'-TCATCGGGGAGAAACTGTTC-3'	5'-CATGTAGGGCTGGAACCTGT-3'	58C
Runx 1	5'-TACCTGGGATCCATCACCTC-3'	5'-GACGGCAGAGTAGGGAACTG-3'	59C
Ahr	5'-AGCAGCTGTGTAGATGGTG-3'	5'-CTGAGCAGTCCCCTGTAAGC-3'	60C
Irf4	5'-GCAGCTCACTTTGGATGACA-3'	5'-CCAAACGTCACAGGACATTG-3'	58C
Batf	5'-TGACGCTCAACGTGAGAAG-3'	5'-GAGCTGCGTTCTGTTTCTCC-3'	59C
Socs3	5'-AGCTCCAAAAGCGAGTACCA-3'	5'-TGACGCTCAACGTGAAGAAG-3'	59C
Ccr6	5'-TCCAGGCAACCAATCTTTC-3'	5'-GATGAACCACACTGCCACAC-3'	59C
Ccl20	5'-CGACTGTTGCCTCTCTCGTACA-3'	5'-CACCCAGTTCTGCTTTGGAT-3'	58C
EGR-1	5'-AGGAGATGATGCTGAGC-3'	5'-GAGGATTGGTCATGCTCACGA-3'	58C
EGR-3	5-TACCCCAATCGGCCTAGCAAGA-3'	5'-CAGGGTCGCTCGGTGTTCGA-3'	58C
IL-6	5'-TGGTCTTCTGGAGTACCATAGC-	5'-ACTCCTCTGTGACTCCAGC-3'	58C

	3’		
IFNg	5’-CCATCAGCAACAACATAAGC-3’	5’-AGCTCATTGAATGCTTGGCG-3’	58C
IL-4	5’-CAAACGTCCTCACAGCAACG-3’	5’ -CTTGGACTCATTCATGGTGC-3’	57C
Gm-CSF	5'-TGGTCTACAGCCTCTCAGCA-3’	5'-CCGTAGACCCTGCTCGAATA-3’	57C
E2f2	5'-TTGGATCCCAGTCAATCCCT-3’	5'-AAGTGATAGTCAAGGGCCTC-3’	58C
IL-10	5'-GGTTGCCAAGCCTTATCGGA-3'	5'-ACCTGCTCCACTGCCTTGCT-3'	59C
IL-15	5'-GATGAACATTTGGACAATGCG-3'	5'-AGAAACGTGCTCTACCTTGC-3'	60C
RORGT	5'-GGAGCTCTGCCAGAATGACC-3’	5'-CAA GGC TCG AAA CAG CTC CAC-3’	58C

Table 2-4. Forward and Reverse human primers used for Real Time quantitative PCR

Primer	Sense	Antisense	Annealing Temperature
IL-17	5'-ACCAATCCCAAAAGGTCCTC-3’	5'-CCCACGGACACCAGTATCTT-3’	58C
EGR-3	5'-AGCAGCGACTCGGTAGTCC-3’	5'-TGATGTTGTCCTGGCACCAGT-3’	58C
EGR-2	5’-CTTTGACCAGATGAACGGAG-3’	5’-CCCATGTAAGTGAAGGTCTG -3’	58C
EGR-1	5'-TGACCGCAGAGTCTTTTCCT-3’	5'-AGCGGCCAGTATAGGTGATG-3’	57C
Beta-Actin	5'-AGAAAATCTGGCACCACACC-3’	5'-AGAGGCGTACAGGGATAGCA-3’	58C
B2M	5' -AGGTATCCAGCGTACTCCA -3’	5'-TCAATGTCGGATGGATGAAA-3’	58C
IL-2	5'-CACAAACAGTGCACCTACTTCA-3’	5'-TCCGGTGAGTTTGGGATTC-3’	57C
IL-10	5'-TTTAGGGTTACCTGGGTTGC-3’	5'-CCTGATGTCTGGGTCTTGG-3’	59C

2.8 Flow cytometry

Flow cytometry is a powerful tool for the multi-parameter analysis of cells. This system senses cells as they pass through a liquid stream through a laser or light beam that emits coherent light at a specified wavelength. Scattered and emitted fluorescent light is collected by two lenses, one set in front of the light source (forward scatter) and one set at right angles (side scatter). Intensity of fluorescence is measured in specific bands. This technique shows the expression of cellular markers by a mixed population of cells. We used antibodies that have high affinity for the protein of interest and are usually conjugated to a fluorescent marker to enable detection. The computer then plots this data so that the expression of the markers by the cells can be quantified.

In addition the computer can be directed to show only those cells in a certain population; a process termed gating. In this study, we gated on $CD4^+$ populations to investigate the expression of various markers. Single cell suspensions were analysed on a LSRII (BD Immunocytometry Systems) and the data were analysed using FlowJo (Tree Star).

2.8.1 Surface and intracellular staining

$CD4^+$ T cells, Splenocytes and CNS infiltrating mononuclear cells after day 4 of culture were stimulated with PMA 0.5ug/ml and 0.5ug/ml Ionomycin was added in the presence of Golgi stop (1:1000), an inhibitor of cytokine secretion, for four hours at 37ºC. Wells of cells were pooled together with the same conditioning treatment for each WT and EGR-2cKO cells respectively. Cells were then harvested and spun down. Using BD cytofix/cytoperm plus fixation/permeabilization kit, supernatant removed and 300 ul of fixation buffer added to fix the cells and keep in the dark for 20 minutes. Cells were then washed with BD Perm/Wash twice and specific fluorchromes (Fluorescein isothiocyanate (FITC)-conjugated antibodies IFN-g, and IL-4. Phycoerythrin (PE)-conjugated antibodies to IL-17A, IL-17F, CD127, CD25, CD69allophycocyanin (APC)-conjugated antibody to CD4) added to the cells. Total splenocytes incubated next with the specific fluorescently labelled antibodies for 30mins at 4ºC and then washed with Perm/Wash and the cells were then resuspended in PBS ready to be analysed by flow cytometry.

2.9 Microarray

To study whole gene expression of EGR-2 we performed a microarray using RNA from isolated naive $CD4^+$ T cells (using MACS columns) of WT and EGR-2 cKO mice that were stimulated for six hours with plate bound anti-CD3 (5ug/ml) or left unstimulated. Total RNA was first isolated using the trizol reagent as described in section 0 and then further purified using the Qiagen RNAeasy kit RNA clean up protocol according to the manufacturer's instructions. The experiment was performed ten times and the trizol lysates for each condition were pooled.The quantity and quality of the RNA was assessed by nanodrop. Only RNA of acceptable quality that had A260/A280 ratio in the range of 1.7 to 2.1 was used.The integrity of RNA was checked using an Agilent 2100 Bioanalyser with a RNA LabChip Kit. RNA integrity was evaluated using the RNA Integrity Number (RIN) and Poly-A RNA control (See Fig 2.6). The total RNA was analysed using MouseRef-8 v2.0 BeadChip expression array (Illumina). These Bead chip arrays consist of 50-mer oligonucleotide probes directed against ~24,000 well annotatedRefSeq transcripts, allowing us to study a vast number of genes in one array. The content is derived from the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq) database and supplemented with probes derived from the Mouse Exonic Evidence Based Oligonucliotide (MEEBO) set, as well as exemplar protein-coding sequences described in the RIKEN FANTOM2 database. The probe labeling, array hybridization and data processing were carried out at the Microarray facility, Barts and London School of Medicine and Dentistry according to the manufacturer's instructions. Briefly these arrays were manufactured by chemically synthesizing the oligonucleotide probes covalently attached to 3 micron beads which are then

fixed inplace on the array. Total RNA is converted to double-stranded cDNA, followed by an amplification step [*in vitro* transcription] to generate labelled cRNA. Upon introduction of labelled test RNA sequences, the labelled RNA will hybridize to complementary sequences present on the array (cRNA) and the unbound RNA is then washed away. The array is then scanned to detect the bound cRNA via the fluorescent label providing a genome-wide transcriptional profile for that sample (see Figure 2-6). The data was first normalized using the cubic spline method and then returned to us for further analysis.We focused on genes that showed a difference of at least three-fold between wild-type and Egr-2 deficient $CD4^+$ T cells and excluded genes that had a Detection P value greater than 0.01. The differentially expressed genes were first grouped based on self-organizing maps (SOMs) by a clustering algorithm method. The SOM clusters were further clustered by a hierarchical clustering program to confirm the different profile (ArrayExpress accession: E-MEXP-1698,www.ebi.ac.uk/arrayexpress). The heat maps where generated by Dr Alistair Symonds. Results were validated by Real Time RT-PCR using the primers listed above (Table 2-3).

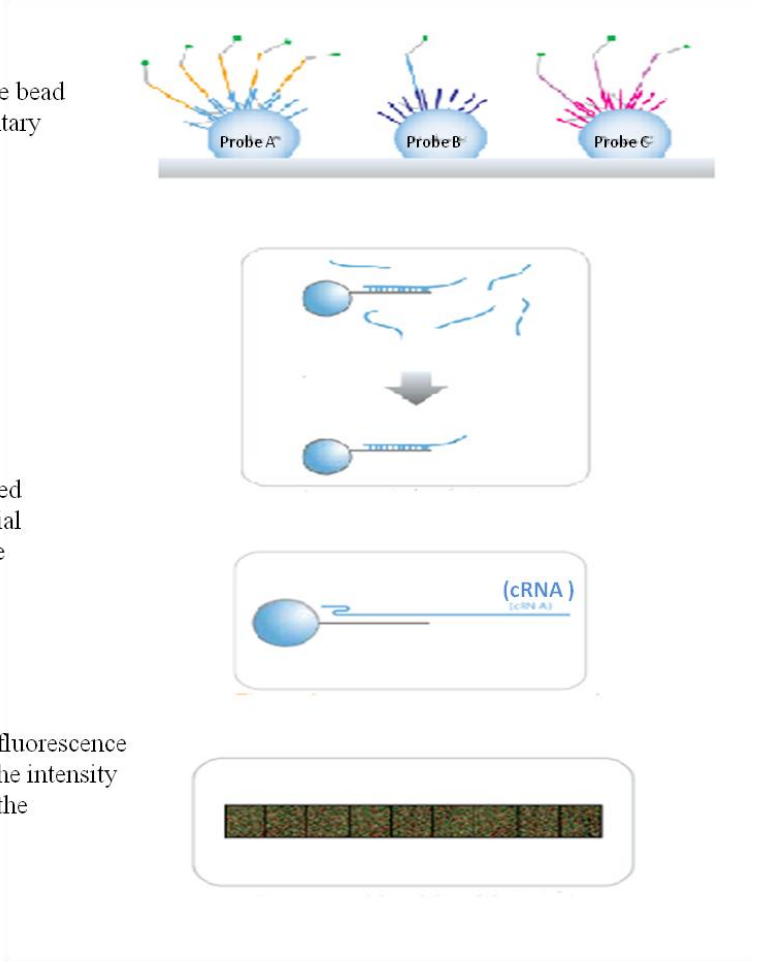
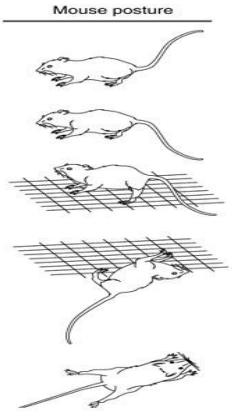


Figure 2-6.Overview of using MouseRef-8 v2.0 BeadChip expression array (Illumina)

Adapted from:http://support.illumina.com/downloads/wggex_direct_hybridization_assay_guide_(11322355_a).html.

2.10 Experimental Autoimmune Encephalomyelitis

To study the effect EGR-2 has on the breakdown in tolerance in mice and to study T-cell-mediated autoimmune disease we induced Experimental Autoimmune Encephalomyelitis (EAE) in EGR-2 cKO mice and WT mice. EAE is an animal model of brain inflammation. It is widely studied as an animal model of the human CNS demyelinating diseases, including multiple sclerosis (Martin 1997; Steinman 1999). We carried out EAE induction using a myelin oligodendrocyte glyco-protein $(MOG)_{35-55}$/ CFA (EK-0111) (Hooke) according to the manufacturer's instruction. This work has been carried out at Brunel University and performed by Emma Ghiffari. Briefly: five twelve-week-old female mice of both WT and EGR-2 cKO mice were immunized first with a pre-filled syringe of MOG_{35-55}in CFA emulsion. The emulsion provides antigen needed to expand and differentiate MOG-specific autoimmune T cells. Tissue dendritic cells pick up the antigen and take it to the draining lymph nodes, where priming of T cells takes place. We performed subcutaneous injection technique where we injected mice subcutaneously on upper and lower back with 0.1 ml of emulsion. Within 2 hours of injection of the emulsion, we intraperitoneally injected the first dose of pertussis toxin at 0.1 ml per mouse. Pertussis toxin enhances EAE development by providing additional adjuvant. PTX injection affects cell trafficking, development of Th17 cells and integrity of the blood-brain barrier. Up to 22-26 hours after injection of the emulsion, we intraperitoneally injected the second dose of pertussis toxin into the mice at 0.1 ml of pertussis toxin. The mice were checked for signs of EAE daily (see EAE scoring Figure 2-7), starting on day 7 after the immunization.



Score	Clinical Observation
0	No obvious changes in motor functions of the mouse in comparison to non-immunized mice.
1	Limp tail
2	Limp tail and weakness of hind legs
3	Limp tail and complete paralysis of hind legs or walking along side of cage and severe head tilting
4	Limp tail, complete hind leg and partial front leg paralysis
5	Complete hind and complete front leg paralysis , no movement around the cage.

Figure 2-7.EAE clinical scoring guide for mice

EFR-2 cKO and WT mice were immunized with MOG peptides, Five mice for each were immunized at 12 weeks, and after seven days the severity of EAE was scored according to standard protocols on a scale of 0 to 4, 0 being normal and 4 being paralysis.

2.10.1 Haematoxylin and eosin staining

30 days after EAE induction, using sacrificed mice, their CNS tissues were fixed with 10% formalin in PBS and embedded in paraffin. Sections are next stained with haematoxylin and eosin. Histological examination of tissue sections were done in a blind manner. In addition, CNS infiltrating mononuclear cells were isolated and analysed for IL-17 and IFNγ producing $CD4^+$ T cells by intracellular cytokine staining.

2.11 Multiple Sclerosis patients

Eleven relapsing and remitting Multiple Sclerosis (MS) patients in remission and five healthy donors were enrolled to this study. All MS patients were retrieved from the MS center Queen Mary University of London, Barts and the Royal London Hospital, London. The study has been approved by our ethics committee, trial number NCT01767701. All patients were diagnosed as having relapsing remitting multiple sclerosis according to the McDonald criteria (Polman, Reingold et al. 2005), were drug naive in relation to disease-modifying therapy and were in clinical remission. Blood was taken according to standard protocols, using heparinized tubes, after signed informed consent was obtained. Patient characteristics are presented below.

Table 2-5 Patient Profile

	SEX	AGE	Disease Course
MS 1	male	29	RRMS
MS 2	female	26	RRMS
MS 3	female	56	RRMS
MS 4	female	32	RRMS
MS 5	male	37	RRMS
MS 6	male	42	RRMS
MS 7	male	38	RRMS
MS 8	male	44	RRMS
MS 9	female	33	RRMS
MS 10	female	45	RRMS
MS 11	female	39	RRMS

RRMS = Relapsing Remitting All patients untreated

Table 2-6 Healthy Controls Profile and Average age of Patient and Controls

Healthy Controls	Sex	Age (years)
1	female	32
2	female	27
3	male	43
4	male	51
5	female	37

Age Years	MS Patients	Healthy Controls
median	38	37
range	26-56	27-51
Sex		
Male	5	2
Female	6	3

2.11.1 Peripheral blood mononuclear cells extraction from patient whole blood

For *in vitro* isolation of patient whole human blood lymphocytes we used Ficoll-Paque ^{TM} PLUS (Amersham Biosciences). This is a density gradient medium that purifies high yields of lymphocytes using a rapid centrifugation procedure based on the method developed by Boyum (1976). Standard protocol was used. Briefly: for lymphocyte separation, defibrinated or anticoagulant-treated blood diluted with an equal amount of serum-free RPMI and layered carefully over Ficoll-Paque PLUS (without intermixing) in a centrifuge tube. After a short centrifugation at room temperature with break off as not to disturb the layers (2500rpmfor 30 min), lymphocytes, together with monocytes and platelets, are harvested from the interface between the Ficoll-Paque PLUS and sample layers (see Figure 2-8). This material is centrifuged twice in PBS to wash the lymphocytes and to remove the platelets.

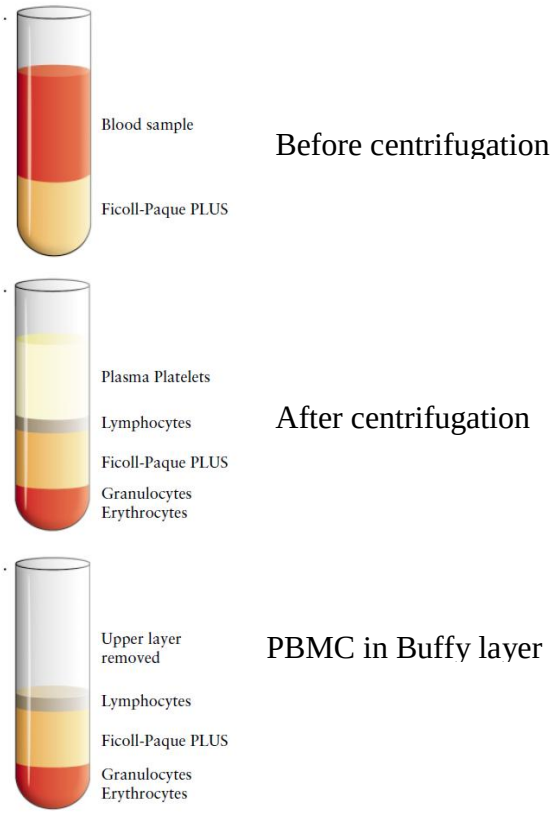


Figure 2-8. Whole human blood lymphocytes we used Ficoll-Paque ™ PLUS

Adapted from Amersham website

$CD4^+$T cells were isolated from blood lymphocytes by positive selection using MACS systems (Miltenyi Biotec) and stimulated *in vitro* for 48 hours with anti-CD3 and anti-CD28 before restimulation for six hours with the same stimuli. Total RNA extracted from these cells, and unstimulated cells, was analysed for Batf, Egr-2 and IL-17A expression by real-time quantitative PCR (RT QPCR).

2.12 Western Blot and Immunoprecipitation

To analyse proteins we preformed Immunoprecipitation and Western Blot techniques. Immunoprecipitation allows for precipitation and isolation of a protein antigen out of solution using specifically binding antibodies to the protein of interest. Cells were first lysed with cold 0.5ml Immunoprecipitation Buffer (150nM Nacl, 10mM Tris-HCL, 1mM EDTA, 1mM EGTA, 0.2 mM sodium ortho-vanadate, 0.2mM PMSF, 1% triton x-100, 0.5% NP-40). Cells were agitated constantly for 30 minutes at 4°C and sonicated on ice for five seconds and repeated four times. They were then centrifuged five minutes at 4°C and assayed for total protein at 1mg/ml. To get rid of protein that binds non-specifically to the beads we used 25ul protein A/G magnetic beads (*New England Biolabs*) to 200ul of cell extract. This was vortexed and incubated for one hour. A magnetic field was applied for 30 seconds to pull beads to the side of the tube. Supernatant was then put in to another microcentrifuge tube and beads discarded. Anti c-jun or antiflag were added at 1:1000 and incubated at 4°C for one hour. Protein A/G magnetic beads were added at 25ul and incubated further one hour. Magnetic field was applied to pull beads to the side of the tube and supernatant was removed. Cells were washed with Immunoprecipitation buffer and magnetic field applied again and supernatant removed and discarded; this was repeated twice.

Bead pellet was then resuspended in 30ul of 3x SDS sample loading buffer (187.5mM Tris-HCL, SDS, 30% glycerol, 150mM DTT, 0.03% bromophenol blue, 2% β-mercaptoethanol). Sample was then incubated at 70°C for five minutes. Magnetic field was then applied to the sample then supernatant was loaded on SDS-PAGE gel and electrophoresed.

Western Blot was performed using a 10% resolving gel; composed of 10% acrylamide: 0.26% bisacrylamide solution (ProtoGel), 0.1% SDS, 0.1% Ammonium Persulphate (APS), 0.04% N,N,N',N' - tetramethylethylenediamine (Temed) and 380mM Tris pH8.8; and a 5% stacking gel; composed of 5% acrylamide: 0.13% bisacrylamide solution (ProtoGel), 0.1% SDS, 0.1% APS, 0.1% Temed and 187.5mM Tris pH6.8. The protein samples were mixed with loading buffer containing SDS and β-2-mercaptoethanol and then heated to 95°C for five minutes to denature and reduce the proteins. The protein samples, and 5µl of Rainbow molecular marker (Amersham), were loaded into the wells of the gel and a potential difference of 100V was applied for 1.5 hours. After separation by electrophoresis the proteins were transferred to a nitrocellulose membrane by a wet transfer protocol. The gel was placed on top of a nitrocellulose membrane (Amersham) and this was sandwiched between two pieces of filter paper. This was placed in a cassette in the wet transfer system and a current of 300mA applied for one hour. The negatively charged proteins move towards the anode but are unable to pass through the nitrocellulose membrane and hence are retained on this surface in the same orientation as in the gel. To prevent antibody binding non-specifically, the membrane was blocked in 5% milk in TBST (Tris Buffered Saline with 0.1% Tween [a detergent]) for one hour at room temperature. The membrane was then incubated overnight at 4°C with the relevant primary antibody against Myc, Bactin, phospho Stat 3, and phosphorylayted c-Fos (*R and D systems*) in 5% milk in TBST at a 1:1000 dilution. These antibodies were raised by immunizing a rabbit with a peptide derived from the protein of interest. Therefore the antibody will bind to any protein containing this sequence present on the membrane. The following day any unbound primary antibody was removed by washing the membrane three times in TBST. The membrane was then incubated

with a polyclonal goat anti-rabbit IgG HRP (horseradish peroxidase) secondary antibody (Biorad) in 5% milk in TBST, at a 1:2000 dilution, for one hour at 4°C with rotation. This secondary antibody, raised in goat against rabbit antibody, is conjugated to the reporter enzyme HRP to enable detection. The membrane was then washed three times in TBST and once in TBS to remove any unbound secondary antibody and then transferred onto cling film. The immune complexes were detected by chemiluminescence using the ECL Plus kit (GE Healthcare) according to the manufacturer's instructions. This reagent contains a substrate that is oxidised, catalysed by HRP, producing a luminescent signal. Following exposure to ECL Plus for five minutes, the luminescence was detected by exposing photographic film to the membrane and then developing the film.

2.13 EMSA

Probes corresponding to a Batf binding site in the IL-17 promoter; TGGTTCTGTGCTGACCTCATTTGAGGATG (nucleotides -155 to -187) (10) were labelled with [α-^{32}P] dCTP using Ready-to-Go DNA labeling beads (Amersham Biosciences UK Ltd., Pollards Wood, Bucks) and used in binding reactions with nuclear extracts. Nuclear extracts were obtained as described in our previous report (26). For supershift reactions anti-Batf were added after 10 minutes of incubation. The samples were electrophoresed on 5% polyacrylamide gels in 0.5 x TBE.

2.14 Reporter gene assay

Mouse Batf, Egr-1, Egr-2, and Egr-3 cDNAs were introduced into pcDNA expression vectors by PCR-cloning approaches. The reporter plasmid IL17-Luc and the indicated expression plasmids were transfected into a specific cell line, HEK293 (Human Embryonic Kidney 293 cells) T cells using FuGENE 6 (Roche). After 36 hours a dual luciferase assay (Promega) was performed according to the manufacturer's protocol. This work was performed by Dr Tizong Miao.

2.15 Lentiviral transduction

The lentiviral constructs used were described previously (Miao, Wu et al. 2006). Egr-2 was introduced into these constructs by a PCR cloning approach. In addition to Egr-2, the construct carries an IRES driven GFP which allows us to isolate transduced cells by fluorescence activated cell sorting. Naive $CD4^+$ cells from EGR-2cKO mice were seeded at 1 x 10^6 per well in a 24-well plate coated with anti-CD3 and anti-CD28. This was supplemented with 2ng/ml TGFβ, 20ng/ml IL-6, 10ug/ml anti-IFNγ and 20ug/ml anti-IL-4 and infected with concentrated lentivirus at a multiplicity of infection (MOI) of 50–100 (~10^5–10^6 transducing units/ng) as previously described (Miao, Wu et al. 2006). Two days after infection, fresh medium, supplemented with 2ng/ml TGFβ, 20ng/ml IL-6, 10ug/ml anti-IFNγ and 20ug/ml anti-IL-4, was added. The cells were harvested after five days and the GFP positive cells were isolated by cell sorting. The isolated cells were used for RT-PCR analysis and analysis of IL-17 producing cells. This work was preformed under the supervision of Dr Tizong Miao.

2.16 RNA silencing

A specific short interfering oligonucleotide (siRNA) targeting the mRNA sequence of Egr-2, siEgr-2-1, GCUGCUAUCCAGAAGGUAU was developed and used. Irrelevant scrambled siRNA obtained from QIAGEN, CAT:1027281, was used as a negative control. Primary naive $CD4^+$ T cells isolated from 6 to 8 week old mice were transfected with 1 μM siRNA using an Amaxa Nucleofector according to the manufacturer's instructions. Cells were stimulated with anti-CD3 and anti-CD28; supplemented with 2ng/ml TGFβ, 20ng/ml IL-6, 10ug/ml anti-IFNγ and 20ug/ml anti-IL-4; for three days before analysis of Egr-2 and IL-17 expression. This work was preformed with Dr Tizong Miao.

2.17 Statistics

Student's unpaired t-test was used to analyse the statistical significance of differences between the groups. Differences with a p-value < 0.05 considered significant.

CHAPTER 3

RESULTS

3 RESULTS

3.0 Egr-2 is selectively deleted in CD4+ T cells from EGR-2 cKO mice

To confirm the absence of Egr-2 in $CD4^+$ T cells from genotyped Egr2-cKO mice and the presence of Egr-2 in WT mice, splenic $CD4^+$ T cells were isolated respectively and stimulated with or without anti-CD3(5ug/ml) and anti-CD28 (3ug/ml). Both at the mRNA level and at the protein level Egr-2 was successfully deleted out in $CD4^+$ T cell of Egr-2 cKO mice in both unstimulated and stimulated conditions (Figure 3-1 A and B). $CD4^+$ T cells from WT mice expressed Egr-2 and were highly expressed upon activation.

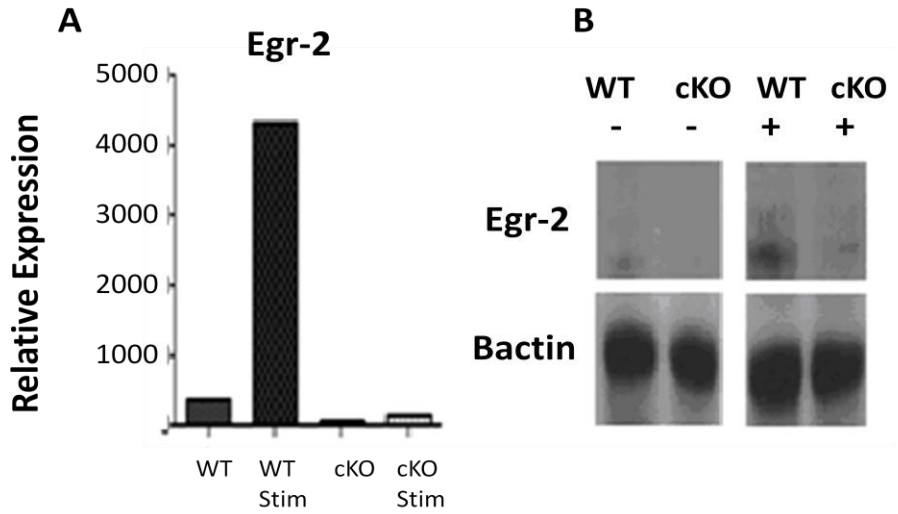


Figure 3-1. Egr-2 is successfully knocked out of $CD4^+$ T cells from EGR-2 cKO mice

(A) , RNA was extracted from $CD4^+$T cells from WT and cKO mice, in either unstimulated or stimulated with anti CD3 and anti CD28. Real time QPCR was preformed to measure EGR-2

expression. (B), To confirm results we also checked the protein level by Western blot for WT and cKO $CD4^+$ T cells with and without anti CD3 and anti CD28.

3.1 No change in $CD4^+$ T cell proliferation without Egr-2 but change in phenotype.

The T cell proliferation in Egr-2 cKO mice was compatible to that in wild-type mice (Figure 3-2 A-C), which is consistent with our previous report (Wang, Zhu et al. 2008). However, Egr-2 cKO mice developed autoimmune syndromes at late age (Zhu, Symonds et al. 2008) indicating a dysregulatory homeostasis (Wang, Zhu et al. 2008). The phenotype of the $CD4^+$ T cells from mice age at two months showed that there were increased populations of $CD25^+CD4^+$ and $CD44^{high}CD4$ cells detected from Egr-2 cKO mice (Figure 3-3), which is consistent with our previous report (Wang, Zhu et al. 2008). The increased effector phenotype of Egr-2 cKO T cells, suggest that T cells are less tolerant under homeostatic conditions.

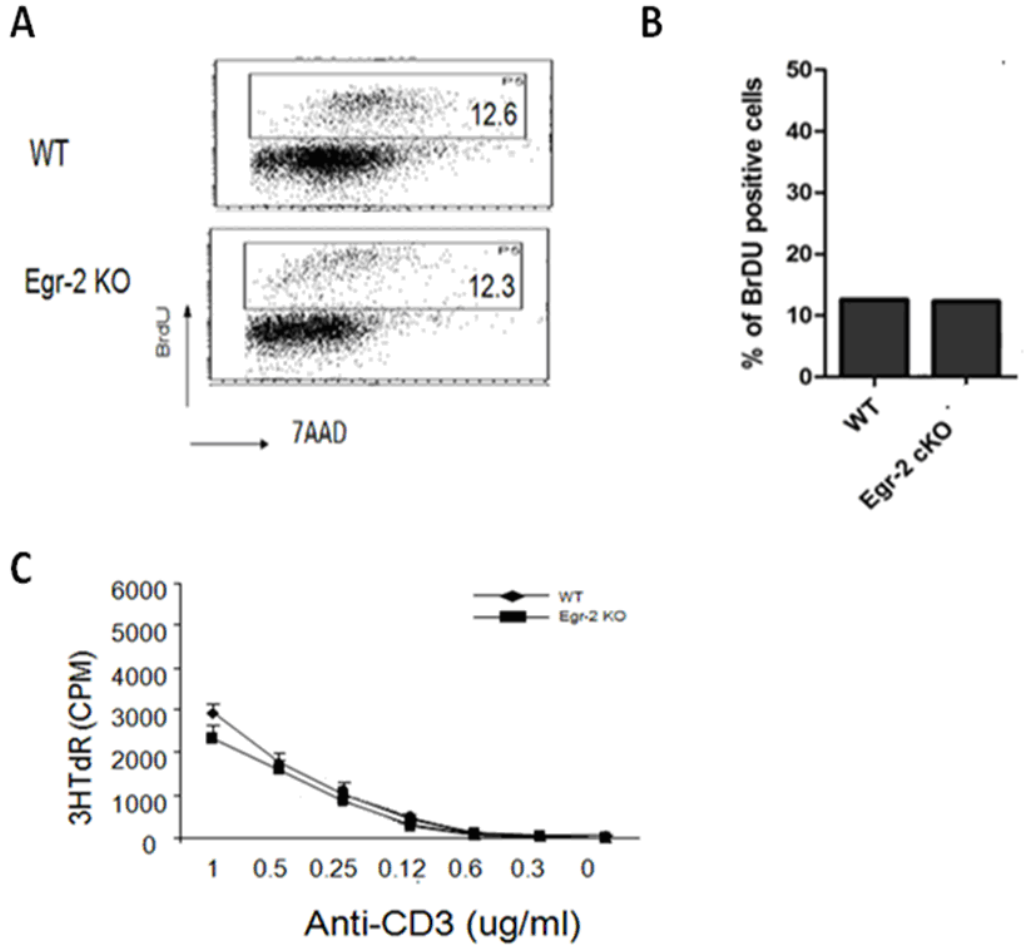


Figure 3-2.No change in T cell proliferation in EGR-2cKO and WT mice

BrdU assay showing cell division over four days of CD4$^+$ T cells from Egr-2KO and WT mice. Cells were treated with 10μM BrdU and incubated for 30 mins. Using a goat anti mouse secondary antibody S phase cells were detected by FACS. Cells were also stained for 7AAD to exclude non-viable cells [(A) and (B)]. Isolated CD4$^+$ T cells from Egr-2cKO and WT mice were stimulated with anti CD3 at the indicated concentrations for 72 hours, 1μCi of [3H]TdR was added to each well for the last eight hours of culture. Tritium incorporation was measured by 54 scintillation counting (C).

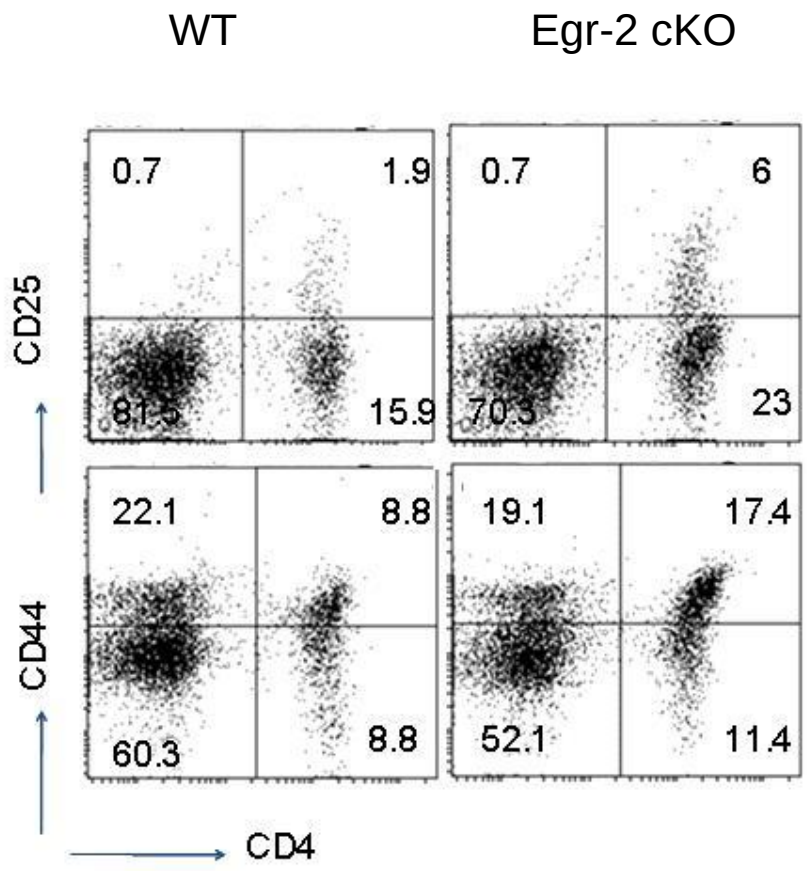


Figure 3-3. Increased percentage of thymic effector phenotype cells in Egr-2 cKO mice

Cell surface staining, anti CD4, anti CD44 and anti CD25, on $CD4^+$ T cells from WT and Egr-2 cKO mice. Egr-2 cKO mice had higher number of $CD4^+CD44^+CD25^+$ thymic effector phenotype cells compared to WT mice.

3.2 Egr-2 controls expression of Th17, but not Th1 or Th2, cytokines in effector CD4 T cells

Th differentiation is driven by the cytokine environment induced by specific antigens or with polarization protocols in vitro (Stockinger, Veldhoen et al. 2007). In the absence of polarization conditions, naive $CD4^+$ T cells are activated and entered into cell cycles under low differentiation state *in vitro*. To assess the impact of Egr-2 on cytokine production by activated naive $CD4^+$ T cells, naïve $CD62L^+CD44^{low}CD4^+$ T cells were isolated by cell sorter and activated *in vitro* by anti-CD3 and anti-CD28 stimulation. As expected, a high level of IL-2 was detected in WT naïve CD4 T cells following anti-CD3 and anti-CD28 stimulation *in vitro* for 16 hours (Figure 3-4).

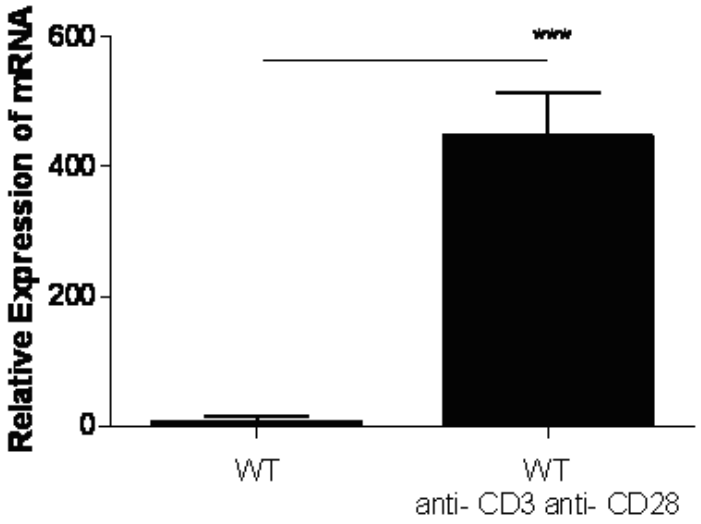


Figure 3-4. IL-2 expression is activated in naive CD4$^+$ T cells upon stimulation

Expression of IL-2 cytokine from naive CD4$^+$ T cells from Wild-type (WT) mice in unstimulated conditions or stimulated with anti-CD3 (5ug/ml) and anti- CD28 (3ug/ml) for 16 hours in vitro. Expression of IL-2 was measured by Real Time qPCR and normalized to house keeping gene β-actin. Data is representative of three independent experiments.

The Th cytokines such as Th1 cytokines IFNγ, Th2 cytokine IL-4 and Th17 cytokine IL-17, were barely detected in the absence of unstimulated polarization conditions (Figure 3-5A and B). In contrast to the wild-type CD4 T cells, a broad spectrum of inflammatory cytokines were detected following anti-CD3 and anti-CD28 stimulation, but in the absence of polarization conditions *in*

vitro (Figure 3-5A) suggesting that Egr-2 represses inflammatory responses following T cell receptor stimulation (TCR) when there is no cytokine environments. Previously, we found that, in association with the development of lupus-like disease, Egr-2 deficiency induced high expression of IL-17 and IFN-γ in effector T cells isolated from aged mice (Zhu, Symonds et al. 2008).

To look at global expression of EGR-2 we preformed Bead Chip microarray, which showed following TCR stimulation, Egr-2 deficient naive $CD4^+$ T cells produced similar expression levels of IL-2 and IFN-γ in comparison to wild-type counter-parts, however, the Th17 associated cytokines such as IL-1, IL-17 and IL-21 were highly expressed in Egr-2-deficient naive $CD4^+$ T cells on stimulation in comparing to the minimum production of Th17 cytokines in wild-type $CD4^+$ T cells (Figure 3-6). The scatterplot shows the closeness of EGR-2 to the other inflammatory cytokines on stimulation (Figure 3-7). From the results IL2, and IFN-γ remain unchanged from unstimulated to stimulated setting , while IL17A and Il17F is highly expressed in cKO upon stimulation and has moved closer to the X axis (Figure 3-7).

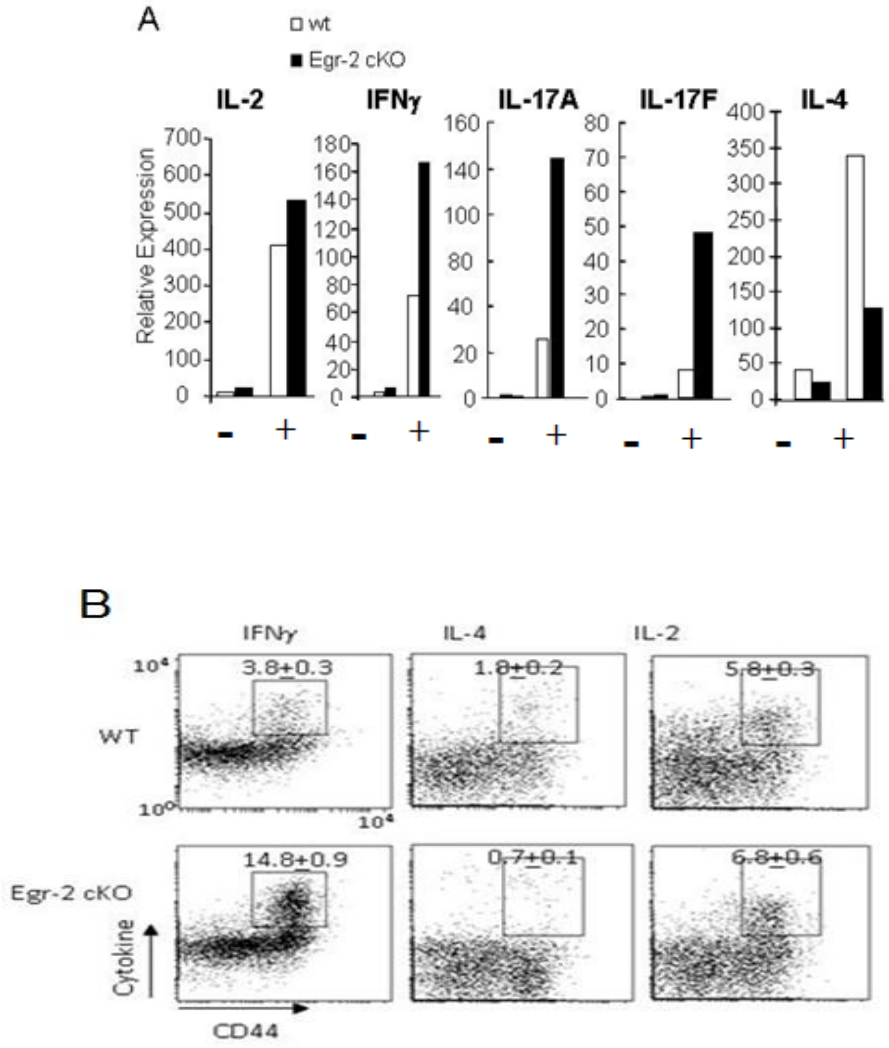
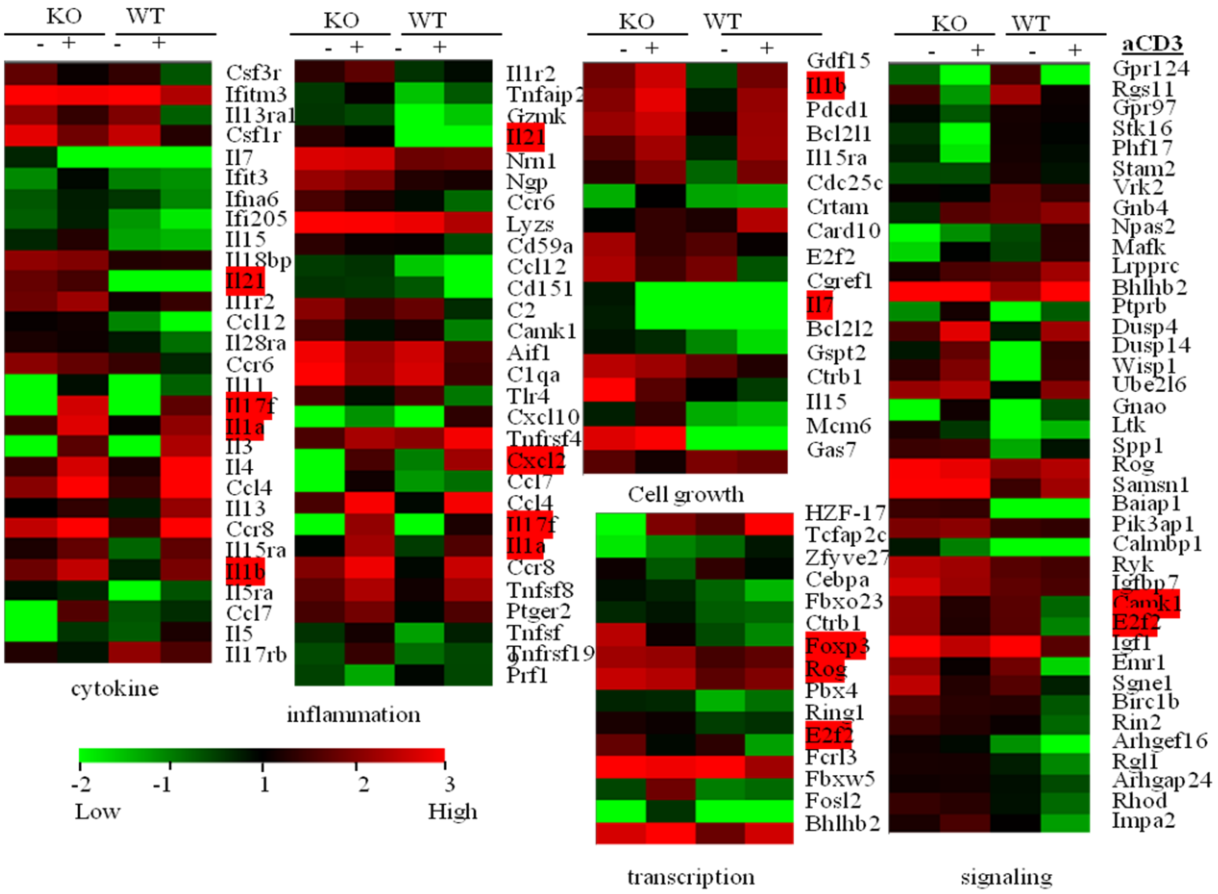


Figure 3-5. Expression of Th cytokines in stimulated and unstimulated conditions in the absence of polarized conditions

$CD4^+$ T cells from WT mice and cKO mice in unstimulated or stimulated conditions with anti-CD3 (5ug/ml) and anti- CD28 (3ug/ml) for 16 hours in vitro. Relative expression of Th cytokines was measured by Real Time qPCR (A) and Surface cell expression of Th cytokines by FACS (B). Data is representative of three independent experiments.

A


KO
WT
- + - +
Csf3r
Ifitm3
Il13ra1
Csf1r
Il7
Ifit3
Ifna6
Ifi205
Il15
Il18bp
Il21
Il1r2
Ccl12
Il28ra
Ccr6
Il1l
Il17f
Il1a
Il3
Il4
Ccl4
Il13
Ccr8
Il15ra
Il1b
Il5ra
Ccl7
Il5
Il17rb
cytokine
Il1r2
Tnfaip2
Gzmk
Il21
Nrn1
Ngp
Ccr6
Lyzs
Cd59a
Ccl12
Cd151
C2
Camk1
Aif1
C1qa
Tlr4
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Tnfrsf4
Cxcl2
Ccl7
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Il17f
Il1a
Ccr8
Tnfsf8
Ptger2
Tnfsf
Tnfrsf19
Prf1
inflammation
-2 -1 1 2 3
Low
High
Gdf15
Il1b
Pdcd1
Bcl2l1
Il15ra
Cdc25c
Crtam
Card10
E2f2
Cgref1
Il7
Bcl2l2
Gspt2
Ctrb1
Il15
Mcm6
Gas7
Cell growth
HZF-17
Tcfap2c
Zfyve27
Cebpa
Fbxo23
Ctrb1
Foxp3
Rog
Pbx4
Ring1
E2f2
Fcrl3
Fbxw5
Fosl2
Bhlhb2
transcription
aCD3
Gpr124
Rgs11
Gpr97
Stk16
Phf17
Stam2
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Rog
Samsn1
Baiap1
Pik3ap1
Calmbp1
Ryk
Igfbp7
Camk1
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Rgl1
Arhgap24
Rhod
Impa2
signaling

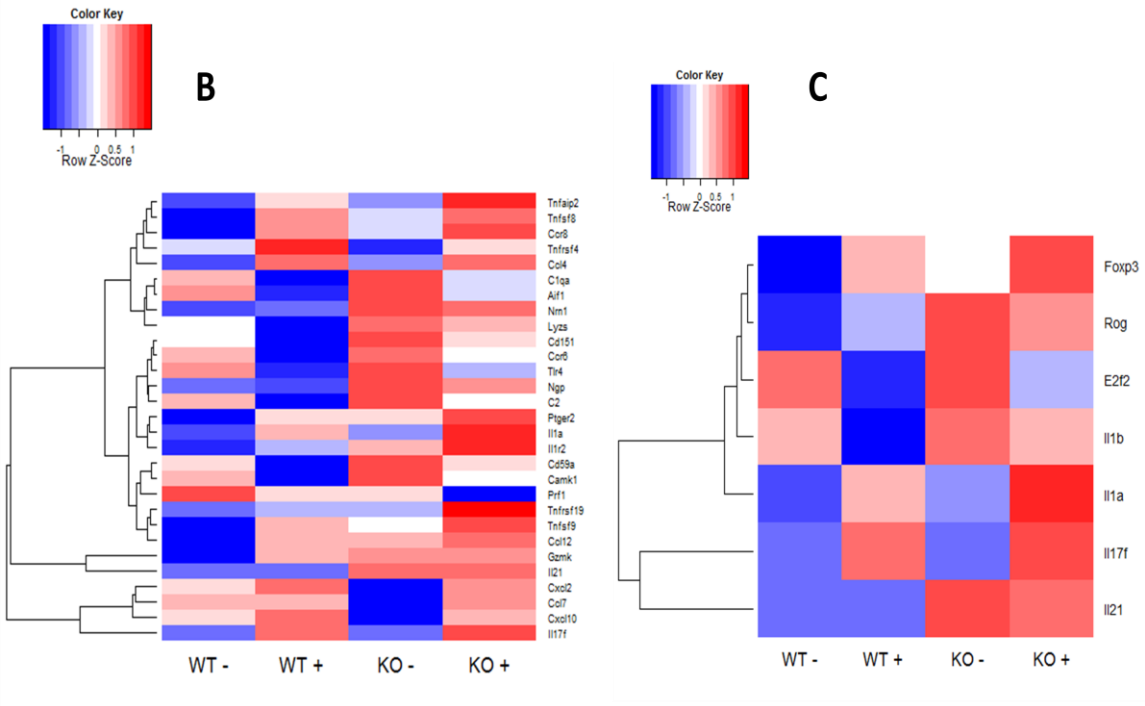

Figure 3-6. Egr-2 deficiency leads to enhanced expression of Th17 cytokines in naive CD4$^+$ T cells in response to TCR stimulation as seen from Beadchip expression microarray.

(A) Naive CD4+ T cells were stimulated with or without anti-CD3 for six hours. Extracted RNA was analysed by genome-wide transcriptional profiles using MouseRef-8 v2.0 BeadChip expression array (Illumina). (A) Altered expression of cytokine and cytokine receptor genes in Egr-2–deficient CD4$^+$T cells as assessed by genome-wide transcriptional analysis. The SOM cluster of cytokines was further clustered by a hierarchical clustering program to show the genes with differential expression profiles. Genes were grouped to into specific cellular pathways with green showing very low intensity expression and red high intensity expression. Genes highlighted in red where genes of interest. We focused on genes that had at least three-fold difference between WT and Egr-2 cKO mice. (B and C) Heat maps showing the Z score, which measures how many deviations away from the mean. Blue indicates negative deivations below the mean and red indicates positive deviations above the mean.

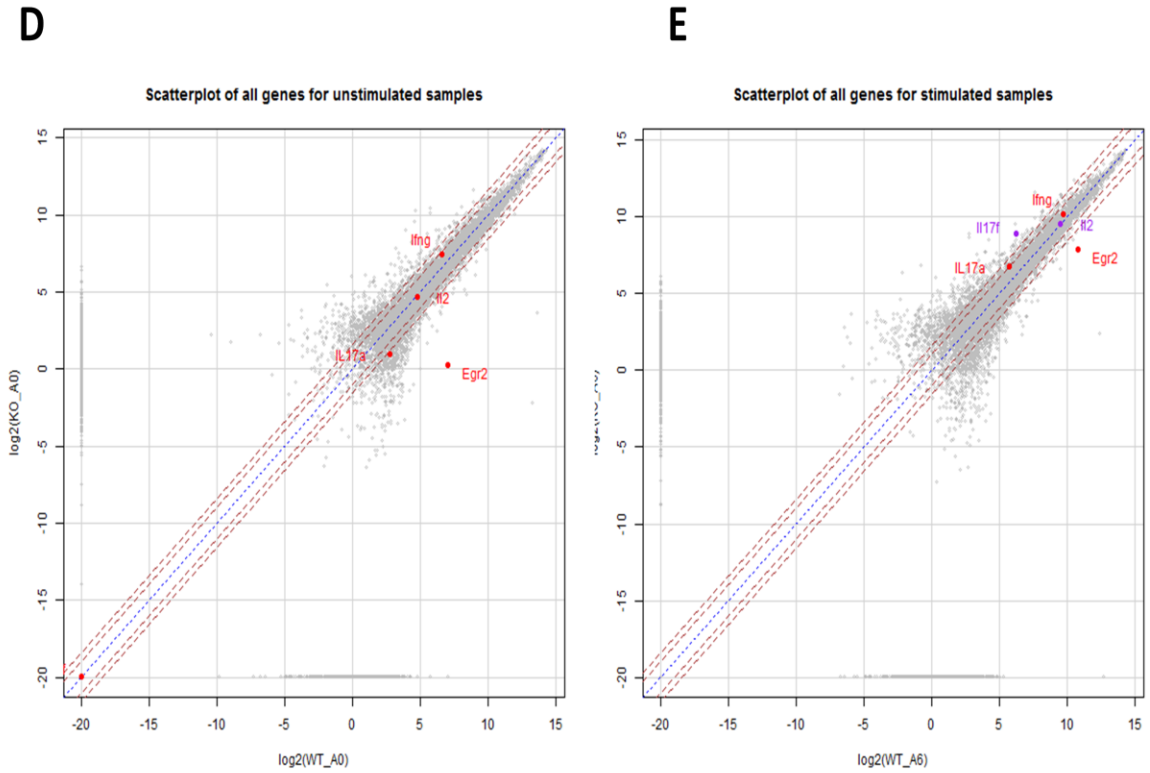

Figure 3-7. Upon stimulation Egr-2 is closely associated to inflammatory cytokines.

Scatter plots (D and E) , to visualize key cytokines and show the spread of genes in unstimulated conditions (D) or stimulated conditions (E) against WT and cKO samples. Each point represents an expression value. The purple dotted diagonal line represents equal expression, The further the gene is to X axis the higher the expression is to cKO, the closer to the Y axis the higher the expression is to WT.

The globe analysis of transcripts can give false results. It is therefore required validation of individual genes by real-time PCR with house keeping gene (β-actin) as a control. We analysed the Th17 cytokines detected by microarray by RT-PCR with primers specific for the regions including last two exons of the cytokine genes. Indeed, relative expression of those Th17 cytokines in Egr-2-deficient $CD4^+$ T cells was significantly increased in comparing to the wild-type $CD4^+$ T cells following TCR stimulation (Figure 3-8).

Therefore, the overall increased transcription of Th17 cytokines in Egr-2-deficient $CD4^+$ T cells was confirmed. Although the transciption of Th17 cytokine genes indicate the Th17 effect, it is important to measure the cytokine proteins in $CD4^+$ T cells to determine weather the cytokine are produced and also the levels of cytokine producing cells. Thus, intracellular cytokines were measured by intracellular staining with specific antibodies. To investigate the IL17-producing $CD4^+$ T cells following TCR stimulation, the stimulated cells were stained intracellularly with anti-IL17A. Again, IL17 positive $CD4^+$ T cells were barely detected in wild-type $CD4^+$ T cells, while the percentage of Egr-2-deficient $CD4^+$ cells that produced IL-17 was also increased (Figure 3-9).

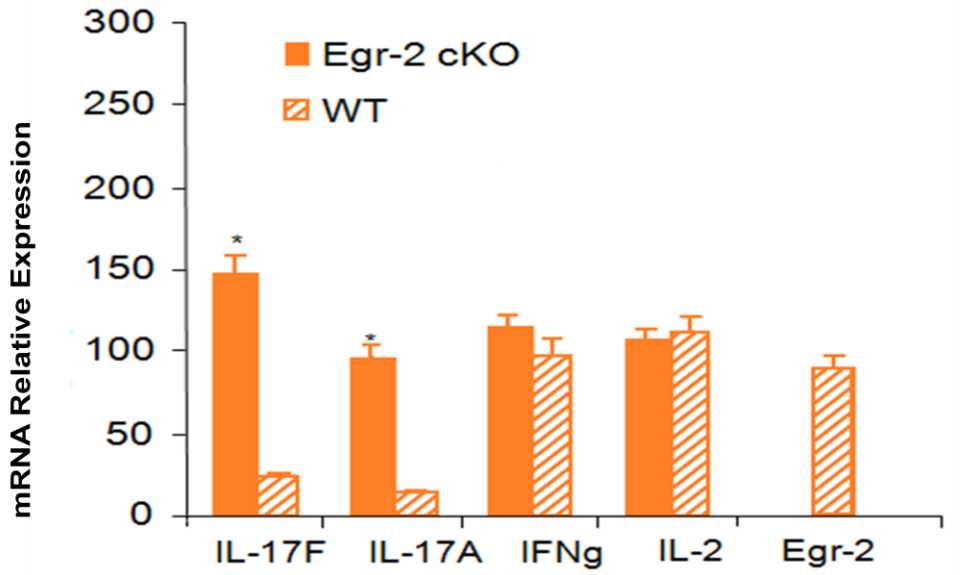

Figure 3-8. Enhanced expression of Th17 cytokines in naive CD4 T cells in response to TCR stimulation

Egr-2 deficient (Egr-2 cKO) and Wild-type (WT)naive CD4+ T cells were stimulated for 16 hours with anti CD3 5ug/ml and anti CD28 3ug/ml. Cytokine expression was measured by RT-qPCR and normalized to housekeeping gene β-actin and represents three independent experiments. P value < 0.05

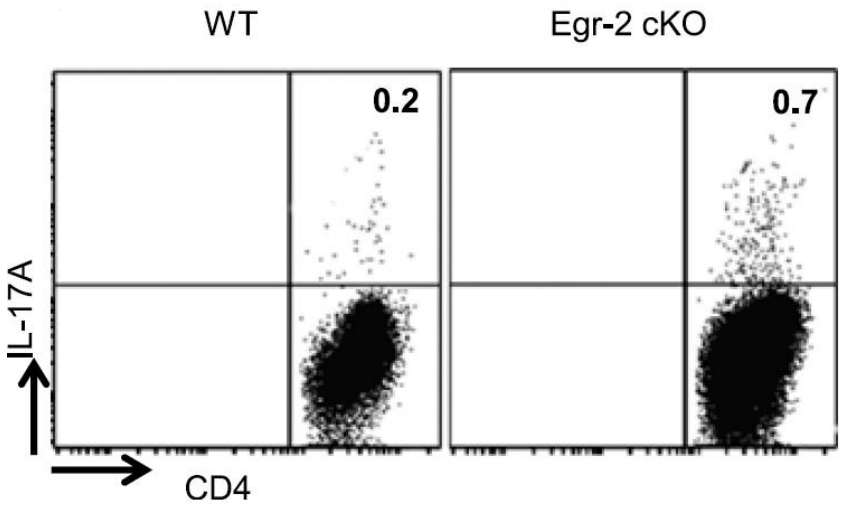

Figure 3-9. IL17-A producing CD4+T cells

Intracellular cytokine staining of CD4+ T cells isolated from WT (Wild type) and EGR-2 cKO mice splenocytes for three hours with 100ng/ml PMA/ionomycin in vitro. This is representative of one of three independent experiments.

We have now discovered a novel function of Egr-2 in the control of Th17 cytokine production during TCR stimulation in $CD4^+$ T cells. Therefore, Egr-2 may be involved in the control of Th17 cytokine expression in effector T cells.

3.3 Egr-2 is induced in naive CD4+ T cells by TGFβ and IL-6

Th differentiation is induced by polarized cytokine environment (Harrington, Mangan et al. 2006). Most of the cytokines responsible for differentiating Th subsets are produced by T cells (Powrie and Coffman 1993). It has been well defined that TGFβ and IL-6 can differentiate activated $CD4^+$ T cells to Th17 cells and these cytokines can be produced by activated T cells (Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006). If Egr-2 is antagonist for Th17 differentiation, it may be present under TGFβ and IL-6 conditions. Egr-2 is however not expressed in naive T cells (Anderson, Manzo et al. 2006). Egr-2 can be induced by antigen *in vitro* and *in vivo* in naive T cells (Harris, Bishop et al. 2004; Safford, Collins et al. 2005; Anderson, Manzo et al. 2006; Zhu, Symonds et al. 2008). These inductions are triggered by the activation of NFAT pathway (Decker, Nehmann et al. 2003). Although an overproduction of inflammatory cytokines has been discovered in Egr-2-deficient T cells (Zhu, Symonds et al. 2008), it is not known if it can be induced by any cytokine in $CD4^+$ T cells. The excessive production of IL-17 suggests that Egr-2 may be expressed under Th17 conditions and negatively regulate Th17 differentiation. Previously, we found that Egr-2 is effectively induced in naive T cells by antigen stimulation *in vivo* (Anderson, Manzo et al. 2006). The selective induction of Th17 cytokines in Egr-2-deficient $CD4^+$ T cells by TCR stimulation indicates that Egr-2 controls the expression of Th17 cytokines in T cells in response to mitogenic antigen stimulation. TGFβ and IL-6 are effective inducers for Th17 differentiation and they signal through Smad and STAT3 pathways, respectively (Zhou, Chong et al. 2009). These pathways are not activated through TCR signaling (Hirahara, Ghoreschi et al. 2010). However, TCR stimulation can induce

both expressions in naive $CD4^+$ T cells (Hirota, Martin et al. 2010). Whether TGFβ and IL6 can induce Egr-2 expression is unknown. We therefore examined the expression of Egr-2 in naive T cells following stimulation with different cytokines in the absence of TCR stimulation in vitro. The results showed that Egr-2 is rapidly induced in naive T cells following TGFβ and IL-6 stimulation (Figure 3-10 A). In contrast, IFNγ, IL-12 and IL-4; cytokines that can induce Th1 and Th2 differentiation, respectively; had little effects on Egr-2 expression in naive $CD4^+$ T cells (Figure 3-10 A).

The transcriptional activation of Egr-2 gene is induced in T cells by anti-CD3 stimulation (Anderson, Manzo et al. 2006). It has been found that this induction is mediated by NFAT and can be inhibited by cyclosporin A (CsA) (Harris, Bishop et al. 2004). It is therefore a mechanism involving in the induction of anergy (Safford, Collins et al. 2005; Zhu, Symonds et al. 2008). Consistent with this, we found that Egr-2 was strongly induced by anti-CD3 in naive $CD4^+$ T cells and the expression of Egr-2 was suppressed by CsA (Figure 3-10 B). As TGFβ and IL-6 signal through Smad and STAT3 pathways, respectively (Zhou, Chong et al. 2009). It is not known if TGFβ and IL-6 induce activation of the NFAT pathway for Egr-2 expression in T cells. To test this, the naive $CD4^+$ T cells from wild-type mice were stimulated with TGFβ and IL-6 in the presence or absence of CsA inhibitor for NFAT signaling. The induction of Egr-2 by TGFβ and IL-6 was not suppressed by CsA (Figure 3-10 B). This result indicates that the induction of Egr-2 in $CD4^+$ T cells by TGFβ and IL-6 is not mediated by NFAT which differs from the induction of Egr-2 in $CD4^+$ T cells by T cell receptor stimulation. It therefore suggests that Egr-2 is induced by different signaling pathways: by TCR stimulation and TGFβ and IL-6. Egr-2 is one

of the Egr family members (Egr-1, -2, -3 and -4:(Swirnoff and Milbrandt 1995). Egr-1, -2 and -3 share structure similarities and have overlapped function within T cell development (Shao, Kono et al. 1997; Carter, Lefebvre et al. 2007). All three are also induced by TCR stimulation through activation of NFAT pathway (Aifantis, Gounari et al. 2001; Lazarevic, Zullo et al. 2009). However, Egr-1 and -3 knockout mice did not display autoimmune syndromes. To investigate whether these two Egr molecules play similar role in Th17 induction, we analysed expression of Egr-1, -2 and -3 in $CD4^+$ T cells under the same conditions of TGFβ and IL-6 stimulation. Although Egr-1 and Egr-3 share similar functions with Egr-2 in the regulation of thymocyte development (Kaye 2000), TGFβ and IL-6 did not induce expression of either of these transcription factors (Figure 3-10 C). These results further suggest that Egr-2 is selectively involved in the regulation of Th17 cytokines in effector $CD4^+$ T cells and Th17 differentiation. The induction of Egr-2 by TGFβ and IL-6 in naive $CD4^+$ T cells during antigen stimulation may provide a feedback mechanism to prevent immune pathology induced by Th17 responses during adaptive immune responses.

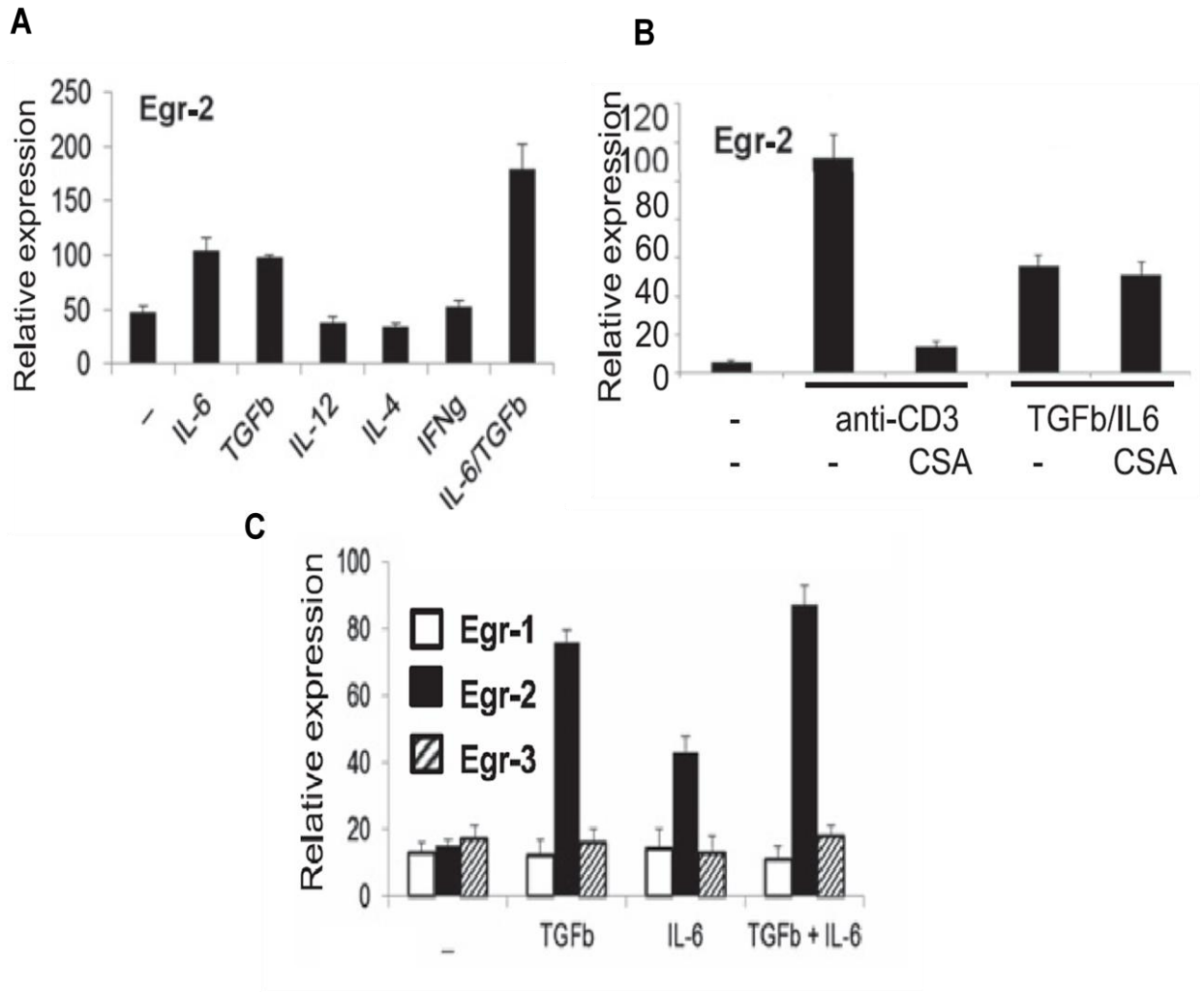

Figure 3-10. Th17 differentiation cytokines increase the expression of Egr-2 specifically compared to the other Egr- subtypes and these cytokines are not inhibited by CSA

(A and B) Expression of Egr-2 in naive CD4 T cells from wild-type mice following stimulation with the indicated cytokines (A) or with 5 mg/ml anti-CD3 and 2 mg/ml anti-CD28 or TGF-b/IL-6 in the absence or presence of 250 ng/ml CsA (B) for 6 h. (C) Expression of Egr-1, Egr-2, and Egr-3 in naive CD4 T cells from wild-type mice following stimulation with TGF-b, IL-6, or both. mRNA expression was measured by RT-qPCR and data represents three independent experiments (A, B and C).

3.4 Egr-2 regulates Th17 differentiation

Differentiation of Th subsets is regulated by the function of distinct transcription factors (Hirahara, Ghoreschi et al. 2010). These include master regulators, RORγt and STAT3 which drive Th17 differentiation (Ivanov 2006; Harris, Grosso et al. 2007). However, recently a number of transcription factors have important roles in Th17 differentiation and plasticity (Brustle, Heink et al. 2007; Schraml, Hildner et al. 2009; Okamoto, Iwai et al. 2010). Egr-2 is induced in T cells in response to TCR stimulation (Anderson, Manzo et al. 2006). Therefore, it is expressed in all types of the Th cells. We have now found that TGFβ and IL-6, but not by the other the cytokines known for Th1 and Th2 differentiation can induce EGR-2, indicating that Egr-2 may selectively control Th17 differentiation. However, although Egr-2 is not induced by Th1 and Th2 cytokines, it does not automatically exclude the function in the control of Th1 and Th2 differentiations. To investigate whether Egr-2 regulates the differentiation of $CD4^+$ T cells, naive $CD4^+$ T cells were isolated from wild-type and Egr-2-deficient mice and were stimulated with anti-CD3 and anti-CD28 under different Th polarization conditions (Stockinger, Veldhoen et al. 2007). In the absence of Egr-2, naive $CD4^+$ T cells did not alter Th1 and Th2 differentiation (Figure 3-11 A). The percentage of Th1 cytokine producing and Th2 cytokine producing cells were compatible to wild-type counter-parts (Figure 3-11 A). Similar to the cytokine producing cells, the levels of cytokine expression in positive Th1 or Th2 cells were also at normal expression (Figure 3-11 A). However, an Egr-2 defect rendered $CD4^+$ T cells more susceptible to differentiation into Th17 cells than naive $CD4^+$ T cells from wild-type mice (Figure 3-13 A). Following Th17 differentiation *in vitro*, the expression of several Th17 cytokines, including IL-

17 and IL-21, was increased in $CD4^+$ cells differentiated from Egr-2 deficient naive $CD4^+$ T cells (Figure 3-11 B).

Although Egr-2, but not Egr-1 and -3, was induced by TGFβ and IL-6, all three Egr molecules were induced in Th1, Th2 and Th17 cells in response to anti-CD3 stimulation (Figure 3-11C). However, in the absence of TCR stimulation, the Th1 and Th2 cytokines did not induce Egr-2 expression, while Th17 cytokines did (Figure 3-11C). In contrast, Egr-2 expression persisted in Th17 cells in the absence of anti-CD3 stimulation (Figure 3-11C), further supporting the notion that Egr-2 is induced by both the Th17 environment and antigen receptor stimulation. Although in the absence of Egr-2, naive $CD4^+$ T cells are susceptible for Th17 differentiation, it may result from altered cytokine production during the differentiation rather than impact of Egr-2 directly.

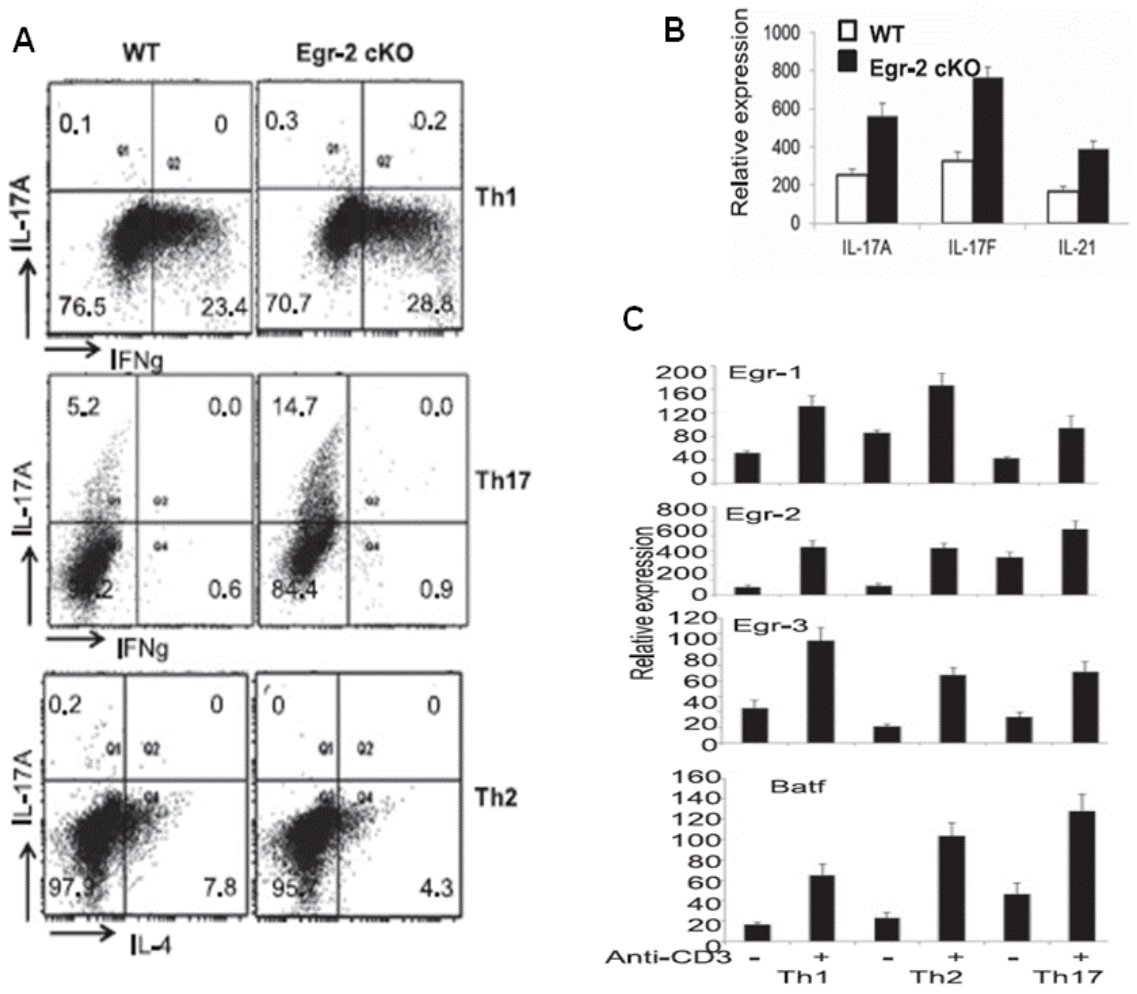

Figure 3-11. Egr-2 controls Th17 differentiation

(A) Naive CD4$^+$ T cells were differentiated into Th subsets Th1, Th2, and Th17 as described in materials and methods. Expression of cytokines was measured by intracellular staining of antibodies to IL17A and IFNγ and IL-4 for confirmation of differentiation to Th17 Th1 and Th2 respectively. (B) Relative mRNA expression of IL-17A, IL-17F and IL-21 from TH17 differentiated cells of naive CD4 T cells WT and EGR-2 cKO mice. (C) Expression of Egr-1, Egr-2, Egr-3, and Batf in Th1, Th2, and Th17 polarized cells from WT mice, stimulated for 6 hours with or without 5 µg/ml anti-CD3 and 2 µg/ml anti-CD28. Data (D) represents 5 experiments (B and C) represent 3 independent experiments.

To further define the role of Egr-2 in the control of IL-17 expression, we applied the lentiviral system to re-introduce Egr-2 expression in Egr-2-deficient $CD4^+$ T cells. The open-reading frame of Egr-2 was cloned by PCR from mRNA of mouse $CD4^+$ T cells following TCR stimulation into a Lentiviral construct in which GFP gene is operated by IRSE (Figure 3-12D). Which allows us to isolate transduced cells by fluorescence –activated cell sorting .The Egr-2 gene was orientated from 5' to 3' under CMV promoter (Figure 3-12E).

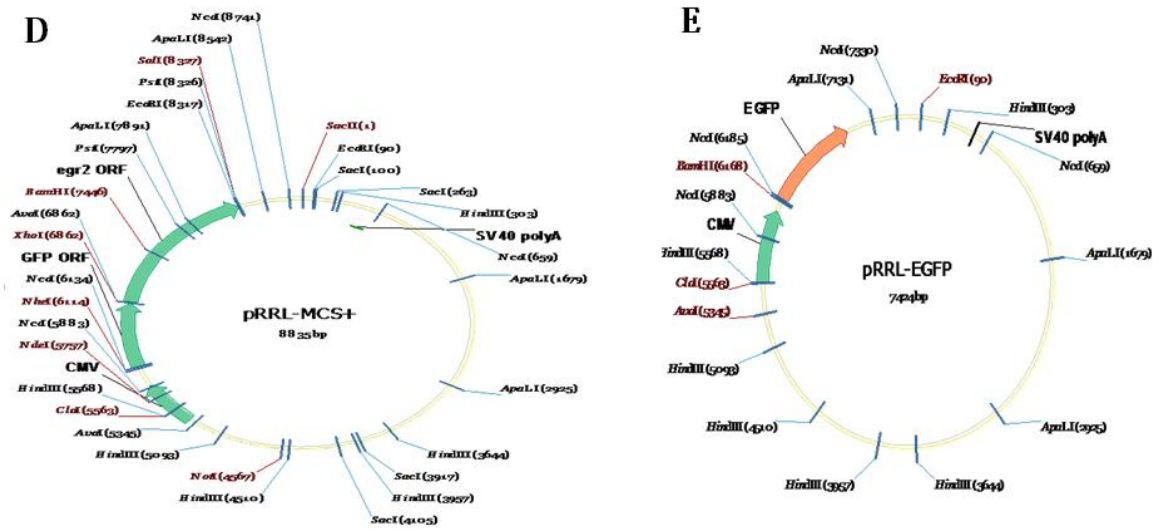

Figure 3-12. Cloned open reading frame of Egr-2 in Lentiviral construct

(D) Egr-2 was introduced into lentiviral construct by PCR cloning approach. In addition to Egr-2, the construct carries an IRES-driven GFP. A Map of vector sites from a plasmid containing the cloned open-reading frame of Egr-2 (E) The Egr-2 gene was orientated from 5' to 3' under CMV promoter Maps were created by Vector NTI by Invitrogen.

The positive clones were sequenced to confirm the integrity of Egr-2 gene. Lentivirus was replicated in packaging cells (Miao, Wu et al. 2006). A stock of 10^{12}/ml was harvested and stored in liquid nitrogen. To increase the transduction efficiency, the Egr-2-lentivirus was incubated with naive $CD4^+$ T cells throughout the in vitro culture. The Egr2 positive cells were identified and isolated based on GFP signal. Lentivirus without insert was served as control. Thus, we reconstituted Egr-2 expression in $CD4^+$ T cells from Egr-2 cKO mice before Th17 differentiation (Figure 3-13 A). The restoration of Egr-2 expression in naive $CD4^+$ T cells from Egr-2-deficient mice reduced the percentage of IL-17 producing $CD4^+$ T cells after Th17 differentiation in comparison to the naive $CD4^+$ T cells transduced with control lentivirus (Figure 3-13 B and C).

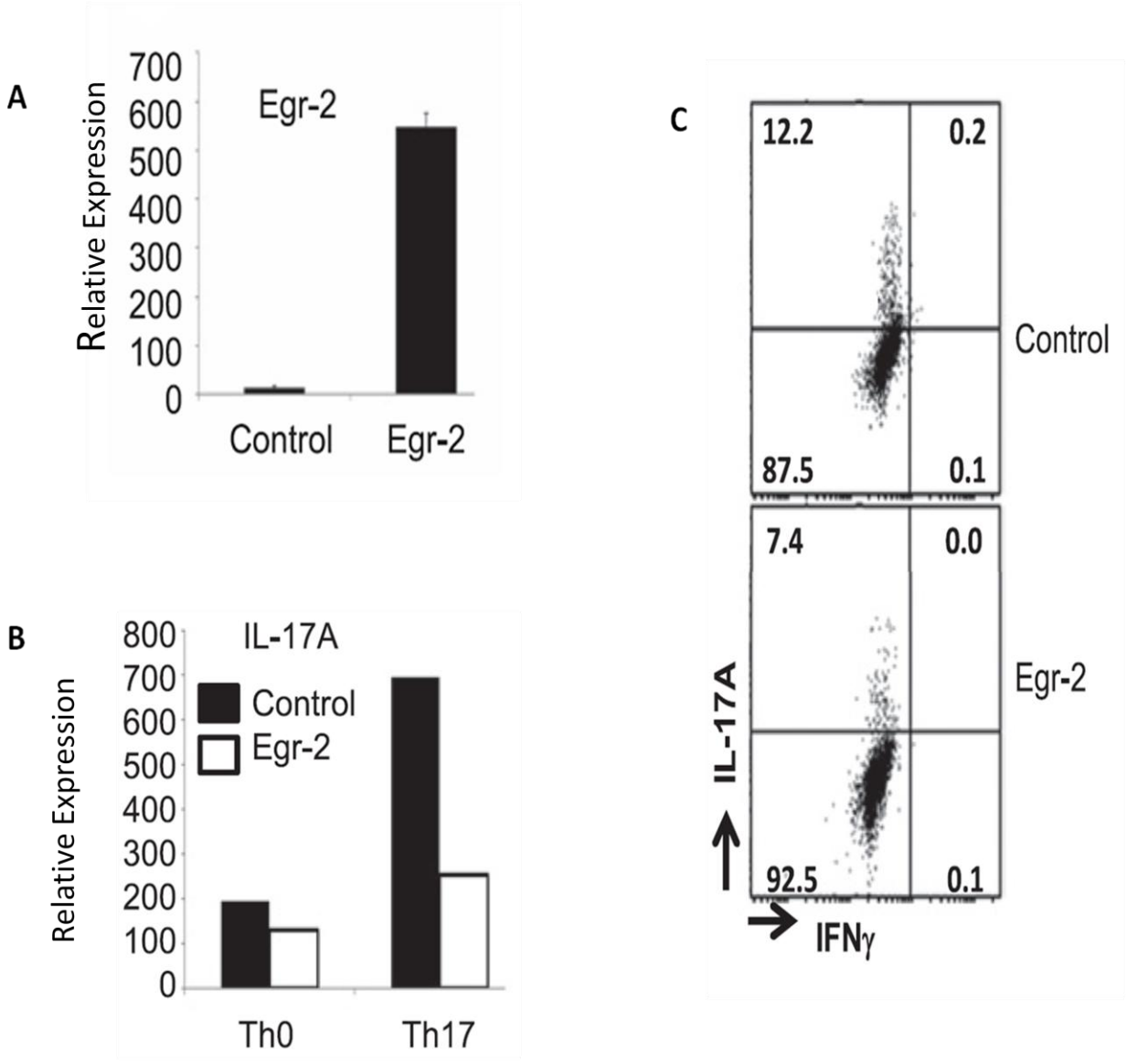

Figure 3-13. Regulating Egr-2 expression alters Th17 expression

(A) *CD4 T cells from Egr-2 cKO mice were transduced with Egr-2-lentivirus-IRES–GFP (Egr-2) or lentivirus-IRES–GFP (control) and the GFP$^+$ cells were analysed for the relative expression of Egr-2 by Real Time QPCR.* ***(B)****These transduced cells were then differentiated in to Th17 cells. The GFP$^+$ cells were analysed for mRNA relative expression of IL-17A by Real Time qPCR*

or (C) for cytokine producing cells of IL17A and IFNγ as measured by FACS. Data (A and B) represents three independent experiments; (C) represents two independent experiments.

To further confirm the function of Egr-2 in the control of Th17 differentiation, the normal expression of Egr-2 in naive $CD4^+$ T cells was knocked down by Egr-2 specific siRNA (Figure 3-14 A). The Egr-2-specific siRNAs selectively reduced Egr-2 expression in wild-type $CD4^+$ T cells following stimulation while control siRNA did not (Figure 3-14 A). Consistently, knockdown of Egr-2 expression by siRNA, in wild-type $CD4^+$ T cells resembled the altered Th17 differentiation of Egr-2-deficient $CD4^+$T cells. With enhanced fraction of IL-17 producing $CD4^+$ T cells following Th17 differentiation (Figure 3-14 A, B and C). Thus, Egr-2 selectively controls Th17 differentiation and expression of Th17 cytokines, which is consistent with the selectively altered expression of Th17 cytokines in naive $CD4^+$ T cells from Egr-2 cKO mice in response to TCR stimulation (Figure 3-5A).

Considering the overlapped function between Egr-2 and Egr-3, we also examined Th17 differentiation in Egr-3-deficient $CD4^+$ T cells *in vitro*. This dysregulation of Th17 differentiation was not observed in Egr-3 deficient naive $CD4^+$ T cells (data not shown). Although the normal phenotype of Th17 differentiation from Egr-3-deficient $CD4^+$ T cells may result from the function of wild-type Egr-2, our results at least demonstrate that among the Egr molecules, Egr-2 predominantly control the Th17 differentiation. Thus, Egr-2 plays an important role in the control of Th17 differentiation.

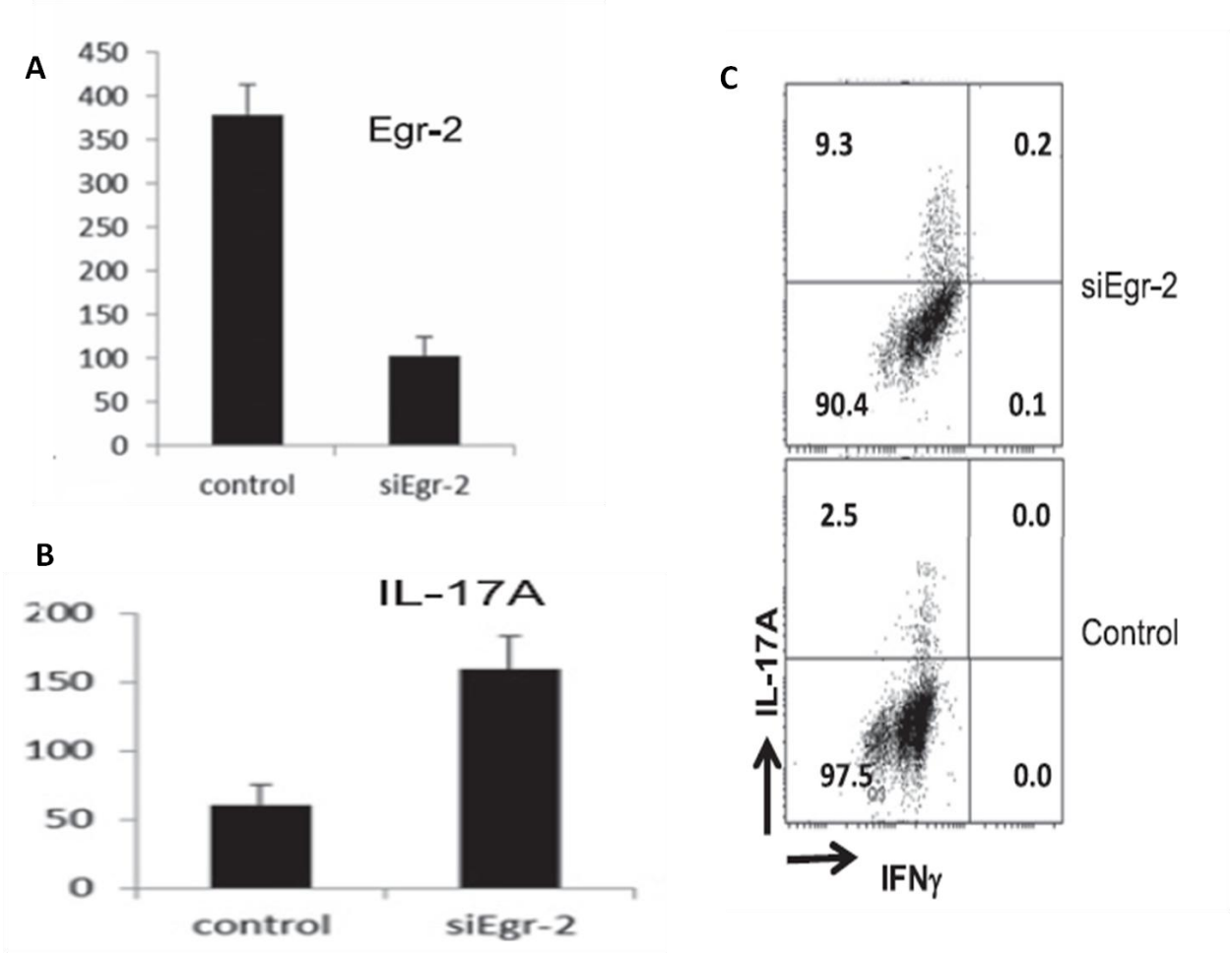

Figure 3-14. Knocking out Egr-2 alters TH17 expression

(A) CD4$^+$ cells from wild-type mice were differentiated into Th17 cells after transfection with siRNAs against Egr-2 or control siRNA. The transfected cells were cultured under Th17 conditions for 3 days (see Materials and Methods) before analysis of Egr-2 expression or (B) IL-17A expression or (C) for cytokine producing cells of IL17A and IFN-γ as measured by FACS Data (A and B) represents three independent experiments; (C) represents two independent experiments.

3.5 Egr-2 interacts with Batf and blocks its binding to the IL-17 promoter

Th17 differentiation is regulated by a number of master regulatory molecules. However, the repressors for Th17 differentiation and plasticity are largely unknown. In order to investigate the mechanisms by which Egr-2 controls Th17 differentiation, we analysed the major regulatory pathways involved in the differentiation of Th17 cells. Despite the increased Th17 differentiation, we did not find differences in the expression levels of the well-defined regulators such as RORγt/RORc, IRF4, aryl hydrocarbon receptor (Ahr), Ικbζ, or Batf by RT-PCR (Figure 3-15) indicating that Egr-2 is not involved in the control of transcriptional activation of these genes. Altered cytokine expression in effector phenotype $CD4^+$ T cells has been found in aged Egr-2 deficient mice (Zhu, Symonds et al. 2008). A few of the inflammatory cytokines sign through STAT3 pathways which are also involved in IL-6 signaling pathway during Th17 differentiation (Harris, Grosso et al. 2007). However, we could not detect altered activation of STAT3 in Egr-2-deficient Th17 cells (data not shown).

When we examined transcriptional activity, we discovered that the binding of Batf to DNA binding sites from the IL-17 promoters in Th17 cells was significantly increased in the absence of Egr-2 (Figure 3-16A), suggesting that Egr-2 may control Batf function in the regulation of IL-17 expression. Batf is required for Th17 differentiation and expression of IL-17 (Schraml, Hildner et al. 2009). The function of Batf is independent to the other Th17 regulators (Schraml, Hildner et al. 2009). Similar to Egr-2, Batf was induced in Th1, Th2, and Th17 cells in response to anti-CD3 stimulation (Figure 3-16 C), suggesting that the antagonistic function of Egr-2 and

Batf in Th17 differentiation is conditional. Despite the increase in binding to Batf sites in the IL-17 promoter, we did not find increased expression of Batf in naive $CD4^+$ T cells or Th17 cells derived from Egr-2 cKO mice (Figure 3-16 B), suggesting that Egr-2 does not transcriptionally regulate Batf. Thus, Egr-2 either directly inhibits Batf function or regulates Batf's repressors in Th17 cells. To investigate whether Egr-2 antagonizes Batf function directly, we analysed Batf immunoprecipitates from wild-type $CD4^+$ T cells after TCR stimulation. The immunoprecipitates of anti-Batf antibody were analysed by immunoblotting with anti-Batf and anti-Egr-2 antibodies, respectively. Both Egr-2 and Batf were discovered in the Batf precipitates (Figure 3-16 C).

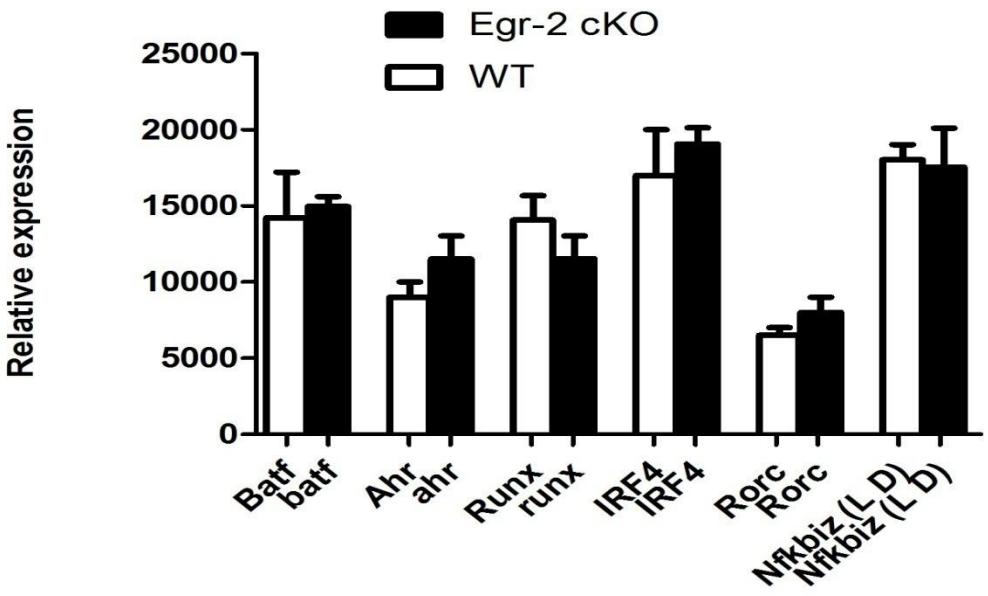

Figure 3-15. No difference in expression levels between WT and Egr-2 cKO mice in well defined transcription factors related to Th17

WT and Egr-2 cKO CD4^{+}T cells were differentiated to Th17 cells and mRNA expression levels were measured by Real Time qPCR for transcription factors, Batf, Ahr, Runx 1, IRF4, Rorc and Nfkbiz (L D) (a gene that encodes IκBζ). Data is representative of three individual experiments.

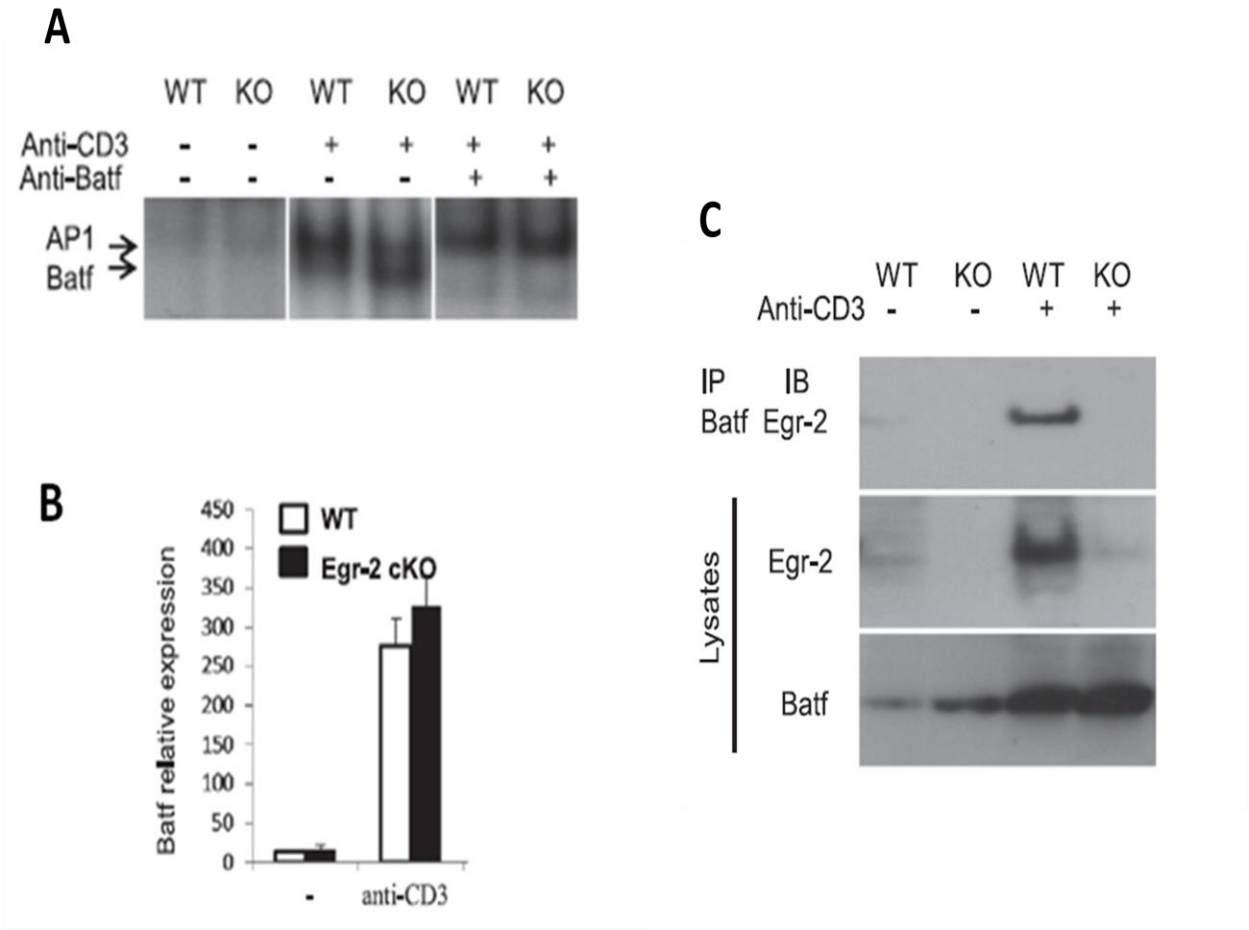

Figure 3-16.Interaction of Batf with EGR-2 blocks IL17 promoter

(A) Interaction of Batf from Th17 cells with a DNA sequence derived from the IL-17 promoter. The differentiated Th17 cells were restimulated with or without anti-CD3 and anti-CD28 for 6 h before nuclear proteins were extracted and analysed by EMSA. (B) Expression of Batf in Th17 cells from wild-type and Egr-2 cKO mice. The differentiated Th17 cells were restimulated with

or without anti-CD3 and anti-CD28 for 6 h. The expression of Batf was analysed by RT-qPCR. (C) Naive CD4$^+$ T cells from WT or Egr-2 cKO mice were stimulated with or without anti-CD3 and anti-CD28 for 36 hrs and then restimulated with the same stimuli for 6 h. The nuclear proteins were extracted and used for immunoprecipitation with anti-Batf. The precipitates were blotted with anti–Egr-2. The data represents two experiments (A and C) and three experiments (B).

To confirm whether other Egr molecules are also involved in the Batf complex, Batf precipitates were blotted by anti-Egr-1 and -3 antibodies, respectively. Neither Egr1 nor Egr3 were found in Batf precipitates (Figure 3-17A).Thus, Egr-2 and Batf directly interact in CD4$^+$ T cells raising the possibility that Egr-2 may regulate Batf by directly blocking its binding to promoters of Th17 cytokines. To further analyse the antagonistic function of Egr-2 in the regulation of Batf mediated IL-17 expression, we took advantage of an available IL17-reporter construct (Okamoto, Iwai et al. 2010). This reporter consists of 25kb region up stream of reading frame of IL17A gene (Okamoto, Iwai et al. 2010). Thus, we were able to measure the activity of an IL-17-promoter reporter gene (Okamoto, Iwai et al. 2010) in the presence of Batf alone or together with Egr-1, Egr-2 or Egr-3. In the presence of Batf, the trans-activation of the reporter gene was detected in comparing to the control reporter (data not shown).

So, Egr-2 directly suppresses Batf function in the induction of IL-17 expression. Batf is also involved in activation of other Th17 cytokine expression such as IL-21 (Schraml, Hildner et al. 2009). Although we did not have a reporter construct for the IL-21 gene, the altered expression of IL-21 in Egr-2-deficientCD4$^+$ T cells following Th17 differentiation indicates that Egr-2 is involved in the control of Th17 cytokine expression resulting in the suppression of Th17 differentiation.

The trans-action of the reporter induced by Batf was suppressed by co-transfection of Egr-2, but not Egr-1 or -3. Egr-2 effectively suppressed Batf mediated trans-activation (Figure 3-17 B).

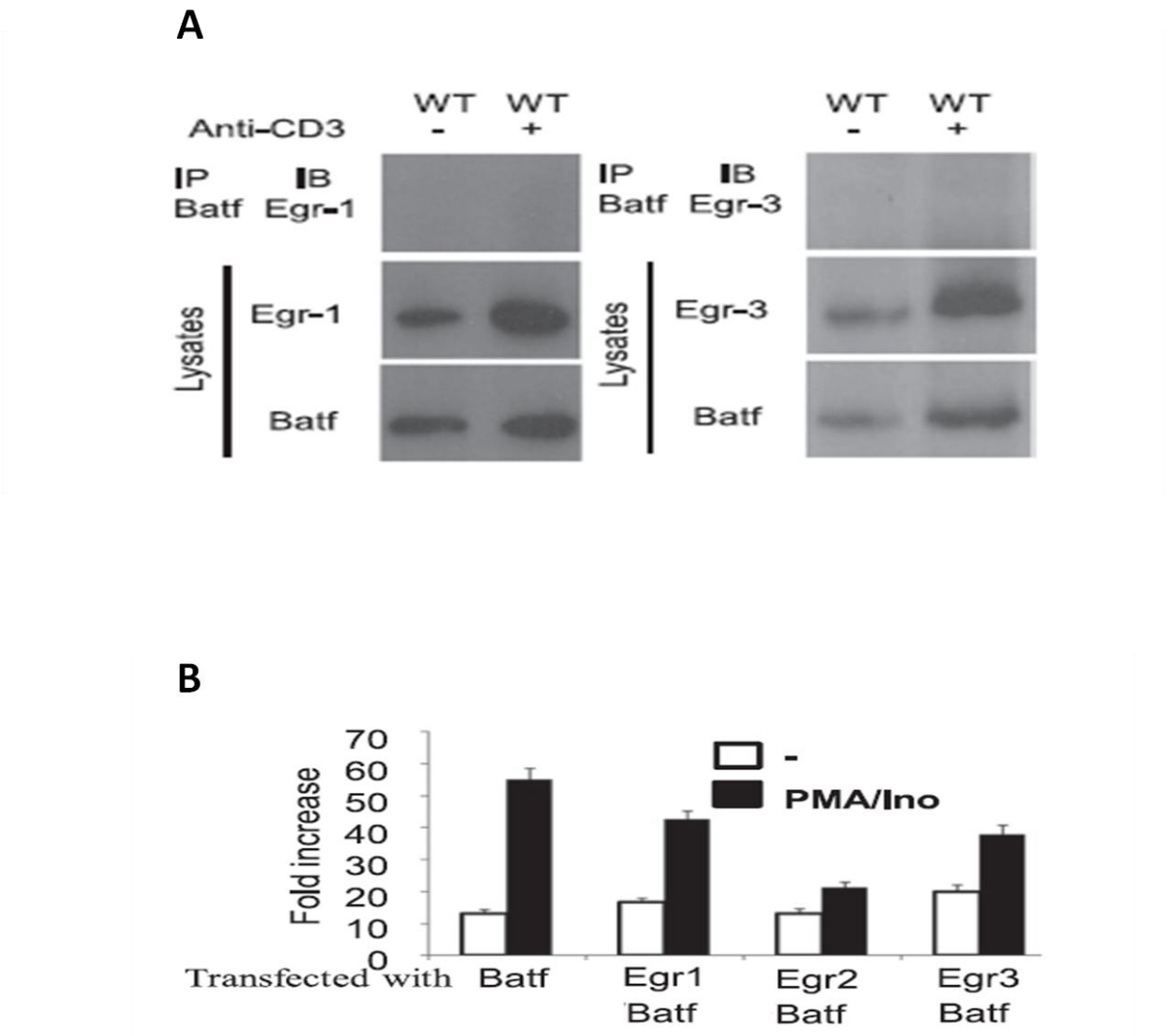

Figure 3-17 . Only Egr-2 not Egr-1or Egr-3 interacts with Batf

(A) *Naive CD4 T cells from wild-type or Egr-2 cKO mice were stimulated with or without anti-CD3 and anti-CD28 for 36 h and then restimulated with the same stimuli for 6 h. The nuclear*

proteins were extracted and used for immunoprecipitation with anti-Batf. The precipitates were blotted with anti-Egr-1 and anti-Egr-3. (B) IL-17 promoter reporter gene assay. The IL-17 promoter-reporter gene was co-transfected with the indicated genes into HEK293T cells. Cells were stimulated with PMA and ionomycin for 30 min before reporter gene activity was measured. The data presented is a fold increase above the background signal from reporter gene transfected cells.

3.6 Egr-2 cKO mice are susceptible to the induction of Experimental Autoimmune Encephalomyelitis (EAE)

Th17 cells are the major pathological T cells in the development of EAE. Experimental autoimmune encephalomyelitis (EAE) is the most commonly used experimental model for the human inflammatory demyelinating disease, multiple sclerosis (MS). EAE is a complex condition in which the interaction between a variety of immune pathological and neuropathological mechanisms leads to an approximation of the key pathological features of MS: inflammation, demyelination, axonal loss and gliosis. The counter-regulatory mechanisms of resolution of inflammation and remyelination also occur in EAE, which, therefore can also serve as a model for these processes. Moreover, EAE is often used as a model of cell-mediated organ-specific autoimmune conditions in general (Constantinescu, Farooqi et al. 2011). It has been reported that Batf deficiency results in resistance to EAE induction owning to the reduction of Th17 cells in disease animals (Schraml, Hildner et al. 2009). To examine whether the control of Th17 differentiation by Egr-2 can affect immune pathology in autoimmunity, we examined disease progression in mice after immunization with the myelin protein in the presence of

complete adjuvants as described. (Schraml, Hildner et al. 2009). The disease progression is monitored according to the clinical score systems (Bettelli, Carrier et al. 2006). Since Egr-2 cKO mice develop spontaneous autoimmunity in later life, naive mice aged 10-12 weeks, well before the onset of autoimmunity, were used for the induction of EAE. We discovered that the onset of the diseases was similar in wild-type and Egr-2 cKO mice (Fig3.12A) suggesting that Egr-2 deficiency in T cells did not affect the autoimmune responses against self-antigens. However, the disease was more severe in Egr-2 cKO mice compared to wild-type mice as demonstrated by higher clinical scores (Figure 3-18 A).

To confirm the cellular mediated autoimmunity, infiltration of mononuclear cells into inflammatory lesions in spinal cords was examined when mice were scored 3 or above (Figure 3-18 B and C).

The infiltration of mononuclear cells were detected in spinal cords of both wild-type and Egr-2 cKO mice (Figure 3-18 B). However, the levels of infiltration in Egr-2 cKO mice were much severer than wild-type mice (Figure 3-18 B). The levels of IL-17 expressing $CD4^+$ T cells were also analysed in mononuclear cells from spinal cords of diseased mice. The percentage of $CD4^+$ T cells in infiltrated mononuclear cells in inflamed spinal cords of Egr2 cKO mice was higher than that of wild-type mice (Figure 3-18B). Importantly, the infiltrated $CD4^+$ T cells expressing IL-17A were more in Egr-2 cKO mice than in wild-type mice (Figure 3-18 C).

Although we cannot rule out the effect of other inflammatory factors, these results suggest that Egr-2 is important for the control of Th17 cell driven pathology and for the control of the development and severity of autoimmune disease. Thus, consistent with the development of

lupus like autoimmune disease in aged Egr-2 cKO mice, these results demonstrate that the regulatory function of Egr-2 in the control of Th17 differentiation and cytokine expression is important for the control of autoimmunity.

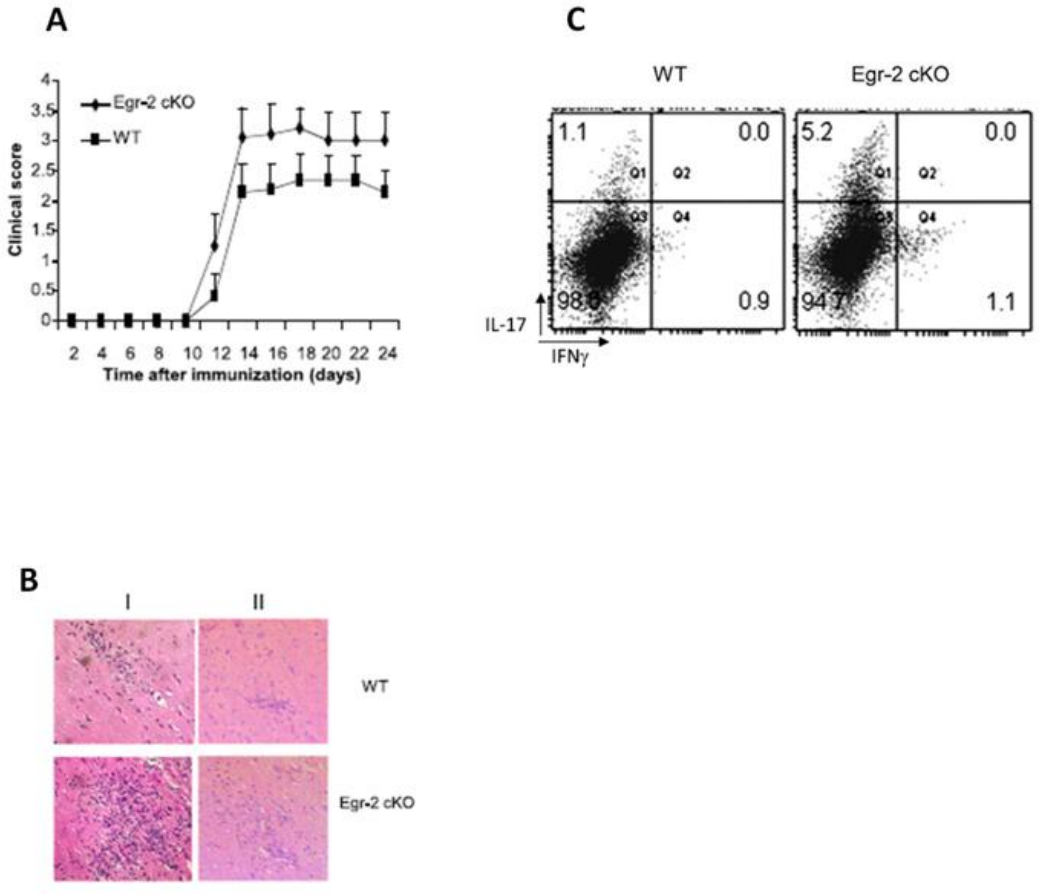

Figure 3-18. EGR-2 cKO mice are more susceptible to EAE induction

(A) Timeline showing the clinical scores of five 12-week-old WT or Egr-2 cKO mice immunized with MOG peptides. The clinical symptoms were scored according to standardprotocols (see Materials and Methods).(B) H&E staining of CNS tissues from two (I and II) diseased mice 30 days after immunization of MOG EAE peptide. Original magnification x10. (C) Mononuclear cells pooled from CNS tissues from five diseased mice in each group were stained with anti

human CD4, anti human IFN-γ, and anti human IL-17A. The data shown are after gating on $CD4^+$ cells. The data are representative of three experiments with five mice for each group.

3.7 Altered expression of Egr-2 and IL-17 in T cells from Multiple Sclerosis patients

The severe EAE observed in Egr-2 cKO mice suggests that Egr-2 maybe involved in the control of multiple sclerosis development. It has been found that IL-17 is highly expressed in central nervous system-infiltrating T cells and glial cells and is associated with active disease in multiple sclerosis (MS) (Tzartos, Friese et al. 2008). Based on the increased production of IL-17 in Egr-2 deficient $CD4^+$ T cells and the high susceptibility of Egr-2 cKO mice to EAE induction, it suggests that Egr-2 may be important for the control of MS development. Egr-2 is expressed at a basal level in naive T cells and is rapidly induced by TCR stimulation. To investigate the possible role of Egr-2 in the control of MS development, we analysed the expression of Egr-2, Batf and IL-17 in T cells from MS patients. Our hypothesis is that the high level of IL-17 positive T cells in MS patients may associate with defective Egr-2 function. To investigate this, we isolated $CD4^+$ T cells from peripheral blood lymphocytes of treatment-naive MS patients. The $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 in vitro for 48 hours or left unstimulated, and then the expression of Egr-2, Batf and IL-17 was analysed by RT-PCR. Consistent with the expression patterns in murine naive T cells, the expression of Egr-2 and Batf in T cells from healthy donors was highly induced in response to TCR stimulation (Figure 3-19 A and C), while IL-17 was barely detected (Figure 3-19 B).

However, in $CD4^+$ T cells from MS patients, although the levels of Batf induced in response to TCR stimulation were similar to that seen in healthy donors, the levels of Egr-2 expression were significantly lower among the patients tested (Figure 3-19 A and C). In contrast to the reduced expression of Egr-2, the mean expression of IL-17 in patient group was increased in T cells from MS patients compared with healthy controls (Figure 3-19B). Due to limited availability of patient $CD4^+$ T cells, we were not be able to manipulate $CD4^+$ T cells from patients for transduction of Egr-2 or knockdown of Batf. Therefore, we do not know whether the downregulation of Egr-2 and increased expression of IL-17 is associated with uncontrolled Batf activity in T cells from MS patients. Nevertheless, our results provide direct evidence that Egr-2 expression in effector T cells may be important for the control of Th17-mediated inflammation which is believed to be part of the mechanism underlying MS development.

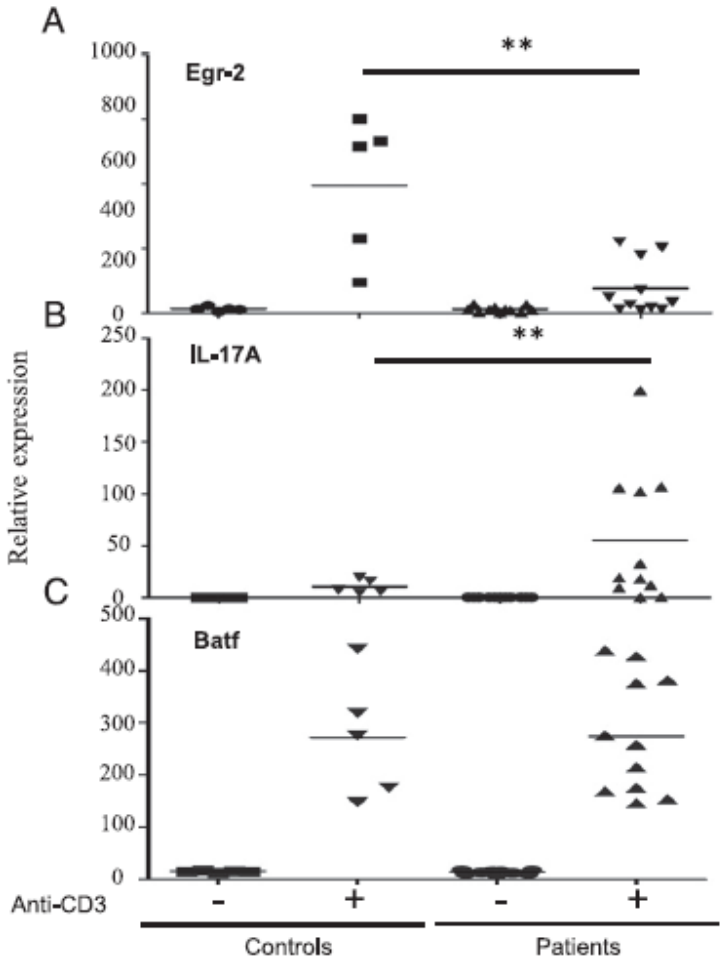

Figure 3-19. Altered expression of Egr-2 and IL17A in CD4 T cells but not Batf in patients with MS

*CD4 T cells were isolated from blood lymphocytes of 11 treatment-naive MS patients and five healthy donors and stimulated withanti-CD3 and anti-CD28 for 48 h. The cells were then restimulated for 6 hand the expression of Egr-2 (A), IL-17A (B), and Batf (C) was analysed by RT-qPCR. The data are represented relative to the expression of mRNA of β-actin and from two experiments. **p, 0.01.*

CHAPTER 4

DISCUSSION

4 DISCUSSION

4.0 Discussion

Regulation of Th differentiation and plasticity is the centre part of adaptive immunity. Although most of the master regulators such as T-bet for Th1, Gata3 for Th2 and RORγt for Th17 have been well defined for their requirement for differentiation, the regulatory mechanisms for these master transcription factors and the molecules control the plasticity of differentiated Th cells are still largely unknown. Th17 is closely associated with autoimmune pathology and found to be present in many autoimmune diseases such as MS, lupus and IBD (Jadidi-Niaragh and Mirshafiey 2011; Henriques, Ines et al. 2012; Hundorfean, Neurath et al. 2012). Despite the requirements of RORgt and STAT3 in the differentiation of Th17, recently, it has been found that IRF4, aryl hydrocarbon receptor (Ahr), Iκbζ, and Batf are also required for Th17 differentiation (Brustle, Heink et al. 2007; Veldhoen, Hirota et al. 2008; Schraml, Hildner et al. 2009; Okamoto, Iwai et al. 2010; Mudter, Yu et al. 2011). However, among them, Batf functions independent on RORγt and STAT3 (Schraml, Hildner et al. 2009). Batf is a transcription factor that directly binds to cis-regulatory regions of multiple Th17 cytokines such as IL-17 and IL-21 during Th17 differentiation (Betz, Jordan-Williams et al. 2010). Egr-2 has been discovered to be involved in T cell tolerance (Harris, Bishop et al. 2004; Safford, Collins et al. 2005; Anderson, Manzo et al. 2006). Its function in T cells is largely unknown (Li, Symonds et al. 2011). Previously, we found that Egr-2 is induced in both tolerant and naive T cells following antigen stimulation *in vivo* and deficiency of Egr-2 in T cells results in the development of lupus-like

disease in later life with accumulation of activated T cells (Zhu, Symonds et al. 2008). However, although Egr-2 is highly induced in naive T cells following antigen stimulation (Anderson, Manzo et al. 2006), a defect in Egr-2 does not increase T cell activation in response to TCR stimulation, suggesting a role in T cell differentiation (Zhu, Symonds et al. 2008). We have now demonstrated that Egr-2 deficiency results in uncontrolled production of Th17 cytokines in naive $CD4^+$ T cells in response to TCR stimulation and renders $CD4^+$ T cells prone to Th17 differentiation, which contributes to increased susceptibility to EAE induction. The importance of Egr-2 in the control of Th17 driven pathology is further supported by the defective induction of Egr-2 and increased expression of IL-17 in T cells from treatment-naive MS patients.

Th17 cells, one of the major Th subsets, can be generated by differentiation of naive $CD4^+$ T cells in response to antigen and Th17 mediators such as IL-6 and TGFβ (Hirota, Martin et al. 2010; Ghoreschi, Laurence et al. 2011). However, in contrast to Th1 and Th2 cytokines, such as IFN-γ and IL-4, which are highly induced in naive T cells in response to T cell receptor ligation, the induction of Th17 cytokines, such as IL-17 and IL-21, was barely detected in activated naive T cells from wild-type mice in the absence of Th17 mediators. This suggest that the expression of Th17 cytokines in effector T cells is tightly regulated. A number of regulatory factors have been discovered for inhibiting expression of Th17 cytokines and regulating Th17 differentiation (Ivanov 2006; Brustle, Heink et al. 2007; Harris, Grosso et al. 2007; Veldhoen, Hirota et al. 2008; Schraml, Hildner et al. 2009; Hirahara, Ghoreschi et al. 2010; Hirota, Martin et al. 2010; Okamoto, Iwai et al. 2010; Ghoreschi, Laurence et al. 2011). Most of them act by inhibiting the expression, or antagonizing the function, of RORγt, such as Runx1, T-bet and by inhibiting

STAT3 activation, such as SOCS3, or by promoting Foxp3 expression, a counter-activator essential for Treg development (Hirahara, Ghoreschi et al. 2010). The RORγt was unchanged in Egr-2-deficient CD4 T cells under Th17 differentiation conditions. Batf is one of the newly discovered transcription factors that have regulatory function in Th17 differentiation (Schraml, Hildner et al. 2009). In contrast to many other regulatory factors, Batf acts directly to Th17 cytokines such as IL17 and IL21 for their transactivation (Betz, Jordan-Williams et al. 2010). It does not however affect the Th1 and Th2 differentiations (Schraml, Hildner et al. 2009), suggesting that its function for Th17 cytokine expression is antagonized in Th1 and Th2 cells. We have now found that Egr-2 interacts with Batf to suppress Th17 cytokine expression. Despite the structure similarity and overlapping function in T cell development (Decker, Nehmann et al. 2003), we could not detect Egr-1 and -3 in anti-Batf precipitates (data not shown) suggesting a selective interaction between Egr-2 and Batf. In contrast to the suppression of RORγt expression in Th1 and Th2 cells, Batf is induced in all types of effector CD4 T cells in response to TCR ligation (Schraml, Hildner et al. 2009), suggesting that its Th17-promoting function is suppressed in effector T cells to avoid unwanted Th17 differentiation. TCR stimulation induces expression of both Egr-2 and Batf in all types of effector Th cells. The induction of Th17 cytokines, such as IL-17 and IL-21, in Egr-2-deficient T cells following TCR stimulation suggests that Egr-2 plays an important role in the control of Th17 cytokine expression in CD4 T cells in response to mitogenic antigen stimulation. The antagonistic function of Egr-2 on Batf in the regulation of Th17 cytokines in effector T cells may be essential for the control of Th17-mediated immunopathology during an adaptive immune response. Although Egr-2 and Batf are induced by TCR stimulation in all types of Th cells, we found that Egr-2 is selectively induced

by TGFβ and IL-6, but not IL-4, IL-12, or IFNγ, indicating a feedback mechanism involving Egr-2 that serves to control Th17 differentiation. TGFβ and IL-6 signal through STAT3 and Smad pathways, respectively (Harris, Grosso et al. 2007; Yang, Panopoulos et al. 2007; Martinez, Zhang et al. 2009), which are distinct from TCR signaling pathways. Induction of Egr-2 by both TCR and TGFβ suggests that Egr-2 induction can be regulated by multiple stimuli with distinct signaling pathways, which may be coordinated in T cells during adaptive immune responses. Although the mechanisms for the induction of Egr-2 expression by TGFβ and IL-6 are unknown, the increased expression of Egr-2 in Th17 cells indicates that the induction of Egr-2 expression and Th17 differentiation by TGFβ and IL-6 are likely to occur via similar mechanisms, which have yet to be fully investigated.

The importance of Egr-2 in the control of immune pathology in autoimmune diseases is highlighted by the susceptibility of Egr-2 cKO mice to the induction of EAE. The increased severity of disease and enhanced infiltration of inflammatory cells in Egr-2 cKO mice is associated with high levels of IL-17 producing CD4 T cells. The high susceptibility of Egr-2 cKO mice to EAE induction is stark contrast to the reduced EAE induction in Batf deficient mice (Schraml, Hildner et al. 2009) indicating that the repression of Batf function by Egr-2 contributes to the development of EAE. EAE is cellular mediated autoimmune diseases with complex pathological changes (Constantinescu, Farooqi et al. 2011). The resistance of RORγt and/or STAT3 knockout models to EAE induction indicates complex regulatory mechanisms for Th17 pathology in EAE (Ivanov 2006; Yang, Panopoulos et al. 2007). It is therefore unlikely that Batf is the most important factor in EAE development. Nevertheless, the resistance for EAE induction

demonstrates the importance of Batf in the development of Th17-mediated autoimmune diseases (Schraml, Hildner et al. 2009). Thus, the susceptible EAE development in Egr2 cKO mice highlights the function of Egr-2 in the control of autoimmune diseases with likelihood mechanisms for inhibiting Batf function.

The defective expression of Egr-2 and its association with increased production of IL-17 in $CD4^+$ T cells from treatment-naive MS patients is consistent with previous reports (Tzartos, Friese et al. 2008) and further indicates that impaired expression of Egr-2 in activated T cells may contribute to MS development. Egr-2 expression was found to be important for T cell tolerance and its expression in anergic T cells is regulated by NFAT (Harris, Bishop et al. 2004; Lazarevic, Zullo et al. 2009). The dysregulated expression of Egr-2 and IL-17, but normal induction of Batf, in $CD4^+$ T cells from MS patients resemble the expression profile of these molecules in $CD4^+$ T cells from Egr-2 cKO mice, suggesting that uncontrolled Batf activity may contribute to the increased IL-17 production and immunopathology in MS.

Taken together, this study has demonstrated a previously unknown function of Egr-2 in the control of Th17 cytokine expression and Th17 differentiation by inhibition of Batf function. The susceptibility of Egr-2 cKO mice to EAE induction and the reduced induction of Egr-2 in T cells from MS patients after TCR stimulation further indicate the importance of Egr-2 in the control of autoimmunity and Th17-mediated inflammation.

THE JOURNAL OF IMMUNOLOGY

Early Growth Response Gene-2 Controls IL-17 Expression and Th17 Differentiation by Negatively Regulating Batf

Tizong Miao, Meera Raymond, Punamdip Bhullar, Emma Ghaffari, Alistair L. J. Symonds, Ute C. Meier, Gavin Giovannoni, Suling Li and Ping Wang

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Early Growth Response Gene-2 Controls IL-17 Expression and Th17 Differentiation by Negatively Regulating Batf

Tizong Miao,*,[1] Meera Raymond,*,[1] Punamdip Bhullar,† Emma Ghaffari,† Alistair L. J. Symonds,* Ute C. Meier,* Gavin Giovannoni,* Suling Li,† and Ping Wang*

Early growth response gene (Egr)-2 is important for the maintenance of T cell homeostasis and controls the development of autoimmune disease. However, the underlying mechanisms are unknown. We have now discovered that Egr-2, which is induced by TGF-β and IL-6, negatively regulates the expression of IL-17, but not IL-2 or IFN-γ, in effector T cells. In the absence of Egr-2, CD4 T cells produce high levels of Th17 cytokines, which renders mice susceptible to experimental autoimmune encephalomyelitis induction. T cells lacking Egr-2 show increased propensity for Th17, but not Th1 or Th2, differentiation. Control of IL-17 expression and Th17 differentiation by Egr-2 is due to inhibition of Batf, a transcription factor that regulates IL-17 expression and Th17 differentiation. Egr-2 interacts with Batf in CD4 T cells and suppresses its interaction with DNA sequences derived from the IL-17 promoter, whereas the activation of STAT3 and expression of retinoic acid–related orphan receptor γt are unchanged in Th17 cells in the absence of Egr-2. Thus, Egr-2 plays an important role to intrinsically control Th17 differentiation. We also found that CD4 T cells from multiple sclerosis patients have reduced expression of Egr-2 and increased expression of IL-17 following stimulation with anti-CD3 in vitro. Collectively, our results demonstrate that Egr-2 is an intrinsic regulator that controls Th17 differentiation by inhibiting Batf activation, which may be important for the control of multiple sclerosis development. ***The Journal of Immunology*****, 2013, 190: 000–000.**

T helper 17 cells differentiated from naive CD4 T cells provide protection in certain infections, but they play important roles in the immunopathology of autoimmune diseases (1), which has been demonstrated in several mouse models of autoimmune disease such as experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease, and collagen-induced arthritis, and they are also implicated in various human autoimmune diseases (2). Th17 differentiation is dependent on TGF-β, together with proinflammatory cytokines such as IL-6, IL-21, IL-23, and IL-1 (3). These cytokines induce expression and/or activation of the transcription factors retinoic acid–related orphan receptor (ROR)γt/RORα, STAT3, IFN regulatory factor-4, aryl hyrdrocarbon receptor, Iκbζ, and Batf, which individually or collectively drive expression of Th17 cytokines (4–10). The differentiation of Th17 cells and expression of Th17 cytokines are regulated to prevent excessive inflammation. However, little is known about the cell-intrinsic mechanisms that control Th17 differentiation and how they contribute to the control of autoimmune diseases.

Previously, we and others have shown that early growth response gene (Egr)-2 is induced in naive and tolerant T cells by Ag in vitro and in vivo (11–13). Egr-2 is a member of the Egr family of zinc finger transcription factors, which consists of four members: Egr-1, Egr-2, Egr-3, and Egr-4 (14). Egr-2 is essential for hindbrain development and myelination of the peripheral nervous system (15). Egr-1, Egr-2, and Egr-3 are expressed in thymocytes (16) and are involved in the development of double-negative thymocytes and in positive selection (17–20). Recently, expression of Egr-1 and Egr-2 has been found in NKT cells, which is required for the development of NKT cells (21, 22). In peripheral T cells, Egr-2 is involved in the induction of Fas ligand and the regulation of homeostasis (23, 24). RNA interference–mediated knockdown of Egr-2 in an established T cell line rendered the cells less susceptible to anergy induction (11), whereas overexpression of Egr-2 reduced T cell activation in vitro through induction of the E3 ligase Cbl-b (11, 12). Egr-2 has been found to be preferentially expressed in LAG3$^+$ regulatory T cells, and forced expression of Egr-2 converted naive CD4 T cells into IL-10–expressing LAG3$^+$ regulatory T cells (25), which further demonstrates the importance of Egr-2 in the regulation of immune homeostasis.

We have previously demonstrated that Egr-2 deficiency in T cells results in the development of autoimmune diseases in late life with hyperproliferation of effector phenotype T cells and accumulation of IFN-γ– and IL-17–producing CD4 T cells (26). However, despite this homeostatic disorder and development of autoimmune diseases, Egr-2 deficiency did not increase the activation or production of IL-2 by naive T cells induced by TCR ligation (26), suggesting that Egr-2 may be involved in the regulation of T cell subsets involved in the immunopathology of autoimmunity.

In this study, we show that Egr-2 regulates Th17 differentiation and expression of IL-17. CD2-specific Egr-2 deficiency resulted in

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The online version of this article contains supplemental material.

Abbreviations used in this article: cKO, conditional knockout; CsA, cyclosporine A; EAE, experimental autoimmune encephalomyelitis; Egr, early growth response gene; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; ROR, retinoic acid–related orphan receptor; RT-qPCR, real-time quantitative PCR; si, small interfering; SOM, self-organizing map.

the development of autoimmune diseases in later life, with accumulation of activated T cells and high levels of IL-17 expression in activated CD4 T cells (26). Egr-2 was induced by TGF-β and IL-6 in naive CD4 T cells. A defect in Egr-2 renders naive CD4 T cells highly prone to Th17 differentiation and increases production of Th17 cytokines. The Egr-2–deficient mice were highly susceptible to the induction of EAE. Importantly, we found that the expression of Egr-2 was reduced in activated T cells from multiple sclerosis (MS) patients. The reduced expression of Egr-2 was associated with increased expression of IL-17. We found that Egr-2 directly interacts with Batf, a Th17 inducer, and blocks its binding to the IL-17 promoter. Thus, Egr-2 induced during Th17 differentiation serves as a negative feedback inhibitor to control Th17-mediated inflammation.

Materials and Methods

Mice

CD2-specific Egr-2$^{-/-}$ mice on the C57BL/6 background were reported in our previous publication (26). C57BL/6 mice were used as controls in all experiments. All mice were maintained in the Biological Services Unit, Brunel University, and used according to established institutional guidelines under the authority of a U.K. Home Office project license.

Abs, reagents, and flow cytometry

Cyclosporin A (CsA) was from Sigma-Aldrich. FITC-conjugated Abs to IFN-γ and IL-4, PE-conjugated Ab to IL-17A, and allophycocyanin-conjugated Ab to CD4 were obtained from BD Biosciences. Egr-1, Egr-2, and Egr-3 Abs were purchased from Covance and Santa Cruz Biotechnology. Ab to pSTAT3 was from Cell Signaling Technology, and anti-BATF was obtained from Santa Cruz Biotechnology. For flow cytometry analysis, single-cell suspensions were analyzed on an LSRII (BD Immunocytometry Systems) and the data were analyzed using FlowJo (Tree Star).

Microarray

Naive CD4 T cells were stimulated with or without anti-CD3 for 6 h. Total RNA was extracted and genome-wild transcriptional profiles were analyzed using MouseRef-8 v2.0 BeadChip expression array (Illumina). The probe labeling, array hybridization, and data processing were carried out at the Microarray Facility, Barts and London School of Medicine and Dentistry, according to the manufacturer's instructions. We focused on genes that showed a difference of at least 3-fold between wild-type and Egr-2–deficient CD4$^+$ T cells and excluded genes that had a detection p value >0.01. The differentially expressed genes were first grouped based on self-organizing maps (SOMs) by a clustering algorithm method. The SOM clusters were further clustered by a hierarchical clustering program to confirm the different profile (ArrayExpress accession E-MEXP-1698; http://www.ebi.ac.uk/arrayexpress).

T cell differentiation

Naive CD4$^+$ T cells were isolated by a CD4$^+$CD62L$^+$ T cell isolation kit II (Miltenyi Biotec). The isolated cells were >95% CD4$^+$CD62L$^+$CD44^{low} CD25$^-$. For differentiation, cells were stimulated with anti-CD3 and anti-CD28 supplemented with 20 ng/ml IL-12 and 20 μg/ml anti–IL-4 for Th1 or 50 ng/ml IL-4 and 10 μg/ml anti–IFN-γ or 2 ng/ml TGF-β, 20 ng/ml IL-6, 10 μg/ml anti–IFN-γ, and 20 μg/ml anti–IL-4 for 3 d. The cells were washed and cultured in Th condition medium in the absence of anti-CD3 and anti-CD28 for 2 d. The differentiated cells were restimulated with or without anti-CD3 and anti-CD28 for 6 h before analysis.

Experimental autoimmune encephalomyelitis

Induction of EAE was carried out using a myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$/CFA emulsion kit (EK-0111; Hooke Laboratories) according to the manufacturer's instructions. The severity of EAE was scored on a scale of 0–4: 0, normal; 1, limp tail; 2, limp tail and weakness of hind legs; 3, limp tail with paralysis of one front and one hind leg; 4, limp tail, complete hind leg and partial front leg paralysis.

Thirty days after EAE induction mice were sacrificed and CNS tissues were fixed with 10% formalin in PBS and embedded in paraffin. Sections were stained with H&E. Histological examination of tissue sections was done in a blind manner. Additionally, CNS-infiltrating mononuclear cells were isolated and analyzed for IL-17– and IFN-γ–producing CD4 T cells by intracellular cytokine staining.

Patients and blood samples

Eleven relapsing and remitting MS patients in remission and five healthy donors were enrolled in this study. All MS patients were retrieved from the MS Center, Royal London Hospital, London. The study has been approved by our Ethics Committee. All patients were diagnosed as having relapsing and remitting MS according to the McDonald criteria (27), were drug naive in relation to disease-modifying therapy, and were in clinical remission. Blood was taken according to standard protocols, using heparinized tubes, after signed informed consent was obtained. CD4 T cells were isolated from blood lymphocytes by positive selection using MACS systems (Miltenyi Biotec) and stimulated in vitro for 48 h with anti-CD3 and anti-CD28 before restimulation for 6 h with the same stimuli. Total RNA extracted from these cells, and unstimulated cells, was analyzed for Batf, Egr-2, and IL-17A expression by real-time quantitative PCR (RT-qPCR). Patient characteristics are presented in Supplemental Table I.

Intracellular cytokine analysis

Splenocytes, CD4 T cells, and CNS-infiltrating mononuclear cells were stimulated with PMA (20 ng/ml) plus ionomycin (0.5 μg/ml) in the presence of brefeldin A for 5 h. After staining with cell surface markers, intracellular cytokine staining was performed with a fixation and permeabilization kit (eBioscience) and IFN-γ, IL-4, and IL-17A Abs in accordance with the manufacturer's instructions.

RT-qPCR

Total RNA was extracted from stimulated or unstimulated CD4$^+$ T cells using an RNeasy kit from Qiagen and reverse transcribed using oligo(dT) primers (Amersham Biosciences). RT-qPCR was performed on a Rotor-Gene system (Corbett Robotics) using SYBR Green PCR Master mix (Qiagen). Primers were as follows: mouse Egr-2, sense, 5′-CTTCAGCCGAAGTGACCACC-3′, antisense, 5′-GCTCTTCCGTTCCTTCTGCC-3′; β-actin, sense, 5′-AATCGTGCGTGACATCAAAG-3′, antisense, 5′-ATGCCACAGGATTCCATACC-3′; IL-2, sense, 5′-GCATGTTCTGGATTTGACTC-3′, antisense, 5′-CAGTTGCTGACTCATCATCG-3′; IL-4, sense, 5′-CAAACGTCCTCACAGCAACG-3′, antisense, 5′-CTTGGACTCATTCATGGTGC-3′; IL-17A, sense, 5′-AGCGTGTCCAAACACTGAGG-3′, antisense, 5′-CTATCAGGGTCTTCATTGCG-3′; IL-17F, sense, 5′-AACCAGGGCATTTCTGTCCC-3′, antisense, 5′-TTTCTTGCTGAATGGCGACG-3′; IFN-γ, sense, 5′-CCATCAGCAACAACATAAGC-3′, antisense, 5′-AGCTCATTGAATGCTTGGCG-3′; IL-21, sense, 5′-ATCCTGAACTTCTATCAGCTCCAC-3′, antisense, 5′-GCATTTAGCTATGTGCTTCTGTTTC-3′; Egr3, sense, 5′-CGACTCGGTAGCCCATTACAATCAGA-3′, antisense, 5′-GAGATCGCCGCAGTTGGAATAAGGAG-3′; Egr1, sense, 5′-ACGACAGCAGTCCCATCTACTCGG-3′, antisense, 5′-GGACTCGACAGGGCAAGCATATGG-3′; BATF, sense, 5′-GAGCTGCGTTCTGTTTCTCC-3′, antisense, 5′-CCAGAAGAGCCGACAGAGAC-3′; human β-actin, sense, 5′-CCCAGCACAATGAAGATCAA-3′, antisense, 5′-ACATCTGCTGGAAGGTGGAC-3′; human Egr-2, sense, 5′-CTTTGACCAGATGAACGGAG-3′, antisense, 5′-CCCATGTAAGTGAAGGTCTG -3′; human Batf, sense, 5′-ACACAGAAGGCCGACACC-3′, antisense, 5′-CTTGATCTCCTTGCGTAGAGC-3′; human IL-17A, sense, 5′-CTCCTGGGAAGACCTCATTG-3′, antisense, 5′-GAGGACCTTTTGGGATTGGT-3′.

The data were analyzed using the Rotor-Gene software. All samples were run in duplicate, and relative mRNA expression levels were obtained by normalizing against the level of β-actin from the same sample under the same program using the following: relative expression = $2^{(Ct\beta\text{-actin} - Ct\text{target})} \times 10{,}000$, where Ct indicates threshold cycle.

EMSA

Probes corresponding to a Batf binding site in the IL-17 promoter (5′-TGGTTCTGTGCTGACCTCATTTGAGGATG-3′; nucleotides −155 to −187) (10) were labeled with α-[^{32}P]dCTP using Ready-to-Go DNA labeling beads (Amersham Biosciences, Buckingham, U.K.) and used in binding reactions with nuclear extracts. Nuclear extracts were obtained as described in our previous report (26). For supershift reactions, anti-Batf was added after 10 min incubation. The samples were electrophoresed on 5% polyacrylamide gels in 0.5× TBE.

Reporter gene assay

Mouse Batf, Egr-1, Egr-2, and Egr-3 cDNAs were introduced into pcDNA expression vectors by PCR cloning approaches. The reporter plasmid IL-17-Luc (9) and the indicated expression plasmids were transfected into HEK293T cells using FuGENE 6 (Roche). After 36 h, a dual luciferase assay (Promega) was performed according to the manufacturer's protocol.

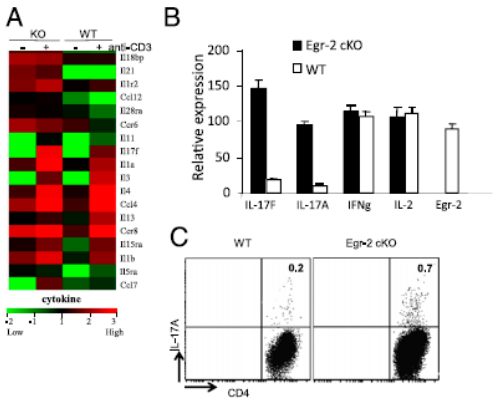

FIGURE 1. Egr-2 deficiency leads to enhanced expression of Th17 cytokines in naive CD4 T cells in response to TCR stimulation. (**A**) Altered expression of cytokine and cytokine receptor genes in Egr-2–deficient CD4 T cells as assessed by genome-wide transcriptional analysis. The SOM cluster of cytokines was further clustered by a hierarchical clustering program to show the genes with differential expression profiles. (**B**) Expression of cytokines in CD4 T cells as measured by RT-qPCR. (**C**) IL-17A–producing CD4 T cells were analyzed by intracellular cytokine staining after stimulation of splenocytes for 3 h with 100 ng/ml PMA/ionomycin in vitro. The PCR data are presented relative to the expression of β-actin mRNA. (B) and (C) are representative of three independent experiments. WT, Wild-type.

Lentiviral transduction

The lentiviral constructs used were described previously (28). Egr-2 was introduced into these constructs by a PCR cloning approach. In addition to Egr-2, the construct carries an IRES-driven GFP that allows us to isolate transduced cells by fluorescence-activated cell sorting. Naive CD4 cells from K2/3 mice at 1×10^6 per well in a 24-well plate coated with anti-CD3 and anti-CD28 and supplemented with 2 ng/ml TGF-β, 20 ng/ml IL-6, 10 μg/ml anti–IFN-γ, and 20 μg/ml anti–IL-4 were infected with concentrated lentivirus at a multiplicity of infection of 50–100 ($\sim 10^5$–10^6 transducing units/ng) as previously described (28). Two days after infection, fresh medium supplemented with 2 ng/ml TGF-β, 20 ng/ml IL-6, 10 μg/ml anti–IFN-γ, and 20 μg/ml anti–IL-4 was added. The cells were harvested after 5 d and the GFP^+ cells were isolated by cell sorting. The isolated cells were used for RT-qPCR analysis and analysis of IL-17–producing cells.

mRNA silencing

A specific small interfering (si) oligonucleotide (siRNA) targeting the mRNA sequence of Egr-2, siEgr-2-1 (5′-GCUGCUAUCCAGAAGGUAU-3′), was used. Irrelevant scrambled siRNA obtained from Qiagen (catalog no. 1027281) was used as a negative control. Primary naive $CD4^+$ T cells isolated from 6- to 8-wk-old mice were transfected with 1 μM siRNA using an Amaxa Nucleofector according to the manufacturer's instructions. Cells were stimulated with anti-CD3 and anti-CD28, which were supplemented with 2 ng/ml TGF-β, 20 ng/ml IL-6, 10 μg/ml anti–IFN-γ, and 20 μg/ml anti–IL-4, for 3 d before analysis of Egr-2 and IL-17 expression.

Statistical analysis

A Student unpaired *t* test was used to analyze the statistical significance of differences between the groups. Differences with a p value <0.05 were considered significant.

Results

Egr-2 controls expression of Th17, but not Th1 or Th2, cytokines in effector CD4 T cells

Previously, we found that, in association with the development of lupus like disease, Egr-2 deficiency induced high expression of IL-17 and IFN-γ in effector T cells in aged mice (26). To investigate the roles of Egr-2 in CD4 T cells, we carried out expression array analysis on unstimulated and anti-CD3– and anti-CD28–

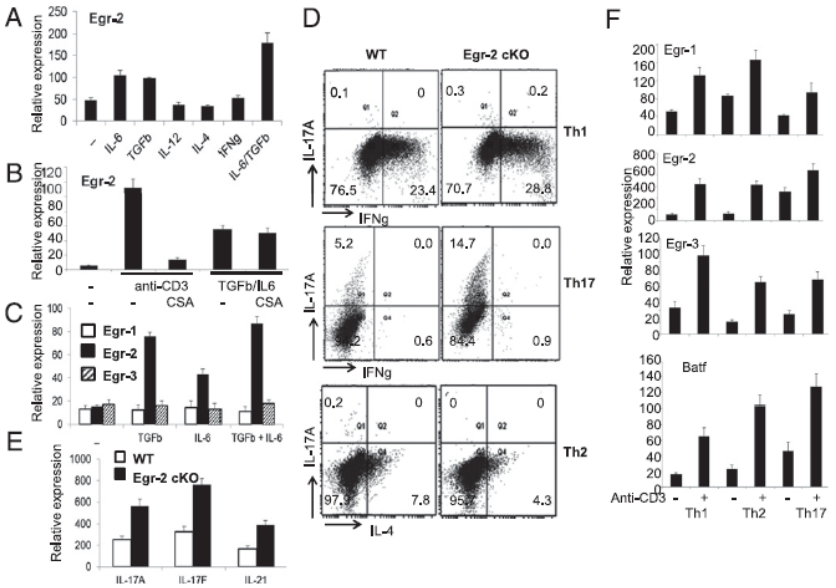

FIGURE 2. Egr-2 controls Th17 differentiation. (**A** and **B**) Expression of Egr-2 in naive CD4 T cells from wild-type mice following stimulation with the indicated cytokines (**A**) or with 5 μg/ml anti-CD3 and 2 μg/ml anti-CD28 or TGF-β/IL-6 in the absence or presence of 250 ng/ml CsA (B) for 6 h. (**C**) Expression of Egr-1, Egr-2, and Egr-3 in naive CD4 T cells from wild-type mice following stimulation with TGF-β, IL-6, or both. (**D**) Th differentiation of naive CD4 T cells from wild-type and Egr-2 cKO mice. Naive CD4 T cells were differentiated to Th cells as described in *Materials and Methods*. The differentiated CD4 cells were stained with the indicated Abs. (**E**) Expression of IL-17A, IL-17F, and IL-21 in Th17 cells differentiated from wild-type or Egr-2–deficient naive CD4 T cells. (**F**) Expression of Egr-1, Egr-2, Egr-3, and Batf in Th1, Th2, and Th17 cells stimulated for 6 h with or without 5 μg/ml anti-CD3 and 2 μg/ml anti-CD28. Data from (A)–(C), (E), and (F) are presented relative to the expression of β-actin mRNA. Data are representative of three (A–C, E, F) or five experiments (D). WT, Wild-type.

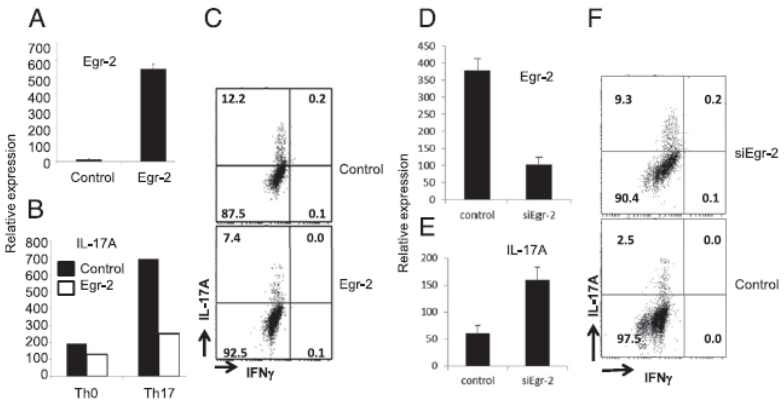

FIGURE 3. Regulation of Egr-2 expression alters Th17 differentiation. (**A**–**C**) CD4 T cells from Egr-2 cKO mice were transduced with Egr-2-lentivirus-IRES–GFP (Egr-2) or lentivirus-IRES–GFP (control) and differentiated into Th17 cells. The GFP$^+$ cells were analyzed for the expression of Egr-2 and IL-17A by PCR (A, B) or cytokine producing cells by flow cytometry (C). (**D**–**F**) CD4 cells from wild-type mice were differentiated into Th17 cells after transfection with siRNAs against Egr-2 or control siRNA. The transfected cells were cultured under Th17 conditions for 3 d (see *Materials and Methods*) before analysis of Egr-2 and IL-17A expression (D, E) by PCR or cytokine producing cells by flow cytometry (F). Results are representative of two independent experiments.

stimulated naive CD4 T cells from Egr-2 conditional knockout (cKO) mice. Among the genes associated with CD4 T cell function, IL-1, IL-17, and IL-21 were highly induced in Egr-2–deficient CD4 T cells whereas the expression of IL-2 and IFN-γ was unchanged in response to TCR stimulation (Fig. 1A, 1B). The selectively increased expression of Th17 cytokines in Egr-2–deficient CD4 T cells was confirmed by RT-qPCR (Fig. 1B). The expression of IL-2 and IFN-γ was effectively induced in both

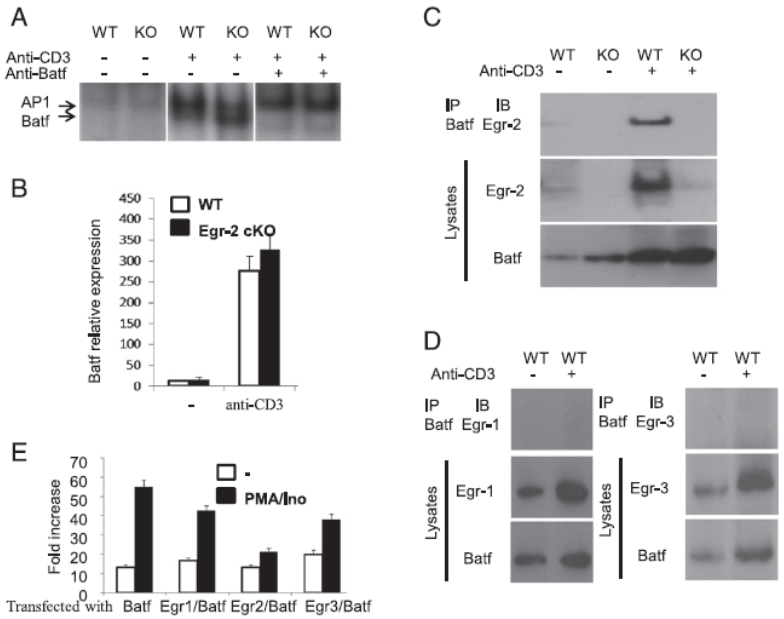

FIGURE 4. Egr-2 interacts with Batf and antagonizes its function in transactivation of the IL-17 promoter. (**A**) Interaction of Batf from Th17 cells with a DNA sequence derived from the IL-17 promoter. The differentiated Th17 cells were restimulated with or without anti-CD3 and anti-CD28 for 6 h before nuclear proteins were extracted and analyzed by EMSA. (**B**) Expression of Batf in Th17 cells from wild-type and Egr-2 cKO mice. The differentiated Th17 cells were restimulated with or without anti-CD3 and anti-CD28 for 6 h. The expression of Batf was analyzed by RT-qPCR and data are presented relative to the expression of β-actin mRNA. (**C** and **D**) Interaction of Batf with Egr-2, but not Egr-1 and Egr-3, in stimulated CD4 T cells. Naive CD4 T cells from wild-type or Egr-2 cKO mice were stimulated with or without anti-CD3 and anti-CD28 for 36 h and then restimulated with the same stimuli for 6 h. The nuclear proteins were extracted and used for immunoprecipitation with anti-Batf. The precipitates were blotted with anti–Egr-2 (C) or anti–Egr-1 and –Egr-3 (D). (**E**) IL-17 promoter reporter gene assay. The IL-17 promoter-reporter gene was cotransfected with the indicated genes into 293 cells. Cells were stimulated with PMA and ionomycin for 30 min before reporter gene activity was measured. The data are presented as fold increase above the background signal from reporter gene–transfected cells. The presented data are representative of two (A, C, D) and three (B, E) experiments. WT, Wild-type.

wild-type and Egr-2–deficient naive CD4 T cells in response to TCR stimulation (Fig. 1B), whereas the expression of Th17 cytokines, including IL-1, IL-17, and IL-21, was highly induced only in Egr-2–deficient CD4 T cells with minimal expression in CD4 naive T cells from wild-type mice (Fig. 1B). Consistent with this, the percentage of Egr-2–deficient CD4 cells that produced IL-17 was also increased compared with wild-type CD4 cells (Fig. 1C). Thus, Egr-2 is involved in the control of Th17 cytokine expression in effector T cells.

Egr-2 is induced in naive CD4 T cells by TGF-β and IL-6

Previously, we found that Egr-2 is effectively induced in naive T cells by Ag stimulation in vivo (13). The selective induction of Th17 cytokines in Egr-2–deficient CD4 T cells by TCR stimulation indicates that Egr-2 controls the expression of Th17 cytokines in T cells in response to mitogenic Ag stimulation. Th17 cytokines are normally induced in naive CD4 T cells in the presence of cytokines that can induce Th17 differentiation such as the well-defined Th17 inducers TGF-β and IL-6 (3). However, whether these stimuli can induce Egr-2 expression is unknown. We therefore examined the expression of Egr-2 in naive T cells following stimulation with different cytokines in vitro. The results showed that Egr-2 is rapidly induced in naive T cells following TGF-β and IL-6 stimulation (Fig. 2A). In contrast, IFN-γ and IL-12/IL-4, cytokines that can induce Th1 and Th2 differentiation, respectively, had little effects on Egr-2 expression in naive CD4 T cells (Fig. 2A). Egr-2 is induced in T cells by anti-CD3 stimulation and this induction is known to be inhibited by CsA (11). Consistent with this, we found that Egr-2 was strongly induced by anti-CD3 in naive T cells and the expression of Egr-2 was suppressed by CsA (Fig. 2B). However, the induction of Egr-2 by TGF-β and IL-6 was not suppressed by CsA (Fig. 2B), suggesting that Egr-2 is induced by different signaling pathways by TCR stimulation and TGF-β and IL-6. Although Egr-1 and Egr-3 share similar functions with Egr-2 in the regulation of thymocyte development (29), TGF-β and IL-6 did not induce expression of either of these transcription factors (Fig. 2C). These results further suggest that Egr-2 is selectively involved in the regulation of Th17 cytokines in effector CD4 T cells and Th17 differentiation.

Egr-2 regulates Th17 differentiation

Egr-2 is induced in T cells in response to TCR stimulation (13); therefore, it is expressed in all types of Th cells. To investigate whether Egr-2 regulates the differentiation of CD4 T cells, naive CD4 T cells were differentiated into various Th subsets under specific polarization conditions. We found that Egr-2–deficient CD4 T cells displayed normal Th1 and Th2 differentiation (Fig. 2D). However, an Egr-2 defect rendered CD4 T cells more susceptible to differentiation into Th17 cells than naive CD4 T cells from wild-type mice (Fig. 2D). The expression of several Th17 cytokines, including IL-17 and IL-21, was increased in Th17 cells differentiated from Egr-2–deficient CD4 T cells (Fig. 2E). Although Egr-2, but not Egr-1 and Egr-3, was induced by TGF-β and IL-6, all three Egr molecules were induced in Th1, Th2, and Th17 cells in response to anti-CD3 stimulation (Fig. 2F). Interestingly, Egr-2 expression persisted in Th17 cells in the absence of anti-CD3 stimulation (Fig. 2F), further supporting the notion that Egr-2 is induced by both the Th17 environment and Ag receptor stimulation. To further define the role of Egr-2 in the control of IL-17 expression, we reconstituted Egr-2 expression in CD4 T cells from Egr-2 cKO mice before Th17 differentiation (Fig. 3A). The restoration of Egr-2 expression reduced the percentage of IL-17–producing CD4 T cells after Th17 differentiation (Fig. 3B, 3C). Conversely, knockdown of Egr-2 expression by siRNA in wild-type CD4 T cells enhanced the fraction of IL-17–producing CD4 T cells following Th17 differentiation (Fig. 3D–F). Thus, Egr-2 selectively controls Th17 differentiation and expression of Th17 cytokines, which is consistent with the selectively altered expression of Th17 cytokines in naive CD4 T cells from Egr-2 cKO mice in response to TCR stimulation (Fig. 1). This dysregulation of Th17 differentiation was not observed in Egr-3–deficient naive CD4 T cells (data not shown). Thus, Egr-2 plays an important role in the control of Th17 differentiation.

Egr-2 interacts with Batf and blocks its binding to the IL-17 promoter

To investigate the mechanisms by which Egr-2 controls Th17 differentiation, we analyzed the major regulatory pathways in-

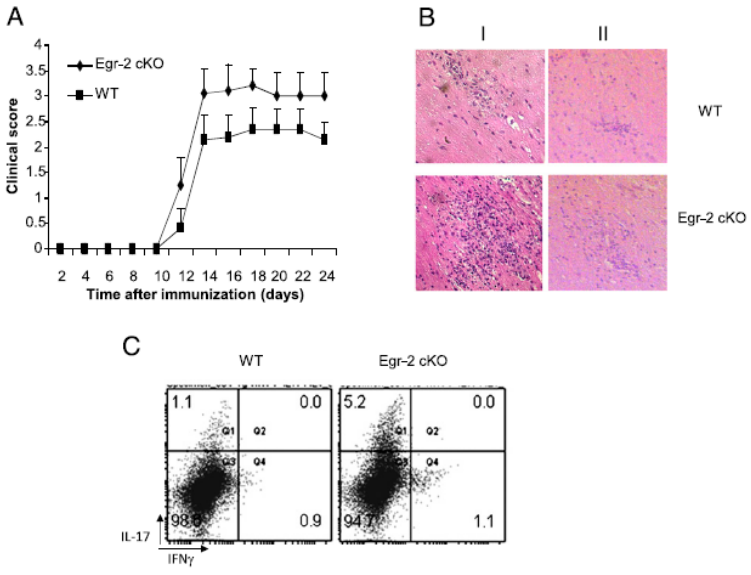

FIGURE 5. Egr-2 deficiency renders mice more susceptible to EAE induction. (**A**) Clinical scores. Five 12-wk-old wild-type or Egr-2 cKO mice were immunized with MOG peptides. The clinical symptoms were scored according to standard protocols (see *Materials and Methods*). (**B**) H&E staining of CNS tissues from two (I and II) diseased mice 30 d after immunization. Original magnification ×10. (**C**) Mononuclear cells pooled from CNS tissues from five diseased mice in each group were stained with CD4, IFN-γ, and IL-17A. The data shown are after gating on $CD4^+$ cells. The data are representative of three experiments with five mice for each group. WT, Wild-type.

volved in the differentiation of Th17 cells. Despite the increased Th17 differentiation, we did not find differences in the expression levels of RORγt/RORα, IFN regulatory factor-4, aryl hydrocarbon receptor, Iκbζ, or Batf or in the activation of STAT3 in Egr-2–deficient Th17 cells (data not shown). However, when we examined transcriptional activity, we found that the binding of Batf to DNA binding sites from the IL-17 promoters in Th17 cells was significantly increased in the absence of Egr-2 (Fig. 4A), suggesting that Egr-2 may control Batf function in the regulation of IL-17 expression. Consistent with previous reports (10), Batf was induced in Th1, Th2, and Th17 cells in response to anti-CD3 stimulation (Fig. 2F). Despite the increase in binding to Batf sites in the IL-17 promoter, we did not find increased expression of Batf in naive CD4 T cells or Th17 cells derived from Egr-2 cKO mice (Fig. 4B), suggesting that Egr-2 does not transcriptionally regulate Batf. To investigate whether Egr-2 antagonizes Batf function directly, we analyzed Batf immunoprecipitates from wild-type CD4 T cells after TCR stimulation. Egr-2 was discovered in the Batf precipitates (Fig. 4C), whereas Egr-1 and Egr-3 were not found (Fig. 4D). Thus, Egr-2 and Batf directly interact in CD4 T cells, raising the possibility that Egr-2 may regulate Batf by directly blocking its binding to promoters of Th17 cytokines. To further analyze the antagonistic function of Egr-2 in the regulation of Batf-mediated IL-17 expression, we measured the activity of an IL-17 promoter reporter gene (9) in the presence of Batf alone or together with Egr-1, Egr-2, or Egr-3. The results showed that Batf transactivated the IL-17 promoter, consistent with previous results (10), whereas Egr-2, but not Egr-1 or Egr-3, effectively suppressed Batf-mediated transactivation (Fig. 4E). Thus, Egr-2 directly suppresses Batf function in the induction of Th17 cytokines.

Egr-2 cKO mice are susceptible to EAE induction

Th17 cells are the major pathological T cells in the development of EAE, and Batf deficiency results in resistance to EAE induction (10). To examine whether the control of Th17 differentiation by Egr-2 can affect immune pathology in autoimmunity, EAE was induced in Egr-2 cKO mice by immunization with MOG peptides. Because Egr-2 cKO mice develop spontaneous autoimmunity in later life, mice aged 10–12 wk, well before the onset of autoimmunity, were used for the induction of EAE. The onset of the diseases was similar in wild-type and Egr-2 cKO mice (Fig. 5A). However, the disease was more severe in Egr-2 cKO mice compared with wild-type mice as demonstrated both by higher clinical scores and increased infiltration of mononuclear cells into inflammatory lesions in spinal cords (Fig. 5A, 5B). Furthermore, the percentage of spinal cord CD4 T cells that was positive for IL-17A was much higher in Egr-2 cKO mice than in wild-type mice (Fig. 5C). These results suggest that Egr-2 is important for the control of Th17 cell–driven pathology and for the control of the development and severity of autoimmune disease. Thus, consistent with the development of lupus-like autoimmune disease in aged Egr-2 cKO mice (26), these results demonstrate that the regulatory function of Egr-2 in the control of Th17 differentiation and cytokine expression is important for the control of autoimmunity.

Altered expression of Egr-2 and IL-17 in T cells from MS patients

It has been found that IL-17 is highly expressed in CNS-infiltrating T cells and glial cells and is associated with active disease in MS (30). The increased production of IL-17 in Egr-2–deficient CD4 T cells and the high susceptibility of Egr-2 cKO mice to EAE induction suggest that Egr-2 may be important for the control of MS development. Egr-2 is expressed at a basal level in naive T cells and is rapidly induced by TCR stimulation. To analyze the expression of Egr-2, Batf, and IL-17 in T cells from MS patients, we isolated CD4 T cells from PBLs of treatment-naive MS patients. The CD4 T cells were stimulated with anti-CD3 and anti-CD28 in vitro for 48 h or left unstimulated, and then the expression of Egr-2, Batf, and IL-17 was analyzed by RT-qPCR. Consistent with the expression patterns in murine naive T cells, the expression of Egr-2 and Batf in T cells from healthy donors was highly induced in response to TCR stimulation (Fig. 6A, 6C), whereas IL-17 was barely detected (Fig. 6B). However, in T cells from MS patients, although the levels of Batf induced in response to TCR stimulation were similar to those seen in healthy donors, the levels of Egr-2 expression were significantly lower (Fig. 6A, 6C). In contrast to the reduced expression of Egr-2, the expression of IL-17 was increased in T cells from MS patients compared with healthy controls (Fig. 6B). Although we do not know whether the downregulation of Egr-2 and increased expression of IL-17 are associated with uncontrolled Batf activity in T cells from MS patients, our results provide direct evidence that Egr-2 expression in effector T cells may be important for the control of Th17-mediated inflammation, which is thought to be part of the mechanism underlying MS development.

Discussion

Previously, we found that Egr-2 is induced in both tolerant and naive T cells following Ag stimulation in vivo, and deficiency of Egr-2 in T cells results in the development of lupus-like disease in later

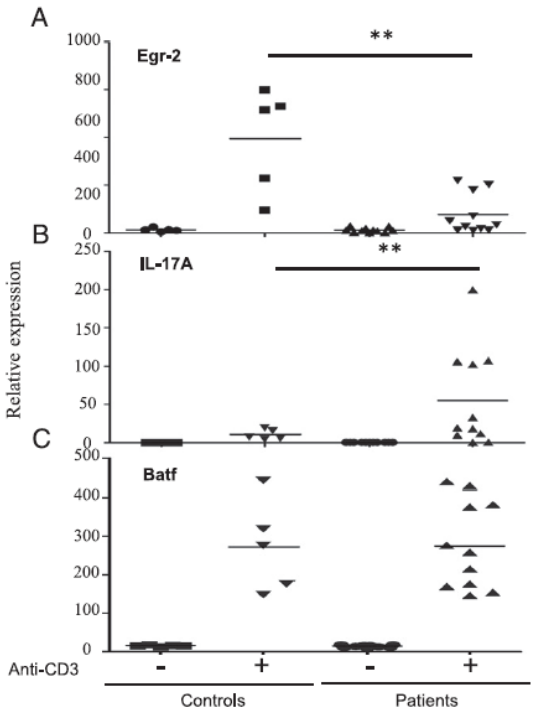

FIGURE 6. Altered expression of Egr-2 and IL-17 in CD4 T cells from MS patients. CD4 T cells were isolated from blood lymphocytes of 11 treatment-naive MS patients and five healthy donors and stimulated with anti-CD3 and anti-CD28 for 48 h. The cells were then restimulated for 6 h and the expression of Egr-2 (**A**), IL-17A (**B**), and Batf (**C**) was analyzed by RT-qPCR. The data are represented relative to the expression of mRNA of β-actin and from two experiments. $^{**}p < 0.01$.

life with accumulation of activated T cells (26). However, although Egr-2 is highly induced in naive T cells following Ag stimulation (13), a defect in Egr-2 does not increase T cell activation in response to TCR stimulation (26). We have now demonstrated that Egr-2 deficiency results in uncontrolled production of Th17 cytokines in naive CD4 T cells in response to TCR stimulation and renders CD4 T cells prone to Th17 differentiation, which contributes to increased susceptibility to EAE induction. The importance of Egr-2 in the control of Th17-driven pathology is further supported by the defective induction of Egr-2 and increased expression of IL-17 in T cells from treatment-naive MS patients.

Th17 cells, one of the major Th subsets, can be generated by differentiation of naive CD4 T cells in response to Ag and Th17 mediators such as IL-6 and TGF-β (1, 2). However, in contrast to Th1 and Th2 cytokines, such as IFN-γ and IL-4, which are highly induced in naive T cells in response to TCR ligation, the induction of Th17 cytokines, such as IL-17 and IL-21, was barely detected in activated naive T cells from wild-type mice in the absence of Th17 mediators, suggesting that the expression of Th17 cytokines in effector T cells is tightly regulated. A number of regulatory factors have been discovered for inhibiting expression of Th17 cytokines and regulating Th17 differentiation (1, 2, 4–10). Most of them act by inhibiting the expression or antagonizing the function of RORγt, such as Runx1 and T-bet, or by inhibiting STAT3 activation, such as SOCS3, or by promoting Foxp3 expression, a counter-activator essential for regulatory T cell development (4). We have found that Egr-2 interacts with Batf to suppress Th17 cytokine expression. However, although deficiency of Egr-2 and Egr-3 in lymphocytes results in much more severe inflammatory autoimmune diseases than does Egr-2 single deficiency (30), we could not detect Egr-1 and Egr-3 in anti-Batf precipitates (data not shown), suggesting that EGR-2 is important for the control of Batf-mediated Th17 differentiations (30). Although it works in synergy with RORγt to induce Th17 differentiation, Batf regulates the expression of Th17 cytokines and Th17 differentiation independently of RORγt (10). In contrast to the suppression of RORγt expression in Th1 and Th2 cells, Batf is induced in all types of effector CD4 T cells in response to TCR ligation (10), suggesting that its Th17 promoting function is suppressed in effector T cells to avoid unwanted Th17 differentiation. TCR stimulation induces expression of both Egr-2 and Batf in all types of effector Th cells. The induction of Th17 cytokines, such as IL-17 and IL-21, in Egr-2–deficient T cells following TCR stimulation suggests that Egr-2 plays an important role in the control of Th17 cytokine expression in CD4 T cells in response to mitogenic Ag stimulation. The antagonistic function of Egr-2 on Batf in the regulation of Th17 cytokines in effector T cells may be essential for the control of Th17-mediated immunopathology during an adaptive immune response. Although Egr-2 and Batf are induced by TCR stimulation in all types of Th cells, we found that Egr-2 is selectively induced by TGF-β and IL-6, but not by IL-4, IL-12, or IFN-γ, indicating a feedback mechanism involving Egr-2 that serves to control Th17 differentiation. Although the mechanisms for the induction of Egr-2 expression by TGF-β and IL-6 are unknown, the increased expression of Egr-2 in Th17 cells indicates that the induction of Egr-2 expression and Th17 differentiation by TGF-β and IL-6 likely occurs via similar mechanisms that have yet to be fully investigated.

The importance of Egr-2 in the control of immune pathology in autoimmune diseases is highlighted by the susceptibility of Egr-2 cKO mice to the induction of EAE. The increased severity of disease and enhanced infiltration of inflammatory cells in Egr-2 cKO mice is associated with high levels of IL-17–producing CD4 T cells. The high susceptibility of Egr-2 cKO mice to EAE induction is in stark contrast to the reduced EAE induction in Batf-deficient mice (10), indicating that the repression of Batf function by Egr-2 contributes to the development of EAE.

The defective expression of Egr-2 and its association with increased production of IL-17 in CD4 T cells from treatment-naive MS patients is consistent with previous reports (31) and further indicates that impaired expression of Egr-2 in activated T cells may contribute to MS development. Egr-2 expression was found to be important for T cell tolerance, and its expression in anergic T cells is regulated by NFAT (11, 12). The dysregulated expression of Egr-2 and IL-17, but normal induction of Batf, in CD4 T cells from MS patients resembles the expression profile of these molecules in CD4 T cells from Egr-2 cKO mice, suggesting that uncontrolled Batf activity may contribute to the increased IL-17 production and immunopathology in MS.

Taken together, we have demonstrated a previously unknown function of Egr-2 in the control of Th17 cytokine expression and Th17 differentiation by inhibition of Batf function. The susceptibility of Egr-2 cKO mice to EAE induction and the reduced induction of Egr-2 in T cells from MS patients after TCR stimulation further indicate the importance of Egr-2 in the control of autoimmunity and Th1-mediated inflammation.

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Disclosures

The authors have no financial conflicts of interest.

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Future Work

If I had more time, I would have liked to have developed the MS pilot study, to increase the number of patients and healthy controls, to also look at the protein level for these samples and other possible biomarkers. I would also like to have performed ELISA's to verify cytokine production from the polarized Th conditioned $CD4^+$ T cells , and CHIP analysis to look at more detail at binding sites.

Publications from my work

Early growth response gene-2 controls IL-17 expression and Th17 differentiation by negatively regulating Batf. (Joint first author paper).

Miao, T., **M. Raymond**, et al. (2013). J Immunol 190(1): 58-65.

The transcription factors Egr2 and Egr3 are essential for the control of inflammation and antigen-induced proliferation of B and T cells.

Li S[1], Miao T, **Sebastian M**, et al (2012)

Early Growth Response Gene-2 (Egr-2) Regulates the Development of B and T Cells.

Li S, Symonds ALJ, Zhu B, Liu M, **Raymond MV,** et al. (2011). PLOS ONE 6(4):

Early Growth Response Gene-2 (EGR-2) Regulates Inflammatory Plasticity of Effector T Cells.

Raymond MV, Maio T, Symonds ALJ, Li S and Wang P. (2011) J.Immunol. 186:167.1 (Abstr)

Conference Presentations

- American Association of Immunology San Francisco 2011: **Research Talk and Poster Presentation.**
- Queen Mary Postgraduate Research Day 2011: **Research Talk**
- Queen Mary Postgraduate Research Day 2010: **1st Prize Winner Poster Presentation**
- European Immunology Summer School Sardinia 2009: **Research Talk and Poster Presentation**

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