January 2009

Batf regulates previously unknown AP-1 target genes to control TH17 differentiation

Barbara Schraml
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

(Program in Immunology)

Dissertation Examination Committee:

Kenneth M. Murphy, Chair
John P. Atkinson
Andrey S. Shaw
Wojciech A. Swat
Marco Colonna
Chyi-Song Hsieh

BATF REGULATES PREVIOUSLY UNKNOWN AP-1 TARGET GENES TO CONTROL TH17 DIFFERENTIATION

by

Barbara Ursula Schraml

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philiosophy

August, 2009

St. Louis, Missouri

ABSTRACT OF THE DISSERTATION

Batf regulates previously unknown AP-1 target genes to control T_H17 differentiation

by

Barbara Ursula Schraml

Doctor of Philosophy in Biology and Biomedical Sciences
(Immunology)

Washington University in Saint Louis, 2009

Professor Kenneth M. Murphy, Chairperson

Activator protein 1 (AP-1) transcription factors are dimers of Jun, Fos, musculoaponeurotic fibrosarcoma (MAF) and activating transcription factor (ATF) family proteins that are characterized by a basic region and a leucine zipper domain. While many AP-1 proteins also contain defined transcriptional activation domains (TADs), some consist only of a basic region and leucine zipper and are thought to function as inhibitors of AP-1 activity. We found that the AP-1 protein Batf, which lacks a TAD, is highly expressed in T helper cells compared to various other immune cells and tissues. IL-17-producing T helper (T_H17) cells are a $CD4^+$ T cell subset that coordinates inflammatory responses in host defense but are pathogenic in autoimmunity. To study the role of *Batf* in T cells, we generated *Batf* deficient mice by gene targeting. *Batf*$^{-/-}$ mice show a highly selective defect in T_H17 differentiation. As a result, *Batf*$^{-/-}$ mice are completely resistant to experimental autoimmune encephalomyelitis. Using gene expression analysis, we found that *Batf*$^{-/-}$ T cells fail to induce known T_H17-specific transcription factors, such as RORγt, and the cytokine IL-21, required for T_H17 differentiation. Neither addition of IL-21 nor overexpression of RORγt fully restores IL-

17 production in *Batf* $^{-/-}$ T cells, suggesting that Batf may be required directly for IL-17 transcription. We found that the *Il17* promoter is Batf-responsive, and upon T_H17 differentiation, Batf binds to several conserved intergenic elements in the *Il17a/f* locus as well as to regions in the *Il17, Il21* and *Il22* promoters. Using bio-computational methods we determined that the Batf-binding element in the *Il17, Il21* and *Il22* promoters differs from canonical symmetric AP-1 elements. Using EMSA analysis we found that Batf forms heterodimers preferentially with JunB during T_H17 differentiation. These results demonstrate that the AP-1 factor Batf regulates previously unknown AP-1 target genes to control T_H17 differentiation and T_H17-mediated autoimmune disease.

ACKNOWLEDGEMENTS

Many people have immeasurably contributed to the work presented here, either by providing help, sharing reagents, engaging in rewarding scientific discussions or simply showing support in and outside of lab. This work could not have been completed without them.

I am indebted to Ken Murphy for giving me the opportunity to train in his lab and for his support over the past few years. Ken's enthusiasm and knowledge about science and immunology as well as his unique views on analyzing, interpreting and presenting data have had an immeasurable influence on me. Ken has taught me to work independently and has given me the flexibility to explore life outside of the lab, which has been extremely important to me. I additionally would like to thank Theresa Murphy for sharing her immense technical expertise and her experience. I have benefited greatly from her advice during many productive discussions about experiments and data analysis.

The Murphy lab members, past and present, have had substantial influence on me as a person and a scientist. There are too many of you that have come and gone in my time here to mention everybody individually. Thank you for providing an extremely supportive environment; I simply could not have done it without you. I will miss our endless scientific discussions and I want to thank you for your enthusiasm and teaching me about science, life and different cultures, particularly during the lunch hours.

I want to also thank a few friends for their continuing support over the years. My friend and mentor Brian Reilly, whose immense enthusiasm for science will always remind me why I chose to become a scientist. My friends Claudia, Balazs, Luca, Helena

and Danielle for always being there for me when I needed them. My “adoptive family” the Missouri Whitewater Association has made my time as a graduate student unforgettable and has helped me immensely by sometimes NOT asking how things in the lab were going. In particular I would like to thank Mike for showing me the world and being not only my shoulder to lean on but also my “substitute” shoulder at times during the last few years.

Most importantly I would like to thank my family for their continuing support during my adventures in the world - far away from home.

“You could not step twice into the same river; for other waters are ever flowing on to you.” (Heraclitus; On the Universe)

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 5

CHAPTER 6

LIST OF ABBREVIATIONS

AHR	Aryl hydrocarbon receptor
APC	Antigen presenting cell
ATF	Activating transcription factor
AP-1	Activator protein 1
Bach2	BTB and CNC homology 2
Batf	Basic leucine zipper transcription factor *ATF*-like
bZIP	Basic leucine zipper
cDC	Conventional dendritic cell
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CNS-2	Conserved non-coding sequence-2
CRE	Cyclic AMP responsive element
CSR	Class switch recombination
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electropehoretic mobility shift analysis IL-2
Foxp3	Forkhead box P3
GFP	Green fluorescent protein
IFN-γ	Interferon gamma

Ig	Immunoglobulin
IL	Interleukin
IRF4	Interferon regulatory factor 4
LP	Lamina propria
LTα	Lymphotoxin alpha
MAF	Musculoaponeurotic fibrosarcoma
MHC	Major histocompatibility complex
MOG_{35-55}	Myelin oligodendrocyte glycoprotein peptide 35-55
NFAT	Nuclear factor of activated T cells
NK	Natural killer cell
NKT	Natural killer T cell
OVA	Ovalbumin
pDC	Plasmacytoid dendritic cell
PMA	Phorbol myristate acetate
qRT-PCR	Quantitative real time polymerase chain reaction
Rag2	Recombination activating gene 2
RORα	Retinoid acid related nuclear orphan receptor alpha
RORγt	Retinoid acid related nuclear orphan receptor gamma T
RUNX1	Runt related transcription factor 1
STAT	Signal transducer and activator of transcription
TAD	Transcriptional activation domain
T-bet	T cell specific T-box transcription factor
TCR	T cell receptor

TD	T-dependent
TGF-β	Transforming growth factor beta
T_H	T helper cell
TI	T-independent
TNF-α	Tumor necrosis factor alpha
TNP	2,4,6,-trinitrophenol
TRE	TPA responsive element
T_{reg}	T regulatory cell

CURRICULUM VITAE

PERSONAL INFORMATION:

Home Address: 1501 Central Ave
St. Louis, MO, 63139
Home/Cell Phone: 314.550.6955
Citizenship: Austria
Date of Birth: March 3, 1980

CURRENT POSITION:

Graduate Student at Washington University in St. Louis (Advisor Kenneth M. Murphy)
Graduate Program in Immunology
Department of Pathology and Immunology

660 S. Euclid Avenue, Campus Box 8118
St. Louis, MO, 63110
Phone: 314.362.2004 Fax: 314.747.4888
Email: bschraml@artsci.wustl.edu

EDUCATION:

Ph.D. Graduate Program in Immunology, Washington University, St. Louis, MO
2003 to present, graduation anticipated June 2009

B.S. in Molecular Biology, Texas Tech University, Lubbock, TX
2000 to 2003; graduated Summa cum Laude

Lamar University, Beaumont, TX
1999 – 2000

RESEARCH EXPERIENCE:

Predoctoral Trainee: Washington University; St. Louis, Missouri.
2003 to present: Laboratory of Dr. Kenneth M. Murphy, M.D., Ph.D.
Batf regulates previously unknown AP-1 target genes to control TH17 differentiation. We genetically deleted *Batf*, a small AP-1 family member, in mice to study its role in determining cellular fate decisions in the immune system.

HHMI Undergraduate Research Fellow: Texas Tech University; Lubbock, Texas.
2001-2003: Laboratory of Dr. Brian Reilly, Ph.D.
Studying the complement system of Channel Catfish (Ictalarus punctatus). Our studies demonstrated the presence of a complement receptor on erythrocytes of rainbow trout but not channel catfish.

Honors and Awards:

▪**Abcam Best Presentation Award,** World Immune Regulation Meeting, Davos, Switzerland, 2009.
▪**Keystone Symposia Scholarship,** TH17 Cells in Health and Disease, 2009**.**
▪**Participant in the RCAI summer program 2007**, Yokohama, Japan.
▪**Earl Camp award** for outstanding graduating student in Biology, Texas Tech University 2003.
▪**Honors College Merit Scholarship**, Texas Tech University, 2002/2003.
▪**Jeannine McHaney Scholarship Award** for academic achievement and athletic excellence, Texas Tech University, 2002 and 2003.

Teaching Experience:

Teaching Assistant: Washington University; Graduate Course in Immunology; "Foundations of Immunology" (Fall 2004).

Publications:

Schraml BU, Hildner K, Ise W, Lee WL, Smith WA, Solomon B, Sahota G, Sim J, Mukasa R, Hatton RD, Stormo GD, Weaver CT, Russell JH, Murphy TL, Murphy KM. The AP-1 transcription factor *Batf* controls TH17 differentiation. *Nature,* in press.

Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, Calderon B, **Schraml BU**, Unanue ER, Diamond MS, Schreiber RD, Murphy TL, Murphy KM. 2008. *Batf3* deficiency reveals a critical role for $CD8\alpha^+$ dendritic cells in cytotoxic T cell immunity. *Science.* Nov 14;322(5904):1097-100**.**

Liszewski MK, Leung MK, **Schraml B**, Goodship TH, Atkinson JP. 2007. Modeling how CD46 deficiency predisposes to atypical hemolytic uremic syndrome. *Mol Immunol.* Mar;44(7):1559-68.

Schraml B, Baker MA, Reilly BD. 2006. A complement receptor for opsonized immune complexes on erythrocytes from *Oncorhynchus mykiss* but not *Ictalarus punctatus* **.** *Mol. Immunol*. Apr; 43(10): 1595-603.

Reviews:

Schraml B, Peng SL. T-B Cell Interactions in Autoimmunity. *Autoantibodies and Autoimmunity* (Ed. KM Pollard) 2006: 85-106, Wiley-VCHVerlag GmbH & Co. KGaA, Weinheim, Germany.

Posters and Oral presentations:

Oral Presentation: (2009) Batf controls TH17 differentiation: An unexpected role as an AP-1 transcriptional activator. World Immune Regulation Meeting, Davos, Switzerland.
Oral Presentation: (2007) Studying the function of Batf in T cells by deletion. RCAI Riken International Summer Program, Yokohama, Japan.
Schraml B, Hildner K, Murphy TL, Murphy KM. (2007) Studying the function of Batf in T cells by deletion. RCAI Riken International Summer Program, Yokohama, Japan.

CHAPTER 1

Introduction

The main function of the immune system is to recognize and eliminate pathogens. This defense against infectious microorganisms is mediated by coordinate responses of innate and adaptive immune cells. The innate immune system is a rapid first line of defense against pathogens. Innate immune cells sense pathogens via germline encoded receptors that recognize pathogen associated molecular patterns present on microbial organisms but not mammalian cells. Recognition of pathogens leads to immediate activation of innate immune cells and release of antimicrobial factors. In contrast, the adaptive immune system is composed of lymphocytes that can recognize a wide spectrum of pathogens and non-microbial derived molecules via highly specific antigen receptors. The adaptive immune response takes days rather than hours to develop and is instructed by the innate immune system to carry out pathogen specific effector functions of the appropriate effector class. Suitable adaptive immune responses ultimately result in the clearance of pathogens and the development of immunological memory, allowing for rapid recall responses the second time a pathogen is encountered.

The ability of the immune systems to self-regulate after pathogens clearance and to regulate responsiveness to self antigens is of equal importance as mounting a strong immune response because uncontrolled immune responses contribute to autoimmune diseases, allergy and cancer development. The main driving force in regulating the expression of genes that promote immune cell development, differentiation, activation and regulation are transcription factors. A better understanding of the transcriptional

networks governing immune cell differentiation will contribute the knowledge necessary to develop novel therapeutic approaches to treat immunological diseases.

Lymphocytes and the adaptive immune response

The adaptive immune system can be divided into humoral and cell mediated immune responses. Humoral immune responses are mediated by B lymphocytes and refer to antibody production. B lymphocytes produce and secrete antigen-specific antibodies that target pathogens for elimination by phagocytic cells or neutralize pathogens to prevent cell entry (Murphy et al., 2008). The nature of the humoral immune response is determined by the specific B cell subsets involved as well as the composition of the antigen. B cell responses include a variety of strictly controlled processes involved in the generation of antibody producing plasma cells, which secrete antigen specific antibodies with high affinity and distinct effector function, as well as the generation of memory B cells, armed for rapid release of antibodies upon reencountering antigen (Murphy et al., 2008).

Cell-mediated immune responses involve the activation of antigen specific $CD4^+$ and $CD8^+$ T lymphocytes and subsequential differentiation into functionally distinct effector T cells (Murphy et al., 2008). Activation of T cells involves the presentation of pathogen-derived peptides in the context of MHC molecules on the surface of antigen presenting cells (APC), since T cell receptors cannot recognize antigen in its native form. Upon activation, $CD8^+$ T cells differentiate into cytotoxic T cells, which kill target cells that display pathogen-derived peptides from cytosolic pathogens, such as viruses, on their surface via MHCI. Activated $CD4^+$ T cells differentiate into multiple subsets effector T

cells that orchestrate the adaptive immune response through the secretion of cytokines and other mechanisms. There are at least three classes of T helper (T_H) cells known to date distinguished by their signature cytokines; T_H1, T_H2 and the recently identified T_H17 (Harrington et al., 2005) cells. In addition to differentiating into T helper subsets, naïve $CD4^+$ T cells can develop into regulatory T cells (T_{reg}) that are characterized by their ability to suppress T cell responses. At least one class of T_{reg} cells develops in the thymus (natural T_{reg}), but other classes of T_{reg} cells develop in the periphery (adaptive T_{reg}) (Weaver et al., 2006).

T helper cell subsets in host defense

T helper cells orchestrate immune responses through the secretion of their signature cytokines: T_H1 cells are characterized by the production of IFN-γ and LTα, T_H2 cells secrete IL-4, IL-5 and IL-13 and T_H17 cells secrete IL-17A (also called IL-17), IL-17F and IL-22 (Weaver et al., 2006; Ouyang et al., 2008b). T_H1 cells are the main players in cellular immunity against intracellular bacteria, viruses and tumors, while T_H2 cells direct the humoral immune response to extracellular pathogens, such as helminthes. T_H17 cells mediate acute inflammatory responses and promote resistance to extracellular bacteria and fungi, particularly at mucosal surfaces. In addition to their importance in primary immune responses T helper cells produce large amounts of IL-2, which is required for the expansion of $CD8^+$ memory T cells during secondary infections (Murphy and Reiner, 2002; Weaver et al., 2006). Uncontrolled helper T cell responses can lead to the development of atopic diseases such as asthma (T_H2 mediated) or the development of autoimmune diseases (T_H1 and T_H17 mediated). T_{reg} cells suppress immune responses

and prevent autoimmunity at least in part through the production of inhibitory cytokines such as IL-10 and TGF-β, as well as by inducing cytokine-deprivation-mediated apoptosis through the consumption of IL-2 (Vignali et al., 2008).

In summary, T helper cells balance the immune response during infections and appropriate T helper cell responses determine the outcome of infectious and inflammatory responses.

T_H17 cells and autoimmunity

Uncontrolled activation of T cells and uncontrolled production of effector T cell cytokines in response to self-antigens can lead to T cell mediated autoimmune diseases. Until recently, T_H1 cells were thought to be the major pathogenic T cell subset in these diseases, based on observations that blocking the T_H1 promoting cytokine IL-12 led to amelioration or cure of various autoimmune diseases. For instance, mice deficient for the p40 subunit of IL-12 or treated with neutralizing antibodies to IL-12 were protected from experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (McGeachy and Cua, 2007). In apparent contradiction with these results targeting IFN-γ did not affect EAE development but in fact, IFN-γ deficient mice developed more severe disease indicating a protective role of IFN-γ in EAE (Willenborg et al., 1996). Since IL-12 is a heterodimer of a unique p35 subunit and a p40 subunit shared with IL-23 (Hunter, 2005), studies targeting the IL-12 specific subunit provided conclusive evidence that T_H1 cells were negligible for the development of EAE (Gran et al., 2002). Instead, targeting IL-23 (p19 subunit) protected against EAE and lead to the identification of IL-23 as a crucial factor in EAE pathology through its role in maintaining the T_H17 lineage

(Langrish et al., 2005). Additionally, mice deficient in transcription factors (Ivanov et al., 2006; Brustle et al., 2007) and cytokines (Korn et al., 2007) required for T_H17 differentiation are protected from disease or develop less severe disease than wild type mice, providing additional evidence for the importance of T_H17 cells in EAE pathology. However, even though the importance of T_H17 cells in disease pathology is recognized, the major T_H17 effector cytokines IL-17A, IL-17F and IL-22 contribute minimally to the disease (Kreymborg et al., 2007; Haak et al., 2009), indicating the existence of other effector pathways contributing to disease.

T_H17 cells and associated effector cytokines have been implicated in several other autoinflammatory disorders or models of autoinflammatory disorders, previously thought to be mainly T_H1-mediated, including arthritis, inflammatory bowel disease, uveitis and psoriasis (McGeachy and Cua, 2007). In psoriasis patients IL-23 expression is readily detected in psoriatic lesions and decreased after commonly used treatments (Torti and Feldman, 2007). In murine models, IL-22 deficiency or neutralization of IL-22 protect against dermal inflammation (Zheng et al., 2007; Ma et al., 2008), while injection of mice with IL-23 promotes skin inflammation (Kopp et al., 2003). In several models of inflammatory bowel disease neutralization of IL-17, IL-6 or IL-23 and loss of IL-21 or IL-23 lead to disease amelioration (Yen et al., 2006; Fina et al., 2008; Elson et al., 2007). Thus, deciphering the molecular mechanisms underlying the differentiation of the inflammatory T_H17 cell lineage will increase the understanding of inflammatory responses to foreign and self-antigens. This fundamental knowledge will provide the basis for the development of novel therapeutic approaches to treat autoinflammatory disorders.

Transcriptional regulation of T_H1 and T_H2 development

The development of T_H1 and T_H2 effector cells is initially linked to instructive cytokine signals that induce the expression of cell specific transcription factors. Engagement of the T cell receptor on naïve T cells in conjunction with IFN-γ leads to the activation of signal transducer and activator of transcription (STAT) 1. STAT1 induces the expression of the T_H1 specific T box family transcription factor T-bet. T-bet induces remodeling of the IFN-γ locus and likely stabilizes its own expression either through an intrinsic autocatalytic loop or an autocrine cytokine feedback loop involving IFN-γ signaling. T-bet also induces the transcription of the IL-12 receptor β2 subunit resulting in surface expression of a functional IL-12 receptor. IL-12 via STAT4 contributes to IFN-γ transcription and induces the expression of IL-18 receptor. Once T_H cells are committed to their fate they readily produce signature cytokines upon TCR stimulation without the need for exogenous cytokines signals. In T_H1 cells, the responsiveness to IL-18 potentiates an alternative, TCR independent pathway to induce cytokine expression, in response to IL-12 and IL-18 (Weaver et al., 2006; Murphy and Reiner, 2002).

IL-4 promotes T_H2 development through activation of STAT6 evident by the fact that STAT6 deficient T cells exhibit a severe reduction in T_H2 differentiation. STAT6 induces low expression of the T_H2 specific transcription factor Gata3, which stabilizes its own expression in a cell intrinsic feedback loop and regulates multiple T_H2 associated cytokines by inducing epigenetic changes in the T_H2 cytokine cluster (IL-4, IL-5 and IL-13) (Ho et al., 2009). Gata3 is capable of inducing T_H2 development in a STAT6 independent manner at least in part by inducing the expression of other T_H2-associated

transcription factors such as the AP-1 family member c-Maf, which directly regulates IL-4 transcription in conjunction with Gata3 (Ho et al., 1998; Murphy and Reiner, 2002).

T_H1 and T_H2 differentiation is stringently counter-regulated to ensure exclusive commitment to either lineage and once naïve T cells commit to the T_H1 of T_H2 lineage, they exhibit stable phenotypes, exclusively expressing their signature cytokines. Early T_H2 signaling induces a rapid feedback loop ensuring stable expression of Gata3, thus promoting T_H2 differentiation. This stable Gata3 expression additionally blocks T_H1 differentiation, since Gata3 inhibits expression of IFN-γ. Similarly, in T_H1 cells T-bet inhibits the expression of T_H2-associated factors and cytokines (Weaver et al., 2006; Murphy and Reiner, 2002).

Transcriptional regulation of T_H17 differentiation

T_H17 cells differentiate in response to the cytokines IL-6 and TGF-β (Bettelli et al., 2006; Korn et al., 2007; Nurieva et al., 2007; Wei et al., 2007; Zhou et al., 2007; Manel et al., 2008), while TGF-β signaling alone, in the absence of proinflammatory cytokines such as IL-6, induces the differentiation of T_{reg} cells in an IL-2 dependent manner (Davidson et al., 2007). T_H17 and T_{reg} cells develop via reciprocal pathways, in which IL-6 inhibits TGF-β-mediated induction of the T_{reg} specific transcription factor Foxp3 (Bettelli et al., 2006). T_H17 and T_{reg} cell development were initially thought to be mutually exclusive but recent data suggest the existence of a bi-potential precursor, primed towards one or the other lineage depending on the strength of TGF-β signaling (Zhou et al., 2008). This shared requirement for TGF-β in the development of the inflammatory T_H17 and the suppressive T_{reg} lineages is intriguing as it implies a close

relationship of two cells with apparent opposite effector functions thus highlighting the necessity for balance in the adaptive immune response to pathogens.

In addition to TGF-β and IL-6, T_H17 differentiation requires IL-21, an early target of IL-6 signaling in T cells, which regulates T_H17 differentiation via an autocrine feedback loop (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007; Wei et al., 2007). Additionally, IL-23 is required for the maintenance of T_H17 cells (Langrish et al., 2005; Veldhoen et al., 2006a). While IL-6, IL-21 and IL-23 are required for T_H17 differentiation and maintenance, proinflammatory cytokines such as IL-1β and TNF-α are not required but can further promote T_H17 differentiation through unknown mechanisms (Veldhoen et al., 2006a).

Similar to T_H1 and T_H2 cell differentiation, the differentiation of T_H17 cells is counterregulated by effector cytokines from other T_H subsets, such as IFN-γ, IL-4 and IL-13. Additionally, the cytokines IL-2, IL-10 and IL-27, have been described to inhibit T_H17 differentiation (Harrington et al., 2005; Park et al., 2005; Laurence et al., 2007; Gu et al., 2008; Newcomb et al., 2009; Awasthi et al., 2007; Diveu et al., 2009; Fitzgerald et al., 2007). In contrast to T_H1 and T_H2 cells, which exhibit stable cytokine secretion profiles once they are committed to their lineage, IL-17 double producing cells that also secrete IFN-γ are readily detected *in vitro* and *in vivo*. As a result T_H1 and T_H17 cells were thought to develop from a common precursor; however, lineage tracing experiments demonstrated that T_H17 cells develop as a lineage distinct from T_H1 cells (Veldhoen et al., 2006b). Nevertheless, recent data demonstrated that T_H17 cells exhibit developmental plasticity and unstable cytokine expression and that the maintenance of a stable T_H17

phenotype is strongly dependent on continuous IL-23 signaling (Shi et al., 2008; Lee et al., 2009).

IL-6, IL-21 and IL-23 each activate STAT3 (Ghilardi and Ouyang, 2007), which is required for T_H17 differentiation and potentially directly induces IL-17 production, since it binds directly to the *Il17* promoter (Laurence et al., 2007; Yang et al., 2007; Mathur et al., 2007). Combined IL-6 and TGF-β signals induce the expression of the retinoid acid related nuclear orphan receptor (ROR) RORγt (Ivanov et al., 2006), a T_H17 specific transcription factor. RORγt is sufficient to induce IL-17 production in wild type T cells and co-expressed with IL-17 in lamina propria T cells, which constitutively express IL-17. Since RORγt deficiency blocks T_H17 differentiation *in vitro* (Ivanov et al., 2006) RORγt is considered the main regulator of T_H17 cells, similar to T-bet and Gata3 in T_H1 and T_H2 cells respectively. However, RORγt deficient mice are only partially resistant to EAE and residual IL-17 production can be observed in RORγt deficient T cells *in vivo* after EAE induction (Ivanov et al., 2006). This residual IL-17 production in RORγt deficient T cells has been attributed to functional redundancy between RORγt and RORα, another ROR family member expressed in T_H17 cells (Yang et al., 2008). Yet, RORα is dispensable for T_H17 differentiation since T cells deficient in RORα show only a mild reduction in IL-17 production (Yang et al., 2008). More strikingly, *Irf4*$^{-/-}$ T cells exhibit an absolute block in T_H17 development and are completely resistant to EAE (Brustle et al., 2007). While *Irf4*$^{-/-}$ T cells exhibit decreased expression of RORγt, overexpression of RORγt in *Irf4*$^{-/-}$ T cells only partially restores IL-17 production (Brustle et al., 2007). Collectively, these data indicate that RORγt is unlikely the sole regulator of T_H17 differentiation.

Consistently, additional factors have been identified to contribute to T_H17 differentiation. RUNX1 overexpression increases IL-17 production in wild type T cells and RUNX1 and RORγt synergize in *Il17* reporter assays (Zhang et al., 2008a). Additionally, the aryl hydrocarbon receptor (AHR) is a T_H17 specific ligand-dependent transcription factor that binds synthetic ligands, such as aromatic hydrocarbons, and naturally occurring ligands, such as tryptophan metabolites (Veldhoen et al., 2008). AHR deficient T cells lack IL-22 production but exhibit relatively normal IL-17 production, indicating that AHR is responsible for the transcriptional regulation of *Il22*, but not *Il17* (Veldhoen et al., 2008; Quintana et al., 2008). Nevertheless, AHR ligands are necessary for optimal differentiation of T_H17 cells (Veldhoen et al., 2009). To summarize, multiple transcription factors have been shown to control T_H17 differentiation, but their transcriptional hierarchy and cooperativity are incompletely understood.

The AP-1 family of transcription factors

AP-1 proteins are members of the Jun (Jun, JunB, JunD), Fos (Fos, FosB Fra1, Fra2), musculoaponeurotic fibrosarcoma (MAF) and activating transcription factor (ATF) families. They are basic leucine zipper (bZIP) proteins that are characterized by a basic region for DNA binding and leucine zipper that mediates dimerization (Vinson et al., 2006). Most AP-1 proteins contain defined transcriptional activation domains (TADs), but some consist only of a basic region and leucine zipper and are thought to function as inhibitors of AP-1 activity (Blank, 2008; Williams et al., 2001; Echlin et al., 2000; Iacobelli et al., 2000; Dorsey et al., 1995; Thornton et al., 2006). AP-1 family members require dimerization to form functional transcription factor complexes. While Fos family

members can only heterodimerize with Jun proteins, Jun proteins can homo- or heterodimerize to form active transcription factors. Additionally, AP-1 proteins often cooperate with other transcription factors to induce target genes. AP-1 complexes generally bind to dyad symmetric DNA sequences, such as TGANTCA (TRE element) and TGANNTCA (CRE element) and AP-1 proteins are also regulated by upstream kinases connecting them to various signal transduction pathways (Ryseck and Bravo, 1991; Eferl and Wagner, 2003; Wagner and Eferl, 2005).

The functions of individual family members have been elucidated using genetically modified mice. These studies revealed essential functions of AP-1 in various processes, including embryonic development, behavior, bone formation as well as immune cell development and function (Eferl and Wagner, 2003).

AP-1 dependent regulation of T cell differentiation and cytokine production

AP-1 family proteins regulate multiple stages of T cell development, differentiation and cytokine production (Hess et al., 2004). Many cytokine promoters are regulated by AP-1 often in cooperation with other transcription factors (Macian et al., 2001); for example the extensively studied IL-2 promoter is regulated by AP-1 in conjunction with NFAT (Garrity et al., 1994; Jain et al., 1992). AP-1 transcriptional activity has been analyzed in T cells using AP-1-luciferase transgenic mice. These studies revealed higher AP-1 activity in T_H2 cells compared to T_H1 cells (Rincon et al., 1997), despite expression of AP-1 proteins in both effector subsets. JunB is preferentially expressed in T_H2 cells compared to T_H1 cells, whereas the expression of JunD and c-Jun is similar between the two subsets (Rincon et al., 1997). Overexpression of JunB leads to

ectopic expression of T_H2 cytokines in T cells cultured under T_H1 conditions (Li et al., 1999), whereas JunB deficient T cells exhibit inefficient skewing towards a T_H2 phenotype (Hartenstein et al., 2002). In contrast, overexpression of JunD suppresses T_H2 cytokine expression and JunD deficiency leads to increased T_H1 and T_H2 cytokine production (Meixner et al., 2004). Therefore, JunB promotes T_H2 differentiation, while JunD acts as a negative regulator of cytokine production in T cells. In addition to Jun family members several MAF family members control T cell function and cytokine production. c-Maf regulates IL-4 production in T_H2 cells (Ho et al., 1996) and transgenic overexpression of MafK, a potential AP-1 transcriptional inhibitor, suppresses T cell proliferation (Yoh et al., 2001). In summary, AP-1 family members regulate T helper cell development, maintenance and cytokine production, thus controlling the balance of T cell differentiation during specific responses to pathogens.

Batf as a regulator of AP-1 transcriptional activity

Batf is a member of the AP-1 family of proteins but unlike most other AP-1 proteins, *Batf* is composed only of a basic region and leucine zipper and lacks obvious transactivation domains (TADs) (Dorsey et al., 1995). Batf forms heterodimers with Jun but not Fos proteins (Echlin et al., 2000; Dorsey et al., 1995; Senga et al., 2002; Hasegawa et al., 1996). In reporter assays *Batf* can inhibit AP-1 dependent luciferase activity *in vitro* and *in vivo* as well as cellular transformation by Fos (Thornton et al., 2006; Williams et al., 2001; Echlin et al., 2000). Thus, Batf has been suggested to function as an endogenous repressor of AP-1 activity, by forming transcriptionally inert

complexes with Jun proteins that exhibit identical DNA binding specificity as the Jun/Fos complexes (Echlin et al., 2000; Thornton et al., 2006; Williams et al., 2001).

Batf was initially cloned from a B cell line (Dorsey et al., 1995), but is also expressed in T cells and induced by activation through the T cell receptor (Williams et al., 2001). Subsequent studies analyzed *Batf* function by overexpression as a transgene under the proximal lck promoter which confers expression at early stages of T cell development. In this context, *Batf* overexpression severely reduced NKT cell development (Williams et al., 2003; Zullo et al., 2007) and thymocyte proliferation. It is not clear from these studies, whether the reduced thymocyte proliferation in *Batf* transgenic mice is due to a T cell intrinsic defect or secondary to the absence of NKT cell derived cytokines, since total thymocytes were analyzed (Williams et al., 2003). Nevertheless, *Batf* is a potential inhibitor of AP-1 transcription expressed in T cells that affects T cell differentiation by overexpression.

In the studies presented here, we identify a critical role for *Batf* in T_H17 differentiation and we found that *Batf* is expressed highly in T helper cells compared to various other immune cells and tissues. We generated *Batf* deficient mice by gene targeting. *Batf*$^{-/-}$ mice show a highly selective defect in T_H17 differentiation and are resistant to experimental autoimmune encephalomyelitis. Using gene expression analysis, we found that *Batf*$^{-/-}$ T cells fail to induce known T_H17-specific transcription factors, such as RORγt, and the cytokine IL-21, required for T_H17 differentiation. Neither addition of IL-21 nor overexpression of RORγt fully restores IL-17 production in *Batf*$^{-/-}$ T cells, suggesting that Batf may be required directly for IL-17 transcription. We found

that the *Il17* promoter is Batf-responsive, and upon T_H17 differentiation, Batf binds to several conserved intergenic elements in the *Il17a/f* locus and to regions in the *Il17, Il21* and *Il22* promoters. Using bio-computational methods we determined that the Batf-binding element in the *Il17, Il21* and *Il22* promoters differs from canonical symmetric AP-1 elements. Using EMSA analysis we found that Batf forms heterodimers preferentially with JunB during T_H17 differentiation. These results demonstrate that Batf regulates previously unknown AP-1 target genes to control T_H17 differentiation and T_H17-mediated autoimmune disease.

CHAPTER 2

Experimental Methods

Generation of mice. Murine *Batf* exons 1–2 were deleted by homologous recombination via a targeting vector constructed in pLNTK(Gorman et al., 1996) using a 1 kb genomic fragment (left arm) upstream of the *Batf* exon 1 and a 3.6 kb genomic fragment (right arm) downstream of exon 2. The left arm was generated by PCR from genomic DNA with the use of the following oligonucleotides: left arm forward (5'-ATTA**CTCGAG**TGAAACAAACAGGCAGTCGCAGTG) and left arm reverse (5'-ATTA**CTCGAG**CCTACTACCTTTCAGGGCTACTGC). The right arm was generated by PCR with the use of the following oligonucleotides: right arm forward (5'-ATTA**GTCGAC**GCATTCTTCATGGTCCTTAGCCTTGG) and right arm reverse (5'-ATTA**GTCGAC**CAGAGAATGAGAAATGTTGGAGG). EDJ22 embryonic stem cells were transfected with linearized targeting vector and targeted clones were identified by Southern blot analysis using probes A and B located 5' to the left arm and 3' to the right arm respectively. Probe A was generated using the oligonucleotides 5'-CAACTGGGTCTGAGTCAAGAGGT and 5'-CGTAGCCGCTGATTGTTTTAGAAC to generate a 531bp product. Probe B was generated using the oligonucleotides 5'-ACAGCTTGAACTTCAGAGCCCTCC and 5'-CACATTTAAGTCACAATAACACTGC to generate a 772bp product. The neomycin resistance cassette was deleted from successfully targeted clones by *in vitro* treatment with Adeno-Cre virus (gift from Dr. Barry Sleckman, Washington University, St. Louis, MO) and targeted clones with successful neo deletion were identified by Southern blot

using probes A and B (Supplementary Fig.1b and c). Blastocyst injections were performed with two distinct recombinant clones each of which generated germline transmission of the targeted *Batf* allele. Male chimeras were crossed with 129SvEv females to establish *Batf* mutants on the pure 129SvEv genetic background. All experiments were performed with mice harboring the neo-deleted mutant allele. Homozygous mice were obtained by intercrossing heterozygous siblings and littermates were used as controls in most experiments. For some experiments 129SvEv wild type mice purchased from Taconic served as controls. For experiments with DO11.10 transgenic Batf$^{-/-}$ mice, mice were crossed to BALB/c mice for at least 5 generations and littermate controls were used as control.

For the generation of transgenic mice, *Batf* cDNA was cloned from CD4^{+} T cell mRNA using primers 5'-GGAAGATTAGAACCATGCCTC and 5'-AGAAGGTCAGGGCTGGAAG and subcloned into the GFP-RV retrovirus (Ranganath et al., 1998). An N-terminal FLAG tag was introduced by Quick Change Mutagenesis kit (Stratagene) using the primers 5'-**G**GACTACAAAGACGATGACGACAAGCCTCACAGCTCCGACAGCA and 5'-CTTGTCGTCATCGTCTTTGTAGTCCATGGTTCTAATCTTCCAGATC. The underlined sequence indicates nucleotides used to introduce the FLAG-tag. The FLAG-tagged *Batf* was cloned into the CD2 microinjection cassette(Zhumabekov et al., 1995) via blunt end strategy into Sma1 digested CD2 microinjection cassette. Transgene expression in CD4^{+} T cells was tested by anti-FLAG western blot. CD2-N-FLAG-*Batf* transgenic mice were crossed to C57BL/6 and BALB/c mice for at least 5 generations. Transgene-negative littermates were used as control mice. Mice were bred and

maintained at the animal facilities at Washington University in St. Louis. All animal experiments were approved by the Animal Studies Committee at Washington University.

Visualization of lymph nodes. To visualize superficial inguinal lymph nodes mice were injected with 50μl of 1% Evans Blue dye solution into each hind foot pad. After 1.5 hours mice were sacrificed and lymph nodes were visualized using a dissecting microscope(Sun et al., 2000).

Western Blot analysis. To test for residual Batf protein expression, total splenocytes from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ 129SvEv mice were stimulated with anti-CD3 for 3 days under T_H17 conditions. Cells were then lysed in RIPA buffer, electrophoresed on 15% polyacrylamide gels, transferred to nitrocellulose and analyzed by Western Blot with rabbit anti-murine Batf polyclonal serum and HRP-conjugated anti-rabbit Ig antibody (Jackson ImmunoResearch). Affinity purified rabbit anti-murine Batf polyclonal serum (Brookwood Biomedical; Birmingham, AL) was generated by immunization with full length recombinant Batf protein. Equal protein loading was assessed by subsequent immunoblotting with antibody to β-actin (Santa Cruz Biotechnology) and HRP conjugated anti-mouse antibody (Jackson ImmunoResearch).

For analysis of Batf protein expression in naïve CD4$^+$ T cells, magnetically purified CD4$^+$ T cells from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ 129SvEv mice were isolated. Equal cell numbers were lysed in RIPA buffer and subjected to Western Blot analysis as described above.

For analysis of Batf expression in T_H2 cells, magnetically purified $CD4^+$ T cells from $Batf^{+/+}$ and $Batf^{-/-}$ mice were activated with anti-CD3/CD28 in the presence of IL-4, anti-IL-12 (Tosh), and anti-IFNγ (H22). On day 4 cells were left unstimulated or stimulated with PMA/ionomycin for 4 hrs. Cells were collected by centrifugation, washed with PBS, and resuspended ($100e^6$ cells/ml) in Affymetrix Chip lysis buffer (10mM Tris pH 7.5, 10mM NaCl, 3mM $MgCl_2$, 0.5% IGEPAL, with protease inhibitors (PMSF, aprotinin, leupeptin)). After 5 min at 4^oC, nuclei were collected by centrifugation (800 rcf for 3 min 4^oC) and lysed in RIPA ($100e^6$ cell equivalents/ml) with protease inhibitors. Nuclear lysates were centrifuged for 10 min 4^oC 15000 rcf and diluted with an equal volume of 2x SDS-PAGE sample buffer containing 2-ME. Nuclear extracts from equal cell numbers were subjected to Western Blot analysis using affinity purified rabbit anti-murine Batf polyclonal serum. Equal protein loading was assessed by subsequent immunoblotting with antibody to Lamin B (Santa Cruz Biotechnology) and HRP conjugated anti-goat Ig (Jackson ImmunoResearch).

Immunohistochemistry. To test for cellular localization of Batf, $CD4^+$ T cells from CD2-N-FLAG-*Batf* transgenic mice were isolated by magnetic separation and either left untreated or stimulated with PMA/ionomycin for 4h. Cells were then allowed to settle on poly-L-lysine treated slides, fixed with 4% Formaldehyde, permeabilized with 0.25% TritonX100 and were stained with an anti-FLAG antibody (M2, Sigma Aldrich) according to the manufacturer's recommendations. A goat anti-mouse AF-488 (Invitrogen) antibody was to detect anti-FLAG staining. For analysis of cellular localization of Batf in T_H2 cells, DO11.10 $CD4^+$ T cells from CD2-N-FLAG-*Batf*

transgenic mice were isolated and differentiated with OVA and APC under T_H2 conditions for 7 days. On day 7 cells were either left untreated or stimulated with PMA/ionomycin for 4h. Cells were stained with anti-FLAG antibody as described above. Cells were also stained with anti-CD4APC antibody (BDBiosciences). Confocal images were obtained with the Olympus FV1000 microscope and software using a 60x oil objective. The pinhole was set to 110μm. The excitation/emission settings used for DAPI, Alexa 488 and Alexa 633 were 405/461nm, 488/520nm and 635/668nm respectively.

Flow cytometry. All flow cytometric data was collected on a FACS Calibur or FACS Canto (both BD Biosciences) and analyzed using FloJo analysis software (Tree Star, Inc.).

The following antibodies were purchased from BD Biosciences; anti-CD4-Allophycoerythrin (APC), CD4-Phycoerythrin (PE)/Cy7 (RM4-5), anti-CD8-APC (53-6.7), anti-CD44-APC (IM7), anti-CD62L-PE (Mel14), anti-CD3-APC (145-2C11), anti-IgM-PE (II/41), anti-B220 Fluorescein isothiocyanate (FITC) (RA3-6B2), anti-IL-17-PE (TC11-18H10), anti-IFNγ- PE (XMG1.2), anti-IFNγ-APC, anti-IL-4-APC (11B11), anti-IL-10-APC (JES5-16E3), anti-CD16/32 (2.4G2), anti-CD11c-PE (HL3), anti-CD11b-PECy7 (M1/70)), anti-CD44-APC (1M7), anti-CD25-APC (3C7), anti-phospho Stat3-AlexaFluor 647 (4/P-Stat3), Streptavidin-PeCy7, 7-AAD, AnnexinV-FITC and AnnexinV staining solution.

The following antibodies and solutions were purchased from eBioscience; anti-AA4.1 APC (AA4.1), anti-IgD PE (11-26c), IL-17A-FITC (eBio17B7), anti-Foxp3 (FJK-16s) and Foxp3 staining buffers.

Anti-CD4-FITC and anti-CD8-FITC were purchased from Invitrogen. Anti-Dec205-biotin (MG38) was purchased from Cedarlane. CD1d-PBS57-PE and CD1d-unloaded-PE tetramers were obtained from the tetramer facility at the NIH. Anti-IL-22 (RMF 222CK) was purchased from Antigenix.

Intracellular Staining. For intracellular cytokine staining cells were stained with antibodies to surface markers followed by fixation with 2% formaldehyde for 15 minutes at room temperature. Cells were then washed once in 0.05% saponin and stained with anti-cytokine antibodies in 0.5% saponin as described previously(Berenson et al., 2004). Anti-phospho-STAT3 antibody was purchased from BD Pharmingen and cells were stained according to the manufacturer's recommendations. Briefly, cells were stained for surface markers followed by fixation with 90% methanol at -20^{o}C overnight. Cells were then washed and stained for phospho-STAT3 in PBS containing 3% FCS. For Foxp3 staining, cells were stained using Foxp3 staining buffers (eBioscience) according to the manufacturer's recommendations.

Isolation of dendritic cells for flow cytometry. Spleens were isolated, cut into small pieces and digested with Collagenase B (Roche) and DNase I (Sigma) for 30 min at 37^{o}C. Red blood cells were lysed by incubation with Red Blood Cell Lysis Buffer (Sigma) (1 minute at room temperature). Single cell suspensions were prepared by passing digested spleens through 35μm nylon cell strainers (Fisher Scientific) and were stained with antibodies for analysis by Flow Cytometry.

Isolation of naïve T cells. Splenic single cells suspensions were generated and red blood cells were lysed by incubation with Red Blood Cell Lysis Buffer (Sigma) (1 minute at room temperature). Splenocytes were then negatively depleted of B220^{+} and CD8^{+} cells

using magnetically labeled beads followed by depletion over LD columns (all Miltenyi Biotec). The depleted fraction was then stained with antibodies to CD4, CD62L and CD25 (all BD Biosciences) and $CD4^+CD62L^+CD25^-$ cells were sorted on a MoFlo cytometer. Sort purity was generally >98%. For some experiments, as indicated, $CD4^+$ T cells were isolated from spleens by incubation with anti-CD4 magnetic beads and selection via LS columns (Miltenyi Biotec) according to the manufacturer's recommendations.

Cell culture. For T cell differentiation assays, sorted naïve $CD4^+$ $CD62L^+CD25^-$ T cells or magnetically purified $CD4^+$ T cells were isolated as indicated. Cells were cultured at $0.5x10^6$ cells/well in 48 well plates containing plate-bound anti-CD3 (from ascites) and soluble anti-CD28 (37.5; BioXcell; 4μg/ml). Stimulation of cells without the addition of cytokines was defined as drift condition. Cultures were supplemented with anti-IL-4 (11B11; hybridoma supernatant), IFNγ (Peprotech; 0.1ng/ml) and IL-12 (Genetics Institute; 10U/ml) for T_H1; anti-IFNγ (H22; BioXcell; 10μg/ml), anti-IL-12 (Tosh; BioXcell; 10μg/ml) and IL-4 (Peprotech; 10ng/ml) for T_H2; anti-IL-4, anti-IL-12, anti-IFNγ, IL-6 (Peprotech; 20ng/ml) and TGF-β (Peprotech; 0.5ng/ml) for T_H17 differentiation. In some experiments, cultures were supplemented with IL-21 (50ng/ml; all Peprotech), anti-IL-6 (MP5-20F3; eBioscience; 10μg/ml), anti-TGF-β (1D11, R&D Biosystems, 10μg/ml) or anti-IL-2 (JES6-1A12; BioXcell; 10μg/ml) as indicated. For drift, T_H1 and T_H2 conditions cells were restimulated on day 7 with anti-CD3 and anti-CD28. Brefeldin A was added for the last 4 hours of stimulation. For T_H17 conditions, cells were restimulated on day 3 or day 7 after activation as indicated with Phorbol 12-

myristate 13-acetate (PMA) (50ng/ml; Sigma) and ionomycin (1μM; Sigma) for 4 hours in the presence of Brefeldin A (1μg/ml; Epicentre). Cells were then analyzed by intracellular cytokine staining and flow cytometry.

In some experiments, as indicated, magnetically purified $CD4^+$ T cells from DO11.10 transgenic mice were activated with OVA (3μM) and irradiated splenocytes in the presence of anti-IL-4, anti-IL-12, anti-IFNγ, IL-6 and TGF-β (1ng/ml) to induce T_H17 differentiation.

To induce T_H17 differentiation in total splenocytes, single cells suspensions from spleens were prepared and red blood cells were lysed. Total splenocytes were activated at $4x10^6$ cells/well in 12 well plates containing plate-bound anti-CD3, anti-IL-4 (hybridoma supernatant), anti-IL-12 (10μg/ml), anti-IFNγ (10μg/ml), IL-6 (20ng/ml) and TGF-β (1ng/ml). Cells were restimulated with PMA and ionomycin for 4h in the presence of Brefeldin A before intracellular cytokine staining and analysis by flow cytometry.
For STAT3-phosphorylation assays magnetically purified $CD4^+$ or $CD8^+$ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of IL-6 or IL-21 (50ng/ml) followed by intracellular staining and analysis by flow cytometry.

ELISA. The concentration of IL-21 in supernatants from $CD4^+$ T cells activated for 3 days under TH17 conditions was determined by ELISA (R&D Systems) according to the manufacturer's recommendations.

Isolation of Lamina Propria T cells. For isolation of lamina propria T cells, mice were sacrificed; small intestines removed, placed in cold DMEM media (10%FCS) and cleared

of Peyer's patches and residual mesenteric fat tissue. Intestines were then opened longitudinally, cleared of contents and cut into 0.5cm pieces. The pieces were washed multiple times in cold media and twice in ice cold Citrate BSA (CB-BSA) buffer followed by two 15 minute incubations in CB-BSA with agitation. After each incubation cells were vortexed to remove epithelial cells. The remaining intestinal pieces were then washed twice with cold media before digestion in media containing 75U/ml Collagenase IV (Sigma) at 37^{o}C for 1 hour. The solution was vortexed at 20 min intervals to detach lymphocytes. After one hour the solution was filtered through a 35μm strainer, the pieces were collected and digested a second time. Supernatants from both digestions were combined, washed once, suspended in the 70% fraction of a percoll gradient and overlaid with 37% and 30% percoll gradient fractions. Lymphocytes were collected at the 70-37% interface, washed once in PBS and stimulated with PMA/ionomycin in the presence of Brefeldin A for 3 hours before cells were stained for extracellular markers and intracellular cytokines.

Induction of EAE and disease scoring. Age and sex matched mice (7-10 weeks old) were immunized subcutaneously with 100μg MOG_{35-55} peptide (Sigma) emulsified in CFA (IFA supplemented with 500μg *Mycobacterium tuberculosis*) on day 0. On days 1 and 3, mice were injected with 300ng Pertussis Toxin (List Biological Laboratories) intraperitonally (i.p.). Clinical scores were given on a scale of 1-5 as follows: 0, no overt signs of disease; 1, limp tail or hind limb weakness, but not both; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund state or death by EAE. Mice with a score of 4 were given 300 μl saline solution

subcutaneously to prevent dehydration. Mice with a score of 5 were euthanized. Some mice died during the course of the experiment. Their score of 5 was included in the analysis for the remainder of the experiment. For T cell transfer experiments, $CD4^+$ T cells were isolated from splenic single cell suspensions by magnetic separation with anti-CD4 magnetic beads and positive selection via LS columns (Miltenyi Biotec). $1x10^7$ MACS purified $CD4^+$ T cells were injected i.p. on day -4 followed by EAE induction on day 0 as described above.

Isolation of CNS lymphocytes. Brain and spinal cords were removed from mice after perfusion with 30ml of saline solution. Single cell suspensions were prepared by dispersion through sterile 35μ nylon cell strainers (Fisher Scientific) and mixed at room temperature for 1hr in HBSS containing 0.1% collagenase, 0.1μg/ml TLCK (N-α-tosyl-L-lysine chloromethylketone hydrochloride), and 10μg/ml DNaseI (all Sigma). The resulting suspension was pelleted, resuspended in the 70% fraction of a Percoll gradient and overlaid by additional 37% and 30% layers. The Percoll gradient separation was achieved by centrifugation for 20 min at 2000rpm and lymphocytes were collected at the 70-37% interface. Subsequently cells were activated with PMA and ionomycin for 3-4 hours in the presence of Brefeldin A and intracellular cytokine staining was performed.

Real time PCR. Naïve $CD4^+CD62L^+CD25^-$ T cells were isolated by cell sorting and activated with plate-bound anti-CD3 and soluble anti-CD28 antibodies under T_H17 conditions for 3 days, unless otherwise indicated. Total RNA was isolated from the indicated cells using Quiagen RNeasy Mini Kit and cDNA was synthesized using

SuperscriptIII reverse transcriptase (Invitrogen). Real time PCR analysis was performed using ABI SYBR Green master mix according to the manufacturer's instructions on an ABI7000 machine (Applied Biosystems) using the relative standard curve method. The PCR conditions were 2min at 50^{o}C, 10 min at 95^{o}C followed by 40 2-step cycles of 15s at 95^{o}C and 1min at 60^{o}C.

Primers for RORγt (RORγt forward 5'-CGCTGAGAGGGCTTCAC, RORγt reverse 5'-GCAGGAGTAGGCCACATTACA)(IVANOV ET AL., 2006), IL-21 (IL-21 forward 5'-ATCCTGAACTTCTATCAGCTCCAC, RORα (RORα forward 5'-TCTCCCTGCGCTCTCCGCAC-3', RORα reverse 5' TCCACAGATCTTGCATGGA-3')(YANG ET AL., 2008), IL-21 reverse 5′-GCATTTAGCTATGTGCTTCTGTTTC)(Zhou et al., 2007), IL-22 (IL-22 forward-5' CATGCAGGAGGTGGTACCTT, IL-22 reverse- 5'-CAGACGCAAGCATTTCTCAG)(Chung et al., 2006), IL-23R (IL-23R forward 5'-GCCAAGAAGACCATTCCCGA, IL-23R reverse 5'-TCAGTGCTACAATCTTCTTCAGAGGACA)(MANGAN ET AL., 2006), IRF-4 (IRF-4 forward 5'-GCCCAACAAGCTAGAAAG, IRF-4 reverse: 5'-TCTCTGAGGGTCTGGAAACT) (Negishi et al., 2005) and HPRT as normalization control (HPRT forward 5'-AGCCTAAGATGAGCGCC, HPRT reverse 5'-TTACTAGGCAGATGGCCACA) were used to evaluate relative gene expression.

For analysis of acute phase response proteins, mice were injected intraperitonally with either 0.9% saline solution or IL-6 (0.3μg per mouse) in 0.9% saline solution. Four hours later, total liver RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's recommendations. cDNA was synthesized and real time PCR

performed as described above. Primers used for serum amyloid protein P (SAP forward: 5'-TTTCAGAAGCCTTTTGTCAGA and SAP reverse: 5'-AAGGTCACTGTAGGTTCGGA) (Korbelik et al., 2008), c-reactive protein (CRP forward: 5'- TTCTGGATTGATGGGAAAAGC and CRP reverse: 5'- AAACATTGGGGCTGAGTGTC)(Korbelik et al., 2008), Serum amyloid protein A (SAA forward 5'-TCTCTGGGGCAACATAGTATACCTCTCAT and SAA reverse 5'-TTTATTACCCTCTCCTCCTCAAGCAGTTAC) (Dierssen et al., 2008), fibrinogen β (fibß forward: 5'-ATTAGCCAGCTTACCAGGATGGGACCCAC-3', Fibß reverse: 5'-CAGTAGTAT CTGCCGTTTGGATTGGCTGC-3')(Chauvet et al., 2005), alpha-1-acid glycoprotein (AGP forward: TCT CTG AAC TCC GAG GGC TG AGP reverse: GAGACAGAATCAAAGTGCACAGGA)(Theilgaard-Monch et al., 2005) and HPRT as normalization control (HPRT forward 5'-AGCCTAAGATGAGCGCC, HPRT reverse 5'-TTACTAGGCAGATGGCCACA) were used to evaluate relative gene expression.

Gene expression profiling. Naïve $CD4^+$ $CD62L^+$ $CD25^-$ T cells and $CD4^+$ $CD62L^+$ $CD25^+$ regulatory T cells were isolated from C57BL/6 mice. Naïve $CD4^+$ $CD62L^+$ $CD25^-$ T cells were differentiated under T_H1 and T_H2 conditions for 7 days. After restimulation with anti-CD3 and anti-CD28 for 24h, T_H1 and T_H2 cells were sorted for IFNγ and IL-4 production respectively using cytokine secretion assays (Miltenyi Biotec) according to the Manufacturer's recommendations. For gene expression profiling of T_H17 cells, naïve $CD4^+$ $CD62L^+$ $CD25^-$ T cells were activated for 3 days with anti-CD3 and anti-CD28 in the presence of anti-IL-4, anti-IL-12, anti-IFNγ, anti-IL-2, IL-6 and TGF-β (0.5ng/ml).

For gene expression analysis in *Batf*$^{-/-}$ T cells, naive CD4^{+} CD62L^{+} CD25^{-} T cells from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice were activated for 3 days with anti-CD3 and anti-CD28 in the presence of either anti-IL-4, anti-IL-12, anti-IFNγ, IL-6 and TGF-β (0.5ng/ml); anti-IL-4, anti-IL-12, anti-IFNγ, IL-6 and anti-TGF-β; anti-IL-4, anti-IL-12, anti-IFNγ, anti-IL-6 and TGF-β or anti-IL-4, anti-IL-12, anti-IFNγ, anti-IL-6 and anti-TGF-β. IL-2 was neutralized in all conditions. Total RNA was isolated from cells using Quiagen RNeasy Mini Kit. Biotinylated antisense cRNA was generated using two cycle target preparation kit (Affymetrix). After fragmentation, cRNA was hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 Arrays. Data were normalized and expression values were modeled using DNA-Chip analyzer (dChip) software (www.dChip.org).

Retroviral infection and analysis. mRNA was isolated from 129SvEv total thymocytes using Quiagen RNAeasy Mini Kit and cDNA was amplified by SuperscriptIII (Invitrogen). Murine RORγt transcript was amplified using primers 5'-CTCGAGGTGTGCTGTCCTGGGCTAC and 5'-CTCGAGGGGAGACGGGTCAGAGGG. Underlined nucleotides indicate XhoI overhangs used to clone RORγt into XhoI digested GFP-RV retrovirus(Ranganath et al., 1998) or XhoI digested hCD4-RV(Zhu et al., 2001).

Batf cDNA was cloned from CD4^{+} T cell mRNA using primers 5'-GGAAGATTAGAACCATGCCTC and 5'-AGAAGGTCAGGGCTGGAAG and subcloned into the GFP-RV retrovirus(Ranganath et al., 1998). An N-terminal FLAG tag was introduced by Quick Change Mutagenesis kit (Stratagene) using the primers 5'-**G**GACTACAAAGACGATGACGACAAGCCTCACAGCTCCGACAGCA and 5'-

CTTGTCGTCATCGTCTTTGTAGTCCATGGTTCTAATCTTCCAGATC. The underlined sequence indicates nucleotides used to introduce the FLAG-tag.

The retrovirus based reporter hCD4-pA-GFP-RV(Zhu et al., 2001), in which a cytoplasmic truncated human CD4 (hCD4) marks viral infection and green fluorescence protein (GFP) is used to report promoter activity has been described previously and was modified as follows to generate hCD4-pA-GFP-RV-IL-17p. The 1021bp promoter region of murine IL-17a was generated by PCR from genomic 129SvEv DNA using primers 5'-AGCTTGAACAGGAGCTATCGGTCC and 5'-AAGCTTGAGGTGGATGAAGAGTAGTGC. Underlined nucleotides indicate overhangs containing HindIII restriction sites used to clone the resulting PCR product into hCD4-pA-GFP-RV.

Retroviral vectors were packaged in Phoenix E cells as described previously (Ranganath et al., 1998). Magnetically purified $CD4^+$ T cells were infected with viral supernatants on days 1 and 2 after activation with anti-CD3 and anti-CD28. 3 days after activation cells were restimulated with PMA/ionomycin in the presence of Brefeldin A and analyzed by intracellular cytokine staining and Flow Cytometry. For the experiments in Figure 4, $CD4^+$ T cells from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice were activated under T_H17 conditions and infected with the IL-17 reporter virus. Stably infected T cells were restimulated with PMA/ionomycin for 4h and examined for GFP expression on day 3 after initial activation.

Statistical Analysis. A Student's unpaired two-tailed t-test was used to indicate statistically significant differences between indicated groups. Differences with a *P* value <0.05 were considered significant.

Electrophoretic mobility shift assays. Whole cell extracts were prepared from total splenocytes activated for 3 days with anti-CD3, TGF-β and IL-6 as described previously (Nakshatri and Currie, 1996). For EMSA analysis the AP-1 consensus probe(Echlin et al., 2000) (top: AGCTTCGCTTGATGAGTC and bottom: GCCGACTGAGTAGTTCGC), RORE element in CNS2 of the IL-17 gene(Yang et al., 2008) (top: GAAAGTTTTCTGACCCACTTTAAATCA and bottom: CTTTAACTAAATTTCACCCAGTCTTTT)
and -187 to -155 of the IL-17 promoter (top: GGTTCTGTGCTGACCTCATTTGAGGATG and bottom: AAAAGACTGGGTGAAATTTAGTTAAAG), Eα Y box probe (TCGACATTTTTCTGATTGGTTAAAAGTC) (Szabo et al., 1993) were used after labeling with ^{32}P-dCTP. The probe ($2.5x10^4$cpm per reaction) was used along with 15μg of total cell extracts and 1ug poly diDC as described previously(Szabo et al., 1993).

For competitor-supershift assay, Batf binding to the AP-1 consensus probe(Echlin et al., 2000) was assessed by anti-FLAG supershift. Unlabeled probes from the IL-17a, IL-21 and IL-22 promoters were used to compete for Batf binding to the AP-1 consensus probe. Their sequences are provided below. Single stranded overhangs of the competitor oligos were not filled in. Sequences identified as competitors for Batf binding were used to determine the Batf consensus motif.

For supershift analysis of the EMSA complexes formed on the AP-1 probe, whole cell extracts were prepared as above. 8μg whole cell extracts were incubated for 15min on ice with anti-Batf, anti-Fos (K25), anti-c-Jun (D), anti-c-Jun (N), anti-JunB (C11), anti-JunD (329), anti-ATF-1 (H60) and anti-ATF-3 (C-19) (all Santa Cruz Biotechnology) before $2.5x10^4$cpm of the AP-1 consensus probe was added.
To test whether Batf binding to the AP-1 probe requires stimulation DO11.10 transgenic $CD4^+$ T cells were activated for 3 days with OVA, irradiated APCs, anti-IFNγ/IL-4/IL12, TGF-β and IL-6, followed by a period of 3 days rest in the presence of TGF-β and IL-6. Cells were left untreated or activated with PMA/ionomycin for 4 hrs before whole cell extracts were prepared and used in EMSA analysis as described above.

CONSENSUS program for determination of Batf binding motif. Sequences of the proximal promoter regions of IL-17, IL-21, and IL-22 identified as competitors for Batf binding in the competitor-supershift EMSA assay were input into CONSENSUS version v6d(Hertz and Stormo, 1999). Default program parameters were applied, except for searching the reverse complement of the input sequences (c2) and uniform background nucleotide frequencies. The program was searching potential motif lengths from 5 to 15 using the expected frequency statistic (e-value) and the optimal motif length was determined as 7. The corresponding weight matrix, with a sample size adjusted information content of 4.467, was chosen from the final cycle. The enrichment of the binding motif in the input set was verified using PATSER v3e(Stormo et al., 1982). Using the numerically calculated cutoff score, 38/40 of the input training sequences were identified as containing the motif.

Batf Chromatin immunoprecipitation (ChIP). ChIP was performed as previously described (Hatton et al., 2006) using an affinity purified anti-Batf rabbit polyclonal antibody prepared by Brookwood Biomedical (Birmingham, AL). Briefly, chromatin was prepared from $1x10^7$ $CD4^+$ T cells isolated from C57BL/6 *Batf*$^{+/+}$ mice stimulated under T_H17 polarizing conditions with anti-CD3 (2.5μg/ml) and syngeneic splenic feeder cells, then restimulated or not at the indicated time points with PMA (50ng/ml) and ionomycin (750ng/ml) for 4 h. For experiments assessing early binding of Batf to the DNA $CD4^+$ T cells from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ 129SvEv mice were activated with anti-CD3/CD28 coated beads under T_H17 conditions for 24 hours, then processed for ChIP analysis. Immunoprecipitations were performed with 20 μg/ml anti-Batf rabbit polyclonal antibody using the Chromatin Immunoprecipitation (ChIP) Assay Kit from Millipore (Billerica, MA) according to the manufacturer's recommendations. Immunoprecipitated DNA released from cross-linked proteins was quantitated by real-time PCR as previously reported (Hatton et al., 2006), and was normalized to input DNA. All real-time PCR primers and probes are provided below. The analyzed sites are denoted relative to the ATG start codons for the *Il17a* or *Il17f* gene.

For ChIP analysis of the IL-21 and IL-22 promoters DO11.10 transgenic $CD4^+$ T cells from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ were stimulated with OVA and APC for 3 days, rested for 3 days before restimulation with PMA/ionomycin for 4h on day 5 and processing for ChIP as described above. Real time PCR analysis was performed using ABI SYBR Green master mix according to the manufacturer's instructions on a Step One Plus (Applied

Biosystems) using the relative standard curve method. Results were normalized to input DNA. Sequences of primers used in the analysis are provided below.

ChIP primers

Primers	Chr. 1 location	5'FAM 3' BHQ1 Probes
IL17a -97 **(-97kb)**		
5' AAATGTGAGCCCCAGATCGA 3'	20,623,606-20,623,625	CTGCTGCTGTCCCAGGCACAGTTG
5' GGGACATTTTTTCCACCATGA 3'	20,623,652-20,623,672	
IL17a -60 **(-60kb**		
5' TTGTCCCCTGGCTGTTCCT 3'	20,661,177-20,661,247	CCTTATCCAGCTGTCTTTTTCTCT
5' GGGCTCCCCAAAAATTCACA 3'	20,661,274-20,661,293	
IL17a -37 **(-37kb)**		
5' GTCCCTCTGTTGTTTCCAAGGAT 3'	20,683,616-20,683,638	TCATTGAGTCCTTCCAGCAGAGATTTCAGG
5' GCCATTTCAGCCACTGTGAA 3'	20,683,671-20,683,690	
IL17a -15 **(-15kb)**		
5' TGGCAAATGTTTTGTCAACCA 3'	20,705,507-20,705,527	TTCCTCGATTGCTGTCTACTCATC
5' CATGCAGCCTCTGCTTGAGA 3'	20,705,554-20,705,573	
IL17a -5 **(-5kb)**		
5' CGATACTTTTCAGTGACATCCGTTT 3'	20,715,852-20,715,876	ACTTGAAACCCAGTCAGTTGCTGACCTTGA
5' TGCTGACTTCATCTGATACCCTTAGA 3'	20,715,910-20,715,935	
IL17a promoter **(-243 to -176)**		
5' GAACTTCTGCCCTTCCCATCT 3'	20,720,800-20,720,820	CCTTCGAGACAGATGTTGCCCGTCA
5' CAGCACAGAACCACCCCTTT 3'	20,720,848-20,720,867	
IL17a +9.6 **(+9.6kb)**		
5' ATTTAGGGCACAGGTGACATGA 3'	20,730,688-20,730,709	TGGTTCTCAAAGCATAAACCTCATTC
5' CCACTTCCCCGACCTCACTA 3'	20,730,738-20,730,757	
IL17a +23 **(+23kb)**		
5' CAAATCCGTGTGCCTTCTGTT 3'	20,744,816-20,744,836	CTGCAGTGAGGAAGATGTTTCCAATGAGG
5' AGGTTGACTTCGTCCCTGTGA 3'	20,744,870-20,744,890	
IL17a +28 **(+28kb)**		
5' GTGGCCTACTTCAGGCAGATG 3'	20,749,994-20,750,014	TGAGAAGCCAGCGTCGGGTCC
5' GGAGCCGATGAGAAGCATTC 3'	20,750,039-20,750,058	
IL17a +36 **(+36kb)**		
5' AGATAATGTATCACACAGCCCTGAAG 3'	20,757,551-20,757,576	AGCCAGTGCCTTAATCCATTGGG
5' CATGGTTGTGAAGTTGGTGAGATG 3'	20,757,602-20,757,625	
IL17f promoter **(-408 to -340)**		
5' ACTGCATGACCCGAAAGCA 3'	20,774,671-20,774,688	AACCCACACGCAGAGCATGACAAGAG
5' TTTAATTCCCCCACAAAGCAA 3'	20,774,620-20,774,640	
IL17f -7 **(-7kb)**		
5' TTCCCTTTTCTGCCTTGCA 3'	20,782,972-20,782,990	ACGAAGCACAGGGCTGGGCC
5' TGTGTAACACGCAGAGTGGAATG 3'	20,783,017-20,783,039	
	Chr. 3 location	
IL21 promoter **(-529 to -382)**		
5' GCATAGTCATCACCCCATAAA 3'	37,131,996-37,132,016	
5' TCAGAGAAGTAAACACAAACAC 3'	37,131,869-37,131,890	
	Chr. 10 location	
IL22 promoter **(-600 to -417)**		
5' GCACAGAATATAGGACACGGGT	117,641,447-117,641,468	
5' ACACAGTTTTCAAAGAAAGCCA	117,641,609 117,641,630	

IL-17a promoter Oligos	Sequence 5' to 3'
33-1-top-IL17a	GCACCCAGCACCAGCTGATCAGGACGCG
33-1-bot-IL17a	GTTTGCGCGTCCTGATCAGCTGGTGCTG
46-14-top-IL17a	ACGAGGCACAAGTGCACCCAGCACCAGC
46-14-bot-IL17a	GATCAGCTGGTGCTGGGTGCACTTGTGC
69-37-top-IL17a	GCACTACTCTTCATCCACCTCACACGAG
69-37-bot-IL17a	TGTGCCTCGTGTGAGGTGGATGAAGAGT
83-51-top-IL17a	AAAGAGAGAAAGGAGCACTACTCTTCAT
83-51-bot-IL17a	GGTGGATGAAGAGTAGTGCTCCTTTCTC
100-68-top-IL17a	GTAGTAAAACCGTATAAAAAGAGAGAAA
100-68-bot-IL17a	GCTCCTTTCTCTCTTTTTATACGGTTTT
119-87-top-IL17a	ACGTAAGTGACCACAGAGGTAGTAAAA
119-87-bot-IL17a	TACGGTTTTACTACCTCTGTGGTCACT
140-106-top-IL17a	GTCACCCCCCAACCCACTCTTGACGTAAGT
140-106-bot-IL17a	TGGTCACTTACGTCAAGAGTGGGTTGGGGG
159-127-top-IL17a	GAATCTTTACTCAAATGGTGTCACCCCC
159-127-bot-IL17a	GGTTGGGGGGTGACACCATTTGAGTAAA
169-137-top-IL17a	TTTGAGGATGGAATCTTTACTCAAATGG
169-137-bot-IL17a	TGACACCATTTGAGTAAAGATTCCATCC
187-155-top-IL17a	GGTTCTGTGCTGACCTCATTTGAGGATG
187-155-bot-IL17a	GATTCCATCCTCAAATGAGGTCAGCACA
204-172-top-IL17a	GCCCGTCATAAAGGGGTGGTTCTGTGCT
204-172-bot-IL17a	AGGTCAGCACAGAACCACCCCTTTATGA
215-183-top-IL17a	AGACAGATGTTGCCCGTCATAAAGGGGT
215-183-bot-IL17a	GAACCACCCCTTTATGACGGGCAACATC
235-203-top-IL17a	GCCCTTCCCATCTACCTTCGAGACAGAT
235-203-bot-IL17a	GCAACATCTGTCTCGAAGGTAGATGGGA
250-217-top-IL17a	GCATAGTGAACTTCTGCCCTTCCCATCTA
250-217-bot-IL17a	GAAGGTAGATGGGAAGGGCAGAAGTTCAC
266-234-top-IL17a	GAAGTCATGCTTCTTTGCATAGTGAACT
266-234-bot-IL17a	GCAGAAGTTCACTATGCAAAGAAGCATG
281-249-top-IL17a	CTGTTCAGCTCCCAAGAAGTCATGCTTC
281-249-bot-IL17a	GCAAAGAAGCATGACTTCTTGGGAGCTG
302-269-top-IL17a	CTGAATCACAGCAAAGCATCTCTGTTCAG
302-269-bot-IL17a	GGGAGCTGAACAGAGATGCTTTGCTGTGA
320-286-top-IL17a	GTCCATACACACATGATACTGAATCACAGC
320-286-bot-IL17a	GCTTTGCTGTGATTCAGTATCATGTGTGTA
334-302-top-IL17a	GCAGCTTCAGATATGTCCATACACACAT
334-302-bot-IL17a	GTATCATGTGTGTATGGACATATCTGAA
349-317-top-IL17a	GAGCCCAGCTCTGCAGCAGCTTCAGATA
349-317-bot-IL17a	GGACATATCTGAAGCTGCTGCAGAGCTG
370-337-top-IL17a	GACTCACAAACCATTACTATGGAGCCCAG
370-337-bot-IL17a	CAGAGCTGGGCTCCATAGTAATGGTTTGT
383-351-top-IL17a	GAGACTGTCAAGAGACTCACAAACCATT
383-351-bot-IL17a	ATAGTAATGGTTTGTGAGTCTCTTGACA
400-368-top-IL17a	AAAGTGTGTGTCACTAGGAGACTGTCAA

400-368-bot-IL17a	GTCTCTTGACAGTCTCCTAGTGACACAC
416-384-top-IL17a	GATCAAGTCAAAATTCAAAGTGTGTGTC
416-384-bot-IL17a	CTAGTGACACACACTTTGAATTTTGACT
433-401-top-IL17a	GGTAGAAAAGTGAGAAAGATCAAGTCAA
433-401-bot-IL17a	GAATTTTGACTTGATCTTTCTCACTTTT
445-413-top-IL17a	GCCAGGGAATTTGGTAGAAAAGTGAGAA
445-413-bot-IL17a	GATCTTTCTCACTTTTCTACCAAATTCC
464-432-top-IL17a	GGGCAAGGGATGCTCTCTAGCCAGGGAA
464-432-bot-IL17a	GCAAATTCCCTGGCTAGAGAGCATCCCT
476-44-top-IL17a	GTGGGTTTCTTTGGGCAAGGGATGCTCT
476-44-bot-IL17a	GCTAGAGAGCATCCCTTGCCCAAAGAAA
497-465-top-IL17a	GTTTACATACTAAGACATTGAGTGGGTT
497-465-bot-IL17a	AAAGAAACCCACTCAATGTCTTAGTATG

IL-21 promoter Oligos	Sequence 5' to 3'
33-1-top-IL21	GTCATCAGCTCCTGGAGACTCAGTTCTG
33-1-bottom-IL21	GCCACCAGAACTGAGTCTCCAGGAGCTG
55-22-top-IL21	GTGAGAACCAGACCAAGGCCCTGTCATCA
55-22-bottom-IL21	GGAGCTGATGACAGGGCCTTGGTCTGGTT
67-35-top-IL21	AGTCAGGTTGAAGTGAGAACCAGACCAA
67-35-bottom-IL21	GGGCCTTGGTCTGGTTCTCACTTCAACC
88-56-top-IL21	TAGCGACAACCTGTGCACAGTCAGGT
88-56-bottom-IL21	GTTCAACCTGACTGTGCACAGGTTGT
105-73-top-IL21	GATGAATAAATAGGTAGCCGTAGCGACA
105-73-bottom-IL21	CAGGTTGTCGCTACGGCTACCTATTTAT
120-88-top-IL21	GGCCTCTTCTTGAGGGATGAATAAATAG
120-88-bottom-IL21	GCTACCTATTTATTCATCCCTCAAGAAG
137-105-top-IL21	CTGCAATGGGAGGGCTTGGCCTCTTCTT
137-105-bottom-IL21	GCCTCAAGAAGAGGCCAAGCCCTCCCAT
150-118-top-IL21	AAAGATTTCCAGGCTGCAATGGGAGGGC
150-118-bottom-IL21	GCCAAGCCCTCCCATTGCAGCCTGGAAA
174-142-top-IL21	GTTACTCACACTCATCCACTATACAAAG
174-142-bottom-IL21	GAAATCTTTGTATAGTGGATGAGTGTGA
183-151-top-IL21	GAAAAACGAGTTACTCACACTCATCCAC
183-151-bottom-IL21	GTATAGTGGATGAGTGTGAGTAACTCGT
207-175-top-IL21	CACGTACACCTAGCCAATGGAAAAGAAA
207-175-bottom-IL21	TCGTTTTTCTTTTCCATTGGCTAGGTGT
221-189-top-IL21	TGCCCCCACACGCACACGTACACCTAGC
221-189-bottom-IL21	CATTGGCTAGGTGTACGTGTGCGTGTGG
240-208-top-IL21	TGTGGACTCTATCCATCCCTGCCCCCAC
240-208-bottom-IL21	TGCGTGTGGGGGCAGGGATGGATAGAGT
254-222-top-IL21	GATGGGGCACATTTTGTGGACTCTATCC
254-222-bottom-IL21	GGGATGGATAGAGTCCACAAAATGTGCC
266-234-top-IL21	GTCTAAGATGCAGATGGGGCACATTTTG
266-234-bottom-IL21	GTCCACAAAATGTGCCCCATCTGCATCT
279-247-top-IL21	GTCTCTTTTTCCTGTCTAAGATGCAGAT

279-247-bottom-IL21 GCCCCATCTGCATCTTAGACAGGAAAAA
304-272-top-IL21 GCTGAAAACTGGAATTCACCCATGTGTC
304-272-bottom-IL21 AAAGAGACACATGGGTGAATTCCAGTTT
314-282-top-IL21 CTTGGTGAATGCTGAAAACTGGAATTCA
314-282-bottom-IL21 ATGGGTGAATTCCAGTTTTCAGCATTCA
334-303-top-IL21 GACACACACACACACACACACCTTGGTG
334-303-bottom-IL21 GCATTCACCAAGGTGTGTGTGTGTGTGTG
361-328-top-IL21 GCCACACACACACACACACACACACACA
361-328-bottom-IL21 GTGTGTGTGTGTGTGTGTGTGTGTGT
383-351-top-IL21 GAAATCTGACGGTGCCTCCTGTGCCACA
383-351-bottom-IL21 GTGTGTGTGGCACAGGAGGCACCGTCAG
395-363-top-IL21 GTTTACTTCTCTGAAATCTGACGGTGCC
395-363-bottom-IL21 CAGGAGGCACCGTCAGATTTCAGAGAAG
410-378-top-IL21 GATCAAAGTGTTTGTGTTTACTTCTCTG
410-378-bottom-IL21 GATTTCAGAGAAGTAAACACAAACACTT
422-390-top-IL21 TGCAGAGCAAAAGATCAAAGTGTTTGTG
422-390-bottom-IL21 GTAAACACAAACACTTTGATCTTTTGCT
447-415-top-IL21 GACAAACCAGGTGAGGTGCCAGGGATGC
447-415-bottom-IL21 GCTCTGCATCCCTGGCACCTCACCTGGT
463-429-top-IL21 GCCTTTATGACTGTCAGACAAACCAGGTGA
463-429-bottom-IL21 GCACCTCACCTGGTTTGTCTGACAGTCATA
476-445-top-IL21 GTCATTGCAGAAGTGCCTTTATGACTGT
476-445-bottom-IL21 GTCTGACAGTCATAAAGGCACTTCTGCA
494-462-top-IL21 GCCATGCCGCTGCTTTACTCATTGCAGA
494-462-bottom-IL21 GCACTTCTGCAATGAGTAAAGCAGCGGC
509-477-top-IL21 AAAGTTCCAATAAAGGCCATGCCGCTGC
509-477-bottom-IL21 GTAAAGCAGCGGCATGGCCTTTATTGGA
525-493-top-IL21 AGTCATCACCCCATAAAAAGTTCCAATA
525-493-bottom-IL21 GCCTTTATTGGAACTTTTTATGGGGTGA
543-511-top-IL21 GGTTCAGTCAAAAAGCATAGTCATCACC
543-511-bottom-IL21 TATGGGGTGATGACTATGCTTTTTGACT
558-526-top-IL21 AATGGAGTACAGGATGGTTCAGTCAAAA
558-526-bottom-IL21 ATGCTTTTTGACTGAACCATCCTGTACT
578-546-top-IL21 GTAACCTCTTCCATCATTGCAATGGAGT
578-546-bottom-IL21 CCTGTACTCCATTGCAATGATGGAAGAG
604-573-top-IL21 GCCCATCATTTAATTCTTCCTAAGAAG
604-573-bottom-IL21 GGTTACTTCTTAGGAAGAATTAAATGA
618-586-top-IL21 AGGTTAGAAAACTAGCCCATCATTTAAT
618-586-bottom-IL21 GAAGAATTAAATGATGGGCTAGTTTTCT
639-607-top-IL21 AGGATCTAAAATACTCTTGCTAGGTTAG
639-607-bottom-IL21 GTTTTCTAACCTAGCAAGAGTATTTTAG
657-625-top-IL21 GCACCCTTACAAAAAGATAAGGATCTAA
657-625-bottom-IL21 GTATTTTAGATCCTTATCTTTTTGTAAG
678-646-top-IL21 TGGAAGCAAATCCTATTTTAACACCCTT
678-646-bottom-IL21 TTTGTAAGGGTGTTAAAATAGGATTTGC
705-672-top-IL21 GCTATTTAAAGATACACTGGTGAAAATTG

705-672-bottom-IL21	GCTTCCAATTTTCACCAGTGTATCTTTAA
718-686-top-IL21	AGGCACCATTAGTGCTATTTAAAGATAC
718-686-bottom-IL21	CCAGTGTATCTTTAAATAGCACTAATGG
736-704-top-IL21	GTTACATAAAGTGTCAGGAGGCACCATT
736-704-bottom-IL21	GCACTAATGGTGCCTCCTGACACTTTAT
754-722-top-IL21	GTATTTACAATCCATATTGTTACATAAA
754-722-bottom-IL21	GACACTTTATGTAACAATATGGATTGTA
775-743-top-IL21	AGTTCATCAAAACTGTTTATTGTATTTA
775-743-bottom-IL21	GATTGTAAATACAATAAACAGTTTTGAT
792-760-top-IL21	GAGCACGCTGTCTACTTAGTTCATCAAA
792-760-bottom-IL21	ACAGTTTTGATGAACTAAGTAGACAGCG

IL-22 promoter oligos	Sequence 5' to 3'
33-1-top-IL22	AGTTATCAACTGTTGACACTTGTGCGAT
33-1-bottom-IL22	CAGAGATCGCACAAGTGTCAACAGTTGA
48-16-top-IL22	ACAGGCTCTCCTCTCAGTTATCAACTGT
48-16-bottom-IL22	TGTCAACAGTTGATAACTGAGAGGAGAG
69-37-top-IL22	TTGCCTTTTGCTCTCTCACTAACAGGCT
69-37-bottom-IL22	AGGAGAGCCTGTTAGTGAGAGAGCAAAA
85-53-top-IL22	TGCTCCCCTGATGTTTTTGCCTTTTGCT
85-53-bottom-IL22	GAGAGAGCAAAAGGCAAAAACATCAGGG
107-75-top-IL22	GTACCATGCTACCCGACGAACATGCTCC
107-75-bottom-IL22	TCAGGGGAGCATGTTCGTCGGGTAGCAT
123-91-top-IL22	GACAATCATCTGCTTGGTACCATGCTAC
123-91-bottom-IL22	GTCGGGTAGCATGGTACCAAGCAGATGA
146-114-top-IL22	AGGTAAGCACTCAGACCTCTACAGACAA
146-114-bottom-IL22	GATGATTGTCTGTAGAGGTCTGAGTGCT
160-128-top-IL22	AGAGACACCTAAACAGGTAAGCACTCAG
160-128-bottom-IL22	GAGGTCTGAGTGCTTACCTGTTTAGGTG
181-149-top-IL22	TCTGCCTCTCCCATCACAAGCAGAGACA
181-149-bottom-IL22	TTAGGTGTCTCTGCTTGTGATGGGAGAG
193-161-top-IL22	AAAAGCAGCAACTTCTGCCTCTCCCATC
193-161-bottom-IL22	CTTGTGATGGGAGAGGCAGAAGTTGCTG
214-182-top-IL22	CCTGGTGTCCCGATGGCTATAAAAGCAG
214-182-bottom-IL22	AGTTGCTGCTTTTATAGCCATCGGGACA
233-201-top-IL22	GTCACAATACCAAAAAAACCCTGGTGTC
233-201-bottom-IL22	ATCGGGACACCAGGGTTTTTTTGGTATT
252-220-top-IL22	AATGTCTGATGTCATATCATTCACAATA
252-220-bottom-IL22	TTTGGTATTGTGAATGATATGACATCAG
267-235-top-IL22	GACTGGAAATTAGATAATGTCTGATGTC
267-235-bottom-IL22	GATATGACATCAGACATTATCTAATTTC
293-261-top-IL22	GTGGTTAGGTACTTCTCAGAAGACAGGA
293-261-bottom-IL22	TCCAGTCCTGTCTTCTGAGAAGTACCTA
305-273-top-IL22	TGGCCTCCTATGGTGGTTAGGTACTTCT
305-273-bottom-IL22	TTCTGAGAAGTACCTAACCACCATAGGA
329-297-top-IL22	GGAAGGCTTGGAGGTGGTGTCTTGTGGC

329-297-bottom-IL22 AGGAGGCCACAAGACACCACCTCCAAGC
340-309-top-IL22 GCTCTCAAGGTGGGAAGGCTTGGAGGTG
340-309-bottom-IL22 GACACCACCTCCAAGCCTTCCCACCTTG
366-334-top-IL22 GTGACGTTTTAGGGAAGACTTCCCATCT
366-334-bottom-IL22 TTGAGAGATGGGAAGTCTTCCCTAAAAC
380-348-top-IL22 TGTTGGCCCTCACCGTGACGTTTTAGGG
380-348-bottom-IL22 GTCTTCCCTAAAACGTCACGGTGAGGGC
405-373-top-IL22 CTGGGATTTGTGTGCAAAAGCACCTTGT
405-373-bottom-IL22 GGCCAACAAGGTGCTTTTGCACACAAAT
420-388-top-IL22 GTGTTTAGAAGATTTCTGGGATTTGTGT
420-388-bottom-IL22 TTTGCACACAAATCCCAGAAATCTTCTA
497-465-top-IL22 AATAGCTACGGGAGATCAAAGGCTGCTC
497-465-bottom-IL22 GAGTAGAGCAGCCTTTGATCTCCCGTAG
518-486-top-IL22 CCGTGACCAAAACGCTGACTCAATAGCT
518-486-bottom-IL22 CCCGTAGCTATTGAGTCAGCGTTTTGGT
528-495-top-IL22 GAAAATGAGTCCGTGACCAAAACGCTGAC
528-495-bottom-IL22 ATTGAGTCAGCGTTTTGGTCACGGACTCA
536-504-top-IL22 GTTGGTGGGAAAATGAGTCCGTGACCAA
536-504-bottom-IL22 GCGTTTTGGTCACGGACTCATTTTCCCA
540-506-top-IL22 TGAAGTTGGTGGGAAAATGAGTCCGTGACC
540-506-bottom-IL22 GTTTTGGTCACGGACTCATTTTCCCACCAA
547-513-top-IL22 GAATCTATGAAGTTGGTGGGAAAATGAGTC
547-513-bottom-IL22 TCACGGACTCATTTTCCCACCAACTTCATA
558-527-top-IL22 TAAAGAGATAAGAATCTATGAAGTTGGT
558-527-bottom-IL22 GTCCCACCAACTTCATAGATTCTTATCT
574-543-top-IL22 GTATTTCTGGTCACTTCTAAAGAGATAA
574-543-bottom-IL22 GATTCTTATCTCTTTAGAAGTGACCAGA
595-563-top-IL22 GAATATAGGACACGGGTCTTTTATTTCT
595-563-bottom-IL22 TGACCAGAAATAAAAGACCCGTGTCCTA
612-580-top-IL22 GCTTATTTCAAAGCACAGAATATAGGAC
612-580-bottom-IL22 CCCGTGTCCTATATTCTGTGCTTTGAAA
628-596-top-IL22 CCAAGTTTTCATTATGGCTTATTTCAAA
628-596-bottom-IL22 TGTGCTTTGAAATAAGCCATAATGAAAA
650-619-top-IL22 GATTTTAAAAATTGAAATAATCTCCAAG
650-619-bottom-IL22 GAAAACTTGGAGATTATTTCAATTTTTA
662-630-top-IL22 AGAGATATAATTATTTTAAAAATTGAAA
662-630-bottom-IL22 GATTATTTCAATTTTTAAAATAATTATA
684-652-top-IL22 GGATTCCATATACTAAAAAAATAGAGATA
684-652-bottom-IL22 GATTATATCTCTATTTTTTTAGTATATGG
700-668-top-IL22 AGCTAGTTATAGTTTAGGATTCCATATA
700-668-bottom-IL22 TTTAGTATATGGAATCCTAAACTATAAC

Ap-1 consensus probe(Echlin et al., 2000) Sequence 5' to 3'

Top	AGCTTCGCTTGATGAGTC
Bottom	GCCGACTGAGTAGTTCGC

RORE element(Yang et al., 2008)	Sequence 5' to 3'
Top	GAAAGTTTTCTGACCCACTTTAAATCA
Bottom	CTTTAACTAAATTTCACCCAGTCTTTT

CHAPTER 3

Batf expression, deletion and T cell specific overexpression of *Batf*

Batf is highly expressed in effector T cells

To identify new candidate genes that might regulate $CD4^+$ effector T cell development and maintenance we performed a gene expression profiling experiment using microarray analysis. We compared gene expression patterns of naïve T cells to T helper cells (T_H1, T_H2 and T_H17), T_{reg} cells and a variety of other immune and non-immune tissues. We hypothesized that genes with highly restricted expression across an array of tissues likely exhibit important regulatory functions in the cell types of expression. Thus, we were interested in identifying transcription factors with T_H cell specific expression and we found the basic leucine zipper transcription factor ATF-like (*Batf*) (Dorsey et al., 1995) to be highly expressed in effector T_H1, T_H2 and T_H17 cells, expressed at lower levels in naïve T cells and B cells and at essentially basal levels in other tissues (Figure 1).

Batf is a transcription factor that is located in an evolutionary conserved cluster of genes that likely stems from a gene duplication event and contains multiple AP-1 family members (Rasmussen et al., 2005) (Figure 2a). *Batf3* (*p21-SNFT, JDP1*), the highly homologous gene duplication of *Batf* (Iacobelli et al., 2000) (Figure 2b), also exhibited a very restricted expression pattern in our gene expression profiling experiment (Hildner et al., 2008), indicating that these two genes might exert regulatory functions in very specific cell types. *Batf* and *Batf3* are highly conserved between species, with murine Batf being about 96% homologous to the human protein (Figure 3). The restricted

expression pattern of *Batf* in effector T cells and its high degree of evolutionary conservation lead us to hypothesize that *Batf* likely plays an important role in $CD4^+$ effector T cells.

Batf and *Batf3* are basic leucine zipper (bZIP) proteins that are members of the AP-1 family that includes Jun, Fos, Musculoaponeurotic fibrosarcoma (MAF) and activating transcription factor (ATF) (Table 1). bZIP proteins are characterized by a *C-terminal* leucine zipper and a basic domain. The leucine zipper mediates dimerization and the fully functional dimer binds to the DNA via the *N-terminal* basic region (Vinson et al., 2006) (Figure 4). Most AP-1 proteins contain defined transcriptional activation domains (TADs), but some, including *Batf* and *Batf3*, are composed only of a basic region and leucine zipper and lack obvious TADs. *Batf* can inhibit AP-1 dependent luciferase activity *in vitro* and *in vivo*, as well as cellular transformation by Fos (Thornton et al., 2006; Williams et al., 2001; Iacobelli et al., 2000; Bower et al., 2004; Echlin et al., 2000). *Batf* and *Batf3* form heterodimers with Jun but not Fos proteins (Echlin et al., 2000; Dorsey et al., 1995; Iacobelli et al., 2000) and are thought to function as endogenous repressors of AP-1 activity, by forming transcriptionally inert complexes with Jun proteins that exhibit identical DNA binding specificity as the Jun/Fos complex (Echlin et al., 2000; Thornton et al., 2006; Williams et al., 2001).

AP-1 family proteins regulate T cell differentiation and cytokine production (Hess et al., 2004). In T_H cells AP-1 proteins are differentially expressed and exhibit distinct functions. JunB is predominantly expressed in T_H2 but not T_H1 cells, whereas JunD and c-Jun levels are similar in both subsets (Rincon et al., 1997). Jun B has been shown to promote T_H2 differentiation, whereas JunD seems to generally inhibit cytokine

production by T cells (Li et al., 1999; Hartenstein et al., 2002; Meixner et al., 2004). In addition to Jun family members c-Maf regulates IL-4 production in T_H2 (Ho et al., 1996) and transgenic overexpression of MafK suppresses T cell proliferation (Yoh et al., 2001). In conclusion, AP-1 family members play distinct roles in effector T cell development and maintenance and controlling AP-1 activity during T helper cell differentiation is vital for balancing pathogen specific effector responses.

AP-1 proteins directly regulate many cytokine promoters in effector T cells, often in cooperation with other transcription factors (Macian et al., 2001). The IL-2 promoter is the most extensively studied cytokine promoter regulated by AP-1 proteins in conjunction with NFAT (Garrity et al., 1994; Jain et al., 1992). Interestingly, *Batf3,* the closely related AP-1 family member of *Batf*, can inhibit IL-2 promoter reporter assays (Iacobelli et al., 2000), indicating the potential of these small AP-1 proteins to regulate effector cytokine production. Therefore, we hypothesized that *Batf* might regulate T effector cell differentiation by modulating AP-1 signaling.

Deletion of *Batf* by homologous recombination

To assess the role of *Batf* in T cells, we generated *Batf* $^{-/-}$ mice by homologous recombination (Figure 5). This approach deletes exons 1 and 2 of *Batf* which contain the basic region and most of the leucine zipper. Correctly targeted clones were identified by Southern Blot analysis and the neomycin resistance cassette was deleted *in vitro* using a cre- recombinase expressing adenovirus. *Batf* $^{-/-}$ mice were born at normal Mendelian frequencies and germline transmission was confirmed by Southern Blot (Figure 5b). The

lack of Batf protein in *Batf* $^{-/-}$ mice was confirmed by Western Blot analysis using antibodies specific for Batf (Figure 5c).

First, we assessed the development of the major lymphoid and myeloid lineages in *Batf* $^{-/-}$ mice. *Batf* $^{-/-}$ mice had no abnormalities in thymus or spleen cellularity, and lymph node development (Figure 6). The development of $CD4^+$ and $CD8^+$ T cells in thymus, spleen or lymph nodes was normal (Figure 7a). To assess whether *Batf* $^{-/-}$ T cells had abnormalities in their homeostatic activation, we stained T cells from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice with antibodies to the activation markers CD44 and CD25. We found similar expression of CD44 and CD25 on *Batf* $^{+/+}$ and *Batf* $^{-/-}$ T cells (Figure 7b). Despite reported severe reduction of NKT cell development following transgenic overexpression of *Batf* under control of the lck-promoter (Williams et al., 2003; Zullo et al., 2007), we found normal NKT cell development in *Batf* $^{-/-}$ mice (Figure 7c).

Batf $^{-/-}$ mice had normal B cell development in the spleen and bone marrow (Figure 8). We analyzed the development of B cells in the bone marrow as defined Hardy and colleagues using the surface markers B220, BP1, CD43, CD24, IgM and IgD (Hardy and Hayakawa, 2001). Within the $B220^+CD43^{hi}$ cells the percentages of $BP\text{-}1^-CD24^-$ (Hardy fraction A), $BP\text{-}1^-CD24^+$ (Hardy fraction B), and $BP\text{-}1^+CD24^+$ (Hardy fraction C) subsets were similar between *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice. Within the $B220^+$ $CD43^-$ cells the percentages of IgM^-IgD^- (Hardy fraction D), IgM^+IgD^{lo} (Hardy fraction E), and $IgM^{lo}IgD^{hi}$ (Hardy fraction F) were similar between *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice (Figure 8b). Likewise, the frequency of splenic immature B cells ($AA4.1^+$ $B220^+$), Transitional 1 ($B220^+IgM^{hi}IgD^{lo}$), Transitional 2 ($B220^+IgM^{hi}$, IgD^{hi}) and mature B cells ($AA4.1^-B220^+$;

$B220^{+}IgM^{lo}IgD^{hi}$) was similar between *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice (Figure 8a) indicating normal development and homeostasis of B cells.

Next, we assessed the development of conventional splenic dendritic cell (cDC) subsets, plasmacytoid dendritic cells and neutrophils in the absence of *Batf*. cDCs were identified as $CD11c^{hi}$ cells and further subdivided into $CD4^{+}$ DCs and $CD8^{+}$ DCs, identified as $CD11c^{hi}CD4^{+}CD8^{-}$ and $CD11c^{hi}CD4^{-}CD8\alpha^{+}$ respectively (Figure 9a). $CD8^{+}$ DCs were further identified as $CD11c^{hi}CD8\alpha^{+}$ $Dec205^{+}$ (Figure 9b). All cDC subsets were present at normal ratios in *Batf* $^{-/-}$ mice compared to *Batf* $^{+/+}$ mice. Similarly, plasmacytoid dendritic cells (pDC), identified as $CD11b^{-}CD11c^{lo}B220^{+}Gr1^{+}$, were present at normal ratios in *Batf* $^{-/-}$ mice (Figure 9b). Neutrophils, identified as $CD11b^{+}Gr1^{hi}$ cells, developed normally in *Batf* $^{-/-}$ mice although we observed a small but consistent increase in the frequency of splenic neutrophils (Figure 10a). This increase could be due to a cell intrinsic role of *Batf* in regulating neutrophil development or homeostasis, or alternatively be secondary to irregular neutrophil homing in response to imbalanced T cell cytokine production (Ye et al., 2001; Stark et al., 2005; Tan et al., 2008). To distinguish these possibilities, we assessed splenic neutrophils in *Rag2* $^{-/-}$ mice, which are deficient in B cells, T cells and NKT cells. We found no differences in neutrophil numbers in 5 week old mice, however found a similar increase of neutrophils in 14 week old *Batf* $^{-/-}$*Rag2* $^{-/-}$ mice compared to *Batf* $^{+/+}$*Rag2* $^{-/-}$ mice (Figure 10b). These data indicate a cell-intrinsic role of *Batf* in neutrophil development or homeostasis rather than abnormal neutrophil homing in response to T cell cytokines. Further analysis will be necessary to determine the potential function of *Batf* in neutrophils.

T cell specific overexpression of *Batf*

To facilitate studies pertaining to the function of *Batf* in T cells and test the affect of *Batf* overexpression on T cell differentiation we generated transgenic mice, expressing an N-terminally FLAG-tagged version of *Batf*. This *N-FLAG-Batf* was cloned under the control of the CD2 promoter (Zhumabekov et al., 1995) (Figure 11a). Transgene expression was confirmed by Western blot with an antibody specific to the FLAG epitope (Figure 11b). These mice were further crossed to DO11.10 T cell receptor (TCR) transgenic animals to facilitate T cell differentiation studies.

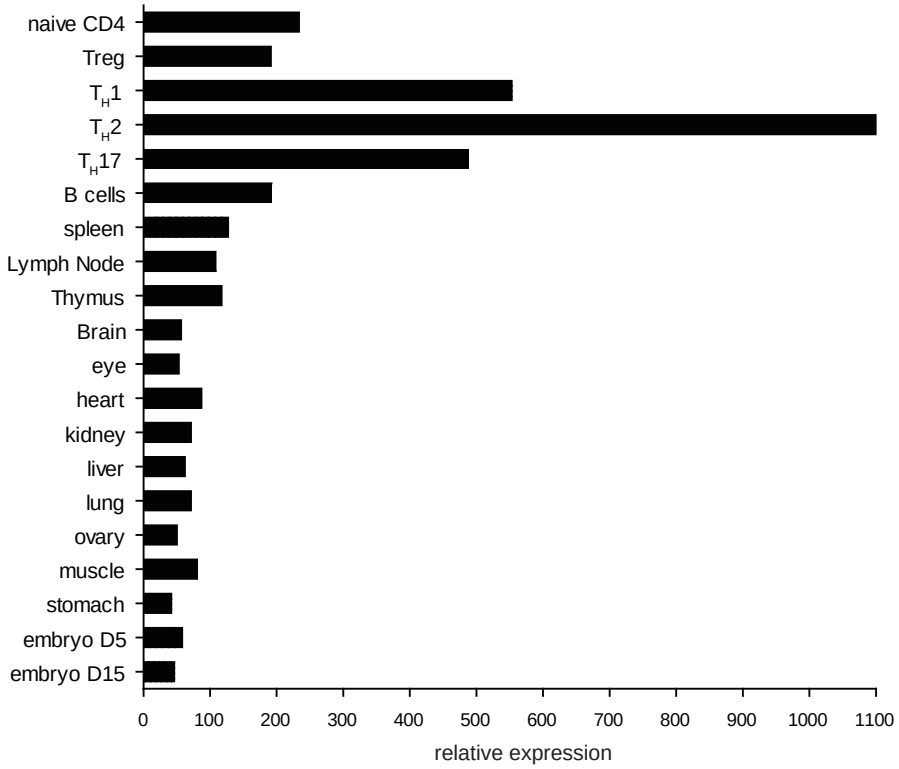
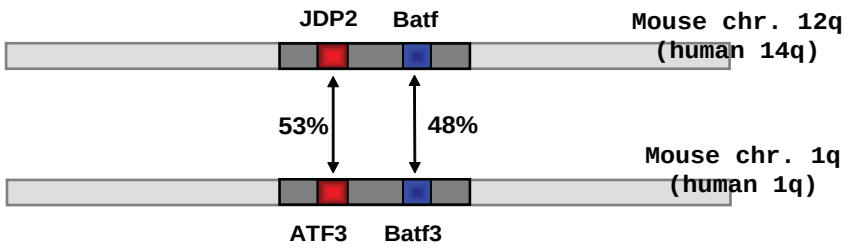


Figure 1. *Batf* is highly expressed in $CD4^+$ effector T cells.
The relative expression of *Batf* in the indicated tissues was determined by Affymetrix gene microarray. The data are presented in arbitrary units and reflect normalized and modeled expression values generated using DNA-Chip analyzer (dChip) software.

a



b

```
Batf3 MSQGPPAVSVLQRSVDAPGNQPQSPKDDDRKVRRREKNRVAAQRSRKKQT
Batf  MPHSSDSSDSSFSRSPPPGKQDSS--DDVRKVQRREKNRIAAQKSRQRQT
      *.:.. : .       .**:* .*  ** ***:******:***:**::**

Batf3 QKADKLHEEHESLEQENSVLRREISKLKEELRHLSEVLKEHEKMCPLLLC
Batf  QKADTLHLESEDLEKQNAALRKEIKQLTEELKYFTSVLSSHEPLCSVLAS
      ****.** * *.**::*:.**:**.:*.***:::::.**..** :*.:* .

Batf3 PMN------FVQLRSDPVASCLPR---
Batf  GTPSPPEVVYSAHAFHQPHISSPRFQP
           :     .    . **
```

Figure 2. *Batf* is located in a cluster of genes that arose from gene duplication. **a,** Representation of the gene cluster containing *Batf*, *Batf3, ATF3* and *JDP2* that likely stems from a gene duplication. The percentage of sequence homology between *Batf* and the closely related *Batf3* is indicated. **b,** Amino acid sequence alignment of Batf and Batf3. Blue amino acids indicate the basic region, red amino acids indicate the leucine zipper.

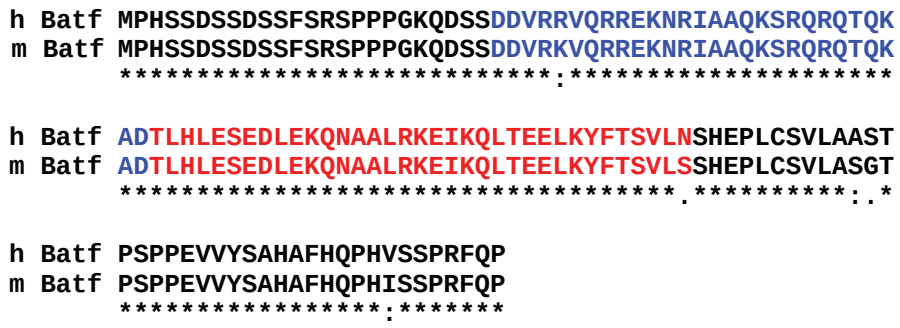


Figure 3. 96% sequence homology of human and mouse Batf.
Amino acid sequence alignment of the human and mouse Batf proteins. Stars indicate identical amino acids, double dots indicate conservative changes, single dots indicate non-conservative changes.

Family	Name	Dimers	DNA binding element
C/EBP	C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, CHOP, HP8	homo- and heterodimers	C/EBP (5'-ATTGCGCAAT-3') CRE, PAR
CREB	ATF-1, CREB, CREM Ia, CREM Ib	Homodimers	CRE (5'-TGACGTCA-3')
OASIS	OASIS, CREB-H, CREB3, CREB4, AC022173	homodimers	CRE
ATF-6	ATF-6, ATF-6β, ZF (Zhangfei), XBP-1	homodimers	CRE ATF/CREB-like
ATF-2	ATF-2, ATF-7, CREBPA	homo- and heterodimers	CRE ATF/CREB-like
ATF-3	ATF-3, JDP2	heterodimers	CRE ATF/CREB-like
ATF-4	ATF-4, ATF-4L1, ATF-5	homo- and heterodimers	CRE ATF/CREB-like
Jun	Jun, JunB, JunD	homo- and heterodimers	TRE (5'TGAGTCA-3')
Fos	Fos, FosB, Fra1, Fra2	heterodimers	TRE (5'TGAGTCA-3')
Batf	Batf, Batf3 (p21-SNFT,JDP1)	heterodimers	TRE (5'TGAGTCA-3')
smMaf	MafG, MafK, MafF	homo- and heterodimers	TRE (5'TGAGTCA-3') ATF/CREB-like
lgMaf	cMaf, MafB, MafA, NRL	heterodimers	TRE (5'TGAGTCA-3') ATF/CREB-like
CNC	BACH1, NFE2L1, NFE2L2, NFE2L3, BACH2	heterodimers	AP-1 like palindromes (5'-T/CGCTGAC/GTCAT/C-3')
PAR	DBP, HLF,TEF, NFIL3	homodimers	PAR (5'-ATTACGTAAT-3')

Table 1. The basic leucine zipper family of proteins (Vinson et al., 2002).
Grouping of human bZIP proteins into families based on their dimerization properties and sequence homology of the leucine zipper region.

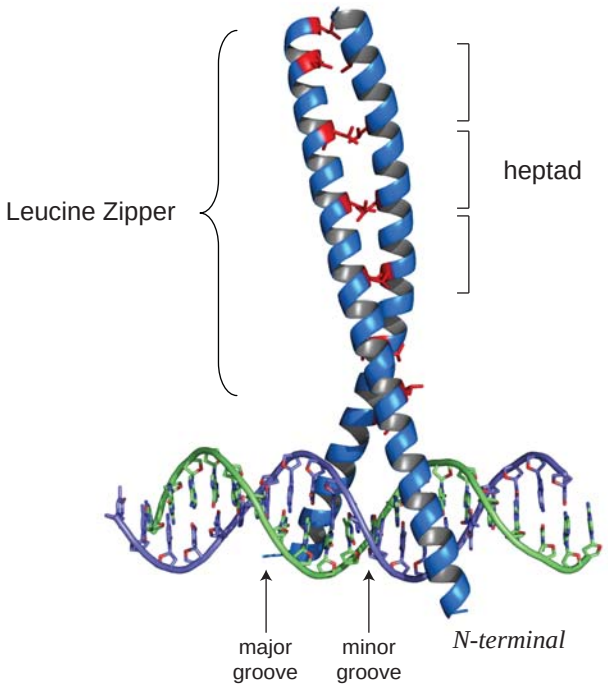


Figure 4. Ribbon diagram of the X-ray crystallography structure of a bZIP homodimer. Representation of a dimer of two basic leucine zipper proteins (blue helix) bound to the DNA. The *N-terminal* DNA recognition helix (basic region) lies in the major groove of the DNA. An almost invariant leucine (red) present every two turns of the *C-terminal* α-helix that forms the leucine zipper and mediates protein-protein interactions.

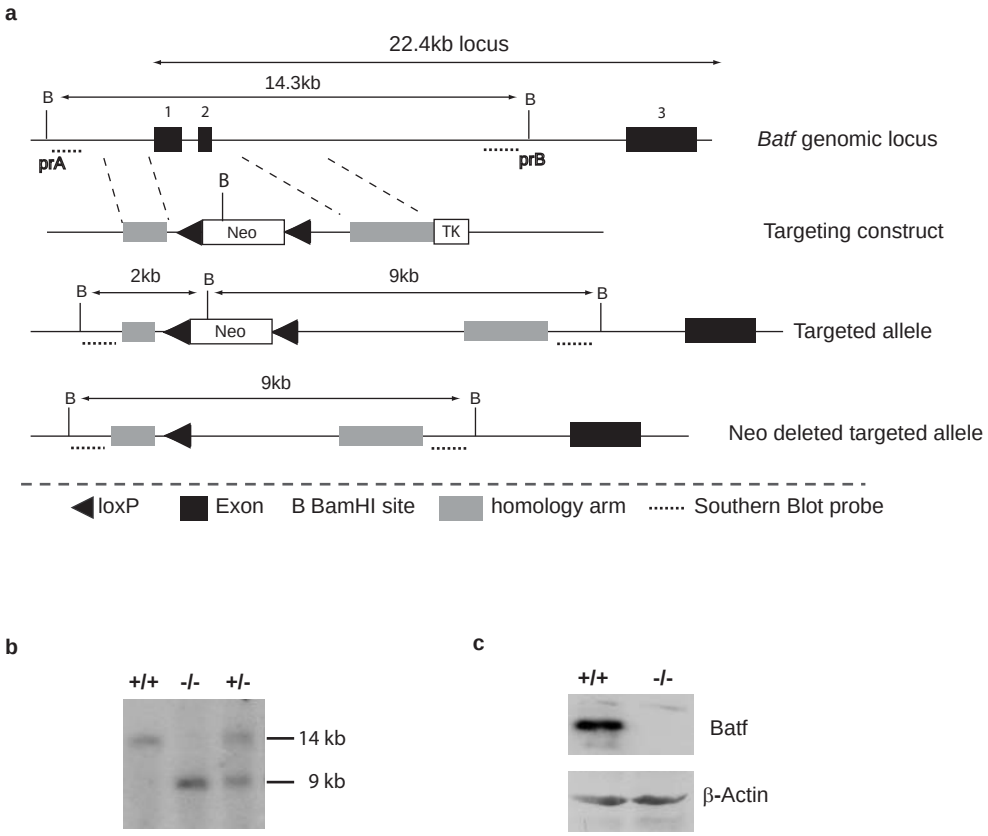


Figure 5. Targeting of the *Batf* locus by homologous recombination.
a, The endogenous genomic *Batf* locus, targeting construct and the mutant allele before and after cre-mediated deletion of the neomycin (neo) cassette are shown. Restriction enzyme digestion of the genomic locus with BamHI results in a 14.3kb wild type fragment detected by Southern Blot probes A and B; in the targeted allele, probe A detects a 2kb and probe B detects a 9kb fragment. In the neomycin-deleted targeted allele, BamHI digestion results in a 9kb fragment that is detected by both the 5' and 3' Southern Blot probes (probes A and B respectively). The neo cassette was deleted by *in vitro* treatment with a Cre-recombinase expressing Adenovirus. **b,** Southern Blot analysis of targeted *Batf* alleles. Probe A was used to hybridize BamHI digested genomic DNA from the indicated genotypes resulting from *Batf*$^{+/-}$ intercrosses. **c,** No residual protein expression in *Batf*$^{-/-}$ mice. Total splenocytes were activated under T_H17 conditions for 3 days. Equal cell numbers were subjected to Western Blot analysis using anti-Batf antibody. The blot was stripped and reblotted with an antibody to β-actin to show equal protein loading

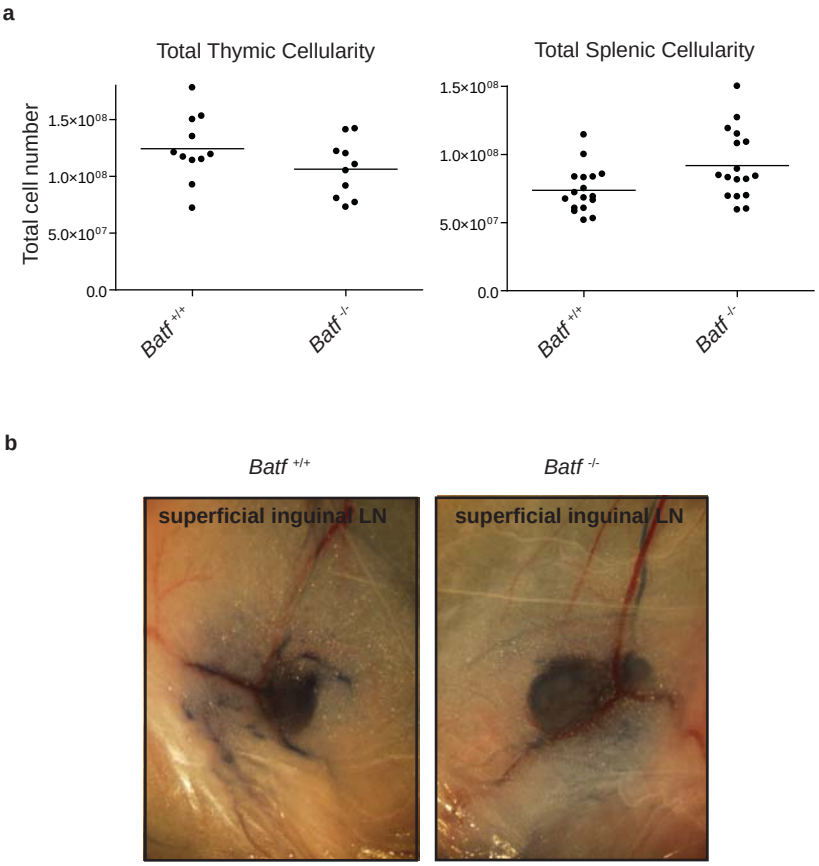


Figure 6. Thymus, spleen and lymph nodes develop normally in *Batf* $^{-/-}$ mice.
a, Total cell numbers of thymus (n=11) and spleen (n=17) from 8-10 week old *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice are shown (horizontal bars indicate mean cell numbers). **b,** *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were injected with Evans Blue dye solution into each hind foot pad. 1.5h later superficial inguinal lymph nodes were visualized using a dissecting microscope. Data are representative of 2 independent experiments.

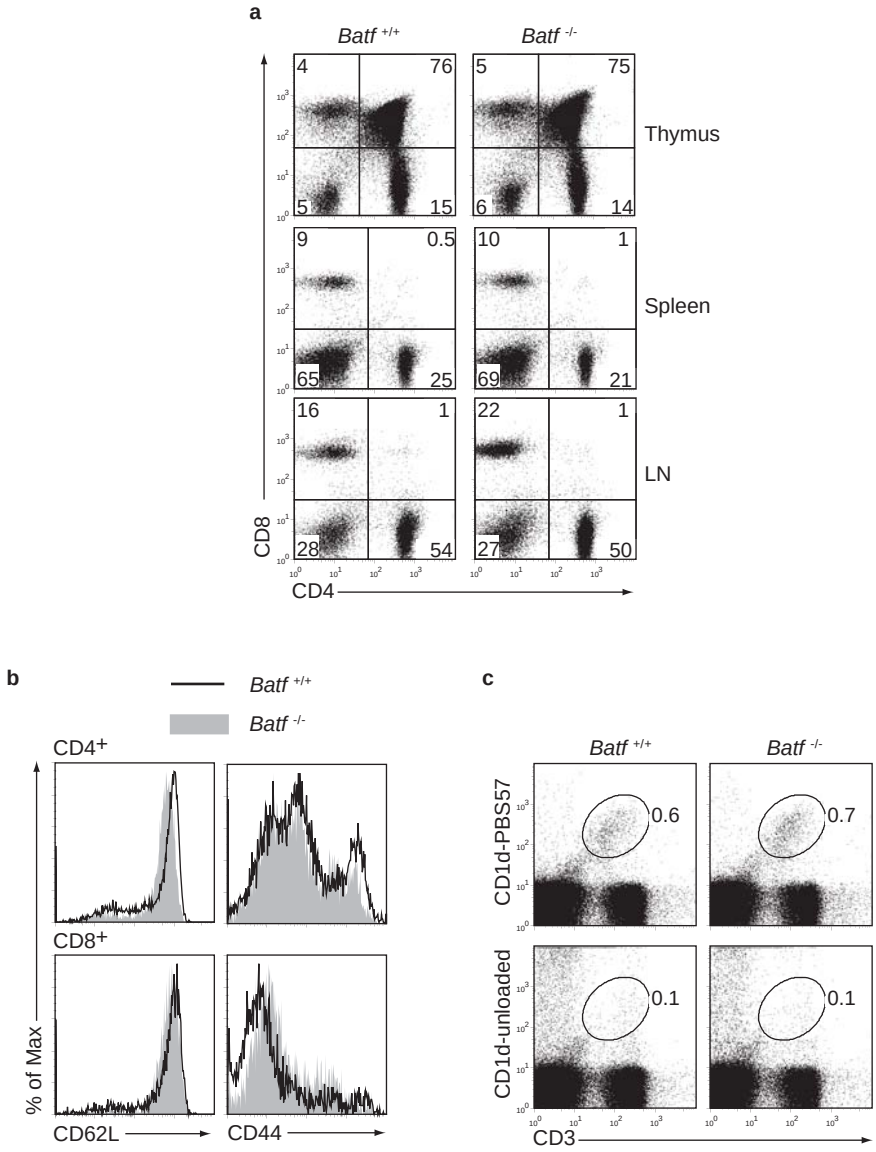


Figure 7. Normal T cell development in *Batf* $^{-/-}$ mice.
a, Thymus, spleen and lymph nodes from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were analyzed for the surface expression of CD4 and CD8 by flow cytometry. The percentages of $CD8^+$, $CD4^+$ and $CD4^+CD8^+$ T cells were similar between *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice. **b,** *Batf* $^{+/+}$ and *Batf* $^{-/-}$ splenic $CD4^+$ and $CD8^+$ cells were analyzed for the surface expression of the activation markers CD62L (left panel) and CD44 (right panel). Data are presented as a histogram overlay of surface expression of CD62L and CD44. **c,** Total splenocytes from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were stained for CD3 in conjunction with unloaded or PBS57-loaded CD1d tetramers. NKT cells are identified as $CD3^+$CD1d-$PBS57^+$.

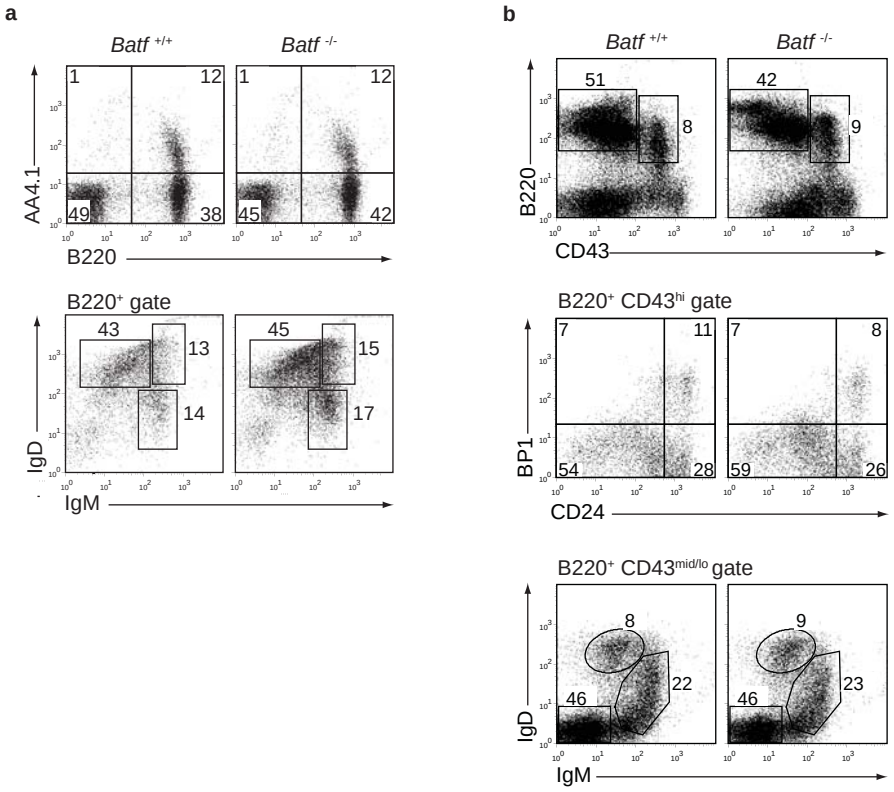


Figure 8. Normal B cell development in *Batf* $^{-/-}$ mice.
a, Total splenocytes were stained with antibodies to B220, AA4.1, IgM and IgD. The percentages of immature B cells (AA4.1^{+} B220^{+}), Transitional 1 (B220^{+}IgM^{hi}IgD^{lo}), Transitional 2 (B220^{+}IgM^{hi}, IgD^{hi}) or mature B cells (AA4.1^{-}B220^{+}; B220^{+}IgM^{lo}IgD^{hi}) were similar between *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice. **b,** Bone marrow cells were stained for the expression of B220, CD43 and either BP1 and CD24 or IgD and IgM. The percentages of cells included in B220^{+}CD43^{hi} subsets: BP-1^{-} CD24^{-}, BP-1^{-}CD24^{+}, and BP-1^{+}CD24^{+} (Hardy fractions A through C respectively) were similar between *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice. Also the percentages of B220^{+} CD43^{-} subsets; IgM^{-}IgD^{-}, IgM^{+}IgD^{lo}, and IgM^{lo}IgD^{hi} (Hardy fractions D through F respectively) were similar between *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice. Numbers indicate percentage of cells in the indicated region. Data are representative of at least 2 independent experiments performed with multiple mice of each genotype.

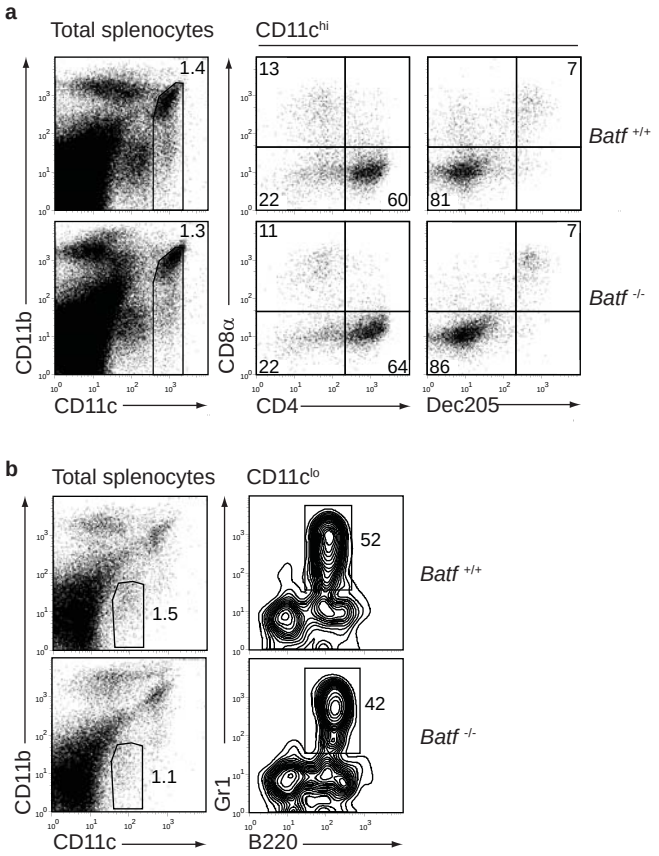


Figure 9. Normal dendritic cell development in *Batf* $^{-/-}$ mice.
a, Total splenocytes were stained with the indicated antibodies. Conventional splenic dendritic cell (cDC) were identified as CD11c^{hi} cells and further subdivided into CD4^{+} DCs and CD8^{+} DCs, identified as CD11c^{hi}CD4^{+}CD8^{-} and CD11c^{hi}CD4^{-}CD8α^{+} respectively. CD8^{+} DCs were further identified as CD11c^{hi}CD8α^{+} Dec205^{+}. **b,** Total splenocytes were stained with antibodies to CD11c, CD11b, Gr1 and B220. Percentages of plasmacytoid dendritic cells (CD11b^{-}CD11c^{lo}B220^{+}Gr1^{+}) were similar between *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice. Numbers indicate the percentage of live cells in each region. Data are representative of at least 2 independent experiments performed with multiple mice of each genotype.

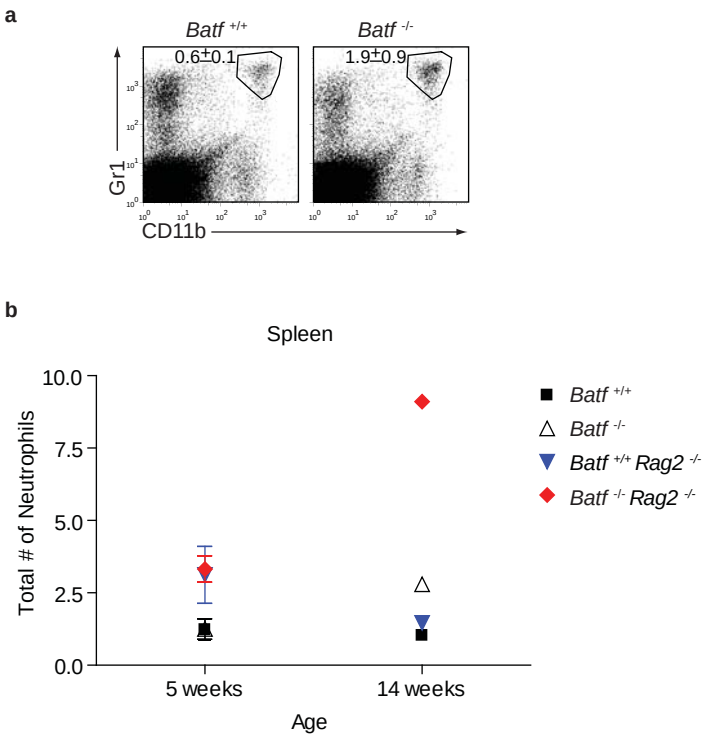


Figure 10. *Batf* $^{-/-}$ mice exhibit a slight increase in splenic neutrophils.
a, Total splenocytes were stained with the indicated antibodies. Neutrophils were identified as Gr1^{hi}CD11b^{+}. Numbers indicate average percentages of neutrophils ± s.d. of four *Batf* $^{+/+}$ and six *Batf* $^{-/-}$ mice from three independent experiments. **b,** Total spleen cells from *Batf* $^{+/+}$, *Batf* $^{-/-}$, *Batf* $^{+/+}$ *Rag2*$^{-/-}$ and *Batf* $^{-/-}$ *Rag2*$^{-/-}$ mice at the indicated ages were counted and stained for Gr1 and CD11b to enumerate the absolute number of splenic neutrophils (Gr1^{hi}CD11b^{+}).

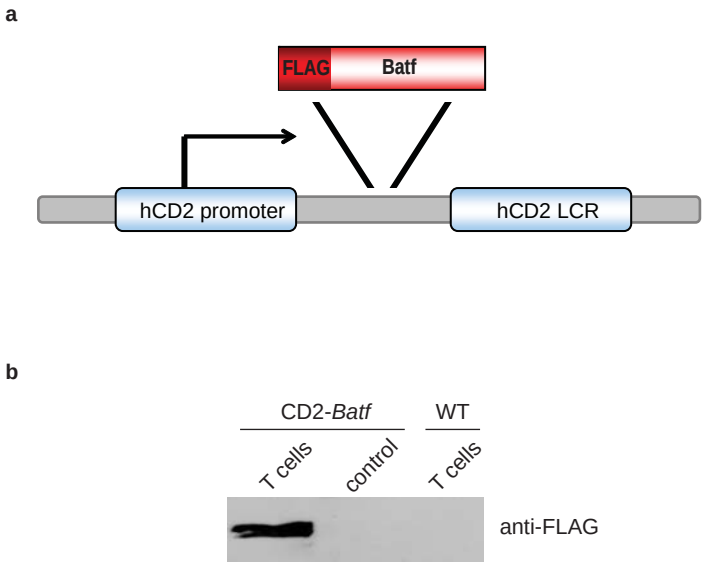


Figure 11. Generation of *CD2-N-FLAG-Batf* transgenic mice.
a, The FLAG-tagged *Batf* was cloned into the CD2 microinjection cassette (Zhumabekov et al., 1995). **b,** Transgene expression in $CD4^+$ and $CD8^+$ splenocytes was assessed by anti-FLAG western blot. (T cells: $CD4^+$ and $CD8^+$ magnetically purified splenocytes; control: splenocytes depleted of $CD4^+$ and $CD8^+$ cells; CD2-Batf: transgenic, WT: non-transgenic littermate)

CHAPTER 4

Batf deficiency specifically ablates T_H17 differentiation

$CD4^+$ Effector T cells orchestrate the immune response through the secretion of their signature cytokines. T_H1 cells are the main players in cellular immunity against intracellular bacteria and viruses, whereas T_H2 cells direct the humoral immune response to extracellular pathogens (Murphy and Reiner, 2002) and T_H17 cells mediate acute inflammatory responses (Weaver et al., 2006). In addition to their importance in primary immune responses T helper cells produce large amounts of IL-2, required for the expansion of $CD8^+$ memory T cells during secondary infections (Murphy and Reiner, 2002; Weaver et al., 2006) and homeostasis of T_{reg} cells (Lohr et al., 2006). Uncontrolled effector T cell responses can lead to the development of atopic diseases such as asthma (T_H2 mediated) or the development of autoimmune diseases (T_H1 and T_H17 mediated) (Murphy and Reiner, 2002). Thus, balancing appropriate effector T cell development and cytokine production is critical during infections and determines the outcome of infectious and inflammatory responses.

Normal T_H1 and T_H2 differentiation of *Batf* $^{-/-}$ T cells

To test whether *Batf* regulates effector T cell differentiation we sorted naïve $CD4^+$ T cells ($CD4^+CD62L^+CD25^-$) from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice. These T cells were activated with anti-CD3 and anti-CD28 antibodies either without the addition of cytokines and antibodies, or under T_H1 (anti-IL-4, IFN-γ, IL-12) and T_H2 (anti-IL-IFN-γ, anti-IL-12, IL-4) conditions. On day 3, cells allowed to rest under skewing conditions. Since fully

differentiated T cells readily produce their effector cytokines upon TCR stimulation (Murphy and Reiner, 2002), we restimulated cells on day 7 with anti-CD3 and anti-CD28 in the absence of exogenous cytokines and antibodies. Under these conditions, *Batf*$^{-/-}$ T cells produced similar levels of IL-4 and IFN-γ as *Batf*$^{+/+}$ T cells (Figure 12a) as well as normal levels of IL-2. Additionally, *Batf*$^{-/-}$ T cells activated without skewing cytokines and antibodies produced similar IL-4 and IFN-γ as *Batf*$^{+/+}$ T cells (Figure 12a). Therefore, *Batf*$^{-/-}$ T cells displayed normal T_H1 and T_H2 differentiation.

Batf $^{-/-}$ T cells fail to produce IL-17

In contrast to normal T_H1 and T_H2 differentiation, *Batf*$^{-/-}$ T cells activated under T_H17 conditions showed a dramatic loss in IL-17 production (Figure 12b). *Batf* $^{+/-}$ T cells produced normal levels of IL-17 (Figure 13a). This remarkably selective defect in one particular pathway of T cell differentiation was somewhat surprising, since *Batf* was prominently expressed in T_H1 and T_H2 cells in our gene expression profiling experiment. To confirm these data, we performed Western blot analysis of naïve T cells and T_H2 cells. Batf protein was low, but present in unactivated naïve T cells (Figure 14a). Resting T_H2 cells expressed abundant Batf protein, which was further induced by activation (Figure 14b). In resting T cells, Batf was present both in the nucleus and cytoplasm, but largely translocated to the nucleus after activation (Figure 15a and b). Notably, we found that Batf was present in T cells in two molecular weight species and that the lower molecular species was predominantly induced by activation (Figure 14). These observations are consistent with previous data demonstrating that Batf can be phosphorylated at serine 43 in the DNA binding domain (Deppmann et al., 2003). This phosphorylation at Serine 43

has been shown to prevent DNA binding without affecting dimerization with Jun (Deppmann et al., 2003). Future experiments need to determine, whether phosphorylation of Batf plays a role for its function in T cells. Nevertheless, our data demonstrate that Batf protein is present in resting naïve and T_H2 cells even though it seems to specifically regulate T_H17 differentiation.

The loss of IL-17 production in *Batf* $^{-/-}$ T cells could conceivably result either from a disregulation in the production of T_H17 suppressing cytokines or from a cell-intrinsic requirement for *Batf* during T_H17 differentiation. Multiple cytokines have been described in the literature to inhibit T_H17 differentiation, including IFN-γ, IL-2, IL-4 and IL-10 (Harrington et al., 2005; Park et al., 2005; Laurence et al., 2007; Gu et al., 2008). We found that *Batf* $^{-/-}$ T cells produced similar levels of IL-2 compared to *Batf* $^{+/+}$ T cells. Additionally, we observed no compensatory changes in the production of IFN-γ or IL-10 in *Batf* $^{-/-}$ T cells (Figure 12b). These data indicate that *Batf* $^{-/-}$ T cell do not produce increased amounts of T_H17 suppressing cytokines, and suggest that *Batf* might directly regulate transcriptional pathways controlling T_H17 differentiation

Proinflammatory cytokines such as IL-1β and TNFα (Veldhoen et al., 2006a), as well as activation by antigen presenting cells (APCs) instead of activation with antibodies to CD3 and CD28 (Ghilardi and Ouyang, 2007) augment T_H17 development through unknown mechanisms. We tested whether addition of IL-1β and TNFα would rescue T_H17 differentiation in *Batf* $^{-/-}$ T cells. As expected, IL-1β and TNFα increased IL-17 production by *Batf* $^{+/+}$ T cells compared to stimulation with IL-6 and TGF-β alone, however, failed to rescue IL-17 production in *Batf* $^{-/-}$ T cells (Figure 13b). Similarly, stimulation of DO11.10 TCR transgenic *Batf* $^{+/+}$ and *Batf* $^{-/-}$ $CD4^+$ T cells with OVA and

irradiated APCs under T_H17 conditions did not restore IL-17 production in *Batf*$^{-/-}$ T cells (Figure 13a), even after repeated rounds of activation under T_H17 conditions (Figure 16a). Thus, *Batf*$^{-/-}$ CD4$^+$ T cells exhibit a severe loss of IL-17 production *in vitro* that cannot be overcome by the addition of proinflammatory cytokines.

Batf regulates IL-17 production in CD8$^+$ T cells

Similarly to CD4$^+$ T cells, IL-17 production can be induced in CD8$^+$ T cells in response to IL-6 and TGF-β (Kryczek et al., 2007; Liu et al., 2007). The physiological role of IL-17 producing CD8$^+$ T cells is not completely understood, but they have been isolated from tumors (Kryczek et al., 2007), have been implicated in a model contact hypersensitivity (He et al., 2006) and are capable of protecting mice against lethal influenza challenge (Hamada et al., 2009).

To test whether IL-17 production in *Batf*$^{-/-}$ CD8$^+$ T cells was normal, we activated total splenocytes from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice with anti-CD3 under T_H17 conditions. *Batf*$^{+/+}$ CD8$^+$ T cells readily produced IL-17 under these conditions, however, *Batf*$^{-/-}$ CD8$^+$ T cells activated under T_H17 conditions showed loss of IL-17 production (Figure 16b), similar to CD4$^+$ T cells. These data indicate a uniform loss of IL-17 production in *Batf*$^{-/-}$ conventional T cells.

Overexpression of *Batf* increases IL-17 production by T cells

If *Batf* was required to induce T_H17 differentiation associated transcriptional programs, overexpression of *Batf* in T cells should promote IL-17 production. To examine *Batf* overexpression we generated transgenic mice expressing FLAG-tagged

Batf under the control of the CD2 promoter (Zhumabekov et al., 1995) (Figure 11a). *Batf*-transgenic DO11.10^{+}CD4^{+} T cells were stimulated with OVA and APCs under T_H17 conditions and analyzed for cytokine production after restimulation with PMA/ionomycin on day 3. *Batf*-transgenic DO11.10^{+}CD4^{+} T cells exhibited significantly increased IL-17 production compared to non-transgenic control T cells (Figure 17a). Likewise, *Batf*-transgenic CD8^{+} T cells produced increased amounts of IL-17 when activated under T_H17 conditions compared to non-transgenic CD8^{+} T cells (Figure 17b), indicating that *Batf* can promote IL-17 production in T cells.

Batf regulates IL-17 production *in vivo*

At mucosal surfaces, such as the intestinal lamina propria (LP), CD4^{+} T cells constitutively express IL-17 (Ivanov et al., 2006). At these sites, T_H17 cells are thought to provide an immediate line of defense against certain bacteria (Ivanov et al., 2007). Indeed recent data suggests that the composition of the intestinal mucosa affects the balance between T_{reg} and T_H17 cells, thus influencing the equilibrium of intestinal tolerance versus immunity (Ivanov et al., 2008).

Since *Batf* $^{-/-}$ T cells fail to produce IL-17 *in vitro*, we analyzed LP T cells for the production of IL-17. *Batf* $^{-/-}$ LP CD4^{+} T cells lacked IL-17 expression; however, spontaneous production of IFNγ was similar to *Batf* $^{+/+}$ LP CD4^{+} T cells (Figure 18). Thus, *Batf* $^{-/-}$ LP CD4^{+} T cells do not exhibit a global deficiency in cytokine production.

Collectively, these data demonstrate a uniform loss of IL-17 production in conventional *Batf* $^{-/-}$ T cells and show that *Batf* $^{-/-}$ T cells selectively fail to differentiate into T_H17 cells.

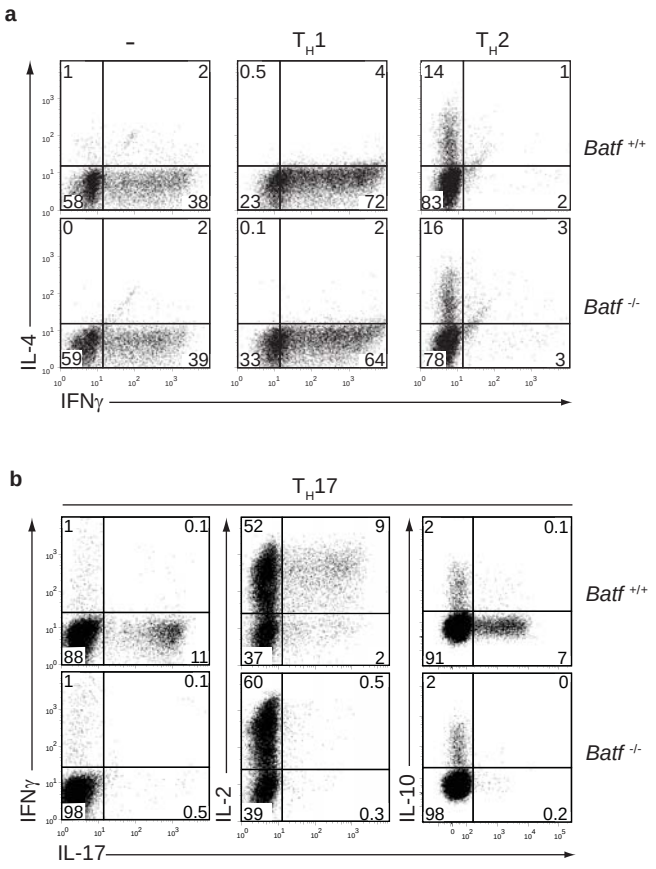


Figure 12. Selective loss of IL-17 production in *Batf* $^{-/-}$ T cells.
a, Naïve CD4^{+}CD62L^{+}CD25^{-} T cells from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were activated with anti-CD3/CD28 alone or under T_H1 or T_H2 conditions. Cells were restimulated on day 7 with anti-CD3/CD28 for 24h and analyzed for IFN-γ and IL-4 production by intracellular staining. **b,** Naïve CD4^{+}CD62L^{+}CD25^{-} T cells from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were activated under T_H17 (TGF-β plus IL-6) conditions. On day 7 (left panel) or day 3 (middle and right panels) cells were restimulated with PMA/ionomycin for 4h and stained for intracellular IL-17, IFN-γ, IL-2 and IL-10. Data are representative of at least 2 independent experiments performed with multiple mice of each genotype.

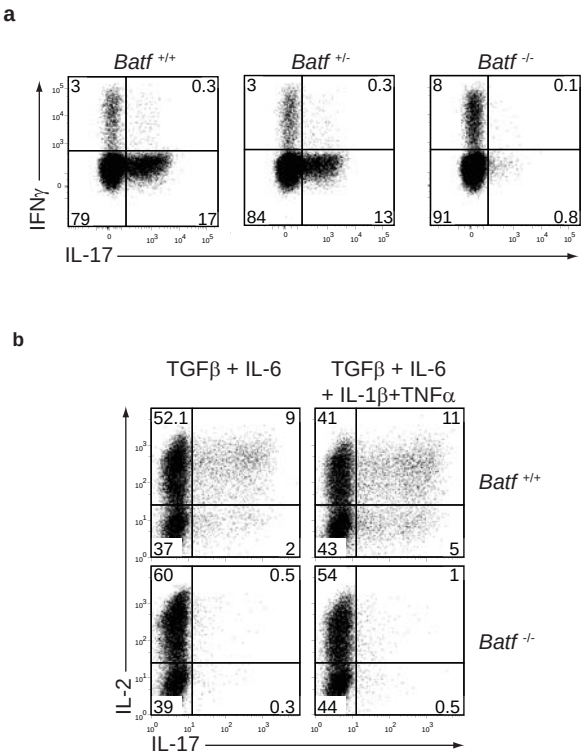


Figure 13. Loss of IL-17 production in *Batf*$^{-/-}$ T cells.
a, DO11.10^{+}CD4^{+} T cells from *Batf*$^{+/+}$, *Batf*$^{+/-}$ and *Batf*$^{-/-}$ mice were activated with OVA and APCs under T_H17 conditions for 3 days and stained for intracellular IL-17 and IFN-γ after restimulation with PMA/ionomycin. **b,** Naïve CD4^{+}CD62L^{+}CD25^{-} T cells from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice were activated with anti-CD3/CD28 under T_H17 in the presence or absence of IL-1β and TNFα and stained for intracellular IL-17 and IFN-γ after restimulation with PMA/ionomycin. Numbers represent the percentage of live cells in the indicated gates. Data are representative of at least 2 independent experiments performed with multiple mice of each genotype.

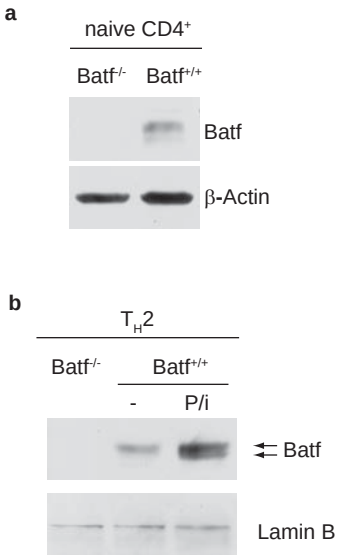


Figure 14: Batf protein is expressed in resting naïve T cells and T_H2 cells.
a, $1.5x10^6$ cell equivalents of $CD4^+$ T cells from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were subjected to Western Blot analysis using anti-Batf antibody. The blots were stripped and reprobed with an antibody to β-actin to show equal protein loading. **b,** $CD4^+$ T cells from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were stimulated with anti-CD3/CD28 under T_H2 conditions. On day 4, cells were left untreated or stimulated with PMA/ionomycin for 4h. Nuclear extracts from $0.5x10^6$ cell equivalents were analyzed for Batf expression by Western Blot. The blots were stripped and reprobed with an anti-Lamin B antibody to show equal protein loading. Data are representative of 2 independent experiments.

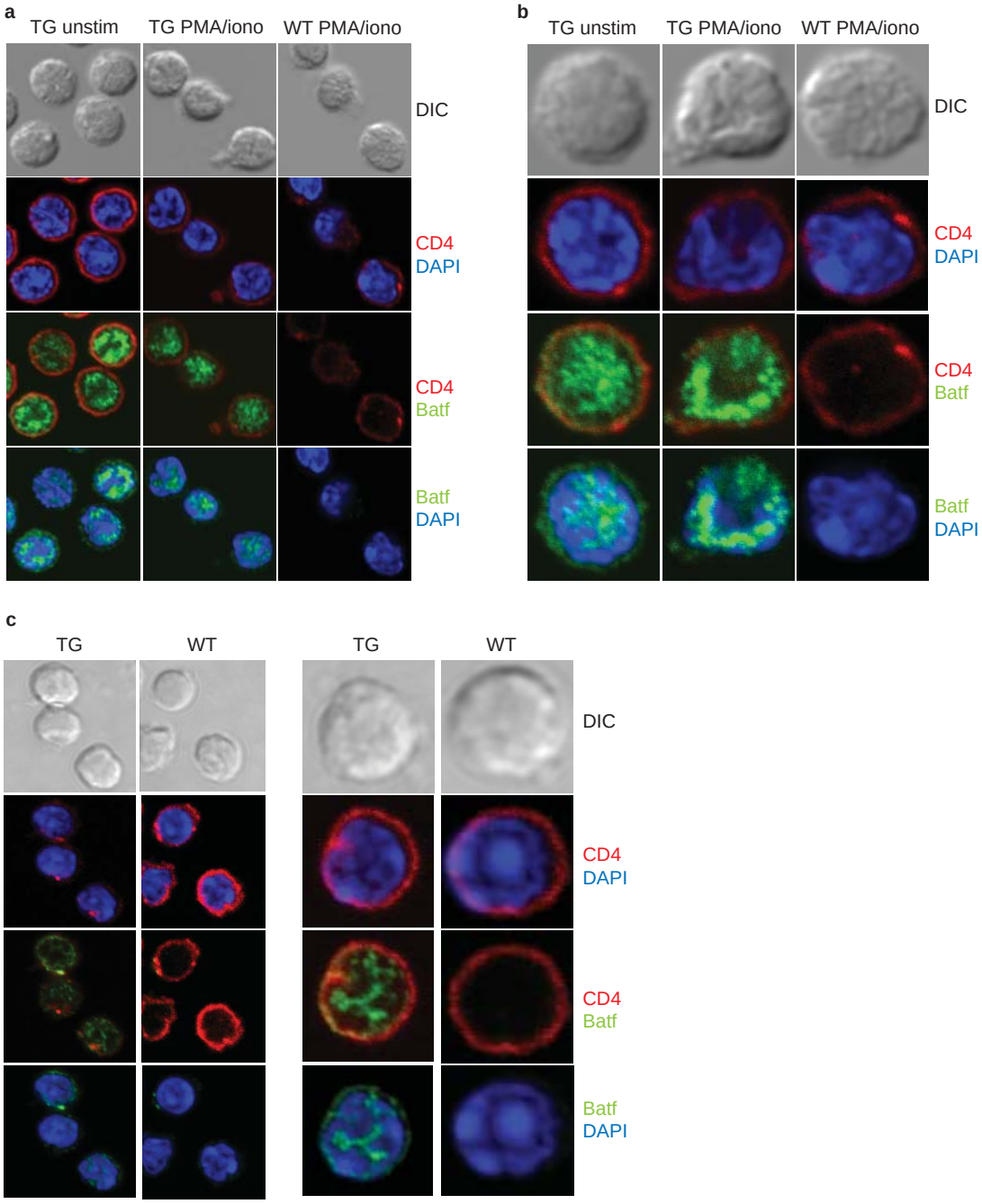


Figure 15. Batf is located in the cytoplasm and nucleus of resting T cells.
a, b, DO11.10^{+}CD4^{+} T cells from *CD2-N-FLAG-Batf* transgenic (TG) or littermate control mice (WT) were cultured with OVA/APC under T_H2 conditions. On day 7, cells were left untreated or stimulated with PMA/ionomycin for 4h, allowed to settle on poly-L-lysine treated slides and stained with antibodies to CD4 and FLAG. DAPI was used as nuclear stain.
b, Higher magnification single cell representation of a cell in **a**. **c,** Naïve DO11.10^{+}CD4^{+} T cells from CD2-N-FLAG-*Batf* transgenic or littermate mice were stained as in **a**. Data are representative of 2 independent experiments.

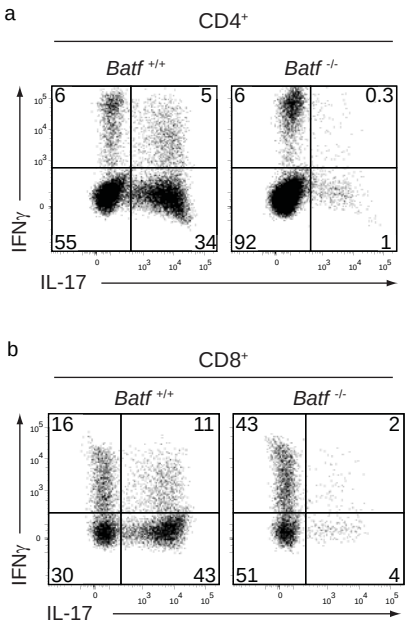


Figure 16. *Batf* regulates IL-17 production in $CD4^+$ and $CD8^+$ T cells.
a, $CD4^+$ T cells from DO11.10 *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were activated with OVA and irradiated APCs under T_H17 conditions. 3 days later, cells were split and allowed to expand for 4 days in the presence of T_H17 inducing cytokines. After 3 rounds of differentiation, cells were restimulated with PMA/ionomycin for 4h and analyzed for CD4, IFN-γ and IL-17 expression by flow cytometry. **b,** Total splenocytes from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were stimulated under T_H17 conditions for 3 days. Cells were restimulated with PMA/ionomycin and analyzed for IL-17 and IFN-γ expression by intracellular cytokine staining. Plots are gated on $CD8^+$ cells. Data are representative of at least 2 independent experiments performed with multiple mice of each genotype.

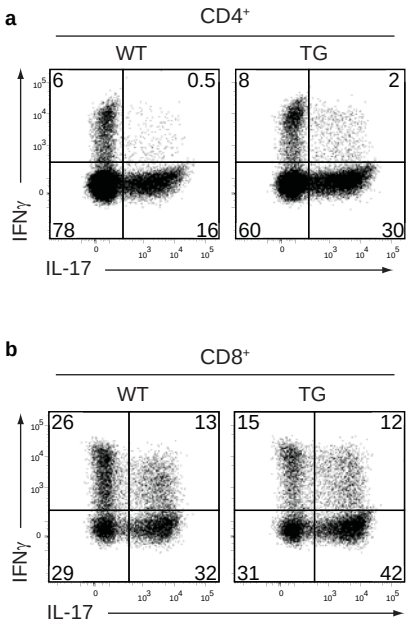


Figure 17. Overexpression of *Batf* increases IL-17 production in $CD4^+$ and $CD8^+$ T cells. **a,** $DO11.10^+CD4^+$ T cells from CD2-N-FLAG-*Batf* transgenic (TG) or transgene-negative (WT) control mice were stimulated with OVA and APC under T_H17 conditions. 3 days later, cells were restimulated with PMA/ionomycin and analyzed for CD4, IFN-γ and IL-17 expression by flow cytometry. **b,** Total splenocytes from CD2-N-FLAG-*Batf* transgenic (TG) or transgene-negative (WT) control were stimulated under T_H17 conditions for 3 days. Cells were restimulated with PMA/ionomycin and analyzed for IL-17 and IFN-γ expression by intracellular cytokine staining. Plots are gated on $CD8^+$ cells. . Data are representative of 2 independent experiments.

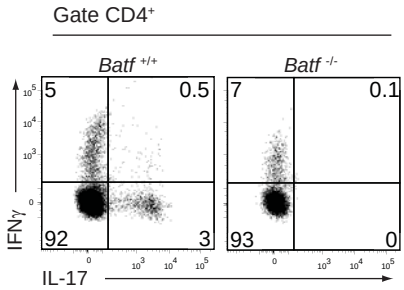


Figure 18. Lamina propria CD4$^+$ T cells in *Batf* $^{-/-}$ mice fail to produce IL-17.
Small intestinal lamina propria cells were isolated from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice, stimulated with PMA/ionomycin for 3h and stained for IL-17 and IFN-γ. Plots are gated on CD4$^+$ lymphocytes. Numbers indicate the percentage of live cells in each indicated gate. Data are representative of 3 independent experiments performed with multiple mice of each genotype.

CHAPTER 5

Batf $^{-/-}$ mice are completely resistant to EAE due to a T cell intrinsic defect

While T_H17 cells provide protection against bacteria at mucosal surfaces, they contribute to the pathology of multiple autoimmune diseases and disease models, including psoriasis, inflammatory bowel disease and experimental autoimmune encephalomyelitis (EAE) (Ouyang et al., 2008a). T_H17 cells were first implicated as major players in EAE when mice deficient for the for the p19 subunit of IL-23, but not the p35 subunit of IL-12 were protected from EAE (Langrish et al., 2005; Park et al., 2005; Yang et al., 2008). Since then, it has been recognized that IL-23 is required for EAE development because of its role in the maintenance of IL-17 producing T cells (Langrish et al., 2005). Additionally, mice deficient for transcription factors that control T_H17 development, such as RORγt and IRF4, are protected from EAE (Ivanov et al., 2006; Brustle et al., 2007). While these data clearly indicate the importance of T_H17 cells in EAE pathogenesis, several studies have unsuccessfully addressed which T_H17 effector cytokines are required for EAE development. Antibody blockade of IL-17A in the setting of *Il17f*-deficiency, or *Il22* deficiency only minimally prevent EAE (Kreymborg et al., 2007; Haak et al., 2009), implying the existence of other T_H17 effector pathways that are required for EAE development.

Batf $^{-/-}$ mice are resistant to MOG_{35-55}-induced EAE

Since *Batf* $^{-/-}$ T cells failed to develop into IL-17-secreting cells, we tested whether *Batf* $^{-/-}$ mice were susceptible to EAE. We immunized *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice with

myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}). Eleven *Batf*$^{+/+}$ mice (n=12) developed EAE, whereas no *Batf*$^{-/-}$ mice (n=13) developed any signs of disease within 40 days after immunization (Figure 19a). During EAE, cytokine-producing CD4^{+} T cells infiltrate the CNS (central nervous system) leading to CNS inflammation and disease manifestations. CNS-infiltrating CD4^{+} T cells in *Batf*$^{+/+}$ mice produced copious amounts of IL-17 and IFN-γ, at times of peak disease (day 10 after EAE induction). In contrast, fewer *Batf*$^{-/-}$ CD4^{+} T cells infiltrated the CNS and produced no IL-17, but made similar amounts of IFN-γ as *Batf* $^{+/+}$ T cells (Figure 19b).

Since fewer *Batf*$^{-/-}$ T cells infiltrated the CNS during EAE, disease resistance in *Batf*$^{-/-}$ mice might be due to a failure of T cells to traffic to the CNS. To address this question we tested whether IL-17-producing T cells were present in spleens of *Batf*$^{-/-}$ mice after EAE induction. Prior to disease onset, IL-17-producing CD4^{+} T cells were present in spleens from *Batf*$^{+/+}$ but not *Batf*$^{-/-}$ mice (Figure 19c), indicating, that IL-17-producing *Batf* $^{-/-}$ T cells do not solely fail to home to the CNS during EAE but do not develop.

Batf $^{-/-}$ T cells do not preferentially develop into Foxp3^{+} cells during EAE

The development of T_H17 and T_{reg} cells is reciprocally regulated (Bettelli et al., 2006); the development of both lineages requires TGF–β but in the presence of IL-6 T_H17 cells develop preferentially. As a result, IL-6 deficient mice are resistant to EAE due to a compensatory increase in Foxp3^{+} T regulatory cells (Korn et al., 2007). Thus, resistance of *Batf*$^{-/-}$ mice to EAE could conceivably result either from the loss of IL-17-producing effector T cells, or from an increase in T_{reg} cells. We analyzed splenic T cells

in *Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice for Foxp3 expression before immunization and 10 or 40 days after immunization with MOG_{35-55} (Figure 20a and b). *Batf*$^{-/-}$ mice had lower basal numbers of splenic Foxp3^{+} T cells compared to *Batf*$^{+/+}$ mice. More importantly, *Batf*$^{-/-}$ mice showed no statistically significant increase in Foxp3^{+} T_{reg} cells after MOG_{35-55} immunization (Figure 20a and b). These data suggest that the resistance of *Batf*$^{-/-}$ mice to EAE results from an absence of T_H17 cells rather than an increase in T_{reg} cells.

Batf$^{-/-}$ mice are protected from EAE due to a T cell intrinsic defect

Since the MOG_{35-55} immunization model of EAE is primarily T-cell dependent (Wolf et al., 1996), the lack of T_H17 development in *Batf*$^{-/-}$ mice likely explains disease resistance. This loss of T_H17 development in *Batf*$^{-/-}$ mice could result either from a defect within T cells or a defect in antigen-presenting cells. To distinguish these possibilities, we carried out an adoptive transfer study. We injected naïve *Batf*$^{+/+}$ CD4^{+} T cells or PBS control buffer into mice before MOG_{35-55} immunization. *Batf*$^{-/-}$ mice receiving PBS control buffer remained resistant to EAE as expected (Figure 21a). In contrast, *Batf*$^{-/-}$ mice receiving naïve *Batf*$^{+/+}$ CD4^{+} T cells developed severe EAE (Figure 21a and Table 2). Although, *Batf*$^{-/-}$ mice that had received *Batf*$^{+/+}$ T cells exhibited a slight delay in disease onset compared to *Batf*$^{+/+}$ mice receiving *Batf*$^{+/+}$ T cells, these differences are not statistically significant (Table 2). Notably, the transfer of *Batf*$^{+/+}$ CD4^{+} T cells into *Batf*$^{-/-}$ mice also restored infiltration of the CNS by IL-17-producing CD4^{+} T cells (Figure 21b).

Since *Batf*$^{-/-}$ T cells fail to develop into T_H17 cells, we hypothesized that *Batf*$^{-/-}$ T cells might exhibit a protective function if transferred into *Batf*$^{+/+}$ mice, conceivably

because of increased production of T_H17 suppressing cytokines by *Batf*$^{-/-}$ T cells (Willenborg et al., 1996) or increased development of Foxp3^{+} T_{reg} cells (Korn et al., 2007). Therefore, we injected naïve *Batf*$^{-/-}$ CD4^{+} T into mice before MOG_{35-55} immunization, however; the transfer of *Batf* $^{-/-}$ CD4^{+} T cells did not protect *Batf* $^{+/+}$ mice from EAE development (Figure 21c).

Collectively, our results indicate that the antigen-presenting environment in *Batf*$^{-/-}$ mice is permissive for T_H17 development, and suggest that resistance to EAE in *Batf*$^{-/-}$ mice is due to a T cell-intrinsic defect in the generation of T_H17 cells.

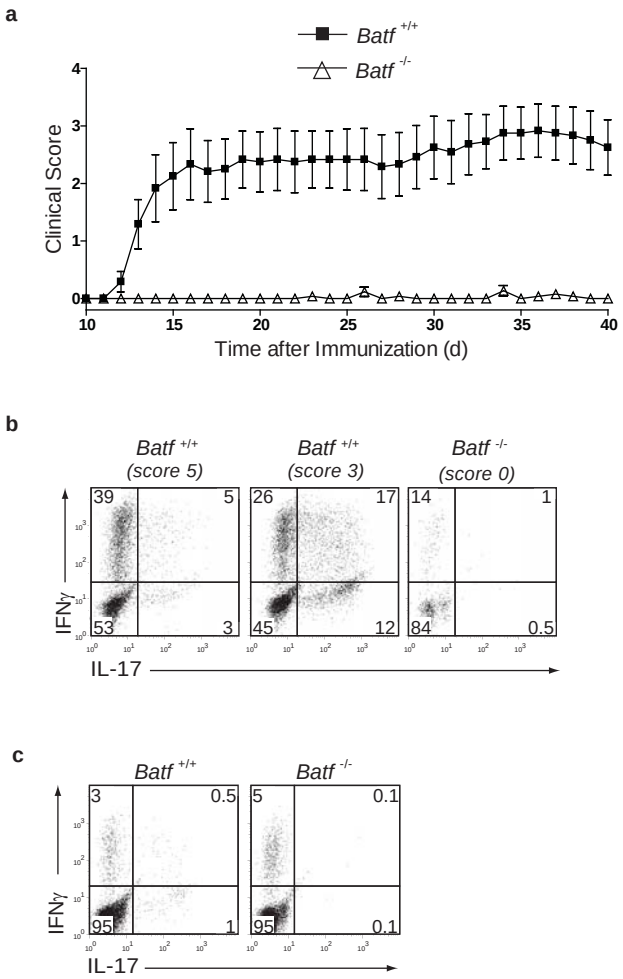


Figure 19. *Batf*$^{-/-}$ mice are resistant to EAE.
a, *Batf*$^{+/+}$ (n=12) and *Batf*$^{-/-}$ (n=13) mice were immunized with MOG_{33-35} peptide as described in Methods. Clinical EAE scores (mean ± s.e.m) representative of two independent experiments are shown. **b,** 13 days after EAE induction, CNS infiltrating lymphocytes were stimulated with PMA/ionomycin for 4h, stained for intracellular IL-17 and IFN-γ and analyzed by flow cytometry. Clinical scores are shown in parentheses. Data are representative of 2-3 mice analyzed per group. **c,** Total splenocytes were isolated from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice 10 days after EAE induction, stimulated with PMA/ionomycin for 3h and analyzed for IL-17 and IFN-γ expression by intracellular cytokine staining. All FACS plots are gated on $CD4^+$ cells. Numbers for FACS plots indicate percentage of cells in each indicated gate.

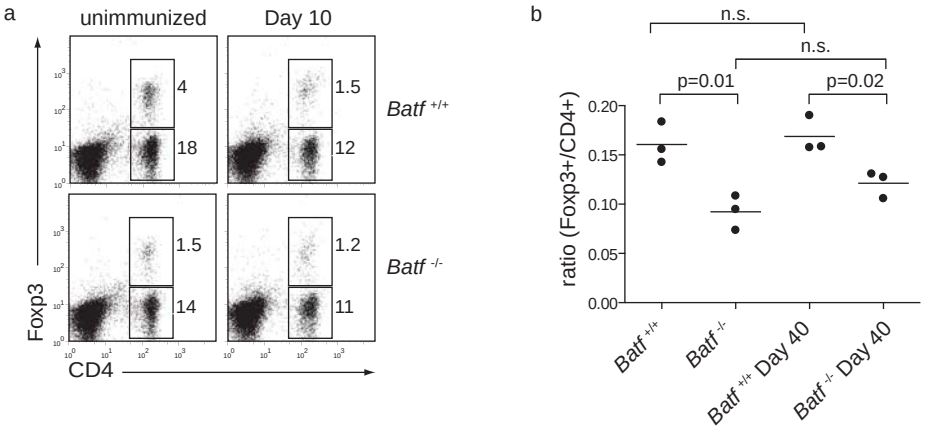


Figure 20. *Batf* $^{-/-}$ mice show no compensatory increase in Foxp3^{+} cells during EAE. **a,** Total splenocytes from unimmunized *Batf* $^{+/+}$ and *Batf* $^{-/-}$ or from mice 10 days after EAE induction were stained for the expression of CD4 and Foxp3 and analyzed by flow cytometry. Numbers indicate percent live cells in each region. **b,** Total splenocytes from unimmunized *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice or from mice 40 days after EAE induction were analyzed for CD4 and Foxp3 expression. The abundance of Foxp3^{+} cells is depicted as the ratio of CD4^{+}Foxp3^{+} cells in the total CD4^{+} T cell compartment. Statistical significance was assessed using an unpaired student's t test. A p-value <0.05 was considered significant. (n.s., not significant)

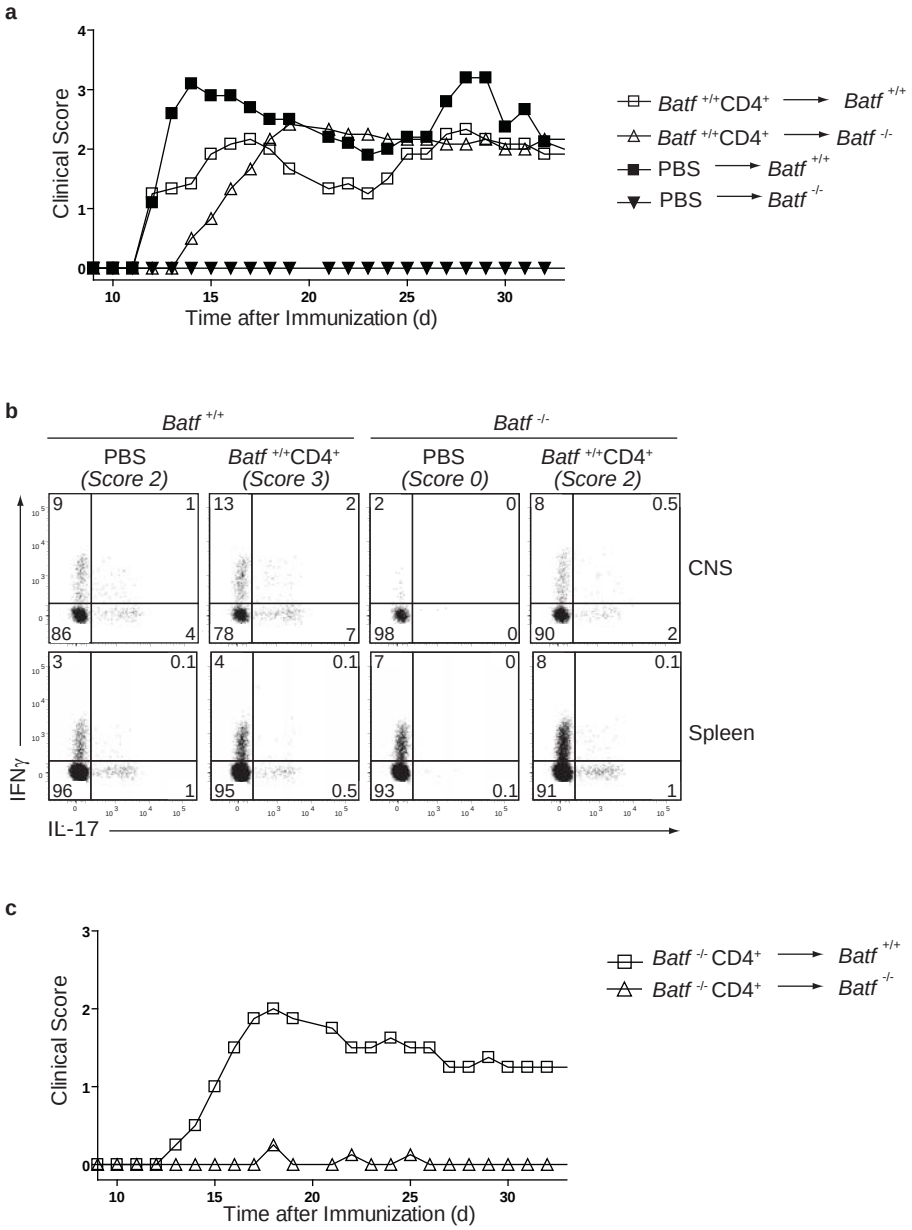


Figure 21. *Batf* $^{-/-}$ mice are resistant to EAE due to a T cell-intrinsic defect. **a,** *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were injected with either control PBS buffer (n=5) or 1x10^7 *Batf* $^{+/+}$ CD4$^+$ T cells (n=6). Four days later, mice were immunized with MOG_{35-55} peptide. **b,** Splenic and CNS infiltrating lymphocytes from experimental animals in **a** were stimulated with PMA/ionomycin for 4h and analyzed for IL-17 and IFN-γ production 40 days after EAE induction. Genotypes and whether mice received PBS or CD4$^+$ T cells are indicated, disease scores are given in parentheses. FACS plots are gated on CD4$^+$ cells and are representative of 2-3 mice analyzed per group. Numbers in FACS plots indicate percentage of live cells in each region. **c,** *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were injected 1x10^7 *Batf* $^{-/-}$ CD4$^+$ T cells (n=4). Four days later, mice were immunized MOG_{33-35} peptide. **a, b,** Mean clinical EAE scores representative of two independent experiments are shown.

Group	Incidence	Mean Max. Score	Mortality	Mean day of onset‡
PBS→*Batf* $^{+/+}$	5 of 5 (100%)	3.4 ± 0.7	1of 5 (20%)	12±0.8¶
PBS→*Batf* $^{-/-}$	0 of 5 (0%)	0	0 of 13 (0%)	NA
Batf $^{+/+}$CD4^{+}→*Batf* $^{+/+}$	5 of 6 (83%)	3.0 ± 0.6	0 of 6 (0%)	13.6±2.3§¶
Batf $^{+/+}$CD4^{+}→*Batf* $^{-/-}$	4 of 6 (66%)	2.4 ± 1.0	2 of 6 (33%)	15.5±1.7§

Table 2. Transfer of *Batf* $^{+/+}$ CD4^{+} T cells restores EAE in *Batf* $^{-/-}$ mice.
Batf $^{+/+}$ and *Batf* $^{-/-}$ mice were injected with either control PBS buffer (n=5) or 1x10^{7} *Batf* $^{+/+}$ CD4^{+} T cells (n=6). Four days later, mice were immunized with MOG_{35-55} as described in Methods. Mean maximum score of disease was calculated and is presented ± s.e.m.
‡ Mean day of onset is presented as mean ± s.d. Only animals positive for disease were included in the analysis. § not significant (p=0.215). ¶ not significant (p=0.232). NA, not applicable.

CHAPTER 6

Batf controls the expression of a subset of IL-6 induced genes

T_H17 cells differentiate in response to the cytokines IL-6 and TGF-β (Bettelli et al., 2006; Korn et al., 2007; Nurieva et al., 2007; Wei et al., 2007; Zhou et al., 2007; Manel et al., 2008) but also require IL-21 and IL-23 for their full development (Korn et al., 2007; Nurieva et al., 2007; Wei et al., 2007; Langrish et al., 2005; Veldhoen et al., 2006a). IL-6, IL-21 and IL-23 each activate STAT3 (Ghilardi and Ouyang, 2007), which is required for T_H17 differentiation and binds directly to the *Il17* promoter (Laurence et al., 2007; Yang et al., 2007; Mathur et al., 2007).

Combined signals from IL-6 and TGF-β induce the expression of the retinoid acid related nuclear orphan receptor (ROR) RORγt (Ivanov et al., 2006), in a STAT3 dependent manner(Yang et al., 2007; Harris et al., 2007). RORγt is sufficient to induce IL-17 production in wild type T cells and RORγt deficiency blocks T_H17 differentiation *in vitro* (Ivanov et al., 2006). RORγt has been considered the main regulator of T_H17 cells, similar to T-bet and Gata3 in T_H1 and T_H2 cells respectively. However, residual IL-17 production can be observed in RORγt deficient T cells *in vivo* after EAE induction (Ivanov et al., 2006). Since RORα and RORγt double deficient do not exhibit this residual IL-17 production, RORα has been suggested to cooperate with RORγt during T_H17 differentiation at the level of *Il17* transcription (Yang et al., 2008). However, RORα deficiency only mildly affects IL-17 production (Yang et al., 2008), indicating that RORα is dispensable for T_H17 differentiation and that RORγt and RORα exhibit mere functional redundancy. More strikingly, *Irf4*$^{-/-}$ T cells exhibit an absolute block in

T_H17 development and decreased expression of RORγt. However, overexpression of RORγt in *Irf4*$^{-/-}$ T cells only partially restores IL-17 production (Brustle et al., 2007), indicating that IRF4 likely regulates additional factors during T_H17 differentiation. More recently, RUNX1 and RORγt have been suggested to synergize to induce *Il17* transcription (Zhang et al., 2008a). The aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor, specifically regulates IL-22 but not IL-17 production in T cells (Veldhoen et al., 2008; Quintana et al., 2008) although AHR ligands are necessary for optimal differentiation of T_H17 cells (Veldhoen et al., 2009). Thus, multiple transcription factors have been suggested to control T_H17 differentiation, although it is unclear how they cooperate to induce T_H17 differentiation.

Proximal IL-6 receptor signaling and TGF-β signaling is normal in *Batf*$^{-/-}$ T cells

Since *Batf* is a transcription factor, it could control T_H17 differentiation either by regulating the expression of components of the IL-6 or TGF-β signaling pathways (Korn et al., 2007; Nurieva et al., 2007), or by regulating the induction of their downstream target genes. To distinguish these alternatives, we determined whether these signaling pathways were intact in *Batf*$^{-/-}$ T cells. IL-6 receptor expression on *Batf*$^{-/-}$ CD4$^+$ T cells was normal (Figure 22a). Further, treatment with IL-6 induced normal levels of STAT3 phosphorylation in both CD4$^+$ and CD8$^+$ *Batf*$^{-/-}$ T cells (Figure 22b), indicating that proximal IL-6 receptor signaling is normal in *Batf*$^{-/-}$ T cells.

Proximal TGF-β signaling also appeared intact based on normal induction of Foxp3 in response to TFG-β in *Batf*$^{-/-}$ CD4$^+$ T cells (Figure 23a). Foxp3 inhibits RORγt function, and one of the roles of IL-6 during T_H17 differentiation is to suppress Foxp3

expression (Bettelli et al., 2006). *Batf*$^{-/-}$ T cells failed to fully downregulate Foxp3 in response to IL-6 when cultured under T_H17 conditions (Figure 23a). Neutralization of IL-2 abrogated increased Foxp3 expression in *Batf*$^{-/-}$ T cells, but failed to restore IL-17 production (Figure 23b). Since TGF-β signaling and proximal IL-6 signaling are intact in *Batf*$^{-/-}$ T cells, these data suggest that *Batf* may be required for the regulation of genes downstream of IL-6.

Batf is required for the induction of IL-21

Consistent with a requirement for *Batf* for the expression of genes downstream of IL-6, induction of IL-21, an early target of IL-6 signaling in CD4$^+$ T cells (Zhou et al., 2007), was significantly reduced in *Batf*$^{-/-}$ CD4$^+$ T cells activated under T_H17 conditions (Figure 24a). Consistently, *Batf*$^{-/-}$ CD4$^+$ T cells activated under T_H17 failed to induce IL-23 receptor expression (Figure 24b), which requires IL-21 signaling (Zhou et al., 2007). This reduced production of IL-21 could potentially explain the absence of T_H17 development in *Batf*$^{-/-}$ T cells, since autocrine IL-21 is required for T_H17 development (Korn et al., 2007; Nurieva et al., 2007; Wei et al., 2007). However, addition of IL-21 failed to rescue T_H17 development in *Batf*$^{-/-}$ T cells (Figure 24c). This absence of IL-17 production in the presence of exogenous IL-21 could be due to a failure to express IL-21 receptor or IL-21 signaling components. However, proximal IL-21 signaling was intact, since *Batf*$^{-/-}$ CD4$^+$ T cells showed normal levels of IL-21-induced STAT3 phosphorylation (Figure 24d). These data indicate normal expression and function of the IL-21 receptor signaling machinery and suggest that *Batf* regulates additional factors besides IL-21 during T_H17 differentiation.

Batf controls the expression of a subset of IL-6-induced genes

To identify additional *Batf* target genes, we performed DNA microarrays and quantitative real time polymerase chain reaction (qRT-PCR) comparing gene expression of *Batf*$^{+/+}$ and *Batf*$^{-/-}$ T cells activated in the presence or absence of IL-6 and/or TGF-β in combination with neutralizing antibodies to IL-6 and/or TGF-β as indicated (Figure 25). To allow identification of *Batf*-dependent T_H17-specific genes, anti-IL-2 antibody was added to avoid contamination with genes specifically expressed in Foxp3^{+} cells (Figure 23a and b). This analysis identified additional *Batf*-dependent genes, some of which are known to regulate T_H17 development (Figure 25 and Figure 27a and b). *Batf*-dependent genes included RORγt (Zhou et al., 2007), RORα (Yang et al., 2008), the aryl hydrocarbon receptor (AHR) (Veldhoen et al., 2008; Kimura et al., 2008; Quintana et al., 2008), IL-22 (Liang et al., 2006) and IL-17. In contrast, IRF4 (Brustle et al., 2007) (Figure 27a) and SOCS gene expression (Figure 26b) were unchanged in *Batf*$^{-/-}$ T cells. Notably, early induction of RORγt and RORα in *Batf*$^{-/-}$ T cells occurred normally but the expression of these genes was not maintained at 62h after stimulation (Figure 27c), indicating that *Batf* is required for the sustained expression of RORγt and RORα rather than their early induction.

Finally, microarray analysis indicated that *Batf* was required for the expression of a subset of IL-6-induced genes (Figure 25) but not TGF-β-induced genes (Figure 26a). Specifically, we found that genes induced in response to either IL-6 alone or IL-6 plus TGF-β in wild type cells clustered into genes that displayed either *Batf*-dependent or *Batf*-independent expression (Figure 25). However, *Batf* deficiency did not affect the

expression of genes induced in response to TGF-β alone (Figure 26a). Notably, *Batf* deficiency did not globally affect IL-6-induced responses in all tissues, since IL-6-induced liver acute phase responses appeared normal in *Batf*$^{-/-}$ mice (Figure 28). These data indicate that *Batf* is required for the induction of numerous IL-6-dependent genes in T cells.

RORγt only partially restores IL-17 production in *Batf*$^{-/-}$ T cells

Since RORγt has been suggested to act directly on the *Il17* promoter (Ichiyama et al., 2008; Zhang et al., 2008b), we tested whether forced RORγt expression would rescue T_H17 development in *Batf*$^{-/-}$ T cells. First, when *Batf*$^{+/+}$ T cells were activated without cytokine additions, forced RORγt overexpression by retrovirus led to 38% IL-17 production, compared to only 1.6% IL-17 production induced by the control retrovirus (Figure 29a and c). This was consistent with previous studies demonstrating that RORγt is sufficient to induce IL-17 production (Ivanov et al., 2006; Brustle et al., 2007). In contrast, in *Batf*$^{-/-}$ T cells activated under these conditions forced RORγt expression led to only 5.7% IL-17 production (Figure 29a and c). When T cells were activated in T_H17-inducing conditions, forced RORγt expression induced only 7.6% IL-17 production in *Batf*$^{-/-}$ T cells, compared to 50% IL-17 production in *Batf*$^{+/+}$ T cells (Figure 29b and c). Thus, RORγt only partially restores T_H17 differentiation in *Batf*$^{-/-}$ T cells.

RORα, a nuclear receptor related to RORγt has been suggested to cooperate with RORγt during T_H17 differentiation, since mice deficient in RORα and RORγt do not exhibit the residual IL-17 production that is observed in the absence of RORγt alone (Yang et al., 2008). However, RORα deficient T cells show only a mild reduction in IL-

17 production (Yang et al., 2008). Even though this indicates that RORα is dispensable for T_H17 differentiation we felt that the potential functional cooperation between RORγt and RORα might explain the absence of IL-17 production in *Batf*$^{-/-}$ T cells, since the expression of both factors is reduced in the absence of Batf. Thus, we tested whether overexpression of both RORα and RORγt would rescue IL-17 production in *Batf*$^{-/-}$ T cells. Even overexpression of both RORα and RORγt failed to fully restore IL-17 production in *Batf*$^{-/-}$ T cells (Figure 29d).

Since RORγt overexpression only partially restored IL-17 production in the absence of *Batf, Batf* and RORγt might cooperate to induce IL-17 production. To test this we carried out dual retroviral infections (Figure 30). Forced expression of both Batf and RORγt in *Batf*$^{-/-}$ T cells induced 26% IL-17 production, compared to only 5% with RORγt alone, and 14% with Batf alone. These data suggest functional synergy between RORγt and Batf in inducing IL-17 production, and also suggest that Batf might directly regulate the transcription of *Il17* and other T_H17-specific genes.

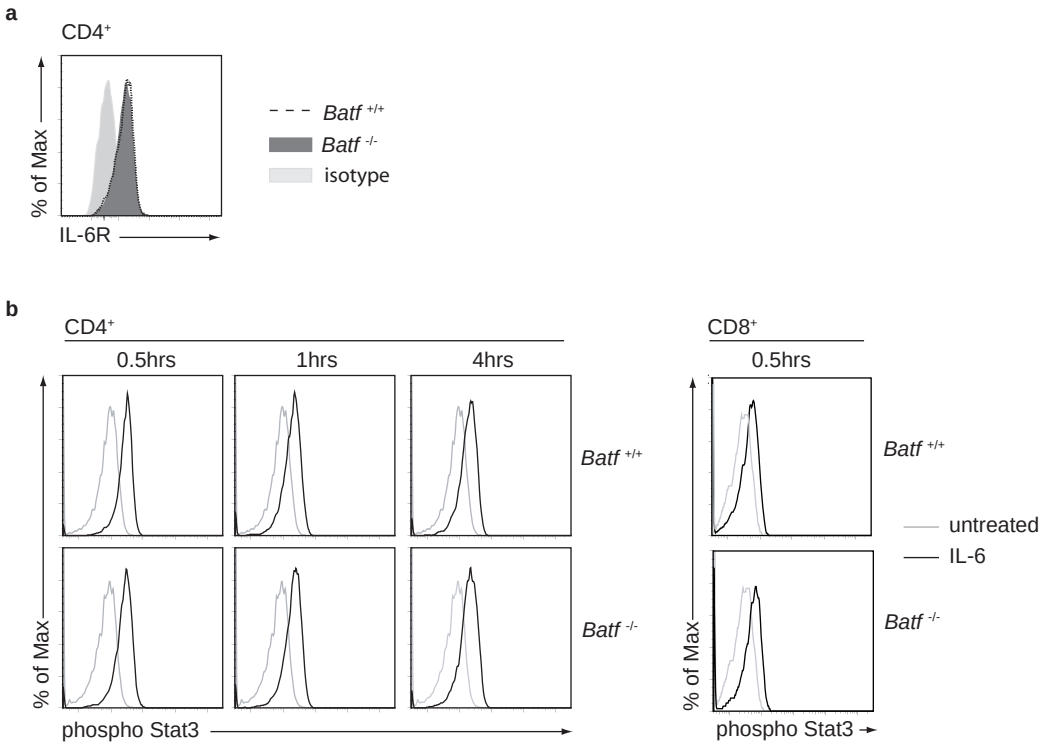


Figure 22. Proximal IL-6 receptor signaling is normal in *Batf*$^{-/-}$ T cells.
a, Splenocytes from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice were stained with antibodies to CD4 and IL-6 receptor (IL-6R). A histogram overlay of IL-6R expression on CD4^{+} cells of the indicated genotypes is shown. **b,** Magnetically purified *Batf*$^{+/+}$ and *Batf*$^{-/-}$ CD4^{+} T cells (left) and CD8^{+} T cells (right) were stimulated with anti-CD3/CD28 in the presence of IL-6 for the indicated times and stained with an antibody to phospho-STAT3 (black lines) by intracellular staining as described in Methods. Untreated cells (grey lines) served as negative control.

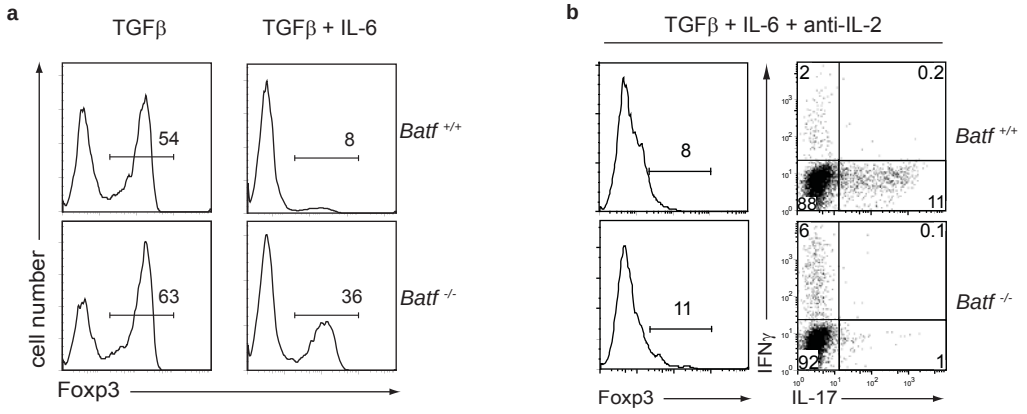


Figure 23. Normal TGF-β signaling in *Batf*$^{-/-}$ T cells.
a, Naïve CD4^{+}CD62L^{+}CD25^{-} T cells from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice were stimulated with TGF-β or TGF-β plus IL-6 for 3 days. Cells were stained for Foxp3 and analyzed by flow cytometry. **b,** Naïve CD4^{+}CD62L^{+}CD25^{-} T cells from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice were stimulated with TGF-β plus IL-6 in the presence of a neutralizing antibody to IL-2 for 3 days. Cells were stained for Foxp3, IL-17 and IFN-γ and analyzed by flow cytometry. Numbers for FACS plots indicate the percentage of live cells in each region. Data are representative of at least 2 independent experiments performed with cells from multiple mice of each genotype.

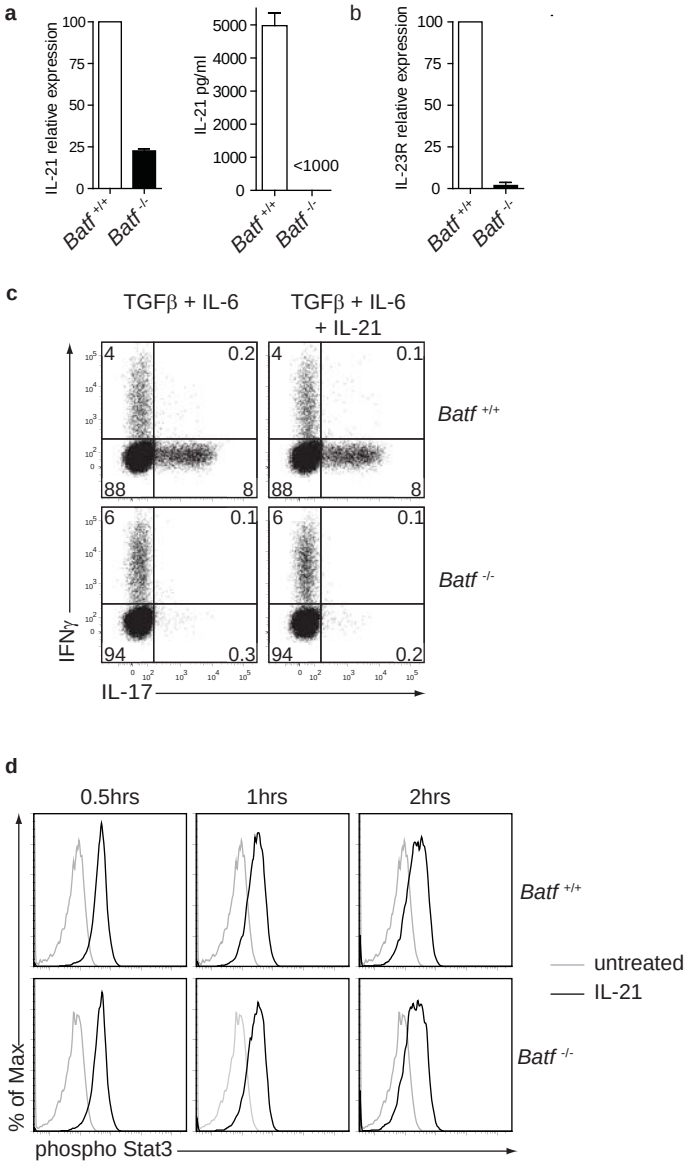


Figure 24. Exogenous IL-21 fails to rescue IL-17 production in *Batf*$^{-/-}$ T cells.
a, IL-21 expression in T cells 3 days after activation with anti-CD3 and anti-CD28 under T_H17 conditions was determined by quantitative real time PCR (left) and ELISA (right). **b,** IL-23 receptor (IL-23R) expression in T cells 3 days after activation with anti-CD3/CD28 under T_H17 conditions was determined by quantitative real time PCR. qRT-PCR data are normalized to HPRT and presented as percent expression relative to *Batf*$^{+/+}$ cells (mean + s.d. of 3 individual mice). **c,** Naive $CD4^+CD62L^+CD25^-$ T cells were activated as in **a** in the presence or absence of IL-21 and stained for intracellular IL-17 and IFN-γ. **d,** Magnetically purified *Batf*$^{+/+}$ and *Batf*$^{-/-}$ $CD4^+$ T cells were stimulated with anti-CD3/CD28 in the presence of IL-21 for the indicated times and stained with an antibody to phospho-STAT3 (black lines) by intracellular staining. Untreated cells (grey lines) served as a negative control.

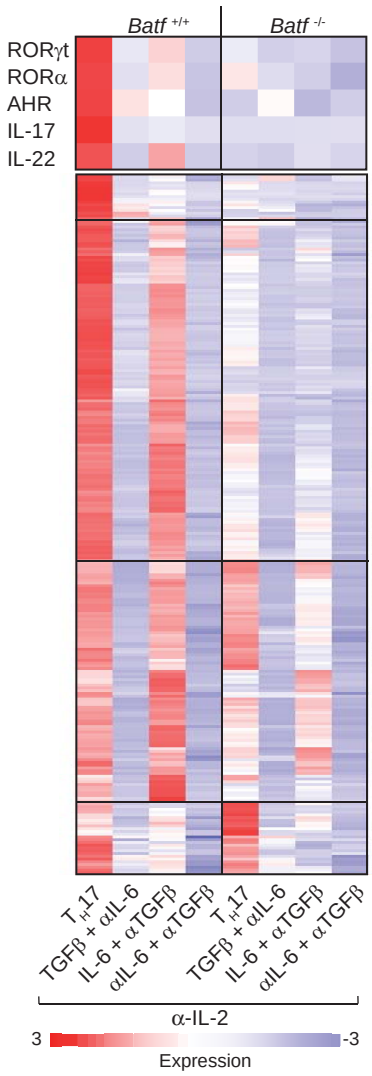


Figure 25. *Batf* controls the expression of a subset of IL-6 dependent genes in during T cell differentiation.
Gene expression microarray analysis of T cells activated with anti-CD3/CD28 for 72h with various combinations the indicated cytokines and antibodies. Shown are representative heat maps of genes at least 5-fold induced under T_H17 conditions compared to neutral conditions in *Batf* $^{+/+}$ T cells. Normalized and modeled expression values were generated and clustered using DNA-Chip analyzer (dChip) software.

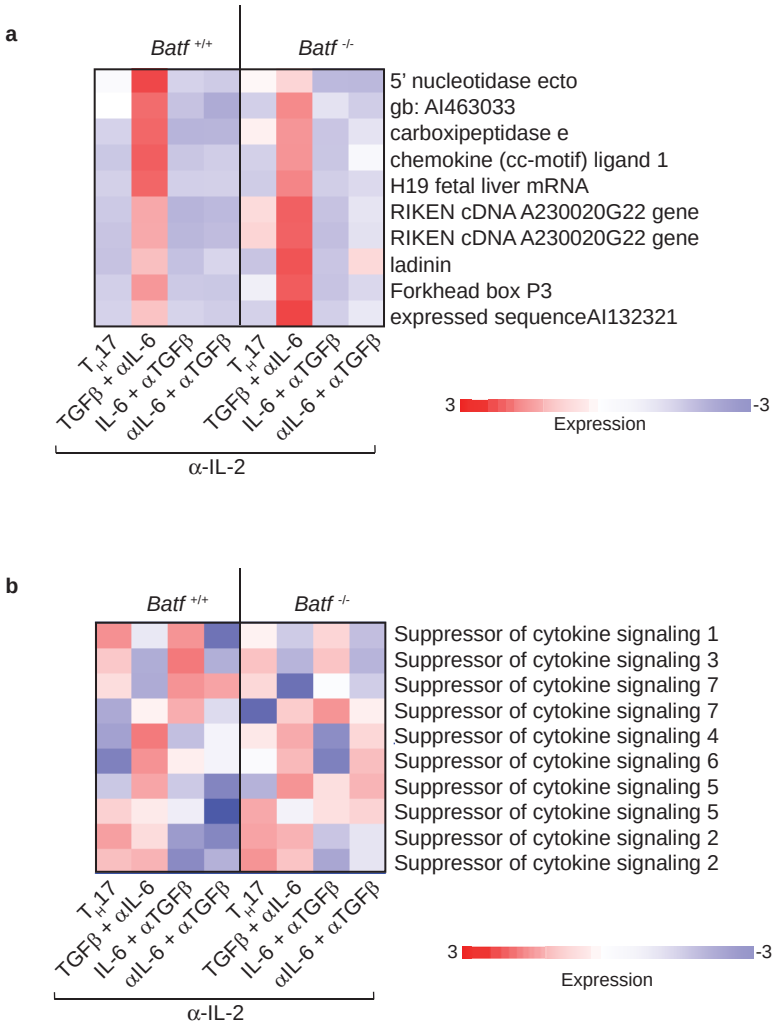


Figure 26. *Batf* does not regulate expression of SOCS genes or genes induced by TGF-β alone

a, b, Gene expression microarray analysis of T cells activated with anti-CD3/CD28 for 72h with various combinations the indicated cytokines and antibodies. **a,** A representative heat map of genes at least 5-fold induced by TGF-β compared to neutral conditions in *Batf* $^{+/+}$ T cells is presented. **b,** Shown is a representative heat map of the expression of Suppressor of cytokine signaling (SOCS) genes in *Batf* $^{+/+}$ and *Batf* $^{-/-}$ T cells. Normalized and modeled expression values were generated using DNA-Chip analyzer (dChip) software.

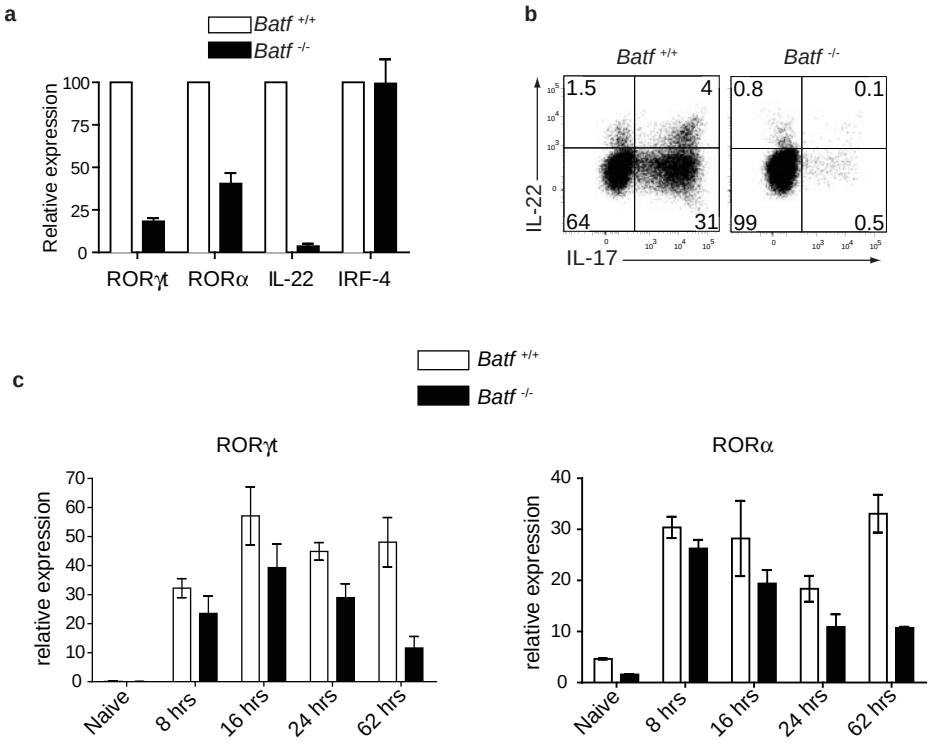


Figure 27. *Batf* is required for the expression of multiple T_H17 associated genes and for sustained expression of RORγt and RORα.
a, *Batf* $^{+/+}$ and *Batf* $^{-/-}$ naïve $CD4^+CD62L^+CD25^-$ T cells were stimulated with anti-CD3/CD28 under T_H17 conditions for 72h and relative expression of RORγt, RORα, IL-22 and IRF-4 in T cells was analyzed by qRT-PCR. **b,** $CD4^+$ T cells from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were activated with anti-CD3/CD28 under T_H17 conditions for 3 days, restimulated with PMA/ionomycin and stained for intracellular IL-17 and IL-22 expression. **c,** *Batf* $^{+/+}$ and *Batf* $^{-/-}$ naïve $CD4^+CD62L^+CD25^-$ T cells were stimulated with anti-CD3/CD28 under T_H17 conditions for 0, 8, 16, 24 and 62h and analyzed for expression of RORγt and RORα by qRT-PCR. qRT-PCR data are normalized to HPRT and presented as percent expression relative to *Batf* $^{+/+}$ cells (mean + s.d. of 3 individual mice).

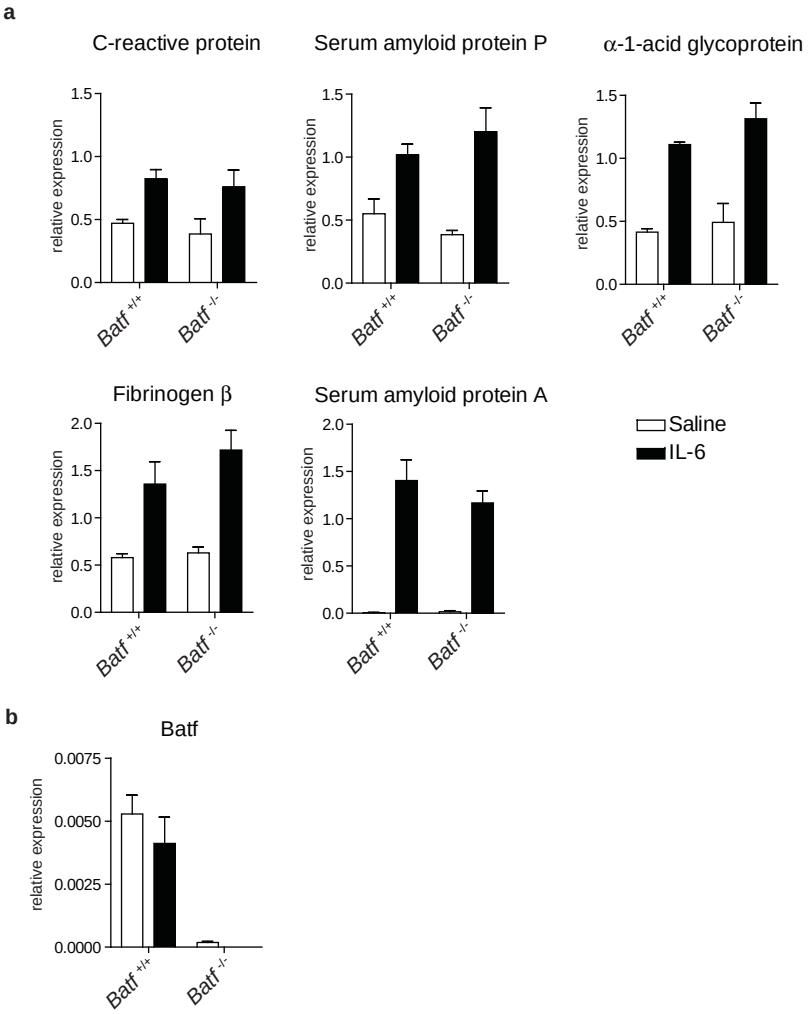


Figure 28. Several aspects of the IL-6-induced liver acute phase response are normal in *Batf* $^{-/-}$ mice.
a, *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice were injected intraperitonally with either 0.3ug IL-6 or saline. 4h after injection the expression of the indicated acute phase proteins in liver was assessed by qRT-PCR. **b,** Relative expression of *Batf* in liver 4h after injection of mice with 0.3ug IL-6 or saline. qRT-PCR data is normalized to HPRT and presented in arbitrary units. Data represent mean + s.d. of 3 individual mice from independent experiments.

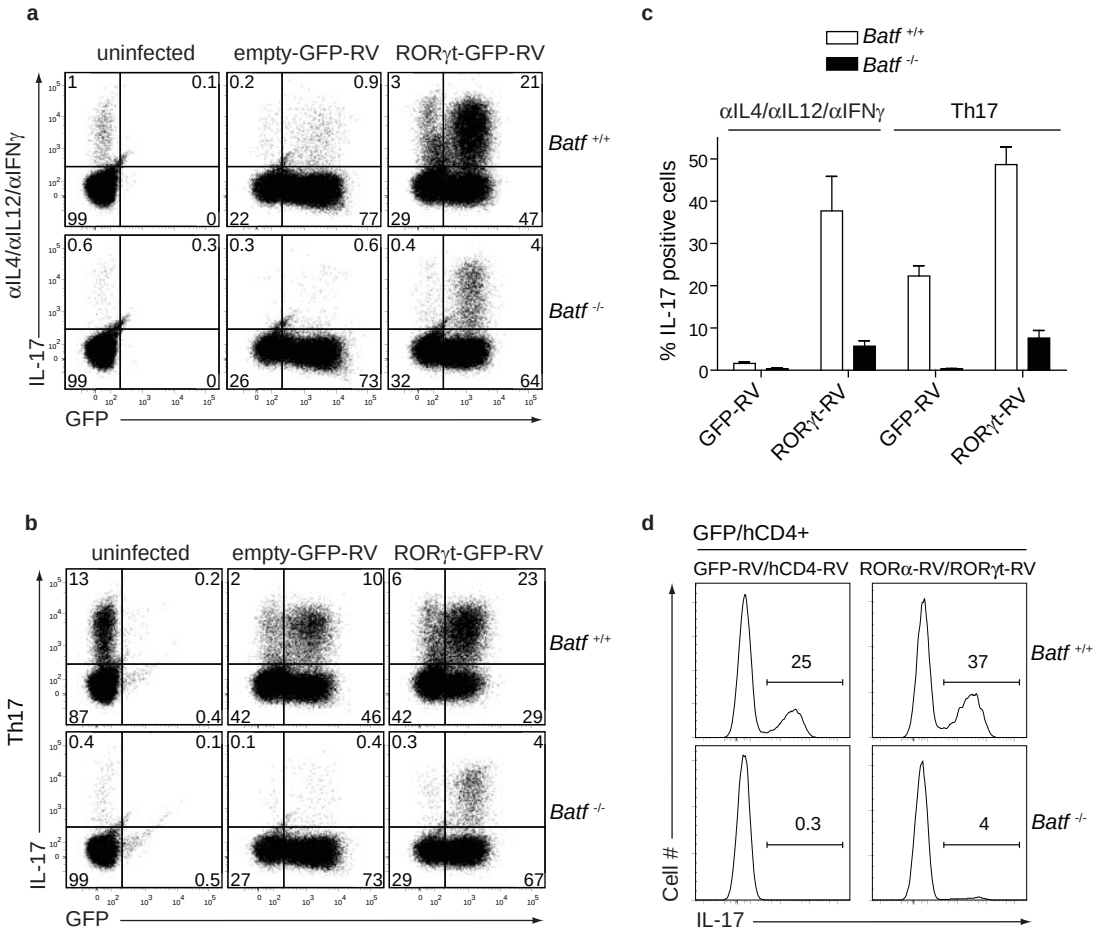


Figure 29. RORγt only partially restores IL-17 production in *Batf*$^{-/-}$ T cells.
a, b, *Batf*$^{+/+}$ and *Batf*$^{-/-}$ CD4^{+} T cells stimulated with anti-CD3/CD28 under the indicated conditions were either left untreated, infected with RORγt expressing IRES-GFP-retrovirus (RORγt-RV) or control retrovirus (GFP-RV). Cells were restimulated with PMA/ionomycin for 4h and analyzed for GFP and IL-17 expression. **c,** The percentage of IL-17 producing cells among stably infected (GFP^{+}) cells treated as in **a** and **b** is depicted (mean + s.d. of 3 independent experiments). **d,** *Batf*$^{+/+}$ and *Batf*$^{-/-}$ CD4^{+} T cells stimulated with anti-CD3/CD28 under T_H17 conditions and infected with RORγt expressing IRES-GFP-retrovirus (RORγt-RV) and RORα expressing IRES-hCD4-retrovirus (RORα-RV) or control retrovirus. Cells were restimulated with PMA/ionomycin for 4h. IL-17 expression in GFP^{+}hCD4^{+} cells is shown. Numbers in FACS plots represent the percentage of live cells in each region.

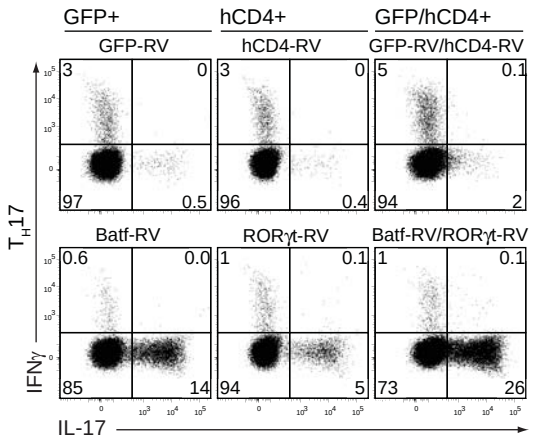


Figure 30. Functional synergy of *Batf* and RORγt during T_H17 differentiation.
a, *Batf*$^{-/-}$ CD4^{+} T cells were stimulated with anti-CD3/CD28 under T_H17 conditions and either infected with Batf-expressing IRES-GFP-retrovirus (Batf-RV), RORγt-expressing IRES-hCD4-retrovirus (RORγt-RV) or both (bottom panel). As a control, cells were infected with empty-control retroviruses as indicated (top panel). On day 3, cells were restimulated with PMA/ionomycin for 4h and analyzed for IL-17 and IFN-γ expression. Data are representative of 2 independent experiments. Representative FACS plots shown are gated as indicated. Numbers represent percentage of live cells in each region.

CHAPTER 7

Batf directly regulates the expression of T_H17 associated cytokines

Little is known about the transcriptional regulation of T_H17 associated genes. RORγt has been suggested to bind directly to the proximal *Il17* promoter and to conserved non-coding sequences upstream of the *Il17* locus (Ichiyama et al., 2008; Zhang et al., 2008b; Akimzhanov et al., 2007). RORγt can induce *Il17* promoter activity in reporter assays (Ichiyama et al., 2008; Zhang et al., 2008b) however, since RORγt deficient T cells show residual IL-17 production (Ivanov et al., 2006) other factors likely contribute to *Il17* gene transcription. RUNX1 cooperates with RORγt at the *Il17* promoter in reporter assays (Zhang et al., 2008b). Additionally, NFAT has been implicated in regulating *Il17* transcription in humans (Liu et al., 2004). Notably, this study found no role for AP-1 transcription factors at the proximal *Il17* promoter, however, promoter analysis was limited to EMSA binding of AP-1 factors to the -0.4kb proximal promoter.

Since *Batf*$^{-/-}$ T cells failed to induce multiple T_H17-associated genes and cytokines and RORγt overexpression only minimally restored IL-17 production in the absence of *Batf*, we hypothesized that *Batf* might regulate *Il17* transcription directly.

The proximal *Il17* promoter is *Batf*-responsive

To test whether *Batf* directly regulates *Il17* expression, we first tested *Il17* promoter activity in primary *Batf*$^{+/+}$ and *Batf*$^{-/-}$ T cells (Zhu et al., 2001) using a reverse strand retroviral reporter in which human CD4 (hCD4) marks viral infection and the *Il17* promoter drives GFP expression (Figure 31). On day 3 of activation under T_H17

conditions we analyzed reporter activity in stably infected (hCD4^{+}) cells. Under these conditions, *Batf*$^{-/-}$ CD4^{+} T cells showed considerably less reporter activity than *Batf*$^{+/+}$ T cells (Figure 31). These data suggest that the 1kb proximal *Il17* promoter is Batf-responsive.

Batf binds several regions in the *Il17a/f* locus

To test whether Batf regulates *Il17* promoter activity and gene expression through direct interactions, we next performed chromatin immunoprecipitation (ChIP) and elecrophoretic mobility shift assays (EMSA). For ChIP analysis, we examined several highly conserved regions within the *Il17a/f* locus (Figure 32a) that we hypothesized to be putative regulatory regions due to their evolutionary sequence conservation. By ChIP analysis, Batf specifically bound to two intergenic regions (+9.6kb and +28kb) as early as 24h after activation (Figure 32c). By day 5 after stimulation, Batf showed strong binding to several intergenic regions (-5kb, +9.6kb and +28kb) and to the proximal *Il17a* and *Il17f* promoters (Figure 32b and d). Interestingly, Batf binding was only slightly augmented by cell stimulation (Figure 32b and d) indicating potential constitutive binding of Batf to the DNA in differentiated T_H17 cells. Notably, Batf binding to the distal conserved regulatory enhancer elements by ChIP was stronger than binding to the proximal promoter elements (Figure 32b and d) and occurred earlier during the course of T cell differentiation (Figure 32c). In summary, Batf binds multiple regions in the *Il17a/f* locus in the natural chromatin state during T_H17 differentiation.

Batf binds multiple sites in the proximal *Il17, Il21 and Il22* promoters

To further define Batf binding to the DNA we performed EMSA analysis by first testing an AP-1 consensus probe (Echlin et al., 2000) (TGAGTCA) for binding Batf in T_H17 cells. This probe formed two complexes in *Batf*$^{+/+}$ T_H17 cell extracts (Figure 33a, lane 1) that were strongly dependent on stimulation (Figure 33c, lanes 1 and 2). Only the upper of these two complexes formed in extracts from *Batf*$^{-/-}$ T cells cultured under T_H17 conditions (Figure 33a, lane 2 and Figure 33c, lanes 3 and 4), suggesting the lower complex contains Batf. Consistently, an anti-Batf antibody blocked the formation of the lower complex (Figure 33a, lane 5). Using T_H17 cell extracts derived from *Batf*-transgenic mice expressing the FLAG-tagged version of Batf, we found that only the lower complex was specifically supershifted by an antibody to the FLAG epitope, but not by a control antibody (Figure 33a, lanes 7-12). Formation of the upper complex was not affected by the anti-FLAG antibody. Thus, Batf is present specifically within the lower complex of the two complexes that bind to the consensus AP-1 probe in T_H17 cells.

Since *Batf* was required for IL-17, IL-21 and IL-22 expression (Figures 24a, 25 and 27a and b), we hypothesized that Batf bound to the promoters of these genes. To test this we preformed a "competitor supershift assay". We used extracts derived from *Batf*-transgenic T_H17 cells in EMSA analysis on the AP-1 probe and identified the Batf containing complex using the anti-FLAG epitope antibody (Figure 34a, lane 4). We then used oligo sequences spanning the proximal promoters of *Il17, Il21 and Il22* as competitors for the formation of this Batf containing complex (Figure 34b-d). This approach identified multiple potential Batf binding sites in all three cytokine promoters,

including the region in the *Il17* promoter binding Batf in ChIP assays (-188 to -210) (Figure 32b and d), confirming the validity of our approach to identify *bona fide* Batf binding sites.

Notably, one of the regions in the *Il17* promoter (-155 to -187) binding Batf by EMSA overlaps with a ROR-responsive element (RORE) suggested to bind RORγt (Ichiyama et al., 2008). This region also contains a sequence (TGACCTCA) closely resembling an AP-1 consensus element (Echlin et al., 2000; Eferl and Wagner, 2003). When used as a competitor, this region (-155 to -187), but not the RORE element in the CNS-2 region of *Il17* (Yang et al., 2008), inhibited the formation of both upper and lower EMSA complexes formed by the AP-1 probe (Figure 33a, lanes 3, 4). Thus, the *Il17* promoter region between -155 and -187 interacts with complexes binding the AP-1 probe independently of its ability to bind RORs.

More importantly, when this region (-155 to -187) was used as an EMSA probe, this element itself formed two complexes in extracts from *Batf*$^{+/+}$ T_H17 cells, which were both augmented by stimulation (Figure 33b, lanes 1-4). Again, the lower complex was selectively inhibited by an anti-Batf antibody and only the upper of the two complexes formed in extracts from *Batf*$^{-/-}$ T cells cultured under T_H17 conditions (Figure 33b, lane 2 and 4), and an anti-Batf antibody blocked the formation of the lower complex (Figure 33b, lane 9). In T_H17 extracts from *Batf*-transgenic T cells only the lower complex was specifically supershifted by an antibody to the FLAG epitope (Figure 33b, lanes 11-14).

To confirm binding of Batf to the *Il21* and *Il22* promoter regions in the context of natural chromatin, we performed ChIP analysis. Indeed, Batf bound to the *Il21* and *Il22*

promoters by ChIP analysis (Figure 35a). Thus Batf binds to multiple regions in the proximal promoters of the *Il17, Il21 and Il22* cytokine genes.

The preferred Batf binding motif differs from a symmetric AP-1 consensus element

We next wondered whether Batf containing transcriptional complexes exhibit identical DNA binding specificity as conventional AP-1 complexes, i.e. whether they bound consensus dyad symmetric AP-1 response elements (TGAN(N)TCA). Thus, we analyzed all Batf-binding sequences in the *Il17, Il21 and Il22* promoters for the presence of a common Batf binding motif using the CONSENSUS program (Hertz and Stormo, 1999). The derived sequence logo (Crooks et al., 2004) (Figure 35b) strongly resembles a canonical AP-1 motif at positions 1 through 3, but exhibits sequence variation in the remaining nucleotides, thus differing from the dyad symmetric AP-1 response element (TGAN(N)TCA).

To potentially identify transcription factors that might cooperate with Batf, we used the CONSENSUS program to obtain information about alternative motifs enriched near the Batf binding sites by masking the regions matching the putative Batf binding motif. This approach identified no secondary motifs to be significantly enriched nearby Batf binding sites. As an alternative approach, we also used the MEME program (meme.sdsc.edu) in a similar manner with default parameters. MEME identified only one motif with significance (e-value of 8.3 e-7), which was essentially the same motif as found by CONSENSUS for Batf binding, containing the same core TGAGTG. Meme found two other motifs, CCTGTGC, which had an e-value of 1.1 e+4, and ACCAAACGCT, which had an e-value of 1.8 e+4. However, e-values for neither of

these motifs were considered significant. Thus, CONSENSUS found no significant enrichment of other sequence motifs or binding sites for other transcription factors in the vicinity of Batf binding elements.

In summary, Batf-binding elements are distributed within the promoters of *Il17, Il21 and Il22* and contain a motif that varies from the conventional AP-1 response element. Even though we found no alternative motifs enriched near Batf binding sites, factors regulating *Il17, Il21 and Il22* may act at sites located outside the regions examined, may have more degenerate target sequences, or may not be common to all cytokine promoters, unlike Batf, which appears common to all of these promoters.

Batf preferentially dimerizes with JunB in the context of T_H17 differentiation

Finally, to test whether in T_H17 cells Batf acts as a homo- or heterodimer with Jun as previously suggested (Echlin et al., 2000; Dorsey et al., 1995), we performed antibody-supershift analysis of EMSA complexes. We used antibodies to Fos (anti-pan-Fos antibody), Jun (anti-pan-Jun antibody, as well as antibodies to c-Jun, JunB and JunD), and antibodies to Batf, ATF1 and ATF3.

The upper complex was specifically supershifted by pan-anti-Fos antibody. The lower complex was specifically supershifted by both a pan-anti-Jun and anti-Batf antibodies. This suggests that the lower complex contains Batf and Jun proteins. Using antibodies to individual Jun family proteins, we found that anti-c-Jun and anti-JunD only slightly affected the lower complex, whereas anti-JunB almost completely inhibited the lower complex (Figure 36). Also, antibodies to ATF1 or ATF3 two ATF, family members related to Batf and expressed in T_H17 cells, had no effect on either complex.

cells that migrate faster on EMSA gels than the Fos-Jun AP-1 complex, producing the distinct lower complex. This is consistent with previous observations that Batf may interact with Jun family proteins (Dorsey et al., 1995; Echlin et al., 2000; Senga et al., 2002).

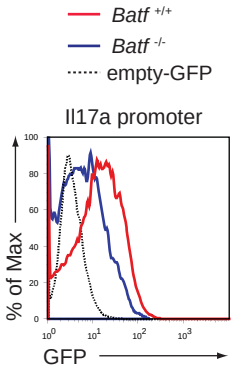


Figure 31. The proximal *Il17* promoter is Batf-responsive.
a, *Batf* $^{+/+}$ and *Batf* $^{-/-}$ CD4^{+} T cells cultured under T_H17 conditions were infected with hCD4-pA-GFP-RV-IL-17p reverse strand reporter virus (Zhu et al., 2001), in which hCD4 marks infected cells and the proximal *Il17a* promoter drives GFP expression. On day 3, cells were restimulated with PMA/ionomycin. Plots are gated on hCD4^{+} cells and analyzed for GFP expression.

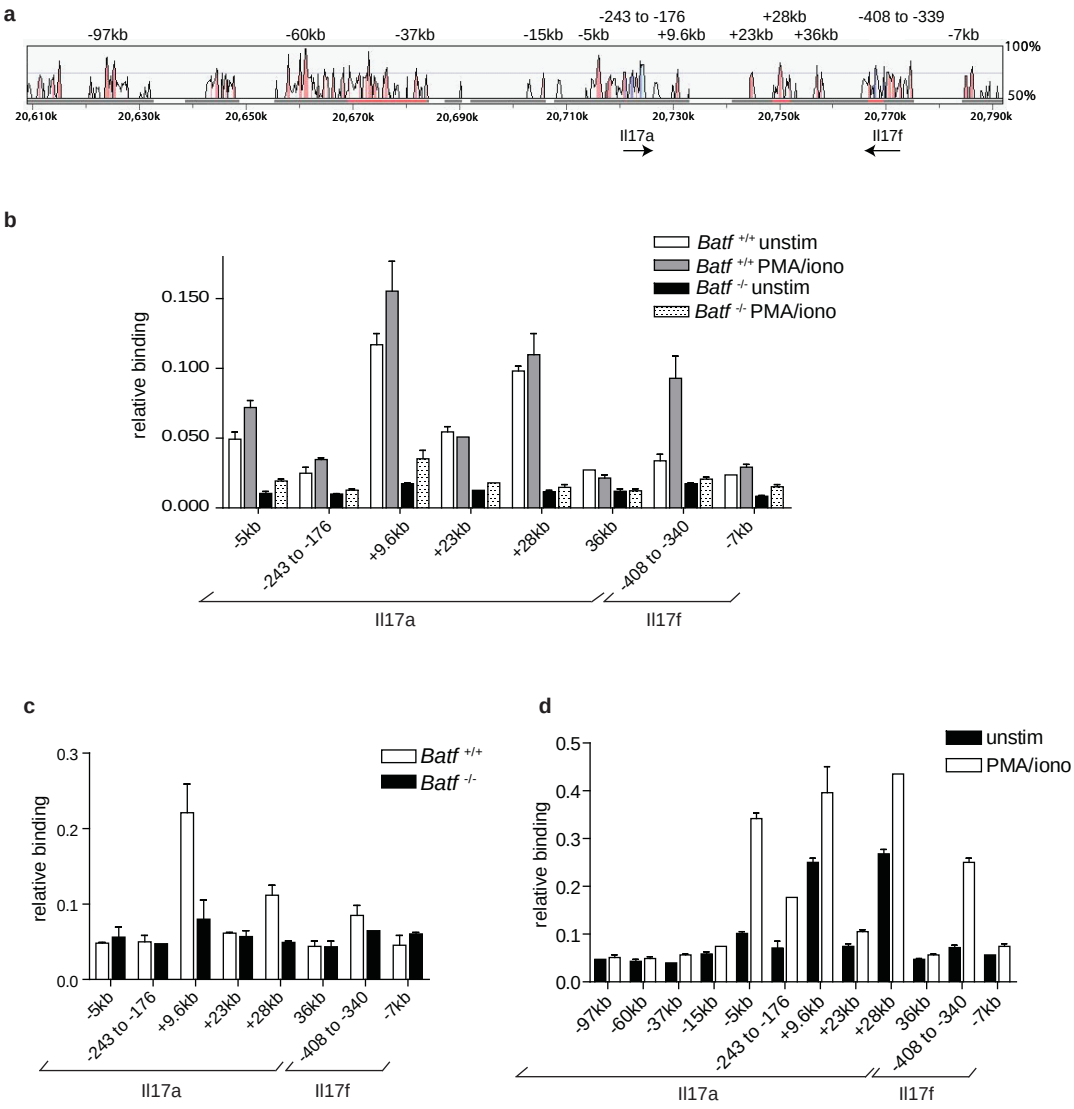


Figure 32. Batf binds multiple regions in the *Il17a/f* locus.
a, Vista blot depicting the sequence conservation of the human and mouse *Il17a/f* gene loci. The locations of primers used for ChIP analysis are indicated and are denoted relative to the ATG for the *Il17a* or *Il17f* genes. **b,** *Batf*$^{+/+}$ and *Batf*$^{-/-}$ CD4^{+} T cells cultured with anti-CD3 and APCs under T_H17 conditions for 5 days were subjected to ChIP analysis of the indicated regions using anti-Batf antibody. **c,** *Batf*$^{+/+}$ or *Batf*$^{-/-}$CD4^{+} T cells were activated with anti-CD3/CD28 under T_H17 conditions for 24h, then subjected to ChIP analysis using anti-Batf polyclonal antibody as in **b**. **d,** *Batf*$^{+/+}$ CD4^{+} T cells from C57Bl/6 mice were treated as in **a**. All ChIP data are presented as relative binding based on normalization to unprecipitated input DNA (mean + s.d. of 2 independent experiments).

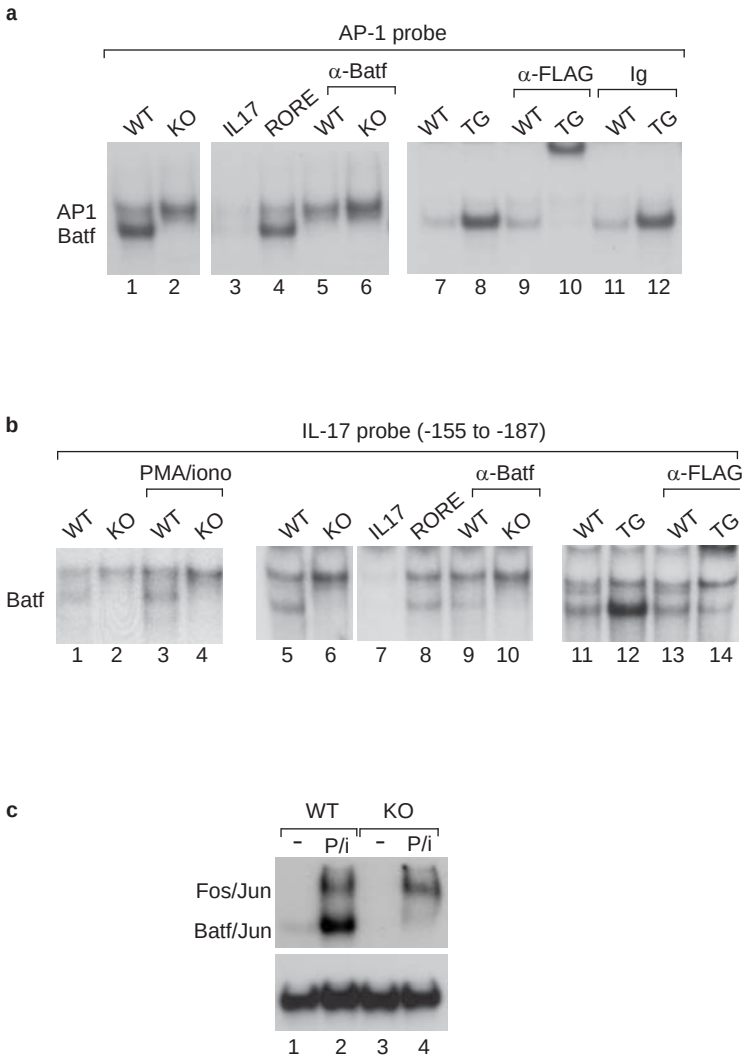


Figure 33. Batf binds to the *Il17a* promoter by EMSA.
a,b, EMSA supershift analysis of whole cell extracts from total splenocytes activated with anti-CD3 under T_H17 conditions for 3 days. Binding to a consensus AP-1 probe (AGCTTCGCTTGATGAGTCAGCCG) (Echlin et al., 2000) **(a, c)** or the IL-17 (-155 to -187) probe **(b)** was analyzed. (*Batf*$^{+/+}$ (WT), *Batf*$^{-/-}$ (KO), *CD2-N-FLAG-Batf* transgenic (TG), IL-17(-155 to -187) and RORE probes were used as competitors). **c,** DO11.10^{+}CD4^{+} T cells from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ littermates were activated with OVA and APCs under T_H17 conditions. On day 7, cells were either left untreated or stimulated with PMA/ionomycin for 4h. Whole cell extracts were analyzed for EMSA binding to the consensus AP-1 probe.

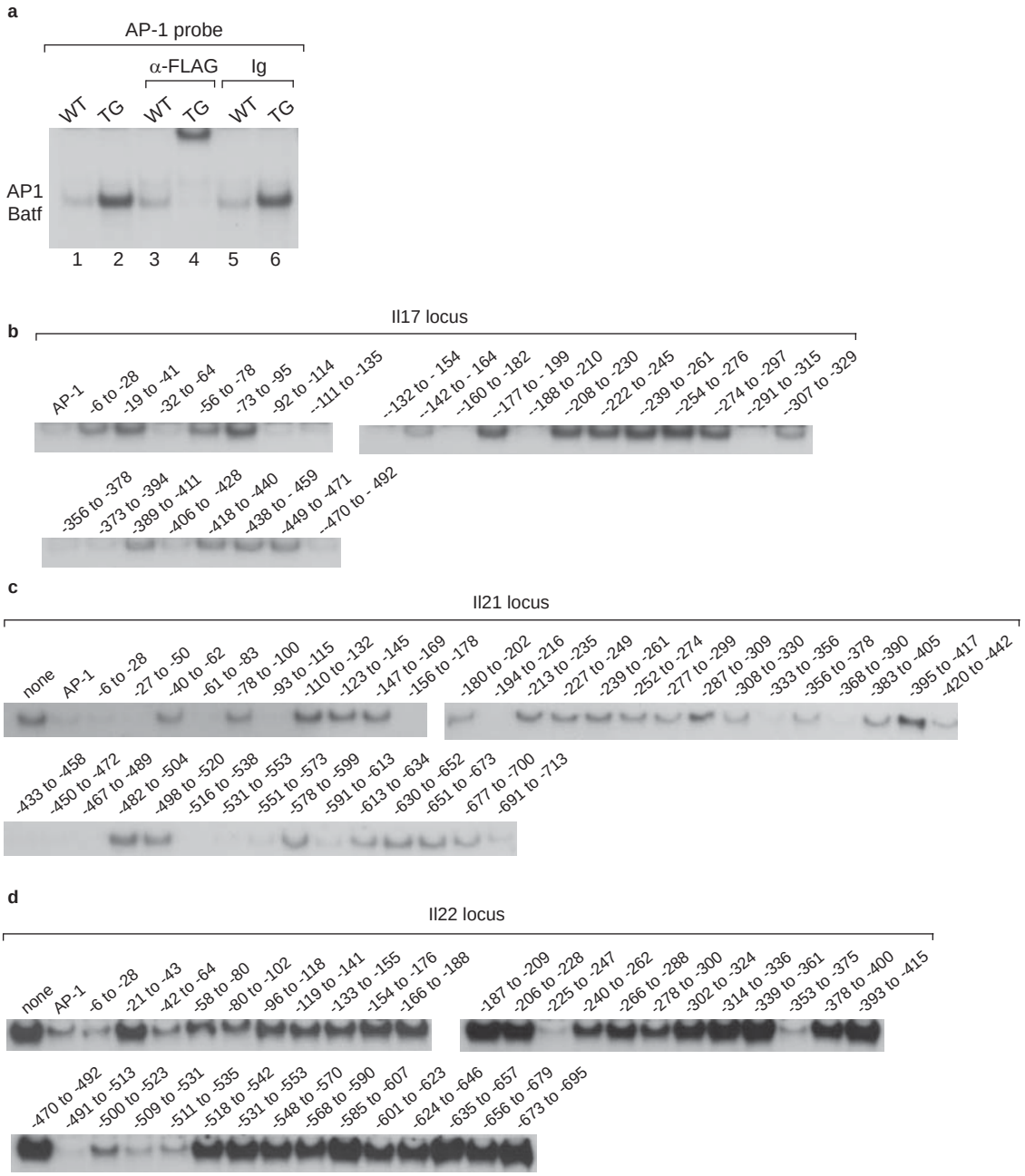


Figure 34. Identification of potential Batf binding sites in the *Il17a*, *Il21* and *Il22* promoters. **a,** Whole cell extracts from total splenocytes activated with anti-CD3 under T_H17 conditions for 3 days were analyzed for binding to the AP-1 probe by EMSA supershift (*CD2-N-FLAG-Batf* transgenic (TG)). **b-d,** EMSA analysis performed as in **a**, Batf containing complexes were identified by supershift with anti-FLAG antibody. Sequences from the *Il17a* **(b)**, *Il21* **(c)** and *Il22* **(d)** promoters were used as inhibitors of Batf containing complexes as described in Methods. Sequences of competitors used are supplied in Chapter 2.

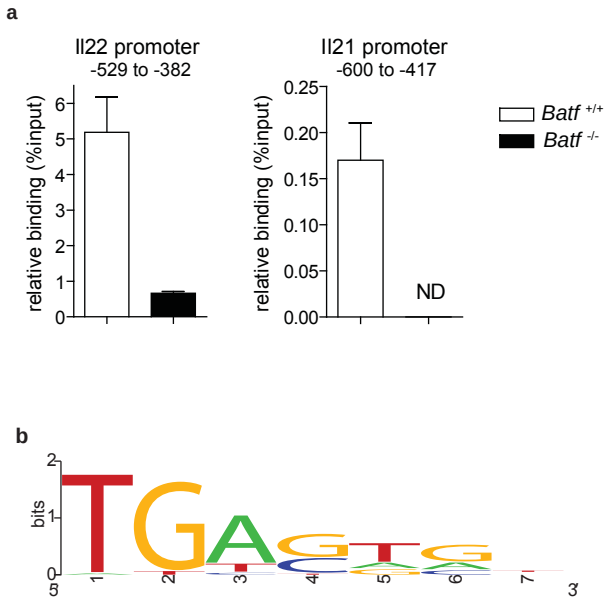


Figure 35. Confirmation of Batf binding to the *Il21* and *Il22* promoters and identification of a Batf binding motif.
a, *Batf*$^{+/+}$ and *Batf*$^{-/-}$ CD4^{+} T cells were stimulated under T_H17 conditions for 5 days. ChIP analysis was performed as described in methods. The analyzed sites are denoted relative to the ATG for the *Il21* or *Il22* genes. Data are presented as relative binding based on normalization to unprecipitated input DNA. **b,** WebLogo (Crooks et al., 2004) presentation of the 7-base Batf-binding motif identified by the CONSENSUS (Hertz and Stormo, 1999) program present in 38/40 Batf-binding regions of the *Il17a, Il21* and *Il22* promoters. The size of each indicated nucleotide is proportional to the frequency of its appearance at each position.

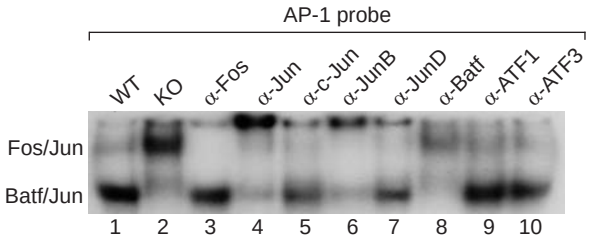


Figure 36. Batf preferentially binds JunB in T_H17 cells.
Total splenocytes from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice were activated with anti-CD3 under T_H17 conditions for 3 days. Whole cell extracts were analyzed by EMSA supershift for binding to the AP-1 consensus probe using antibodies to Batf, pan-Fos, pan-Jun, JunB, JunD, c-Jun, ATF-1 and ATF-2.

Chapter 8

Discussion

The studies presented here identify a critical requirement for *Batf* in T_H17 differentiation and T_H17-mediated autoimmune disease. We found that the AP-1 protein Batf, which lacks a TAD, was highly expressed in T helper cells compared to various other immune cells and tissues. To study the role of *Batf* in T cells, we generated *Batf* deficient mice by gene targeting. *Batf*$^{-/-}$ mice show a highly selective defect in the differentiation of IL-17-producing T helper (T_H17) cells. T_H17 cells are a $CD4^+$ T cell subset that coordinates inflammatory responses in host defense but are pathogenic in autoimmunity (Langrish et al., 2005; Park et al., 2005; Ivanov et al., 2006; Bettelli et al., 2006; Brustle et al., 2007). We found that *Batf*$^{-/-}$ mice are completely resistant to experimental autoimmune encephalomyelitis due to the inability of *Batf* deficient T cells to differentiate into T_H17 cells. Using gene expression analysis, we found that *Batf*$^{-/-}$ T cells fail to induce known T_H17-specific transcription factors, such as RORγt, and the cytokine IL-21, required for T_H17 differentiation. Neither addition of IL-21 nor overexpression of RORγt fully restores IL-17 production in *Batf*$^{-/-}$ T cells, suggesting that Batf may be required directly for IL-17 transcription. We found that the *Il17* promoter is Batf-responsive, and upon T_H17 differentiation, Batf binds to several conserved intergenic elements in the *Il17a/f* locus as well as to regions in the *Il17, Il21 and Il22* promoters. Using bio-computational methods we determined that the Batf-binding element in the *Il17, Il21 and Il22* promoters differs from canonical symmetric AP-1 elements. Using EMSA analysis we found that Batf forms heterodimers

preferentially with JunB during T_H17 differentiation. These results demonstrate that the AP-1 factor *Batf* regulates previously unknown AP-1 target genes to control T_H17 differentiation and T_H17-mediated autoimmune disease.

Batf specifically controls T_H17 differentiation

We found that *Batf* $^{-/-}$ T cells specifically failed to differentiate into T_H17 cells, while T_H1 and T_H2 differentiation was unaffected. Our results demonstrate that *Batf* $^{-/-}$ T cells do not exhibit increased production of cytokines that can suppress T_H17 differentiation, such as IL-2, IFN-γ, IL-4 or IL-10. Thus *Batf* deficiency does not lead to a general imbalance in cytokine production but rather *Batf* seems to directly affect T_H17 differentiation. Consistently, *Batf* deficiency abrogates IL-17 production in $CD4^+$ and $CD8^+$ T cells and the addition of exogenous T_H17-promoting cytokines fails to rescue IL-17 production in *Batf* $^{-/-}$ T cells. Therefore, *Batf* $^{-/-}$ T cells exhibit a remarkably selective defect in T_H17 differentiation.

This selective defect in one particular pathway of T_H differentiation was surprising since *Batf* was also expressed in T_H1 and T_H2 cells. Notably, we found that Batf was present in T cells in two molecular weight species and that the lower molecular weight species was predominantly induced by activation. The presence of two molecular weight species is consistent with previous observations that Batf can be phosphorylated at serine 43 in the DNA binding domain (Deppmann et al., 2003). Phosphorylation of Batf at serine 43 has been suggested to prevent DNA binding without affecting dimerization with Jun proteins (Deppmann et al., 2003), adding complexity to the regulation of AP-1 signaling by Batf. Whether Batf is indeed subject to phosphorylation in T cells and

whether phosphorylation of Batf affects its function in T cells will have to be determined in future experiments. Nevertheless, the occurrence of a lower molecular weight species following activation of T cells suggests that dephosphorylation of Batf in response to T cell activation might regulate Batf function during T cell differentiation.

While *Batf* is not the first transcription factor identified to regulate T_H17 differentiation, *Batf* is unique in that its effects are more specific to T_H17 differentiation. Only four transcription factors have been shown to be required for T_H17 development; IRF4, STAT3, RORγt, and RUNX1 (Laurence et al., 2007; Yang et al., 2007; Ivanov et al., 2006; Brustle et al., 2007; Zhang et al., 2008b). While RORα may influence T_H17 differentiation, RORα deficient mice show essentially normal T_H17 development (Yang et al., 2008), indicating it is not required.

Irf4$^{-/-}$ T cells have a complete block in T_H17 differentiation; however, *Irf4*$^{-/-}$ T cells also show severe defects in T_H2 development (Lohoff et al., 2002; Rengarajan et al., 2002; Hu et al., 2002), plasmacytoid dendritic cell (pDC) and $CD4^+$ dendritic cell development (Suzuki et al., 2004) as well as defects in plasma cell differentiation and B cell class switching (Sciammas et al., 2006). Unlike IRF4, we found no defects in other T helper lineages besides T_H17. *Batf* $^{-/-}$ mice have normal pDC and $CD4^+$ cDC development. Even though *Batf* $^{-/-}$ B cells show a defect in class switching that needs to be further defined (see Appendix 1), plasma cell differentiation occurs. Thus, B cell function is not globally affected in the absence of Batf, as is the case in *Irf4*$^{-/-}$ mice.

STAT3 is activated immediately downstream of the IL-6 receptor and required at multiple stages of hematopoietic cell development. As a result STAT3$^{-/-}$ mice are not viable, and studying its role in T cells required the use of a specific Cre deletor strain.

Deletion of STAT3 during T cell development using an Lck-Cre deletor strain caused the loss of IL-6-induced proliferation of thymocytes (Takeda et al., 1998). Unlike STAT3, *Batf* does not globally affect IL-6 responses, evident by normal liver acute phase response in *Batf* $^{-/-}$ mice. Also, we found that *Batf* is not required for normal development of thymocytes, but functions only in the T_H17 effector response, by regulating a distinct subset of IL-6-dependent genes.

RORγt deficient mice lack lymph nodes and show some residual IL-17 production *in vivo*, with residual disease development in the EAE model (Sun et al., 2000; Ivanov et al., 2006). Unlike RORγt deficient mice, *Batf* $^{-/-}$ mice have normal lymphoid development and architecture and are completely resistant to EAE. Also, *Batf* regulates sustained expression of RORγt and is to our knowledge the first factor shown to directly regulate the expression of *Il17, Il21* and *Il22*.

RUNX1 is required at many stages of development and acts in multiple tissues and lineage decisions, being described recently to be required for the differentiation of haemogenic endothelium into blood cells (Lancrin et al., 2009). RUNX1 was analyzed in T_H17 differentiation only by siRNA knockdown and by overexpression of a dominant negative in mature T cells (Zhang et al., 2008b). Unlike RUNX1, *Batf* $^{-/-}$ mice are viable, fertile, and show a selective defect in T_H17 differentiation.

In summary, *Batf* $^{-/-}$ T cells exhibit a surprisingly selective defect in T_H17 differentiation and the effects of *Batf* are more specific to T_H17 differentiation than those of previously identified factors. Thus, *Batf* $^{-/-}$ mice provide an exceptional model system to selectively study the role of T_H17 cells in T cell mediated immune responses and autoimmune diseases.

Batf $^{-/-}$ mice are completely resistant to EAE

Batf $^{-/-}$ mice were completely resistant to the development of MOG_{35-55} -induced EAE due to defective T_H17 development. The MOG_{35-55} immunization model of EAE is primarily T-cell mediated (Wolf et al., 1996). T_H17 cells were first recognized as the major pathogenic T cell subset when mice deficient for the p19 subunit of IL-23, but not the p35 subunit of IL-12 were found to be protected from disease development (Langrish et al., 2005; Park et al., 2005; Yang et al., 2008). IL-23 is required for the maintenance of IL-17 producing T cells during EAE (Langrish et al., 2005). and mice deficient for transcription factors, such as RORγt and IRF4, that control T_H17 development are protected from EAE (Ivanov et al., 2006; Brustle et al., 2007). However, RORγt deficient mice are only partially resistant to EAE and IL-17 production can be observed in RORγt$^{-/-}$ T cells *in vivo* after EAE induction (Ivanov et al., 2006). In contrast, *Irf4*$^{-/-}$ T cells exhibit an absolute block in T_H17 development resulting in complete resistantance to EAE (Brustle et al., 2007). Intriguingly, the phenotype of *Irf4*$^{-/-}$ mice in EAE is markedly similar to the phenotype we observed in *Batf* $^{-/-}$ mice.

T_H17 and T_{reg} cells develop via reciprocal pathways (Bettelli et al., 2006) and IL-6-deficient mice are resistant to EAE due to a compensatory increase in Foxp3$^+$ T regulatory cells in the absence of T_H17 differentiation (Korn et al., 2007). Strikingly, we found no compensatory increase in the development of Foxp3$^+$ T_{reg} cells in *Batf* $^{-/-}$ mice during EAE, suggesting that *Batf* $^{-/-}$ mice are resistant to EAE due a selective defect in T_H17 differentiation. Consistent with the absence of increased T_{reg} cell development in the absence of *Batf*, the transfer of *Batf* $^{-/-}$ T cells into *Batf* $^{+/+}$ mice did not protect against

EAE development. In contrast, using this same T cell transfer model, we found that the transfer of wild type T cells restored EAE development and T_H17 development in *Batf* $^{-/-}$ mice, indicating that *Batf* $^{-/-}$ mice provide an antigen presenting environment permissive for T_H17 development. Thus *Batf* $^{-/-}$ mice are protected from EAE due to a T cell intrinsic defect that prevents T_H17 development, rather than a defect in T cell priming.

Since the importance of T_H17 cells in EAE pathogenesis is recognized, several studies attempted to address which T_H17 effector cytokines are required for EAE development. IL-23 is critical for EAE development due to its role in maintaining T_H17 cells *in vivo* (Langrish et al., 2005); indicating the requirement for T_H17 cells. However, IL-22 deficiency only minimally prevented EAE (Kreymborg et al., 2007) and antibody blockade of IL-17A in the setting of *Il17f*-deficiency also only minimally affects EAE development (Haak et al., 2009) implying the existence of other T_H17 effector pathways that are required for EAE development. Since *Batf* $^{-/-}$ mice specifically lack the T_H17 lineage, they provide a model allow addressing which T_H17 effector cytokines are required for EAE using adoptive transfer systems.

In summary, the protective effect of *Batf* deficiency on EAE development is striking and very similar to the phenotype observed in *Irf4* $^{-/-}$ mice or mice that are either globally deficient in IL-6 signaling (Korn et al., 2008) or deficient in IL-6 signaling specifically in T cells (Korn et al., 2007). Since T_H1 and T_H2 cell differentiation is normal in the absence of *Batf*, our studies provide strong support that T_H17 cells are important mediators of EAE pathogenesis. Additionally, in contrast to *Irf4* $^{-/-}$ mice, which exhibit abnormal T_H2 development, *Batf* $^{-/-}$ mice provide an excellent model system to selectively study the role of T_H17 cells in T cell-mediated autoimmune disorders.

Batf controls the expression of a subset of IL-6-induced genes

T_H17 cells differentiate in response to TGF-β and the proinflammatory cytokine IL-6, and require IL-21 (Bettelli et al., 2006; Korn et al., 2007; Nurieva et al., 2007; Wei et al., 2007; Zhou et al., 2007; Manel et al., 2008). Our data demonstrate that proximal IL-6 receptor signaling, proximal IL-21 signaling and TGF-β signaling were normal in *Batf* $^{-/-}$ T cells. The TGF-β induced transcription factor Foxp3 inhibits RORγt function and thus T_H17 differentiation (Zhou et al., 2008). IL-6 partly promotes T_H17 differentiation through the inhibition of Foxp3 (Bettelli et al., 2006). Since we found that *Batf* $^{-/-}$ T cells failed to fully downregulate Foxp3 in response to IL-6, *Batf* might function to inhibit Foxp3 expression in T_H17 cells. This was again similar to what has been observed in *Irf4* $^{-/-}$ T cells, which also fail to downregulate Foxp3 in response to IL-6 (Brustle et al., 2007). However, neutralization of IL-2 during T_H17 differentiation abrogated increased Foxp3 expression in *Batf* $^{-/-}$ T cells, without restoring IL-17 production. This indicates that *Batf* affects IL-6-mediated downregulation of Foxp3 but also regulates additional factors downstream of IL-6 directly required for T_H17 differentiation.

Consistently, *Batf* $^{-/-}$ T cells failed to induce the IL-6 target gene IL-21. Further, our gene expression profiling experiments demonstrated that *Batf* was required for the expression of numerous IL-6-dependent T_H17 associated genes. Specifically, we found that genes that were induced in wild type cells in response to either IL-6 alone or IL-6 plus TGF-β clustered into genes that exhibited *Batf*-dependent or *Batf*-independent expression. *Batf*-dependent genes included known T_H17 associated genes, such as RORγt (Zhou et al., 2007), RORα (Yang et al., 2008), the aryl hydrocarbon receptor (AHR)

(Veldhoen et al., 2008; Kimura et al., 2008; Quintana et al., 2008), IL-22 (Liang et al., 2006) and IL-17. Since we found no effect of *Batf* on the expression of genes induced in response to TGF-β alone, we conclude that *Batf* specifically regulates the expression of a subset of IL-6 induced genes in T cells.

A novel role for AP-1 proteins in the regulation of T_H17 differentiation

The transcriptional regulation of T_H17 associated genes is still poorly defined. RORγt has been suggested to bind to the proximal *Il17* promoter and conserved non-coding sequences upstream of the *Il17* locus (Ichiyama et al., 2008; Zhang et al., 2008b; Akimzhanov et al., 2007). Additionally, RORγt can induce *Il17* promoter activity in reporter assays (Ichiyama et al., 2008; Zhang et al., 2008b) however, since RORγt$^{-/-}$ T cells show residual IL-17 production (Ivanov et al., 2006) there are likely other factors that regulate *Il17* gene transcription. Consistently, RUNX1 has been suggested to cooperate with RORγt at the *Il17* promoter (Zhang et al., 2008b), NFAT has been implicated in regulating *Il17* transcription (Liu et al., 2004; Hermann-Kleiter et al., 2008) and STAT3 has been shown to bind to the *Il17* and *Il21* promoters (Wei et al., 2007; Chen et al., 2006).

We have shown that *Batf* is required for T_H17 differentiation and that RORγt overexpression does not fully restore IL-17 production in *Batf*$^{-/-}$ T cells and that the *Il17* promoter is Batf-responsive. Additionally, we found that Batf binds multiple conserved non-coding and promoter regions in the *Il17a/f* locus and the *Il21* and *Il22* proximal promoters in the natural chromatin state during T_H17 differentiation. Additionally, using EMSA analysis, we found that Batf preferentially dimerizes with JunB in T_H17 cells. Our

data indicate direct transcriptional regulation of *Il17, Il21 and Il22* by *Batf* and reveal a yet unrecognized role for AP-1 transcription factors in the regulation of T_H17 associated cytokines.

A potential role for *Batf* as transcriptional activator

Batf and other AP-1 proteins lacking a TAD are thought to act as AP-1 inhibitors by forming transcriptionally inactive heterodimers (Blank, 2008; Williams et al., 2001; Echlin et al., 2000; Iacobelli et al., 2000; Dorsey et al., 1995; Thornton et al., 2006). Our ChIP and EMSA experiments have shown that Batf binds to multiple regions in the *Il17a/f* locus as well as the *Il21* and *Il22* promoters indicating direct transcriptional regulation of *Il17, Il21 and Il22* by *Batf*. Additionally, the *Il17* promoter is Batf responsive and *Batf* is required for the induction of *Il17, Il21 and Il22* indicating that Batf might directly promote transcription of these genes rather than inhibiting their transcription. Consistently, *Batf* $^{-/-}$ T cells lose the expression of many IL-6 induced genes in T cells, whereas *Batf* deficiency leads to increased expression of only few genes. Since Batf preferentially heterodimerizes with JunB during T_H17 differentiation, we hypothesize that Batf in complex with JunB forms an active transcription factor complex in the context of T_H17 differentiation.

Although *Batf* is also expressed in T_H1 and T_H2 cells, it appears that *Batf* is specifically necessary for T_H17 differentiation, but does not induce this process by itself. Rather, our data suggest that *Batf* cooperates with other T_H17-specific factors to regulate target genes. In fact, RORγt only partially restores IL-17 production in the absence of *Batf* and our data shows functional synergy between Batf and RORγt. Notably, *Batf* $^{-/-}$ T

cells exhibit a phenotype markedly similar to *Irf4*$^{-/-}$ T cells, albeit more specific to T_H17 differentiation. Like *Batf* $^{-/-}$ T cells, *Irf4*$^{-/-}$ T cells fail to differentiate into T_H17 cells, fail to downregulate Foxp3 in response to IL-6, and IL-17 production cannot fully be restored by RORγt in the absence of either Batf or IRF4. Thus, Batf and IRF4 might cooperate to induce T_H17 differentiation.

The hypothesis that Batf acts as a transcriptional repressor is based on the underlying assumption that the DNA binding specificity of the 'inhibitory' complex is identical to the AP-1 complex it replaces. Using bioinformatics approaches, we were able to identify a potential Batf-binding motif common to the Batf binding sequences in the *Il17, Il21 and Il22* promoters. This motif strongly resembles a canonical AP-1 motif at positions 1 through 3, but exhibits sequence variation in the remaining nucleotides, thus differing from the dyad symmetric AP-1 response element (TGAN(N)TCA). AP-1 proteins are known to bind to sequences that deviate substantially from consensus AP-1 elements, particularly in promoters of cytokine genes (Rao et al., 1997). This binding to non-consensus sequences often depends on cooperative actions with other transcription factors, such as NFAT or IRF (Rao et al., 1997; Panne et al., 2004). Indeed, a study by Steve Harrison (Panne et al., 2004) defined the structural basis for binding of the Jun-ATF2 heterodimer to a non-symmetric non-consensus TGAC<u>A</u>T<u>AG</u>, which differs from the canonical CRE recognition sequence (TGACGTCA) at the three underlined positions. The crystal structure of the Jun-ATF2 heterodimer and two IRF3 molecules bound to the interferon-β enhanceasome revealed that binding of IRF3 to the DNA induces conformational distortion, bending a non-consensus AP-1 sequence into a structure compatible for Jun-ATF2 binding. Thus, both binding and transcription mediated by AP-

1 factors can depend on flanking sequences and interactions with other factors. Therefore, Batf and IRF4 might cooperate on the DNA in a manner similar to the cooperation of Jun-ATF2 and IRF3 at the interferon-β enhanceasome, enabling the binding of the Batf-JunB heterodimers to non-consensus binding elements.

Although we found no significant enrichment of alternative motifs near Batf binding sites, identification of alternative motifs enriched near Batf binding sites is complicated by the fact that *Il17, Il21 and Il22* are differentially regulated by RORγt and AHR (Veldhoen et al., 2008), which could both conceivably interact with Batf. Such differential regulation would make it more difficult to identify adjacent cooperative cis-elements near the Batf binding sites in our analysis. Additionally, factors regulating *Il17, Il21 and Il22* may act at sites located outside the regions examined, may have more degenerate target sequences, or may not be common to all cytokine promoters, unlike Batf, which appears common to all of these promoters.

The concept that Batf might function as a transcriptional activator is surprising, since Batf lacks an obvious TAD and has been thought to act as AP-1 inhibitor by forming transcriptionally inactive heterodimers (Blank, 2008; Williams et al., 2001; Echlin et al., 2000; Dorsey et al., 1995; Thornton et al., 2006). However, despite the lack of an obvious TAD, Batf might decrease steric hinderance, permitting novel protein-protein interactions of AP-1 with T_H17 specific factors. Alternatively, Batf might change the binding affinity of the AP-1 heterodimeric complex, promoting binding of AP-1 to novel target sequences. Future studies will have to test the ability of Batf to directly induce transcription in reporter assays.

In summary, *Batf* is selectively required for T_H17 development. Since *Batf* is also expressed in T_H1 and T_H2 cells, it likely cooperates with other T_H17-specific factors to regulate target genes. Future work will need to determine whether the actions of *Batf* involve distinct sequence specificity or unique protein-protein interactions with T_H17 specific factors.

Concluding remarks

In the studies presented here, we have shown that *Batf* is required for the differentiation of IL-17-producing T helper (T_H17) cells (Harrington et al., 2005). T_H17 cells comprise a $CD4^+$ T cell subset that coordinates inflammatory responses in host defense but is pathogenic in autoimmunity (Langrish et al., 2005; Ivanov et al., 2006; Bettelli et al., 2006; Brustle et al., 2007). *Batf*$^{-/-}$ mice have normal T_H1 and T_H2 differentiation, but defective T_H17 differentiation, and are resistant to experimental autoimmune encephalomyelitis. *Batf*$^{-/-}$ T cells fail to induce known factors required for T_H17 differentiation, such as RORγt (Ivanov et al., 2006) and the cytokine IL-21 (Korn et al., 2007; Nurieva et al., 2007; Wei et al., 2007; Zhou et al., 2007). Neither addition of IL-21 nor overexpression of RORγt fully restores IL-17 production in *Batf*$^{-/-}$ T cells. The *Il17* promoter is Batf-responsive, and upon T_H17 differentiation, Batf binds conserved intergenic elements in the *Il17a/f* locus and to the *Il17, Il21 and Il22* (Liang et al., 2006) promoters. These results demonstrate that the AP-1 protein Batf plays a critical role in T_H17 differentiation and potentially forms an active transcription factor complex, although future studies will need to demonstrate direct transcriptional activation of *Il17, Il21 and Il22* by Batf.

APPENDIX 1

Batf is required for class switch recombination

Introduction

In addition to cell-mediated immune responses that involve antigen specific $CD4^+$ and $CD8^+$ T lymphocytes, the adaptive immune system entails humoral immune responses, mediated by B lymphocytes. B lymphocytes produce and secrete antigen-specific antibodies that target pathogens for elimination by phagocytic cells or neutralize pathogens to prevent cell entry (Murphy et al., 2008). B cell responses include a variety of processes involved in the generation of antibody producing plasma cells that secrete antigen specific antibodies with high affinity and distinct effector function as well as the generation of memory B cells, armed for rapid release of antibodies upon reencountering antigen. Additionally, the nature of the humoral immune response is determined by the specific B cell subsets involved and the composition of the antigen.

Antigens can be grouped into two categories; those that elicit T-independent (TI) immune responses and those that elicit T dependent (TD) immune responses. TI-1 antigens, such as bacterial associated lipopolysaccharide (LPS), induce B cell activation via germline encoded receptors independent of antigen specificity, leading to B cell proliferation, differentiation and antibody secretion. TI-2 antigens are polysaccharides present on extracellular bacteria that are characterized by repetitive, identical epitopes. These TI antigens induce antigen-specific activation of B cells through their ability to strongly crosslink the B cell receptor and generally involve activation of specific B cell

subsets, B1 B cells and marginal zone B cells. Nevertheless, TI-2 responses require T cell signaling to fully induce B cell responses and proliferation (Alugupalli, 2008).

In contrast to TI immune responses, B cell responses to most protein antigens involve antigen specific T helper cells, thus referred to as TD responses. TD responses require coordinate interactions of DC and antigen specific T and B cells in secondary lymphoid organs. Antigen specific T cells are activated in the T cell zone of secondary lymphoid organs by APCs, such as DCs, that have processed the antigen. These activated T cells travel to the T-B cell border where they interact with B cells that have also taken up and processed the antigen. This interaction involves antigen presentation to T cells via MHCII and costimulatory molecules like CD28 and CD40. This cognate interaction of B cells with activated T cells leads to B cell activation, follicular entry and germinal center formation. Germinal centers are transient structures that foster an environment in which B cells undergo class switch recombination (CSR) and somatic hypermutation allowing for the generation of high affinity antibodies (Klein and Dalla-Favera, 2008; Murphy et al., 2008).

The generation of B cells containing high affinity B cell receptors is stringently controlled during TD immune responses to prevent the generation of autoreactive B cells and entails repeated cognate interactions between B and T cells. Once T cells become activated to move to the B cell zone, they acquire the expression of CXCR5, a chemokine receptor that facilitates entry into the B cell follicle. These T cells are termed follicular helper T (T_{FH}) cells. Whether T_{FH} cells develop as a lineage distinct from T_H1, T_H2, T_H17 or T_{reg} cells remains controversial, however, recent data suggests dependence of CXCR5 expression on IL-21 signaling (Tsuji et al., 2009; Fazilleau et al., 2009; Reinhardt et al.,

2009; Bauquet et al., 2009). Nevertheless, germinal centers also form during TI responses in a T cell independent manner (de Vinuesa et al., 2000).

Class switch recombination is an irreversible somatic mutation mechanism that allows B cells to switch their antigen receptors from IgM and IgD to other classes that have distinct effector functions. CSR can occur in both T cell dependent and independent manner but is ultimately linked to a combination of instructive cytokine and costimulatory signals (Klein and Dalla-Favera, 2008; Longerich et al., 2006). Once B cells have rearranged their B cell receptor, they can differentiate into either plasma cells that are specialized to secrete antibodies, or memory B cells, which become armed to secrete antibodies upon reencountering antigen. Activated B cells that do not enter the germinal center reaction differentiate into IgM secreting plasma cells or memory cells without rearranging their receptor.

Results

Batf $^{-/-}$ mice have decreased basal immunoglobulin levels.

In our studies, we determined that B cell development was normal in *Batf* $^{-/-}$ mice (Chapter 3). Since *Batf* was also expressed at low levels in naïve B cells, we tested whether B cell function was affected in the absence of *Batf*. First, we measured basal serum immunoglobulin isotype levels in unimmunized *Batf* $^{-/-}$ mice of different ages. We found that *Batf* $^{-/-}$ mice had slightly increased IgM serum levels across multiple ages examined (Figure 37). Strikingly, we found that *Batf* $^{-/-}$ mice had severely reduced to non-detectable serum levels of IgA, IgG1, IgG2a and IgG2b isotypes compared to *Batf* $^{+/+}$ mice (Figure 37). Thus, *Batf* $^{-/-}$ B cells show defects in CSR rather than a general defect

in antibody secretion suggesting that *Batf* is required for appropriate antibody responses to pathogens.

Batf $^{-/-}$ B cells fail to mount antigen specific IgG3 responses to a T cell independent antigen

The reduced serum Ig levels in *Batf* $^{-/-}$ mice could result from abnormal generation of T helper cells or T follicular helper cells, or alternatively Batf may be required in a cell intrinsic manner for B cell CSR and germinal center formation. To address the question whether *Batf* was required in a T or B cell intrinsic manner, and whether *Batf* $^{-/-}$ mice are capable of mounting antigen specific antibody responses we first performed immunizations of *Batf* $^{-/-}$ mice with a TI-2 antigen. We immunized *Batf* $^{+/+}$ and *Batf* $^{-/-}$ with 2,4,6,-trinitrophenol (TNP)-conjugated Ficoll (TNP-Ficoll). *Batf* $^{-/-}$ mice showed antigen specific IgM antibodies on days 7 and 14 after immunization. However, *Batf* $^{-/-}$ mice failed to mount and IgG3 response to TNP, whereas *Batf* $^{+/+}$ mice mounted a robust IgG3 response (Figure 38). These data suggest, that *Batf* $^{-/-}$ mice can mount an antigen specific TI-2 response, however, *Batf* $^{-/-}$ mice have impaired class switch recombination

Defective class switch recombination in *Batf* $^{-/-}$ B cells

To assess whether class switch recombination was impaired in *Batf* $^{-/-}$ B cells due to a B cell intrinsic requirement for *Batf* in CSR, we performed *in vitro* CSR experiments. We evaluated class switching in *Batf* $^{-/-}$ B cells in response LPS in combination with various cytokine stimuli. *Batf* $^{-/-}$ B cells failed to induce CSR under all conditions examined (Figure 39 b-d). However, LPS induced secretion of IgM was unaffected in

Batf $^{-/-}$ B cells (Figure 39a), indicating that *Batf* $^{-/-}$ B cells can be activated to secrete antibody. Consistently, CD138 expression, a marker for plasma cells was increased in *Batf* $^{-/-}$ B cells, indicating activation of B cells to differentiate into Ig secreting cells. Collectively, these data indicate that *Batf* is required in a cell intrinsic manner for class switch recombination.

Discussion

Although we found that B cell development was normal in *Batf* $^{-/-}$ mice (Chapter 3), we wanted to determine, whether *Batf* deficiency affects B cell function. We found that *Batf* $^{-/-}$ mice had decreased basal serum Ig levels of class switched Ig isotypes compared to age matched littermate control mice. Notably, this decrease was accompanied by increased serum IgM titers suggesting intact B cell activation and plasma cell differentiation in spite of defective class switching. Consistently, *Batf* $^{-/-}$ mice mounted normal antigen specific IgM responses following immunization with the TI antigen TNP-Ficoll; however, *Batf* $^{-/-}$ mice had defective IgG3 responses. Additionally, *in vitro* analysis of *Batf* $^{-/-}$ B cells demonstrated defective class switching, but normal secretion of IgM in response to activation. Notably, *Batf* $^{-/-}$ B cells preferentially developed into plasma cells following LPS stimulation. These data suggest that *Batf* is required for CSR and potentially germinal center B cell formation.

These observations pose numerous questions and further studies need to define the role of *Batf* in B cell differentiation. Does *Batf* act as a regulator to induce the germinal center B cell transcriptional program or are its functions confined to regulating CSR? Notably, *Bach2* $^{-/-}$ B cells exhibit a phenotype similar to *Batf* $^{-/-}$ B cells. *Bach2* $^{-/-}$ B

cells show a selective defect in germinal center B cell formation and a defect in CSR with normal plasma cell development (Muto et al., 1998). Bach2 seems to be directly involved in both, germinal center B cell formation and CSR. Bach2 is a basic leucine zipper protein that interacts with small Maf proteins. Like *Batf*, small Maf proteins lack obvious TADs and have been considered to act as inhibitors of transcription (Blank, 2008). While these data suggest an important role of AP-1 proteins in B cell differentiation, further studies will need to determine whether the actions of Bach2 and Batf are primarily related to their ability to modulate AP-1 signaling strength, or due to a yet unrecognized specialized ability to induce transcription of target genes.

The defect in CSR in *Batf* $^{-/-}$ B cells is very similar to what has been observed in *Irf4*$^{-/-}$ B cells, which are also deficient in CSR. In T cells, we found that both *Batf* $^{-/-}$ and *Irf4*$^{-/-}$ T cells fail to differentiate into T_H17 cells, thus exhibit very similar phenotypes. However, *Batf* specifically affects the T_H17 lineage, while *Irf4*$^{-/-}$ T cells exhibit additional defects in T_H2 differentiation indicating that the defect observed in *Batf* $^{-/-}$ T cells is more specific than in *Irf4*$^{-/-}$ T cells. *Irf4*$^{-/-}$ B cells exhibit defective plasma cell differentiation in addition to defective class switch recombination (Sciammas et al., 2006; Klein et al., 2006), whereas *Batf* $^{-/-}$ B cells fail to undergo CSR but can develop into plasma cells. These observations indicate similar parallel functions of Batf and IRF4 in B cells but again the defect in *Batf* $^{-/-}$ B cells is more specific than the defects in *Irf4*$^{-/-}$ B cells. Further studies will have to determine, whether Batf and IRF4 might cooperate to induce transcription in activated B cells, in a manner similar to the cooperation of Jun-ATF2 and IRF3 at the interferon-β enhanceasome as discussed in Chapter 8.

Concluding remarks

In the studies presented here, we have shown that *Batf* $^{-/-}$ mice have decreased basal serum immunoglobulin levels and fail to mount antigen specific IgG3 responses after immunization with a TI-2 antigen. Notably, antigen specific IgM responses were normal in *Batf* $^{-/-}$ mice. Further, our studies indicated a B cell intrinsic requirement for *Batf* in CSR. Further studies will need to determine the mechanism by which *Batf* regulates CSR.

Methods

In vitro class switch recombination.

Naïve B cells were isolated from spleens of *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice using CD43 microbeads (Miltenyi Biotech Inc.) according to the manufacturer's recommendation. B cells were then cultured at a density of 1e6 cells/ml in 24 well plates for 7 days. To induce class switch recombination, B cells were stimulated with LPS from from *Escherichia coli* serotype 0127: B8 (Sigma Aldrich) alone or LPS in combination with IL-4 (10ng/ml), IFN-γ (100ng/ml), TGF-β (1ng/ml) (all Peprotech). 7 days later supernatants were harvested and analyzed for Ig isotypes using ELISA (SBA clonotyping System horseradish peroxidase; Southern Biotech) according to the manufacturer's recommendations. CFSE (Sigma-Aldrich) labeling was performed by incubating cells at 20 x 10^6cells/ml in PBS with 1μM CFSE for 8 min at 25 °C. The cells were incubated with an equal volume of fetal calf serum (FCS) for 1 min and were washed twice with media containing 10% FCS before use. Cells were analyzed for CD138 expression four days after activation by flow cytometry.

Determination of basal Serum Immunoglobulin Titers.

For determination of basal serum immunoglobuling titers, serum was collected from sex-matched mice of different ages (2, 5, 9 and 13 months). Basal serum Immunoglobulin levels were determined using ELISA (SBA clonotyping System horseradish peroxidase; Southern Biotech) according to the manufacturer's recommendations.

Immunizations with TNP-Ficoll.

Immunizations were preformed using sex and age matched *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice that were between 8 and 10 weeks old. Mice were immunized with 25ug TNP-Ficoll

(Biosearch Technologies, Inc.). Mice were bled before immunization (day 0) and on days 7 and 14 after immunization. TNP-specific IgM and IgG3 antibody responses were measured by ELISA against plate-bound TNP-conjugated bovine serum albumin using serial dilutions of serum and isotype specific HRP-conjugated secondary antibodies (Southern Biotech). The TNP specific titers were defined as the greatest serial dilution at which the average optical density at 405nm exceeded 1.5 fold of the background optical density.

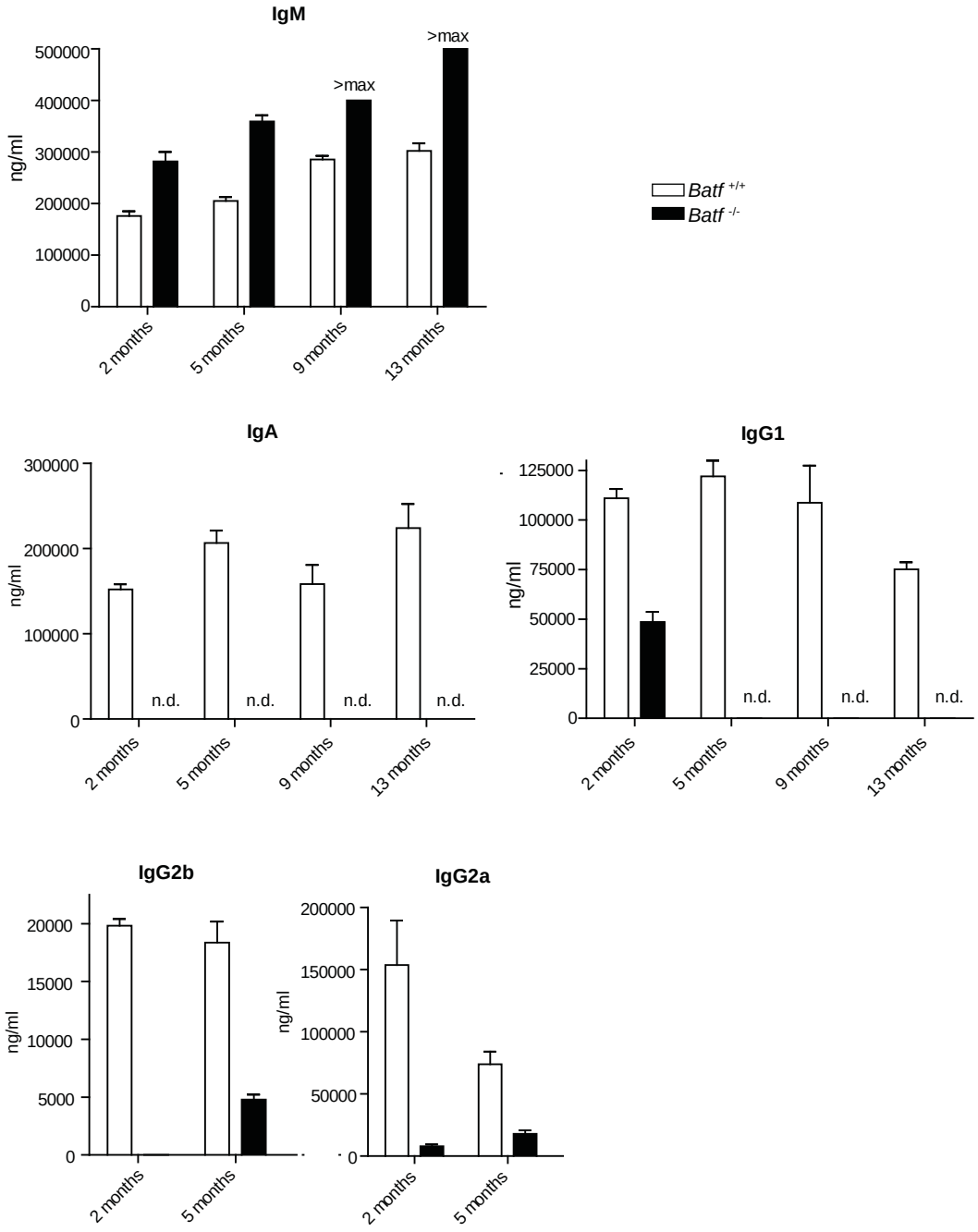


Figure 37. *Batf* $^{-/-}$ mice have decreased basal serum Immunoglobulin concentrations. Sera from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice at the indicated ages were analyzed for serum Ig concentrations using ELISA. Data are presented as mean + s.e.m. of 3-5 mice analyzed per group.

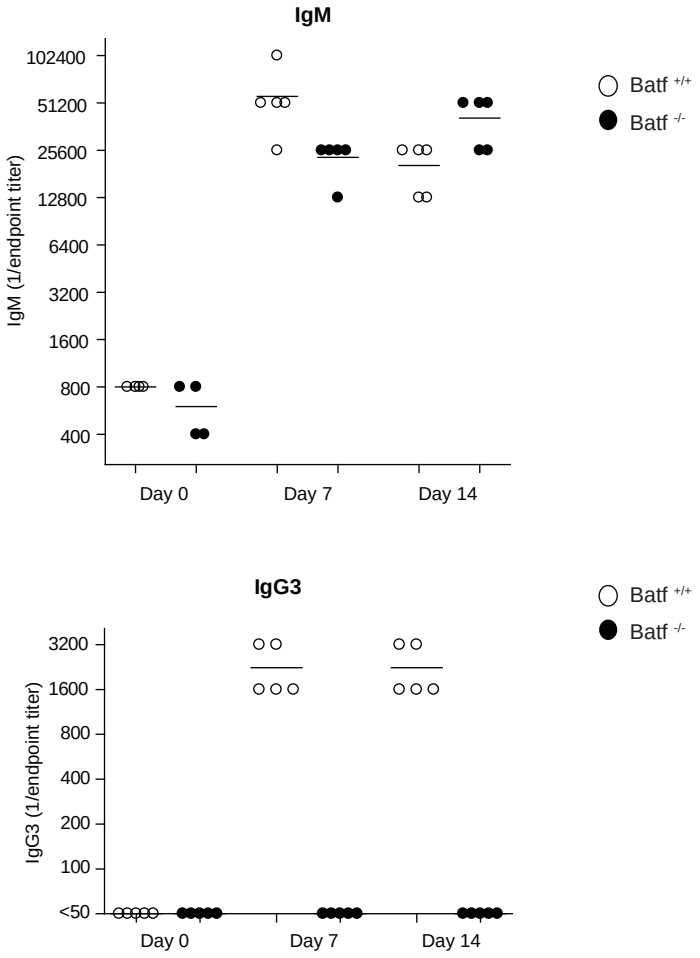


Figure 38. *Batf* $^{-/-}$ mice fail to mount antigen specific IgG3 responses to a T cell independent antigen.
Batf $^{+/+}$ and *Batf* $^{-/-}$ mice were immunized with TNP-Ficoll. Mice were bled before immunization (day 0) and on days 7 and 14 after immunization. TNP-specific IgM and IgG3 antibody responses were measured by ELISA against plate-bound TNP-conjugated bovine serum albumin using serial dilutions of serum. The TNP specific titers were defined as the greatest serial dilution at which the average optical density at 405nm exceeded 1.5 fold of the background optical density. Horizontal bars indicate mean values.

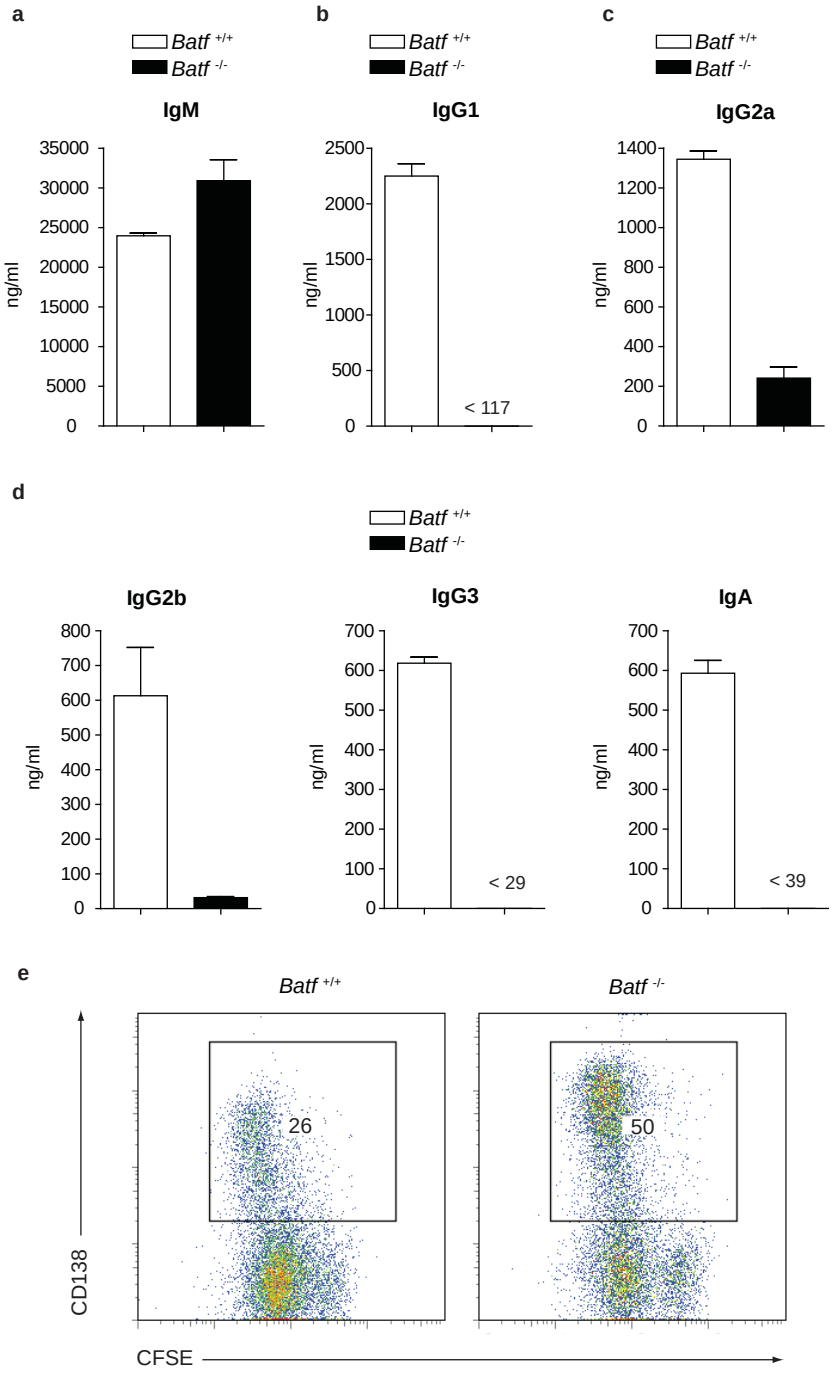


Figure 39. Defective class switch recombination in *Batf* $^{-/-}$ B cells.
a - d, Naïve B cells were isolated from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ mice by negative selection using CD43 magnetic beads. B cells were then activated with either LPS alone (**a**), LPS + IL-4 (**b**), LPS + IFN-γ (**c**), or LPS + TGF-β (**d**). On day 7 supernatants were collected and analyzed for the indicated Ig isotypes by ELISA. **e,** B cells from *Batf* $^{+/+}$ and *Batf* $^{-/-}$ were CFSE labeled and treated with LPS + IL-4. On day 4 after activation B cells were analyzed for CFSE dilution and CD138 expression by Flow cytometry. Number indicated the percentage of live cells in each gate.

REFERENCES

Akimzhanov,A.M., Yang,X.O., and Dong,C. (2007). Chromatin remodeling of interleukin-17 (IL-17)-IL-17F cytokine gene locus during inflammatory helper T cell differentiation. J Biol Chem. *282*, 5969-5972.

Alugupalli,K.R. (2008). A distinct role for B1b lymphocytes in T cell-independent immunity. Specialization and Complementation of Humoral Immune Responses to Infection *319*, 105-130.

Awasthi,A., Carrier,Y., Peron,J.P., Bettelli,E., Kamanaka,M., Flavell,R.A., Kuchroo,V.K., Oukka,M., and Weiner,H.L. (2007). A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. Nat Immunol *8*, 1380-1389.

Bauquet,A.T., Jin,H., Paterson,A.M., Mitsdoerffer,M., Ho,I.C., Sharpe,A.H., and Kuchroo,V.K. (2009). The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nat Immunol *10*, 167-175.

Berenson,L.S., Farrar,J.D., Murphy,T.L., and Murphy,K.M. (2004). Absence of functional STAT4 activation despite detectable tyrosine phosphorylation induced by murine IFN-alpha. Eur. J. Immunol. *34*, 2365-2374.

Bettelli,E., Carrier,Y., Gao,W., Korn,T., Strom,T.B., Oukka,M., Weiner,H.L., and Kuchroo,V.K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature *441*, 235-238.

Blank,V. (2008). Small Maf proteins in mammalian gene control: mere dimerization partners or dynamic transcriptional regulators? J Mol Biol *376*, 913-925.

Bower,K.E., Fritz,J.M., and McGuire,K.L. (2004). Transcriptional repression of MMP-1 by p21SNFT and reduced in vitro invasiveness of hepatocarcinoma cells. Oncogene *23*, 8805-8814.

Brustle,A., Heink,S., Huber,M., Rosenplanter,C., Stadelmann,C., Yu,P., Arpaia,E., Mak,T.W., Kamradt,T., and Lohoff,M. (2007). The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. Nat Immunol *8*, 958-966.

Chauvet,C., Bois-Joyeux,B., Fontaine,C., Gervois,P., Bernard,M.A., Staels,B., and Danan,J.L. (2005). The gene encoding fibrinogen-beta is a target for retinoic acid receptor-related orphan receptor alpha. Mol Endocrinol. *19*, 2517-2526.

Chen,Z., Laurence,A., Kanno,Y., Pacher-Zavisin,M., Zhu,B.M., Tato,C., Yoshimura,A., Hennighausen,L., and O'Shea,J.J. (2006). Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. Proc. Natl. Acad. Sci U. S. A *103*, 8137-8142.

Chung,Y., Yang,X., Chang,S.H., Ma,L., Tian,Q., and Dong,C. (2006). Expression and regulation of IL-22 in the IL-17-producing CD4+ T lymphocytes. Cell Res *16*, 902-907.

Crooks,G.E., Hon,G., Chandonia,J.M., and Brenner,S.E. (2004). WebLogo: a sequence logo generator. Genome Res *14*, 1188-1190.

Davidson,T.S., DiPaolo,R.J., Andersson,J., and Shevach,E.M. (2007). Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. J Immunol *178*, 4022-4026.

de Vinuesa,C.G., Cook,M.C., Ball,J., Drew,M., Sunners,Y., Cascalho,M., Wabl,M., Klaus,G.G., and MacLennan,I.C. (2000). Germinal centers without T cells. J Exp. Med. *191*, 485-494.

Deppmann,C.D., Thornton,T.M., Utama,F.E., and Taparowsky,E.J. (2003). Phosphorylation of BATF regulates DNA binding: a novel mechanism for AP-1 (activator protein-1) regulation. Biochem J *374*, 423-431.

Dierssen,U., Beraza,N., Lutz,H.H., Liedtke,C., Ernst,M., Wasmuth,H.E., and Trautwein,C. (2008). Molecular dissection of gp130-dependent pathways in hepatocytes during liver regeneration. J Biol Chem. *283*, 9886-9895.

Diveu,C., McGeachy,M.J., Boniface,K., Stumhofer,J.S., Sathe,M., Joyce-Shaikh,B., Chen,Y., Tato,C.M., McClanahan,T.K., de Waal,M.R., Hunter,C.A., Cua,D.J., and Kastelein,R.A. (2009). IL-27 blocks RORc expression to inhibit lineage commitment of Th17 cells. J Immunol *182*, 5748-5756.

Dorsey,M.J., Tae,H.J., Sollenberger,K.G., Mascarenhas,N.T., Johansen,L.M., and Taparowsky,E.J. (1995). B-ATF: a novel human bZIP protein that associates with members of the AP-1 transcription factor family. Oncogene *11*, 2255-2265.

Echlin,D.R., Tae,H.J., Mitin,N., and Taparowsky,E.J. (2000). B-ATF functions as a negative regulator of AP-1 mediated transcription and blocks cellular transformation by Ras and Fos. Oncogene *19*, 1752-1763.

Eferl,R. and Wagner,E.F. (2003). AP-1: a double-edged sword in tumorigenesis. Nat Rev Cancer *3*, 859-868.

Elson,C.O., Cong,Y., Weaver,C.T., Schoeb,T.R., McClanahan,T.K., Fick,R.B., and Kastelein,R.A. (2007). Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice. Gastroenterology *132*, 2359-2370.

Fazilleau,N., McHeyzer-Williams,L.J., Rosen,H., and McHeyzer-Williams,M.G. (2009). The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nat Immunol *10*, 375-384.

Fina,D., Sarra,M., Fantini,M.C., Rizzo,A., Caruso,R., Caprioli,F., Stolfi,C., Cardolini,I., Dottori,M., Boirivant,M., Pallone,F., Macdonald,T.T., and Monteleone,G. (2008).

Regulation of gut inflammation and th17 cell response by interleukin-21. Gastroenterology *134*, 1038-1048.

Fitzgerald,D.C., Ciric,B., Touil,T., Harle,H., Grammatikopolou,J., Das,S.J., Gran,B., Zhang,G.X., and Rostami,A. (2007). Suppressive effect of IL-27 on encephalitogenic Th17 cells and the effector phase of experimental autoimmune encephalomyelitis. J Immunol *179*, 3268-3275.

Garrity,P.A., Chen,D., Rothenberg,E.V., and Wold,B.J. (1994). Interleukin-2 transcription is regulated in vivo at the level of coordinated binding of both constitutive and regulated factors. Mol Cell Biol *14*, 2159-2169.

Ghilardi,N. and Ouyang,W. (2007). Targeting the development and effector functions of TH17 cells. Semin. Immunol *19*, 383-393.

Gorman,J.R., van der,S.N., Monroe,R., Cogne,M., Davidson,L., and Alt,F.W. (1996). The Ig(kappa) enhancer influences the ratio of Ig(kappa) versus Ig(lambda) B lymphocytes. Immunity. *5*, 241-252.

Gran,B., Zhang,G.X., Yu,S., Li,J., Chen,X.H., Ventura,E.S., Kamoun,M., and Rostami,A. (2002). IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. J Immunol *169*, 7104-7110.

Gu,Y., Yang,J., Ouyang,X., Liu,W., Li,H., Yang,J., Bromberg,J., Chen,S.H., Mayer,L., Unkeless,J.C., and Xiong,H. (2008). Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. Eur. J Immunol *38*, 1807-1813.

Haak,S., Croxford,A.L., Kreymborg,K., Heppner,F.L., Pouly,S., Becher,B., and Waisman,A. (2009). IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation in mice. J Clin. Invest *119*, 61-69.

Hamada,H., Garcia-Hernandez,M.L., Reome,J.B., Misra,S.K., Strutt,T.M., McKinstry,K.K., Cooper,A.M., Swain,S.L., and Dutton,R.W. (2009). Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge. J Immunol *182*, 3469-3481.

Hardy,R.R. and Hayakawa,K. (2001). B cell development pathways. Annu. Rev Immunol *19*, 595-621.

Harrington,L.E., Hatton,R.D., Mangan,P.R., Turner,H., Murphy,T.L., Murphy,K.M., and Weaver,C.T. (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol *6*, 1123-1132.

Harris,T.J., Grosso,J.F., Yen,H.R., Xin,H., Kortylewski,M., Albesiano,E., Hipkiss,E.L., Getnet,D., Goldberg,M.V., Maris,C.H., Housseau,F., Yu,H., Pardoll,D.M., and Drake,C.G. (2007). Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. J Immunol *179*, 4313-4317.

Hartenstein,B., Teurich,S., Hess,J., Schenkel,J., Schorpp-Kistner,M., and Angel,P. (2002). Th2 cell-specific cytokine expression and allergen-induced airway inflammation depend on JunB. EMBO J *21*, 6321-6329.

Hasegawa,H., Utsunomiya,Y., Kishimoto,K., Tange,Y., Yasukawa,M., and Fujita,S. (1996). SFA-2, a novel bZIP transcription factor induced by human T-cell leukemia virus type I, is highly expressed in mature lymphocytes. Biochem Biophys. Res Commun. *222*, 164-170.

Hatton,R.D., Harrington,L.E., Luther,R.J., Wakefield,T., Janowski,K.M., Oliver,J.R., Lallone,R.L., Murphy,K.M., and Weaver,C.T. (2006). A Distal Conserved Sequence Element Controls Ifng Gene Expression by T Cells and NK Cells. Immunity.

He,D., Wu,L., Kim,H.K., Li,H., Elmets,C.A., and Xu,H. (2006). CD8+ IL-17-producing T cells are important in effector functions for the elicitation of contact hypersensitivity responses. J Immunol *177*, 6852-6858.

Hermann-Kleiter,N., Gruber,T., Lutz-Nicoladoni,C., Thuille,N., Fresser,F., Labi,V., Schiefermeier,N., Warnecke,M., Huber,L., Villunger,A., Eichele,G., Kaminski,S., and Baier,G. (2008). The nuclear orphan receptor NR2F6 suppresses lymphocyte activation and T helper 17-dependent autoimmunity. Immunity *29*, 205-216.

Hertz,G.Z. and Stormo,G.D. (1999). Identifying DNA and protein patterns with statistically significant alignments of multiple sequences. Bioinformatics. *15*, 563-577.

Hess,J., Angel,P., and Schorpp-Kistner,M. (2004). AP-1 subunits: quarrel and harmony among siblings. J Cell Sci *117*, 5965-5973.

Hildner,K., Edelson,B.T., Purtha,W.E., Diamond,M., Matsushita,H., Kohyama,M., Calderon,B., Schraml,B.U., Unanue,E.R., Diamond,M.S., Schreiber,R.D., Murphy,T.L., and Murphy,K.M. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science *322*, 1097-1100.

Ho,I.C., Hodge,M.R., Rooney,J.W., and Glimcher,L.H. (1996). The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. Cell *85*, 973-983.

Ho,I.C., Lo,D., and Glimcher,L.H. (1998). c-maf promotes T helper cell type 2 (Th2) and attenuates Th1 differentiation by both interleukin 4-dependent and -independent mechanisms. J. Exp. Med. *188*, 1859-1866.

Ho,I.C., Tai,T.S., and Pai,S.Y. (2009). GATA3 and the T-cell lineage: essential functions before and after T-helper-2-cell differentiation. Nat Rev Immunol *9*, 125-135.

Hu,C.M., Jang,S.Y., Fanzo,J.C., and Pernis,A.B. (2002). Modulation of T cell cytokine production by interferon regulatory factor-4. J Biol Chem. *277*, 49238-49246.

Hunter,C.A. (2005). New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. Nat Rev Immunol *5*, 521-531.

Iacobelli,M., Wachsman,W., and McGuire,K.L. (2000). Repression of IL-2 promoter activity by the novel basic leucine zipper p21SNFT protein. J Immunol *165*, 860-868.

Ichiyama,K., Yoshida,H., Wakabayashi,Y., Chinen,T., Saeki,K., Nakaya,M., Takaesu,G., Hori,S., Yoshimura,A., and Kobayashi,T. (2008). Foxp3 inhibits RORgammat-mediated IL-17A mRNA transcription through direct interaction with RORgammat. J Biol Chem. *283*, 17003-17008.

Ivanov,I.I., Frutos,R.L., Manel,N., Yoshinaga,K., Rifkin,D.B., Sartor,R.B., Finlay,B.B., and Littman,D.R. (2008). Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host. Microbe *4*, 337-349.

Ivanov,I.I., McKenzie,B.S., Zhou,L., Tadokoro,C.E., Lepelley,A., Lafaille,J.J., Cua,D.J., and Littman,D.R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell *126*, 1121-1133.

Ivanov,I.I., Zhou,L., and Littman,D.R. (2007). Transcriptional regulation of Th17 cell differentiation. Semin. Immunol *19*, 409-417.

Jain,J., Valge-Archer,V.E., and Rao,A. (1992). Analysis of the AP-1 sites in the IL-2 promoter. J Immunol *148*, 1240-1250.

Kimura,A., Naka,T., Nohara,K., Fujii-Kuriyama,Y., and Kishimoto,T. (2008). Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. Proc. Natl. Acad. Sci U. S. A *105*, 9721-9726.

Klein,U., Casola,S., Cattoretti,G., Shen,Q., Lia,M., Mo,T., Ludwig,T., Rajewsky,K., and Dalla-Favera,R. (2006). Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. Nat Immunol *7*, 773-782.

Klein,U. and Dalla-Favera,R. (2008). Germinal centres: role in B-cell physiology and malignancy. Nature Reviews Immunology *8*, 22-33.

Kopp,T., Lenz,P., Bello-Fernandez,C., Kastelein,R.A., Kupper,T.S., and Stingl,G. (2003). IL-23 production by cosecretion of endogenous p19 and transgenic p40 in keratin 14/p40 transgenic mice: evidence for enhanced cutaneous immunity. J Immunol *170*, 5438-5444.

Korbelik,M., Cecic,I., Merchant,S., and Sun,J. (2008). Acute phase response induction by cancer treatment with photodynamic therapy. Int. J Cancer *122*, 1411-1417.

Korn,T., Bettelli,E., Gao,W., Awasthi,A., Jager,A., Strom,T.B., Oukka,M., and Kuchroo,V.K. (2007). IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature *448*, 484-487.

Korn,T., Mitsdoerffer,M., Croxford,A.L., Awasthi,A., Dardalhon,V.A., Galileos,G., Vollmar,P., Stritesky,G.L., Kaplan,M.H., Waisman,A., Kuchroo,V.K., and Oukka,M.

(2008). IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. Proc. Natl. Acad. Sci U. S. A *105*, 18460-18465.

Kreymborg,K., Etzensperger,R., Dumoutier,L., Haak,S., Rebollo,A., Buch,T., Heppner,F.L., Renauld,J.C., and Becher,B. (2007). IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis. J Immunol *179*, 8098-8104.

Kryczek,I., Wei,S., Zou,L., Altuwaijri,S., Szeliga,W., Kolls,J., Chang,A., and Zou,W. (2007). Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. J Immunol *178*, 6730-6733.

Lancrin,C., Sroczynska,P., Stephenson,C., Allen,T., Kouskoff,V., and Lacaud,G. (2009). The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. Nature *457*, 892-895.

Langrish,C.L., Chen,Y., Blumenschein,W.M., Mattson,J., Basham,B., Sedgwick,J.D., McClanahan,T., Kastelein,R.A., and Cua,D.J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp. Med. *201*, 233-240.

Laurence,A., Tato,C.M., Davidson,T.S., Kanno,Y., Chen,Z., Yao,Z., Blank,R.B., Meylan,F., Siegel,R., Hennighausen,L., Shevach,E.M., and O'Shea,J.J. (2007). Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity *26*, 371-381.

Lee,Y.K., Turner,H., Maynard,C.L., Oliver,J.R., Chen,D., Elson,C.O., and Weaver,C.T. (2009). Late developmental plasticity in the T helper 17 lineage. Immunity *30*, 92-107.

Li,B., Tournier,C., Davis,R.J., and Flavell,R.A. (1999). Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. EMBO Journal *18*, 420-432.

Liang,S.C., Tan,X.Y., Luxenberg,D.P., Karim,R., Dunussi-Joannopoulos,K., Collins,M., and Fouser,L.A. (2006). Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J Exp. Med. *203*, 2271-2279.

Liu,S.J., Tsai,J.P., Shen,C.R., Sher,Y.P., Hsieh,C.L., Yeh,Y.C., Chou,A.H., Chang,S.R., Hsiao,K.N., Yu,F.W., and Chen,H.W. (2007). Induction of a distinct CD8 Tnc17 subset by transforming growth factor-beta and interleukin-6. J Leukoc. Biol *82*, 354-360.

Liu,X.K., Lin,X., and Gaffen,S.L. (2004). Crucial role for nuclear factor of activated T cells in T cell receptor-mediated regulation of human interleukin-17. J Biol Chem. *279*, 52762-52771.

Lohoff,M., Mittrucker,H.W., Prechtl,S., Bischof,S., Sommer,F., Kock,S., Ferrick,D.A., Duncan,G.S., Gessner,A., and Mak,T.W. (2002). Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. Proc. Natl. Acad. Sci U. S. A *99*, 11808-11812.

Lohr,J., Knoechel,B., and Abbas,A.K. (2006). Regulatory T cells in the periphery. Immunol Rev *212*, 149-162.

Longerich,S., Basu,U., Alt,F., and Storb,U. (2006). AID in somatic hypermutation and class switch recombination (vol 18, pg 164, 2006). Current Opinion in Immunology *18*, 769.

Ma,H.L., Liang,S., Li,J., Napierata,L., Brown,T., Benoit,S., Senices,M., Gill,D., Dunussi-Joannopoulos,K., Collins,M., Nickerson-Nutter,C., Fouser,L.A., and Young,D.A. (2008). IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. J Clin. Invest *118*, 597-607.

Macian,F., Lopez-Rodriguez,C., and Rao,A. (2001). Partners in transcription: NFAT and AP-1. Oncogene *20*, 2476-2489.

Manel,N., Unutmaz,D., and Littman,D.R. (2008). The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nat Immunol *9*, 641-649.

Mangan,P.R., Harrington,L.E., O'Quinn,D.B., Helms,W.S., Bullard,D.C., Elson,C.O., Hatton,R.D., Wahl,S.M., Schoeb,T.R., and Weaver,C.T. (2006). Transforming growth factor-beta induces development of the T(H)17 lineage. Nature *441*, 231-234.

Mathur,A.N., Chang,H.C., Zisoulis,D.G., Stritesky,G.L., Yu,Q., O'Malley,J.T., Kapur,R., Levy,D.E., Kansas,G.S., and Kaplan,M.H. (2007). Stat3 and Stat4 direct development of IL-17-secreting Th cells. J Immunol *178*, 4901-4907.

McGeachy,M.J. and Cua,D.J. (2007). The link between IL-23 and Th17 cell-mediated immune pathologies. Semin. Immunol *19*, 372-376.

Meixner,A., Karreth,F., Kenner,L., and Wagner,E.F. (2004). JunD regulates lymphocyte proliferation and T helper cell cytokine expression. EMBO J *23*, 1325-1335.

Murphy,K.M. and Reiner,S.L. (2002). The lineage decisions of helper T cells. Nat. Rev. Immunol. *2*, 933-944.

Murphy,K.M., Travers,P., and Walport,M. (2008). Janeway's Immunobiology. Garland Science).

Muto,A., Hoshino,H., Madisen,L., Yanai,N., Obinata,M., Karasuyama,H., Hayashi,N., Nakauchi,H., Yamamoto,M., Groudine,M., and Igarashi,K. (1998). Identification of Bach2 as a B-cell-specific partner for small maf proteins that negatively regulate the immunoglobulin heavy chain gene 3' enhancer. EMBO J *17*, 5734-5743.

Nakshatri,H. and Currie,R.A. (1996). Differential whole-cell extract preparation and electrophoretic mobility shift assay to evaluate the effect of tyrosine phosphatases on DNA binding activity of transcription factors. Anal. Biochem *236*, 178-181.

Negishi,H., Ohba,Y., Yanai,H., Takaoka,A., Honma,K., Yui,K., Matsuyama,T., Taniguchi,T., and Honda,K. (2005). Negative regulation of Toll-like-receptor signaling by IRF-4. Proc. Natl. Acad. Sci U. S. A *102*, 15989-15994.

Newcomb,D.C., Zhou,W., Moore,M.L., Goleniewska,K., Hershey,G.K., Kolls,J.K., and Peebles,R.S., Jr. (2009). A functional IL-13 receptor is expressed on polarized murine CD4+ Th17 cells and IL-13 signaling attenuates Th17 cytokine production. J Immunol *182*, 5317-5321.

Nurieva,R., Yang,X.O., Martinez,G., Zhang,Y., Panopoulos,A.D., Ma,L., Schluns,K., Tian,Q., Watowich,S.S., Jetten,A.M., and Dong,C. (2007). Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature *448*, 480-483.

Ouyang,W., Kolls,J.K., and Zheng,Y. (2008a). The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity *28*, 454-467.

Ouyang,W., Kolls,J.K., and Zheng,Y. (2008b). The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity *28*, 454-467.

Panne,D., Maniatis,T., and Harrison,S.C. (2004). Crystal structure of ATF-2/c-Jun and IRF-3 bound to the interferon-beta enhancer. EMBO J *23*, 4384-4393.

Park,H., Li,Z., Yang,X.O., Chang,S.H., Nurieva,R., Wang,Y.H., Wang,Y., Hood,L., Zhu,Z., Tian,Q., and Dong,C. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol *6*, 1133-1141.

Quintana,F.J., Basso,A.S., Iglesias,A.H., Korn,T., Farez,M.F., Bettelli,E., Caccamo,M., Oukka,M., and Weiner,H.L. (2008). Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. Nature *453*, 65-71.

Ranganath,S., Ouyang,W., Bhattarcharya,D., Sha,W.C., Grupe,A., Peltz,G., and Murphy,K.M. (1998). GATA-3-dependent enhancer activity in IL-4 gene regulation. J. Immunol. *161*, 3822-3826.

Rao,A., Luo,C., and Hogan,P.G. (1997). Transcription factors of the NFAT family: regulation and function. [Review] [220 refs]. Ann. Rev. Immunol. *15*, 707-747.

Rasmussen,M.H., Sorensen,A.B., Morris,D.W., Dutra,J.C., Engelhard,E.K., Wang,C.L., Schmidt,J., and Pedersen,F.S. (2005). Tumor model-specific proviral insertional mutagenesis of the Fos/Jdp2/Batf locus. Virology *337*, 353-364.

Reinhardt,R.L., Liang,H.E., and Locksley,R.M. (2009). Cytokine-secreting follicular T cells shape the antibody repertoire. Nat Immunol *10*, 385-393.

Rengarajan,J., Mowen,K.A., McBride,K.D., Smith,E.D., Singh,H., and Glimcher,L.H. (2002). Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. J. Exp. Med. *195*, 1003-1012.

Rincon,M., Derijard,B., Chow,C.W., Davis,R.J., and Flavell,R.A. (1997). Reprogramming the signalling requirement for AP-1 (activator protein-1) activation during differentiation of precursor CD4+ T-cells into effector Th1 and Th2 cells. Genes Funct. *1*, 51-68.

Ryseck,R.P. and Bravo,R. (1991). c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. Oncogene *6*, 533-542.

Sciammas,R., Shaffer,A.L., Schatz,J.H., Zhao,H., Staudt,L.M., and Singh,H. (2006). Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. Immunity *25*, 225-236.

Senga,T., Iwamoto,T., Humphrey,S.E., Yokota,T., Taparowsky,E.J., and Hamaguchi,M. (2002). Stat3-dependent induction of BATF in M1 mouse myeloid leukemia cells. Oncogene *21*, 8186-8191.

Shi,G., Cox,C.A., Vistica,B.P., Tan,C., Wawrousek,E.F., and Gery,I. (2008). Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. J Immunol *181*, 7205-7213.

Stark,M.A., Huo,Y., Burcin,T.L., Morris,M.A., Olson,T.S., and Ley,K. (2005). Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. Immunity *22*, 285-294.

Stormo,G.D., Schneider,T.D., Gold,L., and Ehrenfeucht,A. (1982). Use of the 'Perceptron' algorithm to distinguish translational initiation sites in E. coli. Nucleic Acids Res *10*, 2997-3011.

Sun,Z., Unutmaz,D., Zou,Y.R., Sunshine,M.J., Pierani,A., Brenner-Morton,S., Mebius,R.E., and Littman,D.R. (2000). Requirement for RORgamma in thymocyte survival and lymphoid organ development. Science *288*, 2369-2373.

Suzuki,S., Honma,K., Matsuyama,T., Suzuki,K., Toriyama,K., Akitoyo,I., Yamamoto,K., Suematsu,T., Nakamura,M., Yui,K., and Kumatori,A. (2004). Critical roles of interferon regulatory factor 4 in CD11bhighCD8alpha- dendritic cell development. Proc. Natl. Acad. Sci U. S. A *101*, 8981-8986.

Szabo,S.J., Gold,J.S., Murphy,T.L., and Murphy,K.M. (1993). Identification of cis-acting regulatory elements controlling interleukin-4 gene expression in T cells: roles for NF-Y and NF-ATc [published erratum appears in Mol Cell Biol 1993 Sep;13(9):5928]. Mol. Cell Biol. *13*, 4793-4805.

Takeda,K., Kaisho,T., Yoshida,N., Takeda,J., Kishimoto,T., and Akira,S. (1998). Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. J Immunol *161*, 4652-4660.

Tan,W., Huang,W., Gu,X., Zhong,Q., Liu,B., and Schwarzenberger,P. (2008). IL-17F/IL-17R interaction stimulates granulopoiesis in mice. Exp. Hematol. *36*, 1417-1427.

Theilgaard-Monch,K., Jacobsen,L.C., Rasmussen,T., Niemann,C.U., Udby,L., Borup,R., Gharib,M., Arkwright,P.D., Gombart,A.F., Calafat,J., Porse,B.T., and Borregaard,N. (2005). Highly glycosylated alpha1-acid glycoprotein is synthesized in myelocytes, stored in secondary granules, and released by activated neutrophils. J Leukoc. Biol *78*, 462-470.

Thornton,T.M., Zullo,A.J., Williams,K.L., and Taparowsky,E.J. (2006). Direct manipulation of activator protein-1 controls thymocyte proliferation in vitro. Eur. J Immunol *36*, 160-169.

Torti,D.C. and Feldman,S.R. (2007). Interleukin-12, interleukin-23, and psoriasis: current prospects. J Am Acad. Dermatol. *57*, 1059-1068.

Tsuji,M., Komatsu,N., Kawamoto,S., Suzuki,K., Kanagawa,O., Honjo,T., Hori,S., and Fagarasan,S. (2009). Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. Science *323*, 1488-1492.

Veldhoen,M., Hirota,K., Christensen,J., O'Garra,A., and Stockinger,B. (2009). Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. J Exp. Med. *206*, 43-49.

Veldhoen,M., Hirota,K., Westendorf,A.M., Buer,J., Dumoutier,L., Renauld,J.C., and Stockinger,B. (2008). The aryl hydrocarbon receptor links T(H)17-cell-mediated autoimmunity to environmental toxins. Nature.

Veldhoen,M., Hocking,R.J., Atkins,C.J., Locksley,R.M., and Stockinger,B. (2006a). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity *24*, 179-189.

Veldhoen,M., Hocking,R.J., Flavell,R.A., and Stockinger,B. (2006b). Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. Nat Immunol *7*, 1151-1156.

Vignali,D.A., Collison,L.W., and Workman,C.J. (2008). How regulatory T cells work. Nat Rev Immunol *8*, 523-532.

Vinson,C., Acharya,A., and Taparowsky,E.J. (2006). Deciphering B-ZIP transcription factor interactions in vitro and in vivo. Biochim. Biophys. Acta *1759*, 4-12.

Wagner,E.F. and Eferl,R. (2005). Fos/AP-1 proteins in bone and the immune system. Immunol Rev *208*, 126-140.

Weaver,C.T., Harrington,L.E., Mangan,P.R., Gavrieli,M., and Murphy,K.M. (2006). Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity *24*, 677-688.

Wei,L., Laurence,A., Elias,K.M., and O'Shea,J.J. (2007). IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. J Biol Chem. *282*, 34605-34610.

Willenborg,D.O., Fordham,S., Bernard,C.C., Cowden,W.B., and Ramshaw,I.A. (1996). IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. J Immunol *157*, 3223-3227.

Williams,K.L., Nanda,I., Lyons,G.E., Kuo,C.T., Schmid,M., Leiden,J.M., Kaplan,M.H., and Taparowsky,E.J. (2001). Characterization of murine BATF: a negative regulator of activator protein-1 activity in the thymus. Eur. J Immunol *31*, 1620-1627.

Williams,K.L., Zullo,A.J., Kaplan,M.H., Brutkiewicz,R.R., Deppmann,C.D., Vinson,C., and Taparowsky,E.J. (2003). BATF transgenic mice reveal a role for activator protein-1 in NKT cell development. J Immunol *170*, 2417-2426.

Wolf,S.D., Dittel,B.N., Hardardottir,F., and Janeway,C.A., Jr. (1996). Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. J Exp. Med. *184*, 2271-2278.

Yang,X.O., Panopoulos,A.D., Nurieva,R., Chang,S.H., Wang,D., Watowich,S.S., and Dong,C. (2007). STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J Biol Chem. *282*, 9358-9363.

Yang,X.O., Pappu,B.P., Nurieva,R., Akimzhanov,A., Kang,H.S., Chung,Y., Ma,L., Shah,B., Panopoulos,A.D., Schluns,K.S., Watowich,S.S., Tian,Q., Jetten,A.M., and Dong,C. (2008). T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity *28*, 29-39.

Ye,P., Rodriguez,F.H., Kanaly,S., Stocking,K.L., Schurr,J., Schwarzenberger,P., Oliver,P., Huang,W., Zhang,P., Zhang,J., Shellito,J.E., Bagby,G.J., Nelson,S., Charrier,K., Peschon,J.J., and Kolls,J.K. (2001). Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp. Med. *194*, 519-527.

Yen,D., Cheung,J., Scheerens,H., Poulet,F., McClanahan,T., McKenzie,B., Kleinschek,M.A., Owyang,A., Mattson,J., Blumenschein,W., Murphy,E., Sathe,M., Cua,D.J., Kastelein,R.A., and Rennick,D. (2006). IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. J Clin. Invest *116*, 1310-1316.

Yoh,K., Sugawara,T., Motohashi,H., Takahama,Y., Koyama,A., Yamamoto,M., and Takahashi,S. (2001). Transgenic over-expression of MafK suppresses T cell proliferation and function in vivo. Genes Cells *6*, 1055-1066.

Zhang,F., Meng,G., and Strober,W. (2008b). Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. Nat Immunol *9*, 1297-1306.

Zhang,F., Meng,G., and Strober,W. (2008a). Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. Nat Immunol *9*, 1297-1306.

Zheng,Y., Danilenko,D.M., Valdez,P., Kasman,I., Eastham-Anderson,J., Wu,J., and Ouyang,W. (2007). Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature *445*, 648-651.

Zhou,L., Ivanov,I.I., Spolski,R., Min,R., Shenderov,K., Egawa,T., Levy,D.E., Leonard,W.J., and Littman,D.R. (2007). IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol *8*, 967-974.

Zhou,L., Lopes,J.E., Chong,M.M., Ivanov,I.I., Min,R., Victora,G.D., Shen,Y., Du,J., Rubtsov,Y.P., Rudensky,A.Y., Ziegler,S.F., and Littman,D.R. (2008). TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature.

Zhu,H., Yang,J., Murphy,T.L., Ouyang,W., Wagner,F., Saparov,A., Weaver,C.T., and Murphy,K.M. (2001). Unexpected characteristics of the IFN-gamma reporters in nontransformed T cells. J. Immunol. *167*, 855-865.

Zhumabekov,T., Corbella,P., Tolaini,M., and Kioussis,D. (1995). Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. J Immunol Methods *185*, 133-140.

Zullo,A.J., Benlagha,K., Bendelac,A., and Taparowsky,E.J. (2007). Sensitivity of NK1.1-negative NKT cells to transgenic BATF defines a role for activator protein-1 in the expansion and maturation of immature NKT cells in the thymus. J Immunol *178*, 58-66.