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

Barbara Schraml
Washington University in St. Louis

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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 Philosophy

August, 2009

St. Louis, Missouri
ABSTRACT OF THE DISSERTATION

Batf regulates previously unknown AP-1 target genes to control Th17 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 (Th17) 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 Th17 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 Th17-specific transcription factors, such as RORγt, and the cytokine IL-21, required for Th17 differentiation. Neither addition of IL-21 nor overexpression of RORγt fully restores IL-
17 production in Batf<sup>−/−</sup> 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<sub>H</sub>17 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<sub>H</sub>17 differentiation. These results demonstrate that the AP-1 factor Batf regulates previously unknown AP-1 target genes to control T<sub>H</sub>17 differentiation and T<sub>H</sub>17-mediated autoimmune disease.
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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.

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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)
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<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
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<td>Bach2</td>
<td>BTB and CNC homology 2</td>
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<td>Batf</td>
<td>Basic leucine zipper transcription factor $ATF$-like</td>
</tr>
<tr>
<td>bZIP</td>
<td>Basic leucine zipper</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional dendritic cell</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CNS-2</td>
<td>Conserved non-coding sequence-2</td>
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<td>CRE</td>
<td>Cyclic AMP responsive element</td>
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<td>CSR</td>
<td>Class switch recombination</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift analysis IL-2</td>
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<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>IFN-$\gamma$</td>
<td>Interferon gamma</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IRF4</td>
<td>Interferon regulatory factor 4</td>
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<tr>
<td>LP</td>
<td>Lamina propria</td>
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<tr>
<td>LTα</td>
<td>Lymphotoxin alpha</td>
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<td>MAF</td>
<td>Musculoaponeurotic fibrosarcoma</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MOG&lt;sub&gt;35-55&lt;/sub&gt;</td>
<td>Myelin oligodendrocyte glycoprotein peptide 35-55</td>
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<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
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<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
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<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
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<td>Rag2</td>
<td>Recombination activating gene 2</td>
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<td>RORα</td>
<td>Retinoid acid related nuclear orphan receptor alpha</td>
</tr>
<tr>
<td>RORγt</td>
<td>Retinoid acid related nuclear orphan receptor gamma T</td>
</tr>
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<td>RUNX1</td>
<td>Runt related transcription factor 1</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>TAD</td>
<td>Transcriptional activation domain</td>
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<td>T-bet</td>
<td>T cell specific T-box transcription factor</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TD</td>
<td>T-dependent</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TH</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TI</td>
<td>T-independent</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNP</td>
<td>2,4,6,-trinitrophenol</td>
</tr>
<tr>
<td>TRE</td>
<td>TPA responsive element</td>
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<tr>
<td>Treg</td>
<td>T regulatory cell</td>
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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 subsequent 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<sub>H</sub>) cells known to date distinguished by their signature cytokines; T<sub>H1</sub>, T<sub>H2</sub> and the recently identified T<sub>H17</sub> (Harrington et al., 2005) cells. In addition to differentiating into T helper subsets, naïve CD4<sup>+</sup> T cells can develop into regulatory T cells (T<sub>reg</sub>) that are characterized by their ability to suppress T cell responses. At least one class of T<sub>reg</sub> cells develops in the thymus (natural T<sub>reg</sub>), but other classes of T<sub>reg</sub> cells develop in the periphery (adaptive T<sub>reg</sub>) (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<sub>H1</sub> cells are characterized by the production of IFN-γ and LTα, T<sub>H2</sub> cells secrete IL-4, IL-5 and IL-13 and T<sub>H17</sub> cells secrete IL-17A (also called IL-17), IL-17F and IL-22 (Weaver et al., 2006; Ouyang et al., 2008b). T<sub>H1</sub> cells are the main players in cellular immunity against intracellular bacteria, viruses and tumors, while T<sub>H2</sub> cells direct the humoral immune response to extracellular pathogens, such as helminthes. T<sub>H17</sub> 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<sup>+</sup> 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<sub>H2</sub> mediated) or the development of autoimmune diseases (T<sub>H1</sub> and T<sub>H17</sub> mediated). T<sub>reg</sub> 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.

**TH17 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, TH1 cells were thought to be the major pathogenic T cell subset in these diseases, based on observations that blocking the TH1 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 TH1 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 TH17 lineage.
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-$\gamma$ 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-$\gamma$ locus and likely stabilizes its own expression either through an intrinsic autocatalytic loop or an autocrine cytokine feedback loop involving IFN-$\gamma$ signaling. T-bet also induces the transcription of the IL-12 receptor $\beta$2 subunit resulting in surface expression of a functional IL-12 receptor. IL-12 via STAT4 contributes to IFN-$\gamma$ transcription and induces the expression of IL-18 receptor. Once $T_{h1}$ 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\textsubscript{H}1 and T\textsubscript{H}2 differentiation is stringently counter-regulated to ensure exclusive commitment to either lineage and once naïve T cells commit to the T\textsubscript{H}1 of T\textsubscript{H}2 lineage, they exhibit stable phenotypes, exclusively expressing their signature cytokines. Early T\textsubscript{H}2 signaling induces a rapid feedback loop ensuring stable expression of Gata3, thus promoting T\textsubscript{H}2 differentiation. This stable Gata3 expression additionally blocks T\textsubscript{H}1 differentiation, since Gata3 inhibits expression of IFN-γ. Similarly, in T\textsubscript{H}1 cells T-bet inhibits the expression of T\textsubscript{H}2-associated factors and cytokines (Weaver et al., 2006; Murphy and Reiner, 2002).

**Transcriptional regulation of T\textsubscript{H}17 differentiation**

T\textsubscript{H}17 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\textsubscript{reg} cells in an IL-2 dependent manner (Davidson et al., 2007). T\textsubscript{H}17 and T\textsubscript{reg} cells develop via reciprocal pathways, in which IL-6 inhibits TGF-β-mediated induction of the T\textsubscript{reg} specific transcription factor Foxp3 (Bettelli et al., 2006). T\textsubscript{H}17 and T\textsubscript{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\textsubscript{H}17 and the suppressive T\textsubscript{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\textsubscript{H}17 differentiation requires IL-21, an early target of IL-6 signaling in T cells, which regulates T\textsubscript{H}17 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\textsubscript{H}17 cells (Langrish et al., 2005; Veldhoen et al., 2006a). While IL-6, IL-21 and IL-23 are required for T\textsubscript{H}17 differentiation and maintenance, proinflammatory cytokines such as IL-1\textbeta and TNF-\alpha are not required but can further promote T\textsubscript{H}17 differentiation through unknown mechanisms (Veldhoen et al., 2006a).

Similar to T\textsubscript{H}1 and T\textsubscript{H}2 cell differentiation, the differentiation of T\textsubscript{H}17 cells is counterregulated by effector cytokines from other T\textsubscript{H} subsets, such as IFN-\gamma, IL-4 and IL-13. Additionally, the cytokines IL-2, IL-10 and IL-27, have been described to inhibit T\textsubscript{H}17 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\textsubscript{H}1 and T\textsubscript{H}2 cells, which exhibit stable cytokine secretion profiles once they are committed to their lineage, IL-17 double producing cells that also secrete IFN-\gamma are readily detected in vitro and in vivo. As a result T\textsubscript{H}1 and T\textsubscript{H}17 cells were thought to develop from a common precursor; however, lineage tracing experiments demonstrated that T\textsubscript{H}17 cells develop as a lineage distinct from T\textsubscript{H}1 cells (Veldhoen et al., 2006b). Nevertheless, recent data demonstrated that T\textsubscript{H}17 cells exhibit developmental plasticity and unstable cytokine expression and that the maintenance of a stable T\textsubscript{H}17
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 \textit{Il17} promoter (Laurence et al., 2007; Yang et al., 2007; Mathur et al., 2007). Combined IL-6 and TGF-\beta signals induce the expression of the retinoid acid related nuclear orphan receptor (ROR) ROR\gamma t (Ivanov et al., 2006), a T_{H17} specific transcription factor. ROR\gamma t 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 ROR\gamma t deficiency blocks T_{H17} differentiation \textit{in vitro} (Ivanov et al., 2006) ROR\gamma t is considered the main regulator of T_{H17} cells, similar to T-bet and Gata3 in T_{H1} and T_{H2} cells respectively. However, ROR\gamma t deficient mice are only partially resistant to EAE and residual IL-17 production can be observed in ROR\gamma t deficient T cells \textit{in vivo} after EAE induction (Ivanov et al., 2006). This residual IL-17 production in ROR\gamma t deficient T cells has been attributed to functional redundancy between ROR\gamma t and ROR\alpha, another ROR family member expressed in T_{H17} cells (Yang et al., 2008). Yet, ROR\alpha is dispensable for T_{H17} differentiation since T cells deficient in ROR\alpha show only a mild reduction in IL-17 production (Yang et al., 2008). More strikingly, \textit{Irf4}\textsuperscript{-/-} T cells exhibit an absolute block in T_{H17} development and are completely resistant to EAE (Brustle et al., 2007). While \textit{Irf4}\textsuperscript{-/-} T cells exhibit decreased expression of ROR\gamma t, overexpression of ROR\gamma t in \textit{Irf4}\textsuperscript{-/-} T cells only partially restores IL-17 production (Brustle et al., 2007). Collectively, these data indicate that ROR\gamma t is unlikely the sole regulator of T_{H17} differentiation.
Consistently, additional factors have been identified to contribute to TH17 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 TH17 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 TH17 cells (Veldhoen et al., 2009). To summarize, multiple transcription factors have been shown to control TH17 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 Th2 cytokines in T cells cultured under Th1 conditions (Li et al., 1999), whereas JunB deficient T cells exhibit inefficient skewing towards a Th2 phenotype (Hartenstein et al., 2002). In contrast, overexpression of JunD suppresses Th2 cytokine expression and JunD deficiency leads to increased Th1 and Th2 cytokine production (Meixner et al., 2004). Therefore, JunB promotes Th2 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 Th2 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 Th17 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 Th17 differentiation and are resistant to experimental autoimmune encephalomyelitis. Using gene expression analysis, we found that Batf−/− T cells fail to induce known Th17-specific transcription factors, such as RORγt, and the cytokine IL-21, required for Th17 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’-ATTACTCGAGTGAAACAAACAGGCGTCGAGTG) and left arm reverse (5’-ATTACTCGAGCCTACTACCTTTTCAGGGCTACTGC). The right arm was generated by PCR with the use of the following oligonucleotides: right arm forward (5’-ATTAGTGCACGCATTCTTTCATGGTCCTTAGCCTTGG) and right arm reverse (5’-ATTAGTGCACCCAGAGAATGAGAAATGTTGGAGG). 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’-ACAGCTTTGAACTTCAGGAGCCCTCC and 5’-CACATTTAAGTCAACAATAACACTGC 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’-

GGACTACAAGACGATGACGACAAGCCTCACAGCTCCGACAGCA and 5’-

CTTGTGTCATCGTCTTTGATGTCATGGTTCTATCTTCAGATC. 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 SmaI 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<sup>+/+</sup> and Batf<sup>−/−</sup> 129SvEv mice were stimulated with anti-CD3 for 3 days under TH17 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<sup>+</sup> T cells, magnetically purified CD4<sup>+</sup> T cells from Batf<sup>+/+</sup> and Batf<sup>−/−</sup> 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 TH2 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 (100e6 cells/ml) in Affymetrix Chip lysis buffer (10mM Tris pH 7.5, 10mM NaCl, 3mM MgCl₂, 0.5% IGEPAL, with protease inhibitors (PMSF, aprotinin, leupeptin)). After 5 min at 4°C, nuclei were collected by centrifugation (800 rcf for 3 min 4°C) and lysed in RIPA (100e6 cell equivalents/ml) with protease inhibitors. Nuclear lysates were centrifuged for 10 min 4°C 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 T₇2 cells, DO11.10 CD4+ T cells from CD2-N-FLAG-Batf
Transgenic mice were isolated and differentiated with OVA and APC under Th2 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-CD4-APC antibody (BD Biosciences). 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-Allophycocyanin (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°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°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 0.5x10⁶ 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 Th1; anti-IFNγ (H22; BioXcell; 10μg/ml), anti-IL-12 (Tosh; BioXcell; 10μg/ml) and IL-4 (Peprotech; 10ng/ml) for Th2; anti-IL-4, anti-IL-12, anti-IFNγ, IL-6 (Peprotech; 20ng/ml) and TGF-β (Peprotech; 0.5ng/ml) for Th17 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, Th1 and Th2 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 Th17 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 4x10⁶ 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°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 MOG35-55 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 TH17 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°C, 10 min at 95°C followed by 40 2-step cycles of 15s at 95°C and 1min at 60°C.

Primers for RORγt (RORγt forward 5’-CGCTGAGAGGGCTTCAC, RORγt reverse 5’-GCAGGAGTAGGCCACATTACA)(IVANOV ET AL., 2006), IL-21 (IL-21 forward 5’-ATCCTGAACCTTCTATCAGCTCCAC, RORα ( RORα forward 5’-TCTCCCTGCGCTCTCCGCAC-3’, RORα reverse 5’ TCCACAGATCTTTGCATGGA-3’)(YANG ET AL., 2008), IL-21 reverse 5’-GCATTTAGCTATGTGCTTCTGTTTC(Zhou et al., 2007), IL-22 (IL-22 forward-5’CATGCAGGAGGTGTACCTT, IL-22 reverse- 5’-CAGACGCAAGCATTTCTCAG)(Chung et al., 2006), IL-23R (IL-23R forward 5’-GCCAAGAAGACCATTCCCGA, IL-23R reverse 5’-TCAGTGCTACAATCTTCTTCAGGACA)(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’-TTACTAGGCAGATGCGCCACA) 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'-AAACATTGGGGCTGAGTGC)(Korbelik et al., 2008), Serum amyloid protein A (SAA forward 5'-TCTCTGGGGCAACATAGTATACCTCAT and SAA reverse 5'-TTATTACCCTCTCCCTCAAGCAGTTAC) (Dierssen et al., 2008), fibrinogen β (fibβ forward: 5'-ATTAGCCAGCTTACCAGGATGGGACCAC-3', fibβ reverse: 5'-CAGTAGATCTGCCGTTTGGATTGGCTGC-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 TH1 and TH2 conditions for 7 days. After restimulation with anti-CD3 and anti-CD28 for 24h, TH1 and TH2 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 TH17 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<sup>−/−</sup> T cells, naive CD4<sup>+</sup> CD62L<sup>+</sup> CD25<sup>−</sup> T cells from Batf<sup>+/+</sup> and Batf<sup>−/−</sup> 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 (0.5 ng/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’-

CTCGAGGTGTGCTGCTCTGGGCTAC 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<sup>+</sup> 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’-

GGACTACAAAGACGATGACGACAAGCCTCACAGCTCCGACAGCA and 5’-
CTTGTGTCGTCATCGTCTTTGTAGTCATGGTCTAATCTTCCAGATC. 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’- AGCTTTGAGAAGCTATCGGTCC and 5’- AAGCTTTGAGGATGAGTAGTAGTGCTCC and 5’-

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 TH17 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-\( \beta \) and IL-6 as described previously (Nakshatri and Currie, 1996). For EMSA analysis the AP-1 consensus probe (Echlin et al., 2000) (top: AGCTTCGCTTTGATGAGTC and bottom: GCCGACTGAGTAGTTCGC), RORE element in CNS2 of the IL-17 gene (Yang et al., 2008) (top: GAAAGTTTTTCTGACCCACTTTAAATCA and bottom: CTTTAACTAAATTTCACCCAGTCTTTT) and -187 to -155 of the IL-17 promoter (top: GGTTCCTGTGCTGTACCTCATTGTGGATG and bottom: AAAAGACTGGGTGAATTATTAGTTAAG), E\( \alpha \) Y box probe (TCGACATTTTTCTGATTTGAAGTAAAGTC) (Szabo et al., 1993) were used after labeling with \( ^{32} \)P-dCTP. The probe (2.5x10^4 cpm per reaction) was used along with 15\( \mu \)g of total cell extracts and 1\( \mu \)g 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 $1 \times 10^7$ CD4$^+$ T cells isolated from C57BL/6 Batf$^{+/+}$ mice stimulated under Th17 polarizing conditions with anti-CD3 (2.5 μg/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 Th17 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

<table>
<thead>
<tr>
<th>Primers</th>
<th>Chr. 1 location</th>
<th>5'FAM 3' BHQ1 Probes</th>
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<tbody>
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IL-17a promoter Oligos  Sequence 5' to 3'
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46-14-top-IL17a  ACGAGGCAACTGTCACCAGCACCAGC
46-14-bot-IL17a  GATCACTTTGCTTGGGGTCACTTGTGCT
69-37-top-IL17a  GCACTACTCTTCATCCACCTACACGAG
69-37-bot-IL17a  TGTGCTCTGAGTGAGTGGAGTGAGAGTAGAG
83-51-top-IL17a  AAAGAGAGAGAGAGACTCCTTTATACGGGT
83-51-bot-IL17a  GCTTCCTTCTTCTTTATACGGGT
100-68-top-IL17a GTAGTAAAAACCGTATAAAAGAGAGAA
100-68-bot-IL17a GCCCTCTTCTCTTTATACGGGT
119-87-top-IL17a TACGGTTTTACTACCTGTCAGCT
119-87-bot-IL17a GTACAAGTAGCAGAGGAGTCTGAGTA
140-106-top-IL17a TGGGATCATCGTCAAGAGTGGGGT
140-106-bot-IL17a GATCACTCTGACTTGTGAGAGT
159-127-top-IL17a GAATCTTTACTCAAATGGTG
159-127-bot-IL17a GGTTGGGGGGTAGCTCTTTCCTCTCC
169-137-top-IL17a TGTGGAGTAATCCTTTACTCAATG
169-137-bot-IL17a GCCCTTTCCATCTTTCCAGAC
187-155-top-IL17a GGTCTGCTGCTCCTCTGTGAGGATG
187-155-bot-IL17a GATTCATCCTCCTAAATGAGTGCAAGCA
204-172-top-IL17a GCCCGTCATAAAGGGTGTTCTGTGGC
204-172-bot-IL17a AGATGTCAGCAGAGACTCCGCTCATT
215-183-top-IL17a AGACAAGATGTTGCGGGCTCATAAAAGG
215-183-bot-IL17a AAGACAACCCCTTATGACGAGGCAACAT
235-203-top-IL17a GCCCTTCCATCTTTCCAGAC
235-203-bot-IL17a GCAACATCTGTCTCGAAAGTGATGGGA
250-217-top-IL17a GCATAGTGAACTTCTTGAAC
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266-234-top-IL17a GAAATGCATCCTTCTTCCCCAT
266-234-bot-IL17a GGACAAGGATCGACATCTGTCCAC
281-249-top-IL17a CTGTTCACGCCTCAAGAACAGAT
281-249-bot-IL17a GCCAAAGAGCATGCTTGGGAGCTG
302-269-top-IL17a CTGAACTGAGACACCATCTGTGTCAG
302-269-bot-IL17a GAGACGTAGAGATGCTGGCTGTA
320-286-top-IL17a GTCCACAGACTGATGTTCTCCACAG
320-286-bot-IL17a GCTTCTGTGTGACATCTGACGT
334-302-top-IL17a GCAGCTCCAGATATGTCCACACACAC
334-302-bot-IL17a GTACCATGCTGTAGAGACATCTGAA
349-317-top-IL17a GAGCCAGCTGCAGAGCTCAGTGA
349-317-bot-IL17a GGCAATCTGAGAGTGCTGACGAGAT
370-337-top-IL17a GACTCACAACACATTACAGTGGAGACCCAG
370-337-bot-IL17a CAGAGCCTGGCTCCATAGTAATTGTGTTT
383-351-top-IL17a GAGACTGCTCAAGAGACACTCAAACAG
383-351-bot-IL17a ATAGTAATGGTTGTGAGTCTCTTGGACA
400-368-top-IL17a AAGAGTGTTGTGACTAGGAGACTGTCAC
IL-21 promoter Oligos
Sequence 5' to 3'

33-1-top-IL21  GTCATCAGCTCCTGGAGACTCAGTTCTG
33-1-bottom-IL21 GGCACCAGAAGCTCCTGGAGACTCAGTTCTG
55-22-top-IL21 GTGAGAAACCAGACCAAGGCCCTGTCATCA
55-22-bottom-IL21 GGAGCTGTGCACAGGGCCTTGGTCTGGTT
67-35-top-IL21 AGTCAGGTTGAAGTGAGAACCCACTCAATGTCTTAGTATG
67-35-bottom-IL21 GGGCCTTGGTCTGGTTCTCACTTCAACC
88-56-top-IL21 TAGCGACAACCTGTGCACAGTCAGGT
88-56-bottom-IL21 GTTCAACCATGACCTGACAGGTCTGTG
105-73-top-IL21 GATGAATAAATAGGTAGCCGTAGCGACA
105-73-bottom-IL21 CAGGTTTGCTGCTACGGCTACCTATTTAT
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137-105-top-IL21 CTGCAATGGGAGGGCTTGGCCTCTTCTT
137-105-bottom-IL21 GCCTCAAGAAGGCCAAGCCCTCCCAT
150-118-top-IL21 AAGAATTTTCCAGCTGCAATGGGAGGGC
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174-142-top-IL21 GTTACTCAACTCTCCTCCACTATACA
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183-151-top-IL21 GAAAAACGAGTTACTCAACTCATACC
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207-175-top-IL21 CACGTAACACTAGCAGAAAGAAAGA
207-175-bottom-IL21 TGCGCTATTCTTCTTCTGAGGTG
221-189-top-IL21 TGCCCAACACGCACAGCTACACCTAGC
221-189-bottom-IL21 CATTGGCTAGGTAGTCTCGTGCTGG
240-208-top-IL21 TGGTGGACTCTCTACCTCCCTGGCCAC
240-208-bottom-IL21 TGCGTGTGGGGGCAGGGATGGATAGAGT
254-222-top-IL21 GATGGGAGACATTTTGTGAGTCTACCTCC
254-222-bottom-IL21 GGGATGGATAGTGGCTACCAAAATGTG
266-234-top-IL21 GTTCAGGATGACTGAGGGGCAACTTCTT
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279-247-top-IL21 GTTCTTTTCTCTGTCTAAGATGACAGAT

400-368-bot-IL17a  GTCTCTTGGACAGTCTCCTAGTGACACAC
416-384-top-IL17a  GATCAAGTCAAAATTCAAGATGTGTC
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464-432-top-IL17a  GGGCAAGGGATGCTCTCTAGCCAGGGAA
464-432-bot-IL17a  GCAAATTCCCTGGCTAGAGAGCATCCCT
476-44-top-IL17a  GTGGGTTTCTTTGGGCAAGGGAGCTGG
476-44-bot-IL17a  GATCTTTTCTACAAATTCCCTGGCTAGAGAGCATCCCT
497-465-top-IL17a  GTTACATACTAAGCATTGAGTTG
497-465-bot-IL17a  AAAGAACCACACTCAATGTCTTAGTATG
Ap-1 consensus probe (Echlin et al., 2000) Sequence 5' to 3'

Top  AGCTTCGCTTGATGAGTC
Bottom GCCGACTGAGTAGTTCGC

329-297-bottom-IL22 AGGAGGCCACAAGACACCACCTCCAAGC
340-309-top-IL22 GCTCTCAAGGTGGGAAGGCTTGGAGGTG
340-309-bottom-IL22 GACACCACCTCCAAGCCTTCCCACCTTG
366-334-top-IL22 TTGAAGAGATGGGAAGTCTTCCCACCTTG
366-334-bottom-IL22 GTGAGCTATTCTTGAGTTTGTGT
405-373-top-IL22 CGGTGCTATTCTTGAGTTTGTGT
405-373-bottom-IL22 CCCGTAGCTATTGGAGTCAGCGTTGGT
420-388-top-IL22 CGGTGCTATTCTTGAGTTTGTGT
420-388-bottom-IL22 CCCGTAGCTATTGGAGTCAGCGTTGGT
497-465-top-IL22 ATAGCAGGAGGAGATCAAAGGCTGCTC
497-465-bottom-IL22 GAGTAGAGCAGCCTTTTGATCTCGACG
518-486-top-IL22 AGCTTCGCTTGATGAGTC
518-486-bottom-IL22 GCCGACTGAGTAGTTCGC
528-495-top-IL22 GGGGAAAATGAGTCCGTGACCAAACGCTGAC
528-495-bottom-IL22 GGGGAAAATGAGTCCGTGACCAAACGCTGAC
536-504-top-IL22 AGCTTCGCTTGATGAGTC
536-504-bottom-IL22 GCCGACTGAGTAGTTCGC
547-513-top-IL22 AGCTTCGCTTGATGAGTC
547-513-bottom-IL22 GCCGACTGAGTAGTTCGC
558-527-top-IL22 AGCTTCGCTTGATGAGTC
558-527-bottom-IL22 GCCGACTGAGTAGTTCGC
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662-630-bottom-IL22 GCCGACTGAGTAGTTCGC
684-652-top-IL22 AGCTTCGCTTGATGAGTC
684-652-bottom-IL22 GCCGACTGAGTAGTTCGC
700-668-top-IL22 AGCTTCGCTTGATGAGTC
700-668-bottom-IL22 GCCGACTGAGTAGTTCGC
RORE element (Yang et al., 2008)  Sequence 5' to 3'

Top         GAAAGTTTTCTGACCCACTTTAAATCA
Bottom      CTTTAACCTAAATTTCCACCCAGTCTTTT
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 (TH1, TH2 and TH17), T\(_{\text{reg}}\) 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 TH 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 TH1, TH2 and TH17 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, JDPI), 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 Th2 (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-1\(^{-}\)CD24\(^{-}\) (Hardy fraction A), BP-1\(^{-}\)CD24\(^{+}\) (Hardy fraction B), and BP-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\(^{hi}\)IgM\(^{hi}\)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.
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.
<table>
<thead>
<tr>
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<th>Dimers</th>
<th>DNA binding element</th>
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</thead>
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<td>homo- and heterodimers</td>
<td>C/EBP (5'-ATTGCGCAAT-3') CRE, PAR</td>
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<td>homodimers</td>
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</table>

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,17 conditions for 3 days. Equal cell numbers were subjected to Western Blot analysis using anti-Batf antibody. The blot was stripped and rebotted 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+IgMhi Igdlo), Transitional 2 (B220+IgMhi Igdhi) or mature B cells (AA4.1-B220+; B220+IgMloIgdhi) 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+CD43hi 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−IgDlo, and IgMloIgdhi (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<sup>−/−</sup> mice.

**a**, Total splenocytes were stained with the indicated antibodies. Conventional splenic dendritic cell (cDC) were identified as CD11c<sup>hi</sup> cells and further subdivided into CD4<sup>+</sup> DCs and CD8<sup>+</sup> DCs, identified as CD11c<sup>hi</sup>CD4<sup>+</sup>CD8<sup>−</sup> and CD11c<sup>hi</sup>CD4<sup>−</sup>CD8α<sup>+</sup> respectively. CD8<sup>+</sup> DCs were further identified as CD11c<sup>hi</sup>CD8α<sup>+</sup> Dec205<sup>+</sup>. **b**, Total splenocytes were stained with antibodies to CD11c, CD11b, Gr1 and B220. Percentages of plasmacytoid dendritic cells (CD11bc<sup>−</sup>CD11cloB220<sup>+</sup>Gr1<sup>+</sup>) were similar between Batf<sup>+/+</sup> and Batf<sup>−/−</sup> 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\textsubscript{H}17 differentiation

CD4\textsuperscript{+} Effector T cells orchestrate the immune response through the secretion of their signature cytokines. T\textsubscript{H}1 cells are the main players in cellular immunity against intracellular bacteria and viruses, whereas T\textsubscript{H}2 cells direct the humoral immune response to extracellular pathogens (Murphy and Reiner, 2002) and T\textsubscript{H}17 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\textsuperscript{+} memory T cells during secondary infections (Murphy and Reiner, 2002; Weaver et al., 2006) and homeostasis of T\textsubscript{reg} cells (Lohr et al., 2006). Uncontrolled effector T cell responses can lead to the development of atopic diseases such as asthma (T\textsubscript{H}2 mediated) or the development of autoimmune diseases (T\textsubscript{H}1 and T\textsubscript{H}17 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\textsubscript{H}1 and T\textsubscript{H}2 differentiation of Batf\textsuperscript{-/-} T cells

To test whether Batf regulates effector T cell differentiation we sorted naïve CD4\textsuperscript{+} T cells (CD4\textsuperscript{+}CD62L\textsuperscript{+}CD25\textsuperscript{-}) from Batf\textsuperscript{+/+} and Batf\textsuperscript{-/-} mice. These T cells were activated with anti-CD3 and anti-CD28 antibodies either without the addition of cytokines and antibodies, or under T\textsubscript{H}1 (anti-IL-4, IFN-\gamma, IL-12) and T\textsubscript{H}2 (anti-IL-IFN-\gamma, 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 TH1 and TH2 differentiation.

**Batf−/− T cells fail to produce IL-17**

In contrast to normal TH1 and TH2 differentiation, Batf−/− T cells activated under TH17 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 TH1 and TH2 cells in our gene expression profiling experiment. To confirm these data, we performed Western blot analysis of naïve T cells and TH2 cells. Batf protein was low, but present in unactivated naïve T cells (Figure 14a). Resting TH2 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<sub>H2</sub> cells even though it seems to specifically
regulate T<sub>H17</sub> differentiation.

The loss of IL-17 production in Batf<sup>-/-</sup> T cells could conceivably result either
from a disregulation in the production of T<sub>H17</sub> suppressing cytokines or from a cell-
inntrinsic requirement for Batf during T<sub>H17</sub> differentiation. Multiple cytokines have been
described in the literature to inhibit T<sub>H17</sub> 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<sup>-/-</sup> T cells produced similar levels of IL-2 compared to Batf<sup>+/+</sup> T cells.
Additionally, we observed no compensatory changes in the production of IFN-γ or IL-10
in Batf<sup>-/-</sup> T cells (Figure 12b). These data indicate that Batf<sup>-/-</sup> T cell do not produce
increased amounts of T<sub>H17</sub> suppressing cytokines, and suggest that Batf might directly
regulate transcriptional pathways controlling T<sub>H17</sub> 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<sub>H17</sub> development through
unknown mechanisms. We tested whether addition of IL-1β and TNFα would rescue
T<sub>H17</sub> differentiation in Batf<sup>-/-</sup> T cells. As expected, IL-1β and TNFα increased IL-17
production by Batf<sup>+/+</sup> T cells compared to stimulation with IL-6 and TGF-β alone,
however, failed to rescue IL-17 production in Batf<sup>-/-</sup> T cells (Figure 13b). Similarly,
stimulation of DO11.10 TCR transgenic Batf<sup>+/+</sup> and Batf<sup>-/-</sup> CD4<sup>+</sup> 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\textsubscript{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\textsubscript{H17} conditions compared to non-transgenic CD8+ T cells (Figure 17b), indicating that Batf can promote IL-17 production in T cells.

\textbf{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\textsubscript{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\textsubscript{reg} and T\textsubscript{H17} cells, thus influencing the equilibrium of intestinal tolerance versus immunity (Ivanov et al., 2008).

Since Batf\textsuperscript{-/-} T cells fail to produce IL-17 \textit{in vitro}, we analyzed LP T cells for the production of IL-17. Batf\textsuperscript{-/-} LP CD4+ T cells lacked IL-17 expression; however, spontaneous production of IFN\textgamma was similar to Batf\textsuperscript{+/+} LP CD4+ T cells (Figure 18). Thus, Batf\textsuperscript{-/-} 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\textsuperscript{-/-} T cells and show that Batf\textsuperscript{-/-} T cells selectively fail to differentiate into T\textsubscript{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-$\gamma$ and IL-4 production by intracellular staining. 

*b*, Naïve CD4$^+$CD62L$^+$CD25$^-$ T cells from Batf$^{+/+}$ and Batf$^{-/-}$ mice were activated under TH17 (TGF-$\beta$ 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-$\gamma$, 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<sup>+</sup> T cells. 

**a,** DO11.10<sup>+</sup>CD4<sup>+</sup> T cells from Batf<sup>+/+</sup>, Batf<sup>+-</sup> and Batf<sup>-/-</sup> mice were activated with OVA and APCs under Th17 conditions for 3 days and stained for intracellular IL-17 and IFN-γ after restimulation with PMA/ionomycin. **b,** Naïve CD4<sup>+</sup>CD62L<sup>-</sup>CD25<sup>-</sup> T cells from Batf<sup>+/+</sup> and Batf<sup>-/-</sup> mice were activated with anti-CD3/CD28 under Th17 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\textsubscript{H}2 cells. 

**a**, 1.5x10^6 cell equivalents of CD4\textsuperscript{+} T cells from Batf\textsuperscript{+/-} and Batf\textsuperscript{-/-} 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\textsuperscript{+} T cells from Batf\textsuperscript{+/-} and Batf\textsuperscript{-/-} mice were stimulated with anti-CD3/CD28 under T\textsubscript{H}2 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 Th2 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 Th17 conditions. 3 days later, cells were split and allowed to expand for 4 days in the presence of Th17 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 Th17 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 Th17 conditions. 3 days later, cells were restimulated with PMA/ionomycin and analyzed for CD4, IFN-$\gamma$ 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 Th17 conditions for 3 days. Cells were restimulated with PMA/ionomycin and analyzed for IL-17 and IFN-$\gamma$ 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.
Batf<sup>−/−</sup> mice are completely resistant to EAE due to a T cell intrinsic defect

While T<sub>H</sub>17 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<sub>H</sub>17 cells were first implicated as major players in EAE when mice deficient 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<sub>H</sub>17 development, such as ROR<sub>γ</sub><sub>t</sub> and IRF4, are protected from EAE (Ivanov et al., 2006; Brustle et al., 2007). While these data clearly indicate the importance of T<sub>H</sub>17 cells in EAE pathogenesis, several studies have unsuccessfully addressed which T<sub>H</sub>17 effector cytokines are required for EAE development. Antibody blockade of IL-17A in the setting of <i>Il17f</i>-deficiency, or <i>Il22</i> deficiency only minimally prevent EAE (Kreyemborg et al., 2007; Haak et al., 2009), implying the existence of other T<sub>H</sub>17 effector pathways that are required for EAE development.

Batf<sup>−/−</sup> mice are resistant to MOG<sub>35-55</sub>-induced EAE

Since Batf<sup>−/−</sup> T cells failed to develop into IL-17-secreting cells, we tested whether Batf<sup>−/−</sup> mice were susceptible to EAE. We immunized Batf<sup>+/+</sup> and Batf<sup>−/−</sup> mice with
myelin oligodendrocyte glycoprotein peptide 35-55 (MOG35-55). 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_{H}17 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_{H}17 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 \textit{Batf}^{+/+} and \textit{Batf}^{-/-} mice for Foxp3 expression before immunization and 10 or 40 days after immunization with MOG_{35-55} (Figure 20a and b). \textit{Batf}^{-/-} mice had lower basal numbers of splenic Foxp3$^+$ T cells compared to \textit{Batf}^{+/+} mice. More importantly, \textit{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 \textit{Batf}^{-/-} mice to EAE results from an absence of T_{H17} cells rather than an increase in T_{reg} cells.

\textit{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 \textit{Batf}^{-/-} mice likely explains disease resistance. This loss of T_{H17} development in \textit{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 \textit{Batf}^{+/+} CD4$^+$ T cells or PBS control buffer into mice before MOG_{35-55} immunization. \textit{Batf}^{-/-} mice receiving PBS control buffer remained resistant to EAE as expected (Figure 21a). In contrast, \textit{Batf}^{-/-} mice receiving naïve \textit{Batf}^{+/+} CD4$^+$ T cells developed severe EAE (Figure 21a and Table 2). Although, \textit{Batf}^{-/-} mice that had received \textit{Batf}^{+/+} T cells exhibited a slight delay in disease onset compared to \textit{Batf}^{+/+} mice receiving \textit{Batf}^{-/+} T cells, these differences are not statistically significant (Table 2). Notably, the transfer of \textit{Batf}^{+/+} CD4$^+$ T cells into \textit{Batf}^{-/-} mice also restored infiltration of the CNS by IL-17-producing CD4$^+$ T cells (Figure 21b).

Since \textit{Batf}^{-/-} T cells fail to develop into T_{H17} cells, we hypothesized that \textit{Batf}^{-/-} T cells might exhibit a protective function if transferred into \textit{Batf}^{+/+} mice, conceivably
because of increased production of T\textsubscript{H}17 suppressing cytokines by Batf\textsuperscript{−/−} T cells (Willenborg et al., 1996) or increased development of Foxp3\textsuperscript{+} T\textsubscript{reg} cells (Korn et al., 2007). Therefore, we injected naïve Batf\textsuperscript{−/−} CD4\textsuperscript{+} T into mice before MOG\textsubscript{35-55} immunization, however; the transfer of Batf\textsuperscript{−/−} CD4\textsuperscript{+} T cells did not protect Batf\textsuperscript{+/+} mice from EAE development (Figure 21c).

Collectively, our results indicate that the antigen-presenting environment in Batf\textsuperscript{−/−} mice is permissive for T\textsubscript{H}17 development, and suggest that resistance to EAE in Batf\textsuperscript{−/−} mice is due to a T cell-intrinsic defect in the generation of T\textsubscript{H}17 cells.
**Figure 19.** *Batf*<sup>−/−</sup> mice are resistant to EAE.

**a,** *Batf*<sup>−/−</sup> (<n=12) and *Batf*<sup>+/−</sup> (<n=13) mice were immunized with MOG<sub>33-35</sub> 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*<sup>+/−</sup> and *Batf<sup>−/−</sup> 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<sup>+</sup> cells. Numbers for FACS plots indicate percentage of cells in each indicated gate.
Figure 20. Batf<sup>−/−</sup> mice show no compensatory increase in Foxp3<sup>+</sup> cells during EAE. 

a, Total splenocytes from unimmunized Batf<sup>+/+</sup> and Batf<sup>−/−</sup> 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<sup>+/+</sup> and Batf<sup>−/−</sup> mice or from mice 40 days after EAE induction were analyzed for CD4 and Foxp3 expression. The abundance of Foxp3<sup>+</sup> cells is depicted as the ratio of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in the total CD4<sup>+</sup> 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 1x10⁷ *Batf*⁺/⁺ CD4⁺ T cells (n=6). Four days later, mice were immunized with MOG₃₅-₅₅ 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 1x10⁷ *Batf*⁺/⁻ CD4⁺ T cells (n=4). Four days later, mice were immunized MOG₃₃-₃₅ peptide. a, b, Mean clinical EAE scores representative of two independent experiments are shown.
Table 2. Transfer of Batf\textsuperscript{+/+} CD4\textsuperscript{+} T cells restores EAE in Batf\textsuperscript{-/-} mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Mean Max. Score</th>
<th>Mortality</th>
<th>Mean day of onset\textsuperscript{‡}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS\textrightarrow{}Batf\textsuperscript{+/+}</td>
<td>5 of 5 (100%)</td>
<td>3.4 ± 0.7</td>
<td>1 of 5 (20%)</td>
<td>12±0.8\textsuperscript{¶}</td>
</tr>
<tr>
<td>PBS\textrightarrow{}Batf\textsuperscript{-/-}</td>
<td>0 of 5 (0%)</td>
<td>0</td>
<td>0 of 13 (0%)</td>
<td>NA</td>
</tr>
<tr>
<td>Batf\textsuperscript{+/+}CD4\textsuperscript{+}\textrightarrow{}Batf\textsuperscript{+/+}</td>
<td>5 of 6 (83%)</td>
<td>3.0 ± 0.6</td>
<td>0 of 6 (0%)</td>
<td>13.6±2.3\textsuperscript{¶¶}</td>
</tr>
<tr>
<td>Batf\textsuperscript{+/+}CD4\textsuperscript{+}\textrightarrow{}Batf\textsuperscript{-/-}</td>
<td>4 of 6 (66%)</td>
<td>2.4 ± 1.0</td>
<td>2 of 6 (33%)</td>
<td>15.5±1.7\textsuperscript{§}</td>
</tr>
</tbody>
</table>

Batf\textsuperscript{+/-} and Batf\textsuperscript{-/-} mice were injected with either control PBS buffer (n=5) or 1\times10\textsuperscript{7} Batf\textsuperscript{+/+} CD4\textsuperscript{+} T cells (n=6). Four days later, mice were immunized with MOG\textsubscript{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.
Batf controls the expression of a subset of IL-6 induced genes

T\textsubscript{H}17 cells differentiate in response to the cytokines IL-6 and TGF-\textbeta (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\textsubscript{H}17 differentiation and binds directly to the \textit{Il17} promoter (Laurence et al., 2007; Yang et al., 2007; Mathur et al., 2007).

Combined signals from IL-6 and TGF-\textbeta induce the expression of the retinoid acid related nuclear orphan receptor (ROR) ROR\textgamma\textsubscript{t} (Ivanov et al., 2006), in a STAT3 dependent manner (Yang et al., 2007; Harris et al., 2007). ROR\textgamma\textsubscript{t} is sufficient to induce IL-17 production in wild type T cells and ROR\textgamma\textsubscript{t} deficiency blocks T\textsubscript{H}17 differentiation \textit{in vitro} (Ivanov et al., 2006). ROR\textgamma\textsubscript{t} has been considered the main regulator of T\textsubscript{H}17 cells, similar to T-bet and Gata3 in T\textsubscript{H}1 and T\textsubscript{H}2 cells respectively. However, residual IL-17 production can be observed in ROR\textgamma\textsubscript{t} deficient T cells \textit{in vivo} after EAE induction (Ivanov et al., 2006). Since ROR\alpha and ROR\textgamma\textsubscript{t} double deficient do not exhibit this residual IL-17 production, ROR\alpha has been suggested to cooperate with ROR\textgamma\textsubscript{t} during T\textsubscript{H}17 differentiation at the level of \textit{Il17} transcription (Yang et al., 2008). However, ROR\alpha deficiency only mildly affects IL-17 production (Yang et al., 2008), indicating that ROR\alpha is dispensable for T\textsubscript{H}17 differentiation and that ROR\textgamma\textsubscript{t} and ROR\alpha exhibit mere functional redundancy. More strikingly, \textit{Irf4}\textsuperscript{-/-} 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-\(\beta\) 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\(_{H}17\)-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\(_{H}17\) development (Figure 25 and Figure 27a and b). Batf-dependent genes included ROR\(\gamma\)t (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 ROR\(\gamma\)t 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 ROR\(\gamma\)t 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sub>H17</sub> development in Batf<sup>−/−</sup> T cells. First, when Batf<sup>+/+</sup> 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<sup>−/−</sup> 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<sub>H17</sub>-inducing conditions, forced RORγt expression induced only 7.6% IL-17 production in Batf<sup>−/−</sup> T cells, compared to 50% IL-17 production in Batf<sup>+/+</sup> T cells (Figure 29b and c). Thus, RORγt only partially restores T<sub>H17</sub> differentiation in Batf<sup>−/−</sup> T cells.

RORα, a nuclear receptor related to RORγt has been suggested to cooperate with RORγt during T<sub>H17</sub> 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<sup>−/−</sup> T cells.

**a,** Splenocytes from Batf<sup>+/+</sup> and Batf<sup>−/−</sup> mice were stained with antibodies to CD4 and IL-6 receptor (IL-6R). A histogram overlay of IL-6R expression on CD4<sup>+</sup> cells of the indicated genotypes is shown. **b,** Magnetically purified Batf<sup>+/+</sup> and Batf<sup>−/−</sup> CD4<sup>+</sup> T cells (left) and CD8<sup>+</sup> 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 + 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 + 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 Th17 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 Th17 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 Th17 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 Th17 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 Th17 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 Th17 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 Th17 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<sup>−/−</sup> T cells.  

a, b, Batf<sup>+/+</sup> and Batf<sup>−/−</sup> CD4<sup>+</sup> 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<sup>+</sup>) cells treated as in a and b is depicted (mean + s.d. of 3 independent experiments).  d, Batf<sup>+/+</sup> and Batf<sup>−/−</sup> CD4<sup>+</sup> T cells stimulated with anti-CD3/CD28 under Th17 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<sup>+</sup>hCD4<sup>+</sup> 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 TH17 differentiation.

a, *Batf*−/− CD4+ T cells were stimulated with anti-CD3/CD28 under TH17 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<sup>−/−</sup> CD4<sup>+</sup> T cells showed considerably less reporter activity than Batf<sup>+/+</sup> 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 electrophoretic 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<sub>H17</sub> 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<sub>H17</sub> 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\textsubscript{H}17 cells. This probe formed two complexes in Batf\textsuperscript{+/+} T\textsubscript{H}17 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\textsuperscript{−/−} T cells cultured under T\textsubscript{H}17 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\textsubscript{H}17 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\textsubscript{H}17 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\textsubscript{H}17 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*<sup>+/−</sup> T<sub>H17</sub> 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<sup>−/−</sup>* T cells cultured under T<sub>H17</sub> 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<sub>H17</sub> 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\(_H\)17 differentiation**

Finally, to test whether in T\(_H\)17 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\(_H\)17 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 \textit{Il17} promoter is Batf-responsive. 
\textit{a, Batf}^{++} and \textit{Batf}^{--} CD4\textsuperscript{+} T cells cultured under T\textsubscript{h}17 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 \textit{Il17a} promoter drives GFP expression. On day 3, cells were restimulated with PMA/ionomycin. Plots are gated on hCD4\textsuperscript{+} 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 Th17 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 Th17 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 TH17 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 TH17 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 Th17 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*<sup>+/+</sup> and *Batf*<sup>−/−</sup> CD4<sup>+</sup> T cells were stimulated under Th17 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\(_h\)17 cells. Total splenocytes from Batf\(^{+/+}\) and Batf\(^{-/-}\) mice were activated with anti-CD3 under T\(_h\)17 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\textsubscript{H17} differentiation and T\textsubscript{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\textsuperscript{−/−} mice show a highly selective defect in the differentiation of IL-17-producing T helper (T\textsubscript{H17}) cells. T\textsubscript{H17} cells are a CD4\textsuperscript{+} 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\textsuperscript{−/−} mice are completely resistant to experimental autoimmune encephalomyelitis due to the inability of Batf deficient T cells to differentiate into T\textsubscript{H17} cells. Using gene expression analysis, we found that Batf\textsuperscript{−/−} T cells fail to induce known T\textsubscript{H17}-specific transcription factors, such as ROR\textsubscript{γt}, and the cytokine IL-21, required for T\textsubscript{H17} differentiation. Neither addition of IL-21 nor overexpression of ROR\textsubscript{γt} fully restores IL-17 production in Batf\textsuperscript{−/−} T cells, suggesting that Batf may be required directly for IL-17 transcription. We found that the \textit{Il17} promoter is Batf-responsive, and upon T\textsubscript{H17} differentiation, Batf binds to several conserved intergenic elements in the \textit{Il17a/f} locus as well as to regions in the \textit{Il17}, \textit{Il21} and \textit{Il22} promoters. Using bio-computational methods we determined that the Batf-binding element in the \textit{Il17}, \textit{Il21} and \textit{Il22} promoters differs from canonical symmetric AP-1 elements. Using EMSA analysis we found that Batf forms heterodimers.
preferentially with JunB during T\textsubscript{H}17 differentiation. These results demonstrate that the AP-1 factor Batf regulates previously unknown AP-1 target genes to control T\textsubscript{H}17 differentiation and T\textsubscript{H}17-mediated autoimmune disease.

**Batf specifically controls T\textsubscript{H}17 differentiation**

We found that Batf\textsuperscript{-/-} T cells specifically failed to differentiate into T\textsubscript{H}17 cells, while T\textsubscript{H}1 and T\textsubscript{H}2 differentiation was unaffected. Our results demonstrate that Batf\textsuperscript{-/-} T cells do not exhibit increased production of cytokines that can suppress T\textsubscript{H}17 differentiation, such as IL-2, IFN-\(\gamma\), 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\textsubscript{H}17 differentiation. Consistently, Batf deficiency abrogates IL-17 production in CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and the addition of exogenous T\textsubscript{H}17-promoting cytokines fails to rescue IL-17 production in Batf\textsuperscript{-/-} T cells. Therefore, Batf\textsuperscript{-/-} T cells exhibit a remarkably selective defect in T\textsubscript{H}17 differentiation.

This selective defect in one particular pathway of T\textsubscript{H}1 differentiation was surprising since Batf was also expressed in T\textsubscript{H}1 and T\textsubscript{H}2 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 TH17 differentiation, Batf is unique in that its effects are more specific to TH17 differentiation. Only four transcription factors have been shown to be required for TH17 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 TH17 differentiation, RORα deficient mice show essentially normal TH17 development (Yang et al., 2008), indicating it is not required.

Irf4−/− T cells have a complete block in TH17 differentiation; however, Irf4−/− T cells also show severe defects in TH2 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 TH17. 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 deleter strain.
Deletion of STAT3 during T cell development using an Lck-Cre deleter 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<sup>−/−</sup> mice. Also, we found that Batf is not required for normal development of thymocytes, but functions only in the T<sub>H</sub>17 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<sup>−/−</sup> 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<sub>H</sub>17 differentiation only by siRNA knockdown and by overexpression of a dominant negative in mature T cells (Zhang et al., 2008b). Unlike RUNX1, Batf<sup>−/−</sup> mice are viable, fertile, and show a selective defect in T<sub>H</sub>17 differentiation.

In summary, Batf<sup>−/−</sup> T cells exhibit a surprisingly selective defect in T<sub>H</sub>17 differentiation and the effects of Batf are more specific to T<sub>H</sub>17 differentiation than those of previously identified factors. Thus, Batf<sup>−/−</sup> mice provide an exceptional model system to selectively study the role of T<sub>H</sub>17 cells in T cell mediated immune responses and autoimmune diseases.
**Batf**<sup>−/−</sup> mice are completely resistant to EAE

Batf<sup>−/−</sup> mice were completely resistant to the development of MOG<sub>35-55</sub>-induced EAE due to defective T<sub>H</sub>17 development. The MOG<sub>35-55</sub> immunization model of EAE is primarily T-cell mediated (Wolf et al., 1996). T<sub>H</sub>17 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 ROR<sub>γ</sub>t and IRF4, that control T<sub>H</sub>17 development are protected from EAE (Ivanov et al., 2006; Brustle et al., 2007). However, ROR<sub>γ</sub>t deficient mice are only partially resistant to EAE and IL-17 production can be observed in ROR<sub>γ</sub>t<sup>−/−</sup> T cells in vivo after EAE induction (Ivanov et al., 2006). In contrast, Irf4<sup>−/−</sup> T cells exhibit an absolute block in T<sub>H</sub>17 development resulting in complete resistantance to EAE (Brustle et al., 2007). Intriguingly, the phenotype of Irf4<sup>−/−</sup> mice in EAE is markedly similar to the phenotype we observed in Batf<sup>−/−</sup> mice.

T<sub>H</sub>17 and T<sub>reg</sub> 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<sup>+</sup> T regulatory cells in the absence of T<sub>H</sub>17 differentiation (Korn et al., 2007). Strikingly, we found no compensatory increase in the development of Foxp3<sup>+</sup> T<sub>reg</sub> cells in Batf<sup>−/−</sup> mice during EAE, suggesting that Batf<sup>−/−</sup> mice are resistant to EAE due a selective defect in T<sub>H</sub>17 differentiation. Consistent with the absence of increased T<sub>reg</sub> cell development in the absence of Batf, the transfer of Batf<sup>−/−</sup> T cells into Batf<sup>+/+</sup> 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\textsubscript{H}17 development in Batf\textsuperscript{-/-} mice, indicating that Batf\textsuperscript{-/-} mice provide an antigen presenting environment permissive for T\textsubscript{H}17 development. Thus Batf\textsuperscript{-/-} mice are protected from EAE due to a T cell intrinsic defect that prevents T\textsubscript{H}17 development, rather than a defect in T cell priming.

Since the importance of T\textsubscript{H}17 cells in EAE pathogenesis is recognized, several studies attempted to address which T\textsubscript{H}17 effector cytokines are required for EAE development. IL-23 is critical for EAE development due to its role in maintaining T\textsubscript{H}17 cells \textit{in vivo} (Langrish et al., 2005); indicating the requirement for T\textsubscript{H}17 cells. However, IL-22 deficiency only minimally prevented EAE (Kreymborg et al., 2007) and antibody blockade of IL-17A in the setting of \textit{Il17f}-deficiency also only minimally affects EAE development (Haak et al., 2009) implying the existence of other T\textsubscript{H}17 effector pathways that are required for EAE development. Since Batf\textsuperscript{-/-} mice specifically lack the T\textsubscript{H}17 lineage, they provide a model allow addressing which T\textsubscript{H}17 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\textsuperscript{-/-} 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\textsubscript{H}1 and T\textsubscript{H}2 cell differentiation is normal in the absence of Batf, our studies provide strong support that T\textsubscript{H}17 cells are important mediators of EAE pathogenesis. Additionally, in contrast to Irf4\textsuperscript{-/-} mice, which exhibit abnormal T\textsubscript{H}2 development, Batf\textsuperscript{-/-} mice provide an excellent model system to selectively study the role of T\textsubscript{H}17 cells in T cell-mediated autoimmune disorders.
Batf controls the expression of a subset of IL-6-induced genes

TH17 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 TH17 differentiation (Zhou et al., 2008). IL-6 partly promotes TH17 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 TH17 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 TH17 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 TH17 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 TH17 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 TH17 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 \textit{Il17, Il21 and Il22} by \textit{Batf} and reveal a yet unrecognized role for AP-1 transcription factors in the regulation of \textit{T}_{\text{H}17} associated cytokines.

\textbf{A potential role for \textit{Batf} as transcriptional activator}

\textit{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 \textit{Batf} binds to multiple regions in the \textit{Il17a/f} locus as well as the \textit{Il21} and \textit{Il22} promoters indicating direct transcriptional regulation of \textit{Il17, Il21 and Il22} by \textit{Batf}. Additionally, the \textit{Il17} promoter is \textit{Batf} responsive and \textit{Batf} is required for the induction of \textit{Il17, Il21 and Il22} indicating that \textit{Batf} might directly promote transcription of these genes rather than inhibiting their transcription. Consistently, \textit{Batf}^{-/-} T cells lose the expression of many IL-6 induced genes in T cells, whereas \textit{Batf} deficiency leads to increased expression of only few genes. Since \textit{Batf} preferentially heterodimerizes with JunB during \textit{T}_{\text{H}17} differentiation, we hypothesize that \textit{Batf} in complex with JunB forms an active transcription factor complex in the context of \textit{T}_{\text{H}17} differentiation.

Although \textit{Batf} is also expressed in \textit{T}_{\text{H}1} and \textit{T}_{\text{H}2} cells, it appears that \textit{Batf} is specifically necessary for \textit{T}_{\text{H}17} differentiation, but does not induce this process by itself. Rather, our data suggest that \textit{Batf} cooperates with other \textit{T}_{\text{H}17}-specific factors to regulate target genes. In fact, ROR\textgamma only partially restores IL-17 production in the absence of \textit{Batf} and our data shows functional synergy between \textit{Batf} and ROR\textgamma. Notably, \textit{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 ROR\( \gamma t \) 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 TGACATAG, which differs from the canonical CRE recognition sequence (TGACGTC) at the three underlined positions. The crystal structure of the Jun-ATF2 heterodimer and two IRF3 molecules bound to the interferon-\( \beta \) 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-
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 enhanceosome, 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_{H}17 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 TH17 development. Since *Batf* is also expressed in TH1 and TH2 cells, it likely cooperates with other TH17-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 TH17 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 (TH17) cells (Harrington et al., 2005). TH17 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 TH1 and TH2 differentiation, but defective TH17 differentiation, and are resistant to experimental autoimmune encephalomyelitis. *Batf*⁻/⁻ T cells fail to induce known factors required for TH17 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 TH17 differentiation, Batf binds conserved intergenic elements in the *Il17* 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 TH17 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 \( \text{CD}4^+ \) and \( \text{CD}8^+ \) 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

\textit{Batf}^{−/−} mice have decreased basal immunoglobulin levels.

In our studies, we determined that B cell development was normal in \textit{Batf}^{−/−} mice (Chapter 3). Since \textit{Batf} was also expressed at low levels in naïve B cells, we tested whether B cell function was affected in the absence of \textit{Batf}. First, we measured basal serum immunoglobulin isotype levels in unimmunized \textit{Batf}^{−/−} mice of different ages. We found that \textit{Batf}^{−/−} mice had slightly increased IgM serum levels across multiple ages examined (Figure 37). Strikingly, we found that \textit{Batf}^{−/−} mice had severely reduced to non-detectable serum levels of IgA, IgG1, IgG2a and IgG2b isotypes compared to \textit{Batf}^{+/+} mice (Figure 37). Thus, \textit{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<sup>−/−</sup> B cells (Figure 39a), indicating that Batf<sup>−/−</sup> B cells can be activated to secrete antibody. Consistently, CD138 expression, a marker for plasma cells was increased in Batf<sup>−/−</sup> 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<sup>−/−</sup> mice (Chapter 3), we wanted to determine, whether Batf deficiency affects B cell function. We found that Batf<sup>−/−</sup> 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<sup>−/−</sup> mice mounted normal antigen specific IgM responses following immunization with the TI antigen TNP-Ficoll; however, Batf<sup>−/−</sup> mice had defective IgG3 responses. Additionally, in vitro analysis of Batf<sup>−/−</sup> B cells demonstrated defective class switching, but normal secretion of IgM in response to activation. Notably, Batf<sup>−/−</sup> 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<sup>−/−</sup> B cells exhibit a phenotype similar to Batf<sup>−/−</sup> B cells. Bach2<sup>−/−</sup> 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-\(\gamma\) (100ng/ml), TGF-\(\beta\) (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\(^6\)cells/ml in PBS with 1\(\mu\)M 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 immunoglobulating 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<sup>++</sup> mice fail to mount antigen specific IgG3 responses to a T cell independent antigen. Batf<sup>++</sup> and Batf<sup>⁻⁻</sup> 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, Naive 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.


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