The Role of the Akt2 Isoform in Th17 Differentiation in Vitro and Peripheral Cd4 T Cell Immune Responses in Vivo

Lauren Banks

University of Pennsylvania, lbanks@mail.med.upenn.edu

Follow this and additional works at: http://repository.upenn.edu/edissertations

Part of the Allergy and Immunology Commons, Immunology and Infectious Disease Commons, and the Medical Immunology Commons

Recommended Citation

http://repository.upenn.edu/edissertations/1203

This paper is posted at ScholarlyCommons. http://repository.upenn.edu/edissertations/1203
For more information, please contact libraryrepositorit@pobox.upenn.edu.
The Role of the Akt2 Isoform in Th17 Differentiation in Vitro and Peripheral Cd4 T Cell Immune Responses in Vivo

Abstract
Akt1 and Akt2, isoforms of the serine threonine kinase Akt, are essential for T cell development. However, their role in peripheral T cell differentiation remains undefined. Using germline deletions of either Akt1 or Akt2 in mice, we found that while both are important for Th17 differentiation, the impact of Akt2 loss was greater. Mechanistically, while Akt2-/- Th17 cells were predicted by microarray analysis to have suppressed cholesterol biosynthetic and IL-6 signaling pathways, neither pathway emerged as the primary mechanism through which Akt2 contributes to Th17 differentiation. We also interrogated the role of the well-known Akt target, mTORC1, and found that Akt2-/- Th17 cells were equally sensitive to inhibition of the mTORC1-S6K axis as their WT counterparts. However, Akt2 deficiency was associated with elevated Gfi1 mRNA, which encodes for a known inhibitor of Th17 differentiation, and decreased mir155, which encodes for a miRNA that promotes Th17 differentiation. Furthermore, Akt2 loss has significant in vivo consequences and results in a dampened peripheral T cell response to myelin oligodendrocyte glycoprotein (MOG) immunization. This dampened response was associated with altered Th cell differentiation including a pronounced diminution of inflammatory cytokine production and preferential expansion of regulatory T cells compared to non-T regulatory cells. Using mice with Akt2 loss only in the T cell compartment, Akt2 cKO, we demonstrate that Akt2 promotes Th17 differentiation in both a T cell-intrinsic and T cell-extrinsic manner; Akt2 cKO CD4 T cells had defective IL-17A production in vitro although this defect was milder than that observed in Akt2-/- cells. In vivo, Akt2 cKO splenocytes trended towards decreased cytokine production compared to their WT counterparts after immunization with MOG peptide, suggesting a significant role for Akt2 in non-T cells in shaping the peripheral CD4 T cell response in this model system. Taken together, we identify Akt2 as an important signaling molecule in regulating peripheral CD4 T cell responses. Future studies interrogating the in vivo impact of Akt2 loss in other experimental systems that rely on CD4 T helper cell mediated immunity would provide insight into the contributions of Akt2 to CD4 Th cell differentiation.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Gary A. Koretzky

Second Advisor
Martha S. Jordan

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/1203
Keywords
Akt, CD4 T cells, experimental autoimmune encephalomyelitis, Th17 cells

Subject Categories
Allergy and Immunology | Immunology and Infectious Disease | Medical Immunology

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/1203
THE ROLE OF THE AKT2 ISOFORM IN TH17 DIFFERENTIATION IN VITRO AND PERIPHERAL CD4 T CELL IMMUNE RESPONSES IN VIVO

LAUREN BANKS

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2014

Supervisor of Dissertation

Co-Supervisor of Dissertation

Gary A. Koretzky, M.D., Ph.D.
Adjunct Professor of Medicine

Martha S. Jordan, Ph.D.
Research Assistant Professor of Pathology and Laboratory Medicine

Graduate Group Chairperson

Daniel S. Kessler, Ph.D., Associate Professor of Cell and Developmental Biology

Dissertation Committee

Rebecca G. Wells, M.D. Associate Professor of Medicine
Taku Kambayashi, M.D., Ph.D. Assistant Professor of Pathology and Laboratory Medicine
Paula Oliver, Ph.D. Associate Professor of Pathology and Laboratory Medicine
Dedication

To my parents, Robert Banks and Marge Baker,

and my brothers, Adam and David Banks.
ACKNOWLEDGEMENTS

I would first and foremost like to acknowledge Martha Jordan and Gary Koretzky for not just their dedicated mentorship but also setting an example of profound professional and scientific integrity that I can only hope to live up to. Under their guidance, I have learned to be not just a better scientist but also how to respect the scientific process.

I would also like to acknowledge members of the Koretzky/Jordan Lab who have weathered the storms and “hiccups” with me: Shannon Carty, Mercy Gohil, Rohan Joshi, and Tammarah Sklarz.

I would be remiss if I did not thank and acknowledge Justina Stadanlick for her help with everything; she is nothing short of a lifesaver.

I would also like to thank members the Behrens, Kambayashi, and Oliver Labs who have provided thoughtful and valuable comments and suggestions along the way. In particular, I would like to thank Ed Behrens for his statistical insight. I am also very grateful to the Penn Immunology Flow Core, in particular Ryan Wychowanec, for invaluable technical support.

Lastly, I would like to acknowledge my friends and family for their unyielding support and encouragement. They have had confidence in me especially when it was more challenging for me to have it myself.
ABSTRACT

THE ROLE OF THE AKT2 ISOFORM IN TH17 DIFFERENTIATION 
IN VITRO AND PERIPHERAL CD4 T CELL IMMUNE RESPONSES 
IN VIVO

Lauren Banks
Gary Koretzky
Martha Jordan

Akt1 and Akt2, isoforms of the serine threonine kinase Akt, are essential for T cell development. However, their role in peripheral T cell differentiation remains undefined. Using germline deletions of either Akt1 or Akt2 in mice, we found that while both are important for Th17 differentiation, the impact of Akt2 loss was greater. Mechanistically, while Akt2-/- Th17 cells were predicted by microarray analysis to have suppressed cholesterol biosynthetic and Il-6 signaling pathways, neither pathway emerged as the primary mechanism through which Akt2 contributes to Th17 differentiation. We also interrogated the role of the well-known Akt target, mTORC1, and found that Akt2-/- Th17 cells were equally sensitive to inhibition of the mTORC1-S6K axis as their WT counterparts. However, Akt2 deficiency was associated with elevated Gfi1 mRNA, which encodes for a known inhibitor of Th17 differentiation, and decreased mir155, which encodes for a miRNA that promotes Th17 differentiation. Furthermore, Akt2 loss has significant in vivo consequences and results in a dampened peripheral T cell response to myelin oligodendrocyte glycoprotein (MOG) immunization. This dampened response was associated with altered Th cell differentiation including a
pronounced diminution of inflammatory cytokine production and preferential expansion of regulatory T cells compared to non-T regulatory cells. Using mice with Akt2 loss only in the T cell compartment, Akt2 cKO, we demonstrate that Akt2 promotes Th17 differentiation in both a T cell-intrinsic and T cell-extrinsic manner; Akt2 cKO CD4 T cells had defective IL-17A production *in vitro* although this defect was milder than that observed in Akt2-/- cells. *In vivo*, Akt2 cKO splenocytes trended towards decreased cytokine production compared to their WT counterparts after immunization with MOG peptide, suggesting a significant role for Akt2 in non-T cells in shaping the peripheral CD4 T cell response in this model system. Taken together, we identify Akt2 as an important signaling molecule in regulating peripheral CD4 T cell responses. Future studies interrogating the *in vivo* impact of Akt2 loss in other experimental systems that rely on CD4 T helper cell mediated immunity would provide insight into the contributions of Akt2 to CD4 Th cell differentiation.
# TABLE OF CONTENTS

**DEDICATION** ................................................................. ii

**ACKNOWLEDGEMENTS** ....................................................... iii

**ABSTRACT** ........................................................................ iv

**TABLE OF CONTENTS** .......................................................... vi

- List of Tables........................................................................ ix
- List of Figures.......................................................................... x

**CHAPTER I: INTRODUCTION** .................................................. 1

1.1. Branches of the immune system and their activation ......................... 1

1.2. CD4 T cell activation and differentiation ............................................. 2
   1.2.1. T helper subset definition and function ........................................ 2
   1.2.2. Signaling requirements for CD4 T helper subset differentiation ....... 4
   1.2.3. Transcriptional regulation of CD4 T helper cell differentiation ....... 5

1.3. CD4 T helper cells and immune-mediated diseases.............................. 6
   1.3.1. Airway hyper-responsiveness ...................................................... 6
   1.3.2. Inflammatory bowel disease ....................................................... 7
   1.3.3. Psoriasis ................................................................................ 8
   1.3.4. Rheumatoid arthritis and joint inflammation............................... 9
   1.3.5. Multiple sclerosis ................................................................... 9

1.4. Multiple sclerosis and Th17 cells ...................................................... 10
   1.4.1. Clinical presentation of multiple sclerosis .................................... 10
   1.4.2. Induction and pathophysiology of EAE ...................................... 11
   1.4.3. The use of EAE for identifying treatments for MS ................. 13

1.5. Cell signaling and T cell fate .......................................................... 13
   1.5.1. Cell signaling in T cells ............................................................. 14
   1.5.2. Akt structure and function ....................................................... 15
   1.5.3. Akt in hematopoietic cells ....................................................... 16
   1.5.4. Akt in T helper cell differentiation........................................... 16

**CHAPTER II: GENERAL METHODS** ................................................. 19
CHAPTER III: AKT2 LOSS IMPEDES TH17 DIFFERENTIATION ........................................ 23

3.1. Introduction .................................................................................................................. 23

3.1.1. Signaling requirements for Th17 differentiation ...................................................... 23

3.1.2. Transcriptional regulation of Th17 differentiation .................................................... 25

3.1.3. Akt signaling and Th17 cells ..................................................................................... 28

3.2. Methods ........................................................................................................................ 29

3.3. Results .......................................................................................................................... 31

3.3.1. Akt2 is more important for Th17 differentiation than Atk1 ....................................... 31

3.3.2. Akt2-/- naïve CD4 T cells can polarize towards other Th subsets ......................... 33

3.3.3. Impaired Th17 differentiation in Akt2-/- CD4 T cells is not due to enhanced Treg differentiation ........................................................................................................ 36

3.3.4. Transcription factors important for Th17 differentiation are comparably expressed in Akt2-/- and WT Th17 cells ........................................................................ 37

3.3.5. Akt2 deficiency impairs the expression of Th17 associated cytokines ............ 40

3.3.6. Akt2-/- Th17 cells do not have a defect in proliferation .......................................... 41

3.4. Discussion ..................................................................................................................... 42

CHAPTER IV: INTERROGATING THE MECHANISM THROUGH WHICH AKT2 REGUALTES TH17 DIFFERENTIATION .......................................................... 47

4.1. Introduction .................................................................................................................. 47

4.1.1. mTOR regulation of Th17 differentiation ................................................................. 48

4.1.2. Cytokine signaling required for Th17 differentiation ............................................. 51

4.2. Methods ........................................................................................................................ 52

4.3. Results .......................................................................................................................... 56

4.3.1. Transcriptional profile of Akt2-/- Th17 cells ........................................................... 56

4.3.2. Akt2 does not mediate Th17 differentiation using cholesterol biosynthetic pathway .......................................................................................................................... 58

4.3.3. Akt2 does not promote Th17 differentiation through regulation of the IL-6 signaling pathway .............................................................................................................. 62

4.3.4. Akt2-/- Th17 cells have a trend toward reduced expression of HIF1α targets ........ 65

4.3.5. Akt2-/- Th17 cells are sensitive to mTORC1 inhibition by Rapamycin and S6K inhibition by LyS6K2 ................................................................................................. 67

4.3.6. Akt2-/- CD4 T cells have moderately elevated expression of Gfi1, a negative regulator of Th17 differentiation .................................................................................. 70

4.3.7. Akt2-/- CD4 T cells have moderately elevated expression of mir155 during Th17 differentiation ........................................................................................................ 71

4.4. Discussion ..................................................................................................................... 73
CHAPTER V: AKT2 LOSS DAMPENS PERIPHERAL IMMUNE RESPONSE TO MOG IMMUNIZATION IN VIVO

5.1. Introduction ............................................................................................................... 78
5.2. Methods .................................................................................................................... 80
5.3. Results ...................................................................................................................... 82
   5.3.1. Akt2-/- mice have a dampened peripheral response to MOG immunization ................................ 82
   5.3.2. Akt2-/- mice are not resistant to the development of EAE ........................................... 85
   5.3.3. Akt2 cKO results in modestly impaired in vitro Th17 differentiation but intact peripheral response to MOG immunization in vivo .................................................... 87
5.4 Discussion .................................................................................................................. 90

CHAPTER VI: CONCLUSIONS AND FUTURE DIRECTIONS ............................................................. 95
6.1. Summary of findings ................................................................................................. 95
6.2. The role of Akt2 in CD4 T cell differentiation ........................................................... 97
6.3. Regulation of Th2 differentiation by Akt2 ................................................................. 98
6.4. The role of Akt2 in promoting Th17 differentiation: cell intrinsic vs. cell extrinsic mechanisms ................................................................. 100
6.5. Conclusions ............................................................................................................. 102

REFERENCES .................................................................................................................. 103
LIST OF TABLES

Table 4.1: Canonical pathway analysis........................................................................................................57
Table 4.2: Upstream pathway analysis........................................................................................................57
Table 5.1: EAE clinical score rubric...........................................................................................................86
Table 5.2: Peak clinical score in second set of experiments............................................................87
Table 5.3: Cumulative peak score and incidence..................................................................................87
LIST OF FIGURES

Fig. 3.1: Akt2-/ Th17 cells have impaired IL-17A production.......................... 32
Fig. 3.2: Akt2-/ CD4 T cells efficiently polarize towards Th1 and Th2 cells in vitro... 35
Fig. 3.3: Akt2-/ CD4 T cells do not have enhanced Foxp3 expression or Treg polarization.......................................................... 37
Fig. 3.4: Akt2-/ Th17 cells have an intact Th17 transcriptional program........... 40
Fig. 3.5: Akt2-/ Th17 cells have impaired cytokine expression but no defect in proliferation.......................................................... 42

Fig. 4.1: Akt2 does not regulate Th17 differentiation through the cholesterol biosynthetic pathway.................................................................................. 61
Fig. 4.2: IL-6 signaling is molecularly intact in Akt2-/ Th17 cells.................... 64
Fig. 4.3: Akt2-/ Th17 cells have a trend towards reduced levels of HIF1α target expression.......................................................... 67
Fig. 4.4: Akt2 does not regulate Th17 differentiation solely through the mTORC1 pathway.................................................................................. 69
Fig. 4.5: Akt2-/ CD4 T cells have a modest elevation in Gfi1 mRNA and modest reduction in mir155 mRNA expression........................................... 73

Fig. 5.1: Splenocytes from MOG immunized Akt2-/ mice have defective cytokine production.................................................................................. 83
Fig. 5.2: Akt2-/ splenocytes are hyporesponsive to MOG immunization with preferential expansion of Foxp3+ cells.................................................. 84
Fig. 5.3: Akt2-/ mice have attenuated EAE............................................................ 86
Fig. 5.4: Akt2 cKO mice intermediate defect in IL-17A production in vitro but are responsive to MOG immunization.................................................. 90
1.1. Branches of the immune system and their activation

The immune system has two relatively well-delineated branches, innate and adaptive immunity, that together orchestrate a defense against threats to the well being of an organism. The innate immune system is diverse with many layers, including the physical barriers that demarcate the inside of an organism from the outside, such as skin. However, specific cells of the innate immune system, namely macrophages and dendritic cells, are essential for bridging innate and adaptive immunity and shaping the kind of adaptive immune response that takes place. Innate immune cells are poised to respond to foreign insults without the need for priming, as they express surface molecules known as non-specific pattern recognition receptors (PRR) that detect common molecules associated with pathogens, known as pathogen associated molecular patterns (PAMPs). As such, they act as sentinels that rapidly detect potential hazards and among other functions release molecules such as cytokines and chemokines that help recruit and activate cells of both branches of the immune system. Adaptive immunity draws on antigen-specific responses targeting host defenses against discrete pathogens. Adaptive immune cells require prior education in the periphery as well as additional stimuli from antigen presenting cells, such as dendritic cells, after which they mount a robust counterattack intended to eliminate the offending pathogen while retaining memory for host defense against subsequent encounters.

The adaptive immune system is comprised of the developmentally distinct B and T lymphocytes, which are both derived from a common lymphoid progenitor in the bone marrow. B-lymphocytes, which complete their development in the bone marrow, express an antigen-specific B cell receptor (BCR) whose specificity is further refined in
the periphery after somatic hypermutation. Activated B cells secrete antibodies that bind to and neutralize the corresponding pathogen. This response is also refined in the periphery, as B cells undergo class switching which results in functionally specialized antibodies. The B cell response collectively is referred to as humoral immunity.

T cell precursors leave the bone marrow and home to the thymus to complete their development. One subset of T cells, expressing the αβ T cell receptor (TCR), undergo rigorous positive and negative selection, ultimately producing non-self reactive CD4+ or CD8+ T cells expressing a unique antigen-specific TCR. The vast majority of these cells exit the thymus as naïve T cells that are activated and gain effector function in the periphery in response to two important signals. Signal one is engagement of the TCR with its cognate antigen presented in the context of a major histocompatibility complex (MHC) on the surface of an antigen presenting cell (APC). As this encounter occurs, a naïve T cell must receive a co-stimulatory signal through the binding of CD28 on its surface with either CD80 or CD86 on the surface of the APC to become activated. Without this co-stimulatory signal, T cells become unresponsive (anergic).

1.2. CD4 T cell activation and differentiation

1.2.1. T helper subset definition and function

In addition to signals 1 and 2, the type of effector function that T cells ultimately acquire is modulated by the local cytokine environment generated/provided by cells of the initial innate immune response. As such, naïve CD4 T cells can differentiate into a number of functionally distinct T helper subsets based on the local cytokine milieu upon encountering their cognate antigen and receiving a co-stimulatory signal through CD28. There are several CD4 T cell subsets described to date, but the best characterized are Th1, Th2, Th17, and regulatory T cells (Tregs), each with its own distinct functional niche.
and spectrum of cytokines they produce. Th1 CD4 T cells, important for host defense against intracellular bacteria and viruses, are defined by production of IFNγ, which depends on expression of the Th1 signature transcription factor, T-bet. Th1 cells engage the innate immune system through macrophage activation and contribute to the adaptive immune response through recruitment and activation of cytotoxic CD8+ T cells. The master regulator of Th2 differentiation is the transcription factor GATA3. Some of the important cytokines that Th2 cells produce are IL-4, IL-13, and IL-5, and these cells are crucial for defending against infections by helminthes and extracellular parasites. Th2 cells in turn enhance both adaptive and innate immunity promoting an IgE antibody response from B cells as well as eosinophilia.

Th17 cells and induced-regulatory T cells (Treg), share a developmental axis and are reciprocally regulated (Bettelli et al., 2006). Anti-inflammatory Tregs are identified by the expression of their lineage-specific transcription factor, Foxp3 and immune dampening cytokines. Tregs are essential for containing immune responses and maintaining self-tolerance. Mice deficient in Foxp3 develop profound lethal autoimmunity (Fontenot et al., 2003).

The pro-inflammatory Th17 cells, defined by the production of their effector cytokine, IL-17A, and canonical transcription factor, RORgt, are important for host defense against extracellular bacteria and fungi and are key for neutrophil recruitment. IL-17A mediates protection from pneumococci colonization in mice and mice lacking IL-17A have increased mortality in the setting of infection with Klebsiella pneumonia (Happel et al., 2005; Lu et al., 2008). Segmented filamentous bacteria present in the gut induce a robust IL-17A and Th17 response that is protective against subsequent Citrobacter rodentium infection (Ivanov et al., 2009). Furthermore, mice that do not have the IL-17 receptor have decreased survival in the setting of systemic Candida albicans.
infection (Huang et al., 2004). Thus, induction of an inflammatory environment by extracellular bacteria and fungi can foster Th17 cell differentiation that is important for host defense against these same pathogens.

1.2.2. Signaling requirements for CD4 T helper subset differentiation

The environmental cues important for the peripheral induction and amplification of T helper cell subsets has been well documented. Differentiation of Th1, Th2, and Th17 cells begins with induction of the master transcription factor and lineage cytokine in response to an environmental cytokine signal through a signal transducer and activator of transcription (STAT) family protein followed by a positive feedback loop that amplifies differentiation. Th1 cells differentiate in response to local IL-12, which signals through STAT4 to up-regulate T-bet and IFNγ. IFNγ in turn acts in an autocrine manner to further promote Th1 differentiation primarily by signaling through STAT1. In response to IL-4, STAT5 is activated and promotes the expression of GATA3 and thus initiates Th2 differentiation. IL-4 produced from Th2 cells amplifies differentiation creating a positive feedback loop similar to that seen in Th1 cells.

Paradoxically, despite having opposing functions, both Th17 cells and Tregs differentiate in response to local TGFβ. TGFβ is sufficient to induce Foxp3 expression, which is the lineage-specific transcription factor responsible for Treg differentiation. However, induction of differentiation into Th17 cells requires IL-6 signaling in addition to TGFβ. Complete Th17 differentiation requires sequential IL-6, IL-21, then IL-23 signaling. After initial induction of Rorc (the gene encoding RORγt) in response to TGFβ and STAT3-dependent IL-6 signals, CD4 T cells produce IL-21. IL-21 acts in an autocrine manner similar to IFNγ and IL-4 in Th1 and Th2 cells, respectively, to further promote Th17 differentiation, in part through the up-regulation of the IL-23 receptor. In
response to local IL-23 secreted from dendritic cells, the Th17 phenotype is matured and stabilized. Indeed, evidence connects IL-23 signaling to the up-regulation of IL-22 and GM-CSF in vivo, emphasizing the importance of the later stages of Th17 differentiation in gaining complete effector function (Codarri, Gyülvészi, Tosevski, Hesske, Fontana, Magnenat, Suter, & Becher, 2011a; Zheng et al., 2006). Together, TGFβ, IL-6, IL-21, and lastly IL-23 signaling are thought to lead to mature and terminally differentiated Th17 cells that can produce IL-17F, IL-21, IL-22, GM-CSF.

A role for Il-1b signaling early in Th17 differentiation as also been described. Although the addition of IL-1β is not necessary for in vitro Th17 differentiation of naïve CD4 T cells, enhanced IL-1 signaling in the presence of TGFβ display IL-6 independent induction of Th17 differentiation (J. Chang, Burkett, Borges, Kuchroo, Turka, & Chang, 2013a; Chung et al., 2009; Ikeda et al., 2014). Interestingly, although the differentiation of Th17 cells in humans is similarly dependent on TGFβ and IL-21 or IL-23 are also required, the role of IL-6 is less straightforward. Only in the presence of IL-1β can IL-6 cooperate with TGFβ to induce Th17 differentiation in humans (Manel, Unutmaz, & Littman, 2008; L. Yang et al., 2008a). These data highlight the complex nature of Th17 induction and potentially important distinctions between the differentiation of murine and human peripheral CD4 T cells.

1.2.3. Transcriptional regulation of CD4 T helper cell differentiation

In dissecting the mechanisms that underlie T helper cell differentiation, a complex regulatory landscape has been uncovered such that no subset can be examined in complete isolation, forcing a conceptual shift in our understanding of CD4 T cell differentiation. This is most clearly demonstrated by the transcriptional networks that inform lineage commitment in CD4 T helper cells. Recent work has shown that often,
regulation of one T helper subset also involves simultaneous suppression of
differentiation into other lineages. For instance, Gfi-1, which is up-regulated downstream
of IL-4 signaling and is important for Th2 differentiation and expansion, also inhibits
Th17 and Th1 differentiation through inhibition of IL17a and Ifng expression,
respectively(Ichiyama et al., 2009; Shinnakasu et al., 2008; Zhu, Yamane, & Paul,
2010). GATA3 expression during Th2 differentiation suppresses expression of STAT4,
which is important for Th1 differentiation, but it in turn is suppressed by T-bet during Th1
differentiation (Usui et al., 2003; Usui et al., 2006). T-bet also quells RORγt expression
thereby suppressing the Th17 phenotype. In Th17 cells, RORγt prevents the expression
of Foxp3, as does STAT3 induction downstream of IL-6 signaling. However, in Tregs,
Foxp3 interacts with RORγt inhibiting its function. Thus, the interwoven transcriptional
regulation in CD4 T helper cells underscores the need to more completely define the
mechanisms in place that support T helper cell fate decisions.

1.3. CD4 T helper cells and immune-mediated diseases

1.3.1. Airway hyper-responsiveness

Understanding CD4 T cell differentiation can help reveal potential therapeutic
targets for human disease, as exaggerated T helper responses are implicated in the
pathophysiology of many immune-mediated disorders. Investigations into disease
phenotypes also underscore the complex interplay between CD4 T cell lineages.
Insights into this balancing act have come from studies of airway hyper-responsiveness
(AHR), comprised of diseases such as asthma and COPD that result in airway
inflammation causing obstructive narrowing leading to symptoms such as shortness of
breath and cough. Classically, allergic asthma has been characterized as a Th2-
mediated hyper-IgE disorder associated with eosinophilia. A 1992 study published in
the New England Journal of Medicine examined the cytokine production in the BAL fluid of asthmatic patients (Robinson Douglas S, 2008). The authors concluded that Th2-associated cytokines were elevated compared with normal healthy controls. Studies in murine models of AHR demonstrated that the Th2 cytokine, IL-13, is necessary and sufficient to induce AHR (Walter, McIntire, Berry, McKenzie, Donaldson, DeKruyff, & Umetsu, 2001a). Antigen-specific induction of the IL-13 and another Th2 associated cytokine, IL-5, were also demonstrated to induce an IL-4rα-independent AHR, again, demonstrating the role that Th2 cells play in development of AHR (Webb et al., 2003). Confirming the role of IL-13 in allergic airway disease, a gene-association study found that both IL-13 and IL-4Rα SNPS are associated with asthma and atopy in people (Beghé et al., 2010). However, genome-wide association studies as well as murine models of AHR have also suggested a role for Th17 cells in allergic asthma. An increase in IL-17A production in CD4 T cells from asthmatic patients was seen after in vitro allergen stimulation (Hashimoto, Akiyama, Kobayashi, & Mori, 2005). Work by Kudo et al. showed mice lacking Th17 cells in the lung were resistant to allergen-induced airway hyper-responsiveness, which is consistent with the finding that allergen-sensitization causes Th17 cell recruitment to the lung with associated IL-17A production resulting in AHR in mice (Kudo et al., 2012; Wilson et al., 2009). Thus in both humans and murine models of disease, Th2 and Th17 cells contribute to the development of allergic AHR.

1.3.2. Inflammatory bowel disease

Although a role for Th2 cells in autoimmune inflammatory bowel diseases (IBD) such as ulcerative colitis (UC), has been described, the more important cell types in the immunopathology associated with IBD appear to be Th1 and Th17 cells (Fuss et al., 2004; Heller et al., 2005). IBD is a chronic inflammatory disease of intestinal mucosal
epithelium and is grossly divided into either Crohn’s disease (segmented inflammation involving the full thickness of the intestinal epithelium) or UC (continuous colonic inflammation involving only superficial layers of the intestinal epithelium). Early studies looking at patients with IBD found an increase in the Th1 associated cytokine, IFNγ, in diseased intestinal mucosa (Noguchi et al., 1995). More recently, an examination of cytokine production from ex vivo organ culture of colonic biopsies from IBD patients and normal controls demonstrated an increase in IFNg and Il-17A production in inflamed colon (Rovedatti et al., 2009). A particular role for Th17 cells in the pathogenesis of IBD has been implicated as a result of a genome wide association study that identified variants within the Il23r gene associated with disease (Duerr et al., 2006). Taken together in the context of previous findings of increased IL-17A expression in the serum and mucosa of patients with IBD, it is clear that in humans, Th17 cells are an important player in the development of IBD (Fujino et al, 2003).

1.3.3. Psoriasis

The immunopathology driving the autoimmune skin disease, psoriasis, is also attributed to Th17 cell biology. Psoriasis is characterized by lesions of focal dermal inflammation and acanthosis and is attributed to an aberrantly overactive inflammatory cytokine milieu. Like many organ-specific autoimmune diseases, Th1 cells were thought to be the key cell type responsible for much of the pathology underlying psoriasis due to marked increases in IFNγ found in T cells from both the blood and active lesions of psoriasis patients (Austin et al., 1999). However, a genome-wide study revealed that Il23r, Il23a, and Il12b, all genes associated with IL-23 signaling axis and thus Th17 cells, are associated with psoriasis in humans (Nair et al., 2009). Studies in mice revealed that indeed, IL-23 not only was abundant in psoriatic lesions in humans, but crucial for
the development of dermal inflammation in murine models of induced acanthosis (E. Lee et al., 2004; Zheng et al., 2006). These discoveries have led to successful clinical trials in patients with severe psoriasis treated with antibodies to IL-17.

1.3.4. Rheumatoid arthritis and joint inflammation

Th1 and Th17 cells are also critical in the development of rheumatoid arthritis, a disease characterized by chronic joint inflammation, particularly of synovial membranes, resulting in permanent and debilitating changes in the joint. Even before their recognition as a distinct T helper cell lineage, characterization of IL-17A production in the synovial fluid from patients with rheumatoid arthritis alluded to the importance of Th17 cells in the development of this disease (Chaubaud et al., 1999).

Th1 cells have also been suspected to contribute to the pathophysiology of joint inflammation, as mononuclear cells from the synovial fluid of rheumatoid arthritis patients produced more Th1 associated cytokines than Th2 (Dolhain et al., 1996; Park et al., 2001). However, murine models of experimentally induced arthritis support the finding that Th17 cells remain a key cell type promoting joint inflammation. After the discovery of IL-23 as a heterodimeric protein sharing a subunit with IL-12, one study suggested that IL-23 and IL-12 actually have opposing roles in arthritis, with IL-12 taking on a more protective role and IL-23 a more pathogenic role in autoimmune joint disease (Murphy et al., 2003).

1.3.5. Multiple sclerosis (MS)

Perhaps the relationship between Th17 cells and multiple sclerosis is one of the best characterized of CD4 T helper cells and autoimmune disease. In 1999, after the discovery of human IL-17 but before the appreciation of Th17 cells as a distinct CD4 T
helper cell lineage, it was discovered that the cerebral spinal fluid and peripheral blood of multiple sclerosis patients had increased expression of *Il17a* mRNA (Matusevicius et al., 1999). Th1 cells have also been implicated in promoting multiple sclerosis, as monocytes and lymphocytes from patients with MS were shown to have increased IL-12 and IFNγ, respectively, compared with healthy controls (Comabella et al., 1998). The role of Th17 cells in MS will be discussed in further detail below.

1.4. Multiple sclerosis and Th17 cells

1.4.1. Clinical presentation of multiple sclerosis

Multiple sclerosis is a demyelinating disease characterized by foci of inflammation in the white matter of the CNS that leads to axonal damage and ultimately permanent debilitating neurodegeneration. Clinically, MS is a heterogeneous disease and can enter different phases or shift clinical course over time. The vast majority of patients present with a relapsing-remitting disease in which patients present with acute episodes of neurologic deficits that resolve for a period of time before onset of subsequent episodes affecting a different area of the CNS. It is thought that during this phase, the underlying pathophysiology stems from acute inflammation leading to demyelination and subsequent remyelination. A majority of relapsing-remitting patients transition into a secondary progressive phase where the disease is characterized by incomplete resolution between episodes and advancing debilitation. The hypothesis is that ultimately, the ability for myelin to regenerate becomes exhausted leading to the accumulation of partially resolved lesions and permanent debilitation. As its name suggests, this phase is marked by sclerotic plaques in the white matter of the CNS with the accompanying characteristic neurodegeneration. A third distinct clinical course
experienced by 15-20% of MS patients is primary progressive disease that is similar to secondary progressive disease but without the initial relapsing-remitting phase.

1.4.2. *Induction and pathophysiology of Experimental Allergic Encephalomyelitis (EAE)*

The pathophysiology underlying MS is studied using a well-characterized murine model that ultimately causes a paralytic autoimmune disease mimicking symptoms of human MS. Experimental Allergic Encephalomyelitis (EAE) can be induced by immunization of mice of particular genotypes with defined peptide antigens derived from myelin components that are presented to CD4 cells by Class II MHC. Because this disease is mediated by activated CD4 cells, it is also possible to induce EAE by adoptive transfer of CD4 T cells specific for myelin. There are three commonly used myelin proteins, proteolipid protein (PLP), myelin basic protein (MBP), and myelin oligodendrocyte glycoprotein (MOG), from which the peptides used are derived. In both adoptive transfer and active immunization models, the disease that develops can take on either a classical or non-classical character. Classical EAE mimics spinal cord pathology, and occurs as an ascending paralysis beginning in the tail and eventually affecting the forelimbs. Non-classical EAE presents with ataxia, and symptoms indicating cerebellar and brain lesions. Exactly what drives classical versus non-classical EAE is still in debate, as some have shown that a predominantly Th1-mediated disease results in classical EAE, while a Th17 mediated disease results in non-classical signs of neurological dysfunction (Stromnes, Cerretti, Liggitt, Harris, & Goverman, 2008). However, another group has shown that adoptive transfer of CD4 T cells that produce Th2 cytokines promotes a non-classical EAE (Wensky et al., 2005; Wensky, Marcondes, & Lafaille, 2001).
Regardless, it now accepted that Th17 cells are an important cell type driving the pathology behind EAE, and that IL-17A itself is important for EAE (Komiyama, Nakae, Matsuki, Nambu, Ishigame, Kakuta, Sudo, & Iwakura, 2006a). Furthermore, the EAE model has been helpful in defining the role of IL-23 in Th17 cell biology as a factor important for terminal differentiation and conferring pathogenicity to effector Th17 cells (Cua et al., 2003; Langrish et al., 2005; McGeachy et al., 2009). IL-23 was first reported in 2000, after discovery of a novel protein, p19, which forms a heterodimer with the p40 subunit of IL-12 (Oppmann et al., 2000). Notably, IL-12 is a heterodimer comprised of a p40 and p35 subunit. Thus, mice deficient in IL-23p19 lack IL-23 and mice deficient in IL-12p35 lack IL-12, whereas mice deficient in IL-12-p40 are missing both IL-23 and IL-12. This sparked curiosity throughout the field as to the specific contributions of IL-23 versus IL-12 in autoimmunity and inflammation, given these functionally distinct cytokines share a common subunit, IL-12p40. A seminal study in 2003 documented the importance IL-23 over IL-12 in EAE disease progression by demonstrating that only mice deficient in IL-23p19 or IL-12p40, and not mice deficient in the IL-12-specific IL-12p35, were resistant to EAE after MOG immunization (Cua et al., 2003). Using EAE as a model of in vivo autoimmune inflammation, this novel cytokine, along with Th17 cells, has emerged as an important effector molecule driving autoimmunity in the CNS. While dispensable for in vitro differentiation of murine Th17 cells, it was later shown that IL-23 drives expansion of pathogenic Il-17A producing cells in the setting of PLP immunization and that mice lacking the receptor for IL-23 are not able to generate terminally differentiated Th17 expressing the full cytokine profile of the Th17 lineage (Langrish et al., 2005; McGeachy et al., 2009; L. Zhou et al., 2007). It was also appreciated that IL-23 drives GM-CSF production by Th17 cells thereby increasing their pathogenicity in the CNS (Codarri, Gyülvészi, Tosevski, Hesske, Fontana, Magnenat, Suter, & Becher,
Thus, in addition to being a useful tool allowing for the discovery of targetable elements of MS pathophysiology, EAE has shed valuable light on Th17 cell biology and the key extracellular signals that induce Th17 differentiation.

1.4.3. The use of EAE in identifying treatments for MS

Currently, MS has no cure, but the accepted understanding of the pathophysiology behind accumulating axonal injury and debilitation has lead to pharmacologic treatment approaches aimed largely at reducing inflammation. The concept behind such therapies is that preventing initial myelin and axonal insult will reduce the frequency of demyelination-remyelination cycles, hence delaying the exhaustion of repair mechanisms and thus disease progression. Studies of EAE have informed a number of these efforts, as they have uncovered the role of CD4 T cells in MS. In fact, the use of this model has identified therapeutics now used to treat patients with MS (albeit with limited success), including glatrimar acetate, natalizumab (an antibody to a4b1 integrin that reduces leukocyte trafficking to the CNS, fingolimod (to prevent emigration of lymphocytes from secondary lymphoid organs), cytokine specific antibodies (anti-TNFα and anti IL-12p40, anti-IL-23p19), and anti-CD4 (in order to deplete T cells).

1.5. Cell signaling and T cell fate

Despite our understanding of the environmental cues necessary for T helper cell differentiation, the precise signal transduction pathways through which these signals inform T helper cell fate are not yet fully understood.
1.5.1. Cell signaling in T cells

Considerable work has been done addressing early signals in T helper fate determination. One pathway that has emerged from these studies and now known to be important for Th1, Th2, Th17, and Treg differentiation is the Notch signaling pathway. There are four Notch receptors that are expressed on the cell membrane that bind one of five ligands, which ultimately result in translocation of the Notch intracellular domain to the nucleus where it activates transcription of Notch-dependent genes. Although different Notch ligands have been associated with different T helper cell fates, the exact mechanism by which this signaling pathway induces each distinct T helper cell differentiation is unknown.

However, there is some insight into which signals begin to inform distinct T helper cell fate decisions, such as the specific cytokine signals associated with the differentiation of specific CD4 T cell lineages described above in section 1.2.2. In addition to cytokine signaling, the TCR also plays an important role in early T helper fate decisions. It is known that the strength of the TCR signal, determined in part by its avidity and affinity for its cognate antigen as well as concentration of its cognate antigen, influences peripheral CD4 T cell differentiation. The use of a transgenic TCR on CD4 T cells with two variations of its peptide ligand, one with higher affinity for the TCR than the other, demonstrated that the weaker ligand biases the CD4 T cell towards Th2 differentiation (Tao et al., 1997). Furthermore, using a similar model, the frequency of IL-17A producing cells increased with the concentration of cognate antigen present, suggesting that strong TCR stimulation promotes IL-17A production (Iezzi et al., 2009). Conversely, low doses of peptide appear to foster Foxp3 expression during peripheral Treg differentiation (Turner et al., 2009).

Thus, avidity and affinity of a TCR for its cognate antigen shapes CD4 T cell lineage determination.
TCR signaling is transduced through multiple downstream pathways. Three important pathways are, the mitogen activated protein kinase/extracellular signal-related kinase (MAPK/ERK) pathway, calcium signaling, and the phosphatidylinositol 3 kinase (PI3K)/Akt pathway. While all of these are important for T cell activation, the PI3K/Akt pathway has recently been shown to have a critical role in regulating CD4 T helper cell differentiation, such that understanding the impact of this pathway and Akt on CD4 T cell lineage fate determination has become the focus of an increasing number of studies.

1.5.2. Akt structure and function

Akt is a serine threonine kinase that was discovered as a retroviral proto-oncogene from a murine leukemia virus, AKT8, isolated from the thymi of leukemic mice (Hartley et al., 1977). Akt is important for promoting cell growth, proliferation, metabolism, and survival in many cell types. It has four domains, a pleckstrin homology (PH) domain connected to a kinase domain through an alpha-helical linker and a carboxy-terminal regulatory domain. Downstream of PI3K signaling, Akt is recruited to the membrane through its PH domain and activated through two phosphorylation events: one in the catalytic domain by phosphoinositide-dependent kinase 1 (PDK1) and one in the regulatory domain by mammalian target of Rapamycin complex 2 (mTORC2), which is functionally distinct from mTORC1 (discussed in section 1.5.4).

There are 3 isoforms of Akt (Akt1, Akt2, and Akt3) that are similar but have some isoform-specific functions as suggested by characterization of germline deletions of Akt1 and Akt2. Akt1 deficient mice have a persistent growth defect and remain smaller in size than WT counterparts throughout life (Cho et al., 2001). However, loss of Akt2 (but not Akt1) results in impaired glucose tolerance (Bae, et al., 2003; Cho et al., 2001;
Garofalo et al., 2003). Other isoform specific functions of Akt in different cell types have been described and will be discussed in detail in Chapter 3.

1.5.3. Akt in hematopoietic cells

Loss of both Akt1 and Akt2 has important consequences in the hematopoietic compartment. Lack of both Akt1 and Akt2 in hematopoietic stem cells in mice results in a defect in long-term self-renewal and differentiation into multipotent progenitor cells. This defect was characterized by more cells in the resting phase of the cell cycle as well as increased apoptosis in multipotent progenitor cells (Juntilla et al., 2010). Akt1 and Akt2 double deficient lymphocytes also have defects compared with WT counterparts. B cell precursors fail to develop into marginal zone progenitors resulting in a defect in B cell maturation with an associated dampened proliferative response to B cell receptor engagement (Calamito et al., 2010). It has been shown by multiple groups that deficiency of both Akt1 and Akt2 in thymocytes results in a block in the DN3-DP transition during maturation characterized by increased apoptosis and failure to respond to pre-TCR survival signals (Juntilla et al., 2007; Mao et al., 2007). A role for Akt3 in thymic maturation was less pronounced. It has also been suggested that thymocytes lacking Akt1 and Akt2 have impaired proliferation during b-selection (Mao et al., 2007). Thus, Akt1 and Ak2 are important for proper development within the hematopoietic system.

1.5.4. Akt in T helper cell differentiation

In addition to thymocytes, it has been known for some time that Akt signaling downstream of TCR/co-stimulation is important in mature T cell activation. Constitutively active Akt in T cells promotes TCR/CD28-independent cell growth and increases in
metabolism with associated increases in cytokine production (Rathmell et al., 2003). Consistent with these observations, Akt can specifically induce IL-2 and IFNγ production in CD28-deficient T cells, mimicking effects seen upon co-stimulatory engagement in CD28-sufficient settings (Kane et al., 2001).

More recently, a major role for two well-described targets of Akt, (Foxo proteins and mTORC1) in regulating the Th17/Treg axis has been described. Akt phosphorylates and inhibits Foxo proteins. In T cells, inactivation of Foxo proteins has important consequences for the differentiation of Tregs, as they are critical for the induction of Foxp3 in response to Treg polarizing conditions in vitro. This process was suggested to be Akt-dependent (Harada et al., 2010). Consistent with this finding, Foxo1/3a deficiency also causes defective Treg generation resulting in a lethal systemic inflammatory disorder (Ouyang et al., 2010). With respect to the Akt target, mTORC1, CD4 T cells lacking mTOR (the component shared between mTORC1 and mTORC2) are unable to differentiate into any effector Th cell, but have increased polarization towards Tregs (Delgoffe et al., 2009; Haxhinasto, Mathis, & Benoist, 2008). However, mice deficient in mTORC1 assembly have a functional defect in Tregs despite normal Foxp3 expression, indicating that despite increased numbers of Tregs in mTOR deficient mice, assembly of mTORC1 is required for normal Treg function (Zeng et al., 2013).

These data suggest that the PI3K/Akt pathway is a critical regulator of Treg differentiation and thus the Th17/Treg axis.

A specific contribution of Akt to Th17 differentiation has also been suggested. In human CCR6+ memory T cells, pharmacologic inhibition of either PI3K (upstream of Akt) or Akt itself represses the expression of IL-17A (Wan et al., 2011). Additionally, pharmacologic inhibition of PI3K in murine CD4 T cells impairs in vitro differentiation of Th17 cells (Kurebayashi et al., 2012). Evidence also suggests that mTORC1 promotes
Th17 differentiation, as mice lacking active mTORC1 have impaired Th1 and Th17 differentiation (Delgoffe, Pollizzi, Waickman, Heikamp, Meyers, Horton, Xiao, Worley, & Powell, 2011a). mTORC1-specific inhibition using the drug Rapamycin impairs HIF1α-dependent induction glycolytic genes that promote Th17 differentiation when naïve CD4 T cells are cultured in Th17 polarizing condition (Shi et al., 2011). Taken together, this evidence suggests that Akt may be an important signaling molecule in Th17 differentiation that links engagement of cell surface receptors with transcriptional changes associated with CD4 T helper cell differentiation.

The overall goal of my thesis research was to further interrogate how Akt regulates the differentiation and function of Th17 cells. Based on the observations that Akt inhibits Treg differentiation, inhibition of Akt impairs the ability of human CCR6+ memory T cells to produce IL-17A, and CD4 T cells lacking active the Akt target mTORC1 have a defect in Th17 differentiation, I hypothesized that Akt positively regulates Th17 differentiation. Similarly, I hypothesized that Akt deficiency would impair Th17 differentiation, and that this defect would result in dampened Th17-mediated immune responses in vivo. In the subsequent chapters, I describe both in vitro and in vivo experiments I performed to test these hypotheses and provide evidence that one Akt isoform, Akt2, is crucial for Th17 differentiation in vitro and peripheral CD4 T cell responses in vivo.
Chapter II: General Methods

**Mice**

Akt1\(-/-\) and Akt2\(-/-\) mice were generated on a C57BL/6 background in the laboratory of MJ Birnbaum and described previously (Cho et al., 2001; Cho et al., 2001). Wild type mice were C57BL/6, were bred in our colony or purchased from Jackson Laboratories.

All experiments were performed in accordance with guidelines provided by the University of Pennsylvania Institutional Animal Care and Use Committee under the supervision of the University Laboratory Animal Resources.

**Cell culture**

Cells were cultured at 37°C in IMDM media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin/glutamine (GIBCO cat # 10378-016), and 50µM β-mercaptoethanol. Lymphocytes were harvested from spleen and lymph nodes. Naïve CD4 T cells (CD4+ CD62L\(^{hi}\) CD44\(^{lo}\) CD25\(^{-}\)) were then sorted (experiments involving intracellular flow cytometry, western blots, real-time PCR, or ELISA) or isolated using Miltenyi MACS naïve CD4 T cell isolation kit as per manufacturer’s instructions (for experiments involving real-time PCR). Naïve CD4 T cells were cultured at 2x10\(^6\) cells/ml on plate-bound anti-CD3 (1µg/ml) and anti-CD28 (5µg/ml) in Th17 (20ng/ml IL-6, 5ng/ml TGFβ, 10µg/ml anti-IL-4, 10µg/ml anti-IFNγ for 3.5 days unless otherwise noted) polarizing conditions. Before cytokine analysis by flow cytometry or quantitative real-time PCR, *in vitro* cultures were then cultured in the presence of phorbol-12-myristate-13-acetate (50ng/ml), ionomycin (500ng/ml), and GolgiStop protein transport inhibitor with monensin (BD Pharmingen cat # 554724, 4µl/6ml) for 3-5 hours at 37°C.
Antibodies

Surface staining for flow cytometry: PE-Cy7 anti-CD4 (clone RM4-5, BioLegend); PE-Texas Red anti-CD8 (clone 5H10, Invitrogen); Pacific Blue anti-CD8 (clone 53-6.7, BioLegend); APC-efluor780 anti-TCRβ (clone H57-597, eBiosciences); APC anti-NK1.1 (clone PK136, eBiosciences); APC anti-γδTCR (clone eBioG13, eBiosciences), AF700 anti-CD44 (clone IM7, BioLegend); APC anti-CD62L (clone MEL-14, BD Pharmingen); PE anti-CD25 (clone PC61.5, eBiosciences); PE anti-IL-6ra (clone D7715A7, BioLegend); PE Rat IgG2bx isotype control (Life Technologies). Intracellular staining for flow cytometry: efluor660 and PE anti-IL-17A (ebio17B7, eBiosciences); Percp-Cy5.5 IFNγ (clone XMG1.2, BioLegend); APC, Pacific Blue, and FITC anti-Foxp3 (FJK-16s, eBiosciences).

Flow cytometry

Live cells were identified by forward vs side scatter properties or with LIVE/DEAD Fixable Aqua Dead Cell stain according to manufacturer's instructions (Molecular Probes). Briefly, LIVE/DEAD stain was diluted 1:600 in PBS and incubated with cells for 15 minutes. Cells were washed then incubated with antibodies specific for surface proteins for 20 minutes. Intracellular staining was performed using, eBiosciences Foxp3 Transcription Factor Staining Buffer according to manufacture’s instructions (cat # 00-5523-00). All incubations were performed in the dark at 4°C in FACS buffer (PBS, 2% FBS, 0.02% azide). Cells were analyzed on a BD LSRII Flow Cytometer. Analysis was performed using FlowJo2.
RNA extraction/cDNA synthesis

RNA was isolated using the QIAgen RNeasy Mini Kit (QIAgen, cat # 74104). cDNA was synthesized in 20 µl reactions with 500µM dNTP, 50ng random hexamers, 11µl RNA (50-500ng per reaction), 40U RNaseOUT, 200U SuperScript III, using SuperScript III, 50mM DTT, First-Strand reaction Buffer. Briefly, dNTP, RNA, and random hexamers were mixed and incubated at 65°C for 5 minutes then left on ice for 1 minute. SupserScript III, RNaseOUT, DTT and First-Strand reaction buffer were added and the reaction was incubated at 50°C for 1 hour. SuperScript III was inactivated at 85°C for 5 minutes.

Real-time PCR

Real-time PCR was performed using TaqMan Gene Expression Assays from Life Technologies on ViiA7 Real-Time PCR System. Gene-specific primer and probe sets were purchased from Life Technologies and TaqMan Fast Gene Expression Universal PCR Master Mix was used. Relative quantity was calculated using the comparative CT method (ΔΔCT). Briefly, samples were normalized to β-actin and then set relative to average WT values within each experiment.

Statistical analysis

Paired Student’s T test was used to analyze intracellular flow cytometry for cytokine production of paired observations in in vitro differentiation experiments. For compiled RT-PCR and ELISA data, one sample T test was used to compare ΔΔCT or cytokine amounts to a theoretical mean of 1 or 100 as indicated in figure legend. For drug treatments during in vitro Th17 differentiation experiments, 2-way ANOVA with matching
was used. Student’s T test was used to compare individual ELISA experiments and
where indicated. Statistical analyses were performed using GraphPad PRISM software.
Chapter III:  Akt2 loss impedes Th17 cell differentiation in vitro

3.1. Introduction

3.1.1. Signaling requirements for Th17 differentiation

Two independent groups simultaneously described Th17 cells as a distinct subset of CD4 T helper cells in 2005. Park et al described CD4 T cells from MOG-immunized mice that produced either IL-17A or IFNγ, but not both (H. Park et al., 2005). Harrington et al. described an IL-17 response to in vitro culture of CD4 T cells with IL-23, which was abrogated by the addition of IFNγ (Harrington et al., 2005). Addition of IL-4 to these cultures similarly impaired IL-17A production, even in the presence of anti-IFNγ, suggesting that these cytokines individually inhibited IL-17A production (Harrington et al., 2005). Both groups observed that culturing CD4 T cells in the presence of anti-IFNγ and anti-IL-4 enhanced IL-17A production in vitro (Harrington et al., 2005; H. Park et al., 2005). Together, these findings suggest that unique cytokine signals, distinct from those that induce Th1 or Th2 cells, promote IL-17A production in vitro.

Additionally, the signal transduction and transcriptional requirements of IL-17A production from CD4 T cells are independent of those from Th1 and Th2 cells. STAT proteins are activated in response to specific cytokine stimulation and traffic to the nucleus where they induce transcriptional changes promoting CD4 Th differentiation. STAT4 is downstream of IL-12, which promotes Th1 differentiation, and STAT6 is downstream of IL-4, which promotes Th2 differentiation. Both Park and Harrington described IL-17A production despite the lack of STAT4 or STAT6. Park et al. found that splenocytes from WT, STAT6 deficient, or STAT4 deficient mice immunized with MOG peptide produced similar amounts of IL-17A (Harrington et al., 2005; H. Park et al.,
Harrington et al. found that CD4 T cells from TCR transgenic mice lacking either STAT4 or STAT6 stimulated *in vitro* with their cognate antigen in the presence of IL-23 and anti-IFNγ produced comparable amounts of IL-17A to STAT4 and STAT6 sufficient mice. This group went on to demonstrate that IL-17A production from CD4 T cells was also STAT1 (downstream of IFNγ signaling) and T-bet (the master regulator of Th1 differentiation) independent (Harrington et al., 2005). Collectively, these observations suggest that transcriptional regulation IL-17A producing CD4 T cells is different than that of Th1 or Th2 cells. Given that these cells appeared to have distinct regulation from that of Th1 or Th2 cells, it was speculated that the IL-17A producing cells represented a novel T helper cell lineage.

Later, the specific cytokine signals that promote Th17 differentiation were identified. It was discovered that TGFβ is required for Th17 differentiation, as culturing naïve CD4 T cells in the presence of TGFβ and conditioned media obtained from LPS-stimulated dendritic cells could induce IL-17A production (Mangan et al., 2006; Veldhoen et al., 2006). Since the presence of TGFβ alone up-regulates Foxp3, the lineage-specific transcription factor in Tregs, this observation suggested that another stimulus must function in concert with TGFβ to induce IL-17A. A study describing the reciprocal relationship of Treg and Th17 found that indeed, IL-6 in combination with TGFβ promoted the differentiation of Th17 cells over Tregs (Bettelli et al., 2006).

After initial induction of the Th17 differentiation program, additional signals are required to expand and maintain the differentiating population. Signaling by TGFβ and IL-6 in Th17 cells promotes the expression of IL-21, which then induces the expression of the IL-23 receptor. IL-21 upregulation is critical for Th17 differentiation, evidenced by impaired *in vitro* Th17 differentiation in CD4 T cells from IL-21 deficient mice. These mice also exhibit delayed onset of EAE with attenuated disease (Korn et al., 2007;
Nurieva et al., 2007). IL-23 signaling is essential for the terminal differentiation of Th17 cells, as in the absence of IL-23 receptor Th17 cells have diminished effector function (McGeachy et al., 2009).

Fully differentiated Th17 cells produce not only IL-17-A, but also IL-17F, IL-21, IL-22, and GM-CSF. IL-17A and IL-17F have similar functions, as both are important for neutrophil activation, promoting their recruitment and production of inflammatory cytokines and chemokines. The main role of IL-21 in Th17 cells is to reinforce Th17 differentiation, as it is necessary and sufficient to induce Th17 differentiation in IL-6-/mice (Korn et al., 2007; L. Zhou et al., 2007). IL-22, although not unique to Th17 cells, is induced by IL-23 signaling and has been shown to be an important mediator of inflammation in the skin (Zheng et al., 2006). Also not unique to Th17 cells is the cytokine GM-CSF, which is induced in vitro in the presence of IL-23 and even more so in the presence of anti-IFNγ and Th17 polarizing conditions. Thus, in the context of IL-17A, IL-17F, and IL-21, GM-CSF is considered a Th17 associated cytokine. GM-CSF is an important cytokine for the effector function of Th17 cells, and is involved in the pathogenesis of EAE by sustaining monocyte and dendritic cell mediated neuroinflammation. It is through this panel of cytokines that Th17 cells are able to carry out their effector function, as such, together they are considered Th17-associated cytokines.

3.1.2. Transcriptional regulation of Th17 differentiation

Coordinating the response of CD4 T cells to the inflammatory cytokine milieu that induces Th17 associated cytokines in Th17 cells is the lineage-specific retinoic acid-related orphan receptor (ROR) transcription factor, RORγt. RORγt is induced in Th17 cells after in vitro culture with TGFβ and IL-6. Cells deficient in RORγt fail to express IL-
17A but have intact Th1 polarization, and overexpression of RORγt leads to a specific increase in IL-17A. Mice lacking RORγt have late onset attenuated EAE compared with RORγt sufficient controls (Ivanov et al., 2006). Another ROR protein, RORα, is also more highly expressed in Th17 cells compared with Th1 or Th2 cells (X. O. Yang et al., 2008b). RORα overexpression closely phenocopies overexpression of RORγt with respect to Th17 differentiation. However, while RORα deficiency results in impaired IL-17A expression, the loss is not as dramatic as that observed for RORγt deficiency and some Th17 cytokines are not affected by the loss of RORα (X. O. Yang et al., 2008b).

These data have contributed to the designation of RORγt as the “master” transcription factor of Th17 differentiation as opposed to RORα. Despite this distinction, the absence of both RORα and RORγt completely abrogated Th17 differentiation in mice and conferred complete resistance to EAE, and the combined overexpression of both RORα and RORγt led to synergistic increase in IL-17A production (X. O. Yang et al., 2008b). Thus, both RORγt and RORα are important for Th17 differentiation and act in a synergistic fashion to induce IL-17A expression.

Although not lineage-specific, other transcription factors have emerged as important molecules regulating Th17 differentiation. One such factor is interferon-regulatory factor 4 (IRF4), which is a critically important transcription factor for Th2 differentiation, and induces expression of the Th2 lineage specific transcription factor, GATA3 (Tominaga et al., 2003; Lohoff et al., 2002). However, IL-17A production is completely abrogated in IRF4−/− mice (Brüstle et al., 2007). These mice are resistant to the induction of EAE characterized by less CD4 T cell infiltration of the brain and a specific decrease in IL-17A production in CD4+ splenocytes after immunization with (MOG). Notably, in vitro Th1 differentiation in these mice resulted in a 50% reduction in IFNγ production compared with heterozygous controls. This suggests that IRF4 could
also play a role in Th1 differentiation, but the relevance of IRF4 deficiency in *in vivo* Th1 differentiation is still unclear, as IRF4-/- mice have normal levels of peripheral IFNγ production in response to MOG immunization (Brüstle et al., 2007). Regardless, although not specific to Th17 cells, IRF4 is regarded as an essential transcription factor in Th17 differentiation.

BATF is yet another transcription factor whose expression is not specific to Th17 cells but whose activity is essential to Th17 differentiation. Although highly expressed in Th1, Th2, and Th17 cells, BATF deficient mice have normal Th1 and Th2 differentiation *in vitro* but impaired IL-17A production when cultured in Th17 polarizing conditions. Similar to IRF4 deficient mice, BATF deficient mice are completely resistant to the induction of EAE (Schraml et al., 2009). Interestingly, IRF4 and BATF do not act in isolation; ChIP-Seq experiments suggest that deficiency in BATF results in less IRF4 binding at the *Il17a* locus, and deficiency in IRF4 results in less BATF binding at the *Il17a* locus (Ciofani et al., 2012; Li et al., 2012).

Another transcription factor that has a role in Th17 differentiation is the Aryl hydrocarbon receptor (Ahr). Ahr is a transcription factor whose activation and function is determined upon ligand binding. Ahr is a cytoplasmic receptor that binds molecules composed of aromatic hydrocarbons that are both endogenous and exogenous and upon activation it translocates to the nucleus. Ahr is up-regulated in Th17 cells in response to TGFβ and IL-6, and its expression in the context of the ligand 6-formylindolo[3,2-b]carbazole (FCIZ) can robustly induce IL-17A and IL-22 expression (Duarte et al., 2013; Quintana et al., 2008; Veldhoen et al., 2008). Interestingly, in the presence of another well-characterized Ahr ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), there is induction of Treg differentiation at the expense of Th17 differentiation (Quintana et al., 2008). Thus, Ahr is an example of the extracellular
environment (type of Ahr ligand) directly informing CD4 helper cell differentiation due to the ligand-dependent nature of its activation.

3.1.3. Akt signaling and Th17 cells

Despite our growing understanding of the transcriptional regulation of Th17 differentiation, the precise signal transduction pathways that integrate environmental cues with this transcriptional program are not fully understood. Numerous studies have been performed to identify the key signaling intermediates downstream of antigen or cytokine receptor engagement that may control effector T cell lineage determination. A number of these studies have pointed to Akt, a serine threonine kinase that is activated downstream of TCR, CD28, and IL-6 signaling, as a candidate. Early on, Akt was shown to be essential for stem cell survival and thymic development (Juntilla et al., 2007; Juntilla et al., 2010; Mao et al., 2007). Subsequently, suppression of IL-17A production through pharmacologic inhibition of the PI3K/Akt axis in both human CCR6+ memory T cells and murine CD4 T cells suggests a role for this kinase in Th17 differentiation specifically, as described in Chapter 1 (Kurebayashi et al., 2012; Wan et al., 2011).

Three isoforms (Akt1, Akt2, and Akt3) have been described, each encoded by separate genes and each differentially expressed in tissues. Although Akt isoforms are structurally and often functionally similar with 80% amino acid sequence identity, increasing evidence has emerged suggesting isoform specific functions. Akt1 and Akt2 have differential subcellular localization after insulin stimulation, with an enrichment of Akt2 at the plasma membrane (Gonzalez et al., 2009). Additionally, loss of Akt2, and not Akt1, in mouse embryonic fibroblasts results in enhanced motility and migration through extracellular matrix in response to platelet-derived growth factor (PDGF) stimulation (G. L. Zhou et al., 2006). Also suggesting isoform-specific functions of Akt, deficiency in only
the Akt1 isoform in neonatal thymocytes demonstrated a similar block in thymocyte maturation (Fayard et al., 2007). Thus, given the accumulating evidence of isoform-specific functions of Akt, and that Akt1 and Akt2 (and to a lesser extent Akt3) are important for thymocyte development, the contribution of each isoform to mature T cell function has not been fully defined and is of increasing interest.

This chapter will explore the hypothesis that Akt is important for in vitro Th17 differentiation, and that there are isoform specific contributions of Akt1 and Akt2 (given their dominant role in thymic development) to Th17 differentiation. In vitro differentiated Th17 cells derived from mice with a germline deletion of either Akt1 or Akt2 were compared, and while there was a defect in IL-17A production seen in both genotypes compared to wild type (WT) counterparts, the impact of Akt2 loss was much greater. As such, we focused on the role of Akt2 in Th17 differentiation and sought to characterize the defect in Th17 differentiation in the setting of Akt2 deficiency. We found that Akt2−/− Th17 cells had reduced expression of Th17 lineage cytokines but retained normal expression of key transcription factors essential to Th17 differentiation.

3.2. Methods

In vitro Th polarization culture conditions

Naïve CD4 T cells isolated as in Chapter 2, then cultured at 2x10^6 cells/ml on plate-bound anti-CD3 (1µg/ml) and anti-CD28 (5µg/ml) in Th17 (20ng/ml IL-6, 5ng/ml TGFβ, 10µg/ml anti-IL-4, 10µg/ml anti-IFNγ for 3.5 days), Th1 (IL-12, 10µg/ml anti-IL-4 for 3.5 days), Treg (1ng/ml TGFβ for 2.5 days), or Th2 (10ng/ml IL-4, 5µg/ml anti-IL-12 for 4.5 days) polarizing conditions.
ELISA

For *in vitro* differentiation experiments, supernatants were collected either 3.5 days after culture in Th17 polarizing conditions for analysis by ELISA. ELISAs were performed using Ready-Set-Go ELISA kit (eBiosciences) as per manufacturer's instructions. Samples were read on Spectramax M2e Plate Reader and analyzed using SoftMax Pro Software.

CFSE labeling

CFSE labeling kit was purchased from Inivtrogen Life Sciences, and CFSE dye was reconstituted in 18ul sterile DMSO. CFSE media was prepared by adding CFSE dye at 1:500 to serum free IMDM. Lymphocytes were harvested from the spleen and lymph nodes and then washed three times with serum free IMDM media after red blood cell lysis. Cells were then resuspended at 2 x 10^7 cells/ml in serum free IMDM, then an equal volume of CFSE media was added in the dark. Cells were incubated at room temperature and then one-half the volume of FBS was added to quench the dye such that the total volume was then 3x the initial volume resuspended in. Cells were then washed twice with TCM and enriched for CD4 T cells using Miltenyi MACS CD4 T cell isolation kit as per manufacturer's protocol. CD4 T cells were labeled with anti-CD4, anti-CD8, anti-CD25, anti-CD44, and anti-CD62L and naïve CD4 T cells sorted and collected using FACS Aria. Cells were then cultured in Th17 polarizing conditions (as described in Chapter 2) and then analyzed by flow cytometry.

Real-time PCR

The following TaqMan Gene Expression Assays were used and purchased from Life Technologies: Mm00439618_m1 (*Il17a*), Mm00521423_m1 (*Il17f*), Mm01290062_m1
(Csf2), Mm00519943_m1 (Il23r), Mm00444241_m1 (Il22), Mm00445259_m1 (Il4), Mm00484683_m1 (Gata3), Mm01261022_m1 (Rorc), Mm00479410_m1 (Baff), Mm00516431_m1 (Irf4), Mm00478932_m1 (Ahr), Mm01306721_ma (Ikzf3), Mm00607939_s1 (Actb).

3.3. Results

3.3.1. Akt2 is more important for Th17 differentiation than Akt1

To study the role of Akt in Th17 differentiation, we cultured sorted naïve CD4+ T cells from WT, Akt1-/-, and Akt2-/- mice in the presence of Th17 polarizing cytokines. We first examined relative levels of Il17a message using RT-PCR after 72 hours in Th17 polarizing conditions. We found that both Akt1-/- and Akt2-/- Th17 cells had less Il17a compared with WT, though this decrease was not statistically significant (Fig. 3.1A). However, given the trend towards less IL-17A in Akt1-/- and Akt2-/- Th17 cells, we next examined if Akt loss affected cytokine protein levels. We therefore examined IL-17 protein produced after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of a Golgi transport inhibitor. The combination of PMA and ionomycin robustly stimulates T cells and induces rapid cytokine expression at both the mRNA and protein levels. The addition of a Golgi transport inhibitor prevents secretion of the cytokines made by the cell during stimulation, allowing for detection with the use of intracellular flow cytometry. Using this methodology, we found that under Th17 polarizing conditions, CD4 T cells from both Akt1-/- and Akt2-/- mice generated lower frequencies of IL-17A producing cells compared with cells from WT mice that reached statistical significance as per paired Student’s T-test (Fig. 3.1B); however, Akt2-/- Th17 cells produced markedly less IL-17A compared with both WT and Akt1-/- Th17 cells.
Given the significant impact of Akt2 deficiency on IL-17A production, we focused more intensely on the role of this isoform in Th17 differentiation. Our previous assessment of IL-17A production was restricted to a single point in time. Our next experiments measured IL-17A production over time in Akt2−/− compared to WT cells. For these studies, we measured secreted IL-17A in the supernatant of CD4 T cells cultured in the presence of Th17 polarizing conditions for 4 days. The defect in IL-17A production in Akt2−/− Th17 cells compared with WT was even more pronounced when secreted IL-17A was measured by ELISA (Fig. 3.1C). Taken together, these data suggest that while both Akt1 and Akt2 are important for Th17 differentiation, Akt2 deficiency has a greater impact on IL-17A protein production than Akt1, a defect that becomes more pronounced over the duration of differentiation.
3.3.2. Akt2 deficient naïve CD4 T cells are able to polarize towards other T helper subsets

Given impaired IL-17A production in Akt2-/ Th17 cells, we next asked if the requirement for Akt2 was unique to Th17 cells or rather indicative of an inability of Akt2-/ naïve CD4 T cells to differentiate into T helper cells of any lineage. Naïve CD4 T cells from WT and Akt2-/- mice were cultured in the presence of Th1 polarizing cytokines for 3.5 days and then restimulated with PMA and ionomycin in the presence of a Golgi transport inhibitor for 5 hours. Production of IFNγ was assessed using intracellular flow cytometry. We found that Akt2-/- cells produced a comparable frequency of IFNγ+ cells in the presence of Th1 polarizing conditions compared with WT (Fig. 3.2A). Interestingly, despite similar levels of IFNγ production, we noticed an increase in survival of Akt2-/- cells compared with WT after culture in these conditions (Fig. 3.2B).
We also evaluated the ability of Akt2/- naïve CD4 T cells to polarize towards Th2 cells \textit{in vitro}. The relative levels of \textit{Il4} and \textit{Gata3} mRNA were evaluated after culturing cells in Th2 polarizing conditions for 5 days followed by stimulation with PMA and ionomycin to boost the production \textit{Il4} message, as little was found in the absence of this stimulus. Under Th2 conditions, there was a trend towards increased \textit{Il4} and \textit{Gata3} mRNA in Akt2/- Th2 cells compared with WT, although these differences did not reach statistical significance (Fig. 3.2C and 3.2D). Although cell viability and proliferation was not measured, by cell counts there were more Akt2/- than WT cells recovered despite similar input, suggesting that there may be enhanced proliferation or survival of Akt2/- CD4 T cells cultured in Th2 polarizing conditions (data not shown). Taken together, these data suggest that despite a defect in Th17 differentiation, Akt2/- CD4 T cells are capable of polarization to other Th subsets \textit{in vitro}, and may perhaps survive better than WT cells in non-Th17 polarizing conditions.
Figure 3.2: Akt2−/− CD4 T cells efficiently polarize towards Th1 and Th2 cells in vitro

(A) Flow cytometry analysis of WT and Akt2−/− cultured Th1 (3.5 days) polarizing conditions (n = 4 independent experiments), cells gated on live CD4+ lymphocytes. Closed squares represent WT frequency of IFNγ+ cells and open circles represent Akt2−/− frequency of IFNγ+ cells. Solid line connects paired experiments. (B) Flow cytometry analysis of Live/Dead aqua stain WT (closed squares) and Akt2−/− (open circles) cells cultured in Th1 polarizing conditions for 3.5 days cells. Cells gated on lymphocytes. P values calculated by paired Student’s T test. Akt2−/− (white bar) and WT (black bar) cells were cultured in Th2 polarizing conditions for 4.5 days and (C) Il4 (n = 6 independent experiments) and (D) Gata3 (n = 5 independent experiments) were measured by RT-PCR. CT’s were normalized to βactin then set relative to WT (RQ = 1), graph shows mean ±SEM.
3.3.3. **Impaired Th17 differentiation in Akt2-/- CD4 T cells is not due to enhanced Treg differentiation**

Inasmuch as Th17 and regulatory T cells (Tregs) cells are known to be reciprocally regulated, we investigated whether defective IL-17A production from Akt2-/- cultures was due to augmented Treg differentiation. We assessed levels of Foxp3 (the canonical transcription factor for Tregs) in WT and Akt2-/- Th17-skewed cells and found both populations expressed similarly low levels of Foxp3 indicating that the diminished IL-17 production in Akt2-/- cultures was not due to enhanced Treg differentiation (Fig. 3.3A).

However, to fully characterize the role, if any, of Akt2 in regulating the Th17-Treg axis, it was necessary to independently evaluate Treg differentiation in Treg polarizing conditions. Importantly, it was still possible that in the absence of IL-6, Akt2-/- CD4 T cells may have an increased propensity to become Tregs compared with WT cells such that the *in vitro* Th17 culture conditions may mask a subtler defect in suppressing Foxp3 expression when IL-6 signaling is limiting. To address this question, naive Akt2-/- and WT CD4 T cells were cultured in the presence of TGFβ alone, which promotes the differentiation of Tregs measured by Foxp3 expression. Akt2-/- and WT CD4 T cells similarly up-regulated Foxp3 in these conditions, suggesting that Akt2-/- CD4 T cells do not have enhanced capacity to become Tregs, which would reciprocally impair their ability to become Th17 cells (Fig. 3.3B). Interestingly, similar to *in vitro* polarization towards Th1 cells, there was enhanced survival of Akt2-/- Tregs compared with WT cells in these conditions (Fig. 3.3C).
3.3. Akt2−/− CD4 T cells do not have enhanced Foxp3 expression or Treg polarization

(A) Representative flow cytometry analysis of sorted WT and Akt2−/− naïve CD4 T cells cultured in Th17 polarizing conditions for 3.5 days, gated on live CD4+ lymphocytes (n = 20 independent experiments). (B) Flow cytometry analysis of WT and Akt2−/− cultured in either Treg polarizing conditions for 2.5 days polarizing conditions (n = 4 independent experiments), cells gated on live CD4+ lymphocytes. Closed squares represent WT frequency of Foxp3+ cells and open circles represent Akt2−/− frequency of Foxp3+ cells. Solid line connects paired experiments. (C) Flow cytometry analysis of Live/Dead aqua stain WT (closed squares) and Akt2−/− (open circles) cells cultured in Th1 polarizing conditions for 3.5 days cells. Cells gated on lymphocytes. P values calculated by paired Student’s T test.

3.3.4. Transcription factors important for Th17 differentiation are comparably expressed in Akt2−/− and WT Th17 cells

Given the defect in IL-17A protein production in Akt2−/− cells, we next investigated the impact of Akt2 loss on the Th17 transcriptional program with the expectation that Akt2−/− cells would have reduced expression of important transcription
factors essential for normal Th17 differentiation. TGFβ and IL-6 signaling induce Th17 differentiation through the up-regulation of RORγt, the canonical Th17 transcription factor responsible for initiating and regulating Th17 differentiation. In its absence, there is a specific defect in *in vitro* polarization towards Th17 cells, and overexpression of RORγt is sufficient to induce IL-17A production (Ivanov et al., 2006). However, RORγt is important for induction of Th17 differentiation as well as maturation and expression of the full Th17 cytokine profile, therefore, differences in *Rorc* mRNA at any time point could influence Th17 differentiation (Codarri et al., 2011a). Hence, to capture both induced and sustained *Rorc* levels, we performed a time course experiment evaluating *Rorc* mRNA in WT and Akt2/−/− CD4 T cells after 12, 24, 36, and 48 hours in Th17 polarizing conditions. Akt2/−/− cells up-regulated *Rorc* after 12 hours and maintained comparable if not slightly elevated levels of *Rorc* mRNA throughout differentiation when compared to WT cells (Fig. 3.4A). We performed similar analysis on *Irf4* and found its expression was also comparable to WT at 12 hours, and elevated thereafter, reaching and maintaining statistical significance at 24 hours (Fig. 3.4B). Given the cooperative relationship between BATF and IRF4 in induction of *Il17a*, we also measured the level of BATF in Akt2/−/− and WT cells after 48 hours in Th17 polarizing conditions. Similar to *Rorc* and *Irf4*, we saw an increase in *Batf* in Akt2/−/− cells compared with WT, which was statistically significant (Fig. 3.4C).

The temporal transcriptional profile of *Ahr* was more complicated. *Ahr* is robustly induced in both WT and Akt2/−/− cells after 12 hours in Th17 polarizing conditions. By 48 hours, however, *Ahr* levels begin to fall and does so faster in WT cells than Akt2/−/− cells (Fig. 3.4D). Thus, by 48 hours, there is a statistically significant elevation in *Ahr* expression in Akt2/−/− Th17 cells compared with WT.
Recently, a member of the Ikaros transcription factor family encoded by the *Ikzf3* gene, Aiolos, was reported to be up-regulated specifically in Th17 in part by *Ahr*, and was induced by the Ahr ligand FICZ known to promote Th17 differentiation. Aiolos deficiency resulted in impaired expression of *Il17a* and overexpression of Aiolos was sufficient to induce both *Rorc* and *Il17a* expression (Quintana et al., 2012). To determine if Akt2 deficiency impaired Th17 differentiation by reducing Aiolos expression, we measured *Ikzf3* expression after 48 hours in Th17 polarizing conditions, which is sufficient time for up-regulation of this gene in Th17 cells (Quintana et al., 2012). Consistent with normal if not elevated expression levels of *Ahr* in Akt2-/- Th17 cells, we found that Akt2-/- and WT cells expressed comparable levels of *Ikzf3*, suggesting that Akt2 does not promote Th17 differentiation through modulation of Aiolos expression (Fig. 3.4E).

Taken together, these data suggest that the transcriptional regulatory network is largely intact in Akt2-/- Th17 cells, as transcription factors known to be essential for Th17 differentiation are expressed at levels comparable if not elevated relative to WT Th17 cells. This was surprising, as the defect in IL-17A production seen in Akt2-/- Th17 cells would similarly predict a defect in the Th17 transcriptional program. However, it is still possible that although they are expressed in Akt2-/- Th17 cells, the function of these transcription factors is impaired preventing them from promoting IL-17A production.
3.3.5. Akt2 deficiency impairs the expression of Th17 associated cytokines

In addition to IL-17A, mature Th17 cells also produce IL-17F, IL-22, and GM-CSF and up-regulate the IL-23 receptor (IL-23R) as they differentiate. To more fully characterize the effect of Akt2 deficiency on Th17 differentiation, we measured *Il17f*, *Il22*, *Csf2* (the gene that encodes GM-CSF), and *Il23r* mRNA levels in cells cultured for 2.5 days in Th17 polarizing conditions followed by several hours of stimulation with PMA and ionomycin to boost the cytokine signal. We found that the levels of *Il17f*, *Csf2* and *Il23r* in Akt2-/- Th17 cells were significantly reduced compared with WT cells (Fig. 3.5A). In contrast, WT and Akt2-/- Th17 cells had comparable *Il22* expression (Fig. 3.5A),
although even in WT cells expression levels of *Il22* were very low compared with other cytokines and at times not detectable. The low level of *Il22* induction could be due to the fact that the Th17 polarizing conditions used do not include IL-23, as IL-23 signaling has been demonstrated to be important for the induction of IL-22 in a murine model of Th17-mediated acanthosis Th17 cells (Zheng et al., 2006). These data indicate that Akt2 is required for many, but not all aspects of Th17 differentiation.

### 3.3.6. Akt2 -/- Th17 cells do not have a defect in proliferation

Akt2 has been shown to be important for *in vitro* proliferation of numerous cell types including lymphocytes (Calamito et al., 2010; Juntilla et al., 2010; Skeen et al., 2006), which could account for the reduction in IL-17A in Akt2-/- cells. Thus, to determine if the diminished IL-17A production was merely the result of a proliferation defect we labeled cells with CFSE before culture in Th17 polarizing conditions. This technique allows the measurement of proliferation by flow cytometry. CFSE is a fluorescent dye that is diluted upon cell division, thus increased CFSE dilution marked by lower fluorescence in the CFSE channel is indicative of increased proliferation. In these experiments, CFSE is diluted synchronously, such that fluorescent peaks are generated with each cell division and a higher number of peaks denote further cell division and thus more proliferation. Both WT and Akt2-/- cells exhibited similar dilution of CFSE after *in vitro* differentiation, indicating that Akt2-/- Th17 cells do not have impaired cell division under these conditions (Fig. 3.5B). The slightly offset histograms are likely due to the fact that the initial labeling between the two samples is modestly different. These data indicate that the defect in measured IL-17A production is not due impaired proliferation in the setting of Akt2 deficiency.
3.4. Discussion

In this chapter, I describe an important role for Akt in in vitro Th17 differentiation. Interestingly, while both Akt1 and Akt2 are important for optimal Th17 cell differentiation, loss of Akt2 appears to have a more profound impact on this cell fate determination. We therefore focused on the role of Akt2 in key aspects of Th17 cell differentiation. For these experiments, we initially compared WT and Akt2−/− CD4 T cells in in vitro differentiation assays that drive cells towards the Th1, Th2, Th17 and Treg lineages. We found that Akt2 deficiency specifically impairs Th17 differentiation, preserving the propensity of Akt2−/− naïve CD4 T cells to polarize towards Th1, Th2, and regulatory T cells in vitro. The impact of Akt2 loss on Th17 differentiation was also characterized by
diminished mRNA expression of some Th17 associated cytokines, but surprisingly left expression of the regulatory transcriptional network necessary for Th17 differentiation intact.

Although Akt2 appeared to be more important for Th17 differentiation than Akt1, we still saw a statistically significant decrease in IL-17A production in Akt1/-/-Th17 cells compared with WT. One potential explanation is that there is a non-isoform-specific requirement for Akt kinase activity and both isoforms target the same substrates, but differential subcellular localization of the two isoforms reads out as a greater reliance on Akt2 than Akt1 for Th17 differentiation. In this scenario, Akt2 would more frequently come in contact with the necessary targets to promote Th17 differentiation than Akt1, resulting in a great impact of Akt2 loss on Th17 differentiation. There is evidence that suggests isoform-specific subcellular localization of Akt in adipocytes after insulin signaling, mediated in part by the PH domain of the proteins (Gonzalez et al, 2009). It is plausible that there is also isoform specific localization of Akt in T cells following TCR engagement or after signaling via key cytokine receptors. Future experiments could make use of isoform-specific antibodies to visualize and compare subcellular localization of Akt1 and Akt2 in both resting naïve CD4 T cells and naïve CD4 T cells stimulated with TGFβ and IL-6.

However, it is also possible that the effective kinase activity of Akt1 and Akt2 may not be equivalent. For example, Akt1 and Akt2 may have different affinities for particular substrates, making one isoform more influential for Th17 cell differentiation than the other. It is also possible that there may be unique substrates of Akt1 and Akt2 that differentially contribute to Th17 differentiation. Perhaps there is a greater requirement for substrates of Akt2 than those of Akt1, resulting in a more pronounced defect in Th17 differentiation in the setting of Akt2 deficiency. Future studies identifying
the direct targets and protein interactions of Akt1 and Akt2 would provide insight into mechanisms of isoform-specific functions of Akt.

Although we identified a defect in cytokine production and expression in Akt2−/− Th17 cells compared with WT, surprisingly, there was no defect in expression of key transcription factors for Th17 differentiation. One would expect that as Akt2−/− Th17 cells appear to have the necessary requirements for appropriate transcriptional regulation of Th17 differentiation, they would express similar levels of Th17 associated cytokines compared with Akt2 sufficient cells. A defect in IL-17A production in the setting of normal Rorc expression raises the possibility that it is not the induction of transcription factors that is altered in Akt2−/− cells but their activity. Often, transcription factors act cooperatively to promote gene expression, as seen in the case of BATF and IRF4. There may be a co-factor for RORγt activity that is suppressed, or similarly a transcriptional repressor that is overexpressed, in the absence of Akt2.

However, this does not explain the elevated levels of Batf, Irf4, and Ahr seen in Akt2−/− cells after 48 of culture in Th17 polarizing conditions. Given the defect in differentiation, it is possible Akt2−/− Th17 cells lack a negative feedback signal that reduces expression of Th17 associated transcription factors when they are no longer needed after induction of Th17 differentiation. Such a mechanism could be in place to reduce the likelihood of immunopathology after induction of a Th17 response. Thus, WT cells are able to appropriately reduce expression of Th17 promoting transcription factors, and failure to do so in Akt2−/− cells results in elevated levels of Batf, Irf4, and Ahr mRNA. In this scenario, the altered levels of transcription factor expression in Akt2−/− cells are a consequence of impaired Th17 differentiation rather than a cause.

Expression of Irf4 is not limited to Th17 cells, and it is essential for Th2 differentiation. Given the trend towards increased Il4 and Gata3 expression in Akt2−/−
Th2 cells and the elevated Irf4 expression in Akt2-/- Th17 cells, it is possible that increased Irf4 expression could enhance the propensity for Akt2-/- cells to polarize towards Th2 at the expense of Th17 differentiation. This shift towards Th2 differentiation away from Th17 differentiation could have significant effects in vivo where naïve CD4 T cells are educated in a more complex cytokine milieu. Further characterization of Th2 differentiation in Akt2-/- cells is warranted, as protein levels of Th2 lineage cytokines and survival of in vitro differentiated Th2 cells would more definitively address the question of whether or not Akt2-/- CD4 T cells are better poised to become Th2 cells than WT.

Our in vitro data suggest that the lack of Akt2 may have significant consequences in vivo. Not only is there a defect in effector cytokine production in Th17 cells, but also we found enhanced survival of Akt2-/- Th1 and Treg cells compared with WT. Preferential survival of different T helper lineages in vivo could cause important shifts in the balance of CD4 T helper cells resulting in altered peripheral immune responses in Akt2-/- mice. Although still debated, there is evidence to suggest that the balance between Th1 and Th17 responses could influence the clinical outcome in EAE (Stromnes et al., 2008). A study of collagen-induced joint inflammation in mice demonstrated that IL-12, a potent inducer of Th1 differentiation, protects from immune-mediated joint disease whereas IL-23, important for the maturation and stability of Th17 differentiation, promotes joint inflammation. Thus, a change in the balance of peripheral CD4 T cells could heavily influence the inflammatory environment and as a consequence, both host defense as well as immunopathology. The impact of Akt2 deficiency on in vivo responses to an antigenic challenge will be described in Chapter 5 of this thesis.

Additionally, our in vitro data do not suggest a perturbation of the Th17/Treg axis, although differential survival in the Treg compartment could shift this balance in vivo.
resulting perhaps in reduced susceptibility of Akt2-/- mice to autoimmune inflammation. Thus, it is possible that Akt2 loss has a more striking impact in vivo than was uncovered in in vitro polarization experiments; again, this possibility will be explored in Chapter 5. Taken together, we have identified an important role for Akt in promoting Th17 differentiation in vitro, yet the mechanism by which it does so and the effects in vivo are not known and will be explored in the next chapter (Chapter 4).
Chapter IV: Interrogating the mechanism through which Akt2 regulates Th17 differentiation

4.1. Introduction

Given the role that Akt2 plays in promoting Th17 differentiation, understanding the signal transduction pathways regulated by Akt2 in Th17 cells is important. As Th17 cells drive a variety of autoimmune diseases, identifying the molecular signals underlying their development could reveal therapeutic targets enabling the modulation of Th17 effector responses. To help identify Akt-dependent pathways required for Th17 differentiation, we used two approaches: an unbiased microarray approach and a candidate approach.

The goal of an unbiased microarray approach is to take a broad view of transcriptional changes between two populations, which in this case are WT and Akt2-/- CD4 T cells in Th17 polarizing conditions. Using comparisons of the gene expression signatures between these populations, patterns of differentially regulated genes are identified and compared to known expression profiles associated with signaling pathways. Rather than interrogating specific pathways, this approach broadens the analysis allowing for the identification of pathways not previously appreciated as being regulated by Akt or contributing to Th17 differentiation. However, in experiments broadly analyzing different populations, such as a microarray, it is important to keep in mind the cellular input. In this case, not only are the cells we are analyzing cells from mice of different genotypes, they also have a known difference their capacity for Th17 differentiation which itself may cause important transcriptional changes. Thus, in these experiments, it is important to consider whether observed transcriptional changes cause...
the defect in differentiation appreciated in one genotype, or whether these differences reflect the decreased frequency of differentiation in this genotype. In other words, it may be challenging to distinguish between gene expression patterns that may cause impaired Th17 differentiation in Akt2-/- cells and those that are the result of impaired Th17 differentiation.

Therefore, in addition to a microarray approach, we used a candidate approach to investigate how Akt2 may be regulating Th17 differentiation. As such, we interrogated a well-documented Akt target, mTORC1, as well as important upstream activators of Th17 differentiation, such as cytokine signaling.

4.1.1. mTOR regulation of Th17 differentiation

A well-documented target of Akt signaling that regulates peripheral T cell differentiation is the mechanistic target of Rapamycin (mTOR). CD4 T cells deficient in mTOR fail to differentiate into effector T helper cells but have enhanced Treg differentiation (Delgoffe et al., 2009). There are two complexes that contain mTOR, mTORC1 and mTORC2, which differ in their binding partners. mTORC1 consists of mTOR and the protein Raptor, while mTORC2 consists of mTOR and the protein Rictor (Kim et al., 2002). The difference in these binding partners is sufficient to confer distinct functional properties on the two complexes. mTORC2, classically considered Rapamycin-insensitive, is upstream of Akt and phosphorylates this kinase in its regulatory domain, which may be necessary for full kinase activity (Sarbassov et al., 2005). In the context of T helper cell differentiation, mTORC2 is essential for Th2 differentiation, but dispensable for Th17 differentiation (Lee et al., 2010; Delgoffe et al., 2011). Conflicting reports leave the role of mTORC2 in Th1 differentiation unresolved. However, these studies used different mechanisms to delete Rictor in T cells, which may
in part explain the discrepancies between their findings (Lee et al., 2011; Delgoffe et al., 2009). mTORC1 is a well described Rapamycin-sensitive downstream mediator of Akt signaling that binds to and phosphorylates initiation factor 4B binding protein (4EB-P1) and ribosomal protein S6 kinase (S6K), important for regulating protein translation as well as proliferation, survival, and growth of cells (Kim et al., 2002; Nojima et al., 2003). It has been demonstrated that mTORC1 specifically regulates Th1 and Th17 differentiation. Akt phosphorylates tuberous sclerosis complex 1 (TSC), which is a negative regulator of the small GTPase Rheb that activates mTORC1, therefore, mice lacking Rheb do not have functionally active mTORC1. Indeed, CD4 T cells from these mice have impaired Th17 differentiation, marked by decreased IL-17A and RORγt production, as well as Th1 differentiation. Interestingly, CD4 T cells from Rheb deficient mice have enhanced Th2 differentiation (Delgoffe et al., 2011). These mice are resistant to the classical symptoms of EAE and develop atypical EAE upon immunization with MOG peptide.

Exactly how mTORC1 regulates Th17 differentiation is not fully understood, but recent work has suggested that mTORC1 signaling can inform T helper cell differentiation through its role in up-regulating glycolysis, a process crucial for effector T cell differentiation (Michalek et al., 2011). mTORC1 signaling can increase the translation and activity of hypoxia-inducible factor 1α (HIF1α), an important molecule for regulating glycolysis and cell metabolism (Land & Tee, 2007; Treins, 2002). Though classically associated with oxygen-sensing and promoting cell survival under conditions of oxygen deprivation, HIF1α transcriptional activity is also important for Th17 differentiation, even under normoxic conditions. Induced by STAT3 downstream of IL-6, Rapamycin-sensitive HIF1α expression is increased in Th17 cells compared with other
CD4 Th cells. HIF1α deficient Th17 cells have diminished IL-17A production, attributed to impaired RORγt expression and function (Dang et al., 2011; Shi et al., 2011).

Another mechanism through which mTORC1 may inform Th17 differentiation is through S6K signaling. Recent work has suggested that downstream of mTORC1, S6K may regulate Th17 differentiation in two ways: (1) S6K promotes nuclear localization of RORγt through direct protein-protein interactions and (2) S6K up-regulates of Egr2 expression which in turn suppresses expression of Gfi1, a known antagonist to Th17 differentiation (Kurebayashi et al., 2012). Gfi-1 is a transcription factor transiently induced by TCR signaling. However, its expression is sustained T cells cultured in the presence of IL-4, where it acts to sustain GATA3 expression, promote proliferation, and prevent apoptosis in Th2 cells (Zhu et al., 2002; Shinnakasu et al., 2008). Gif-1 also functions as a suppressor of Th17 differentiation, through inhibition of RORγt activity and IL-17A and IL-17F expression (Ichiyama et al., 2009; Zhu et al., 2009). In contrast to WT CD4 T cells cultured in Th2 polarizing conditions, Gfi-1 deficient cells have histone modifications associated with active transcription at the Rorc and Il23r loci (Zhu et al., 2009).

Lastly, mTORC1 may influence Th17 differentiation and the Th17/Treg axis is through its regulation of lipid metabolism. Downstream of mTORC1, cholesterol biosynthesis can be up-regulated through activation of the transcription factor SREBP1, which coordinates this tightly regulated metabolic process, through both S6K dependent and independent mechanisms (Owen et al., 2012). Evidence suggests that mTORC1-mediated cholesterol and lipid metabolism is important for differentiation and functionality of Tregs (Zeng et al., 2013). Also suggesting that cholesterol biosynthesis may regulate the Th17/Treg axis is the finding that inhibition of cholesterol synthesis impaired in vitro differentiation of Th17 cells (Kagami et al., 2009). In vivo,
administration of atorvastatin, a cholesterol lowering drug, was shown to ameliorate paralysis associated with EAE as well as incidence of relapse (Aktas et al., 2003). Taken together, accumulating evidence suggests that the well known downstream mediator of Akt signaling, mTORC1, may have a mechanistically diverse but very important role in regulating the differentiation of Th17 cells.

4.1.2. Cytokine signaling required for Th17 differentiation

In addition to investigating the effectors of Akt signaling, a full appreciation of how Akt may influence Th17 differentiation also requires consideration of key modulators of CD4 lineage determination, such as cytokine signaling, that lie upstream of Akt may regulate this kinase. Given that naïve CD4 T cells differentiate into Th17 cells in response to TGFβ and IL-6 signaling, these are the major cytokines that could lead to activation of Akt during Th17 differentiation. Naïve CD4 T cells differentiate into Th17 cells after exposure to both TGFβ signaling and IL-6 signaling. Downstream TGFβ signaling is largely mediated by receptor activated Smad proteins (Smad2 and Smad3) that translocate to the nucleus and regulate TGFβ-dependent gene expression. However, no strong direct link between TGFβ signaling and Akt activation has been established in T cells.

In contrast, inflammatory cytokine signaling activates Akt in T cells and mTORC1 signaling has been shown to be important for STAT3 activation, a key molecule activated by IL-6 signaling. IL-6 signals through a heterodimeric receptor composed of IL-6Rα and gp130 (also known as IL6ST) expressed on naïve CD4 T cells. Activation of IL-6R leads to the phosphorylation of STAT3. STAT3 is a transcription factor that both induces and works in concert with RORγt to initiate the Th17 transcriptional program, and thus differentiation. STAT3 also induces the expression of suppressor of cytokine
signaling 3 (SOCS3), which inhibits phosphorylation of STAT3 creating a negative feedback loop. IL-21 and IL-23, cytokines important for the maturation of Th17 cells, also signal through STAT3, making this an important signaling molecule and transcription factor in Th17 differentiation. Importantly, mice lacking active mTORC1 demonstrate reduced phosphorylation of STAT3 in response to IL-6 stimulation with a concomitant increase in SOCS3 expression. Thus, given the relationship between STAT3 activation and IL-6 as well as STAT3 phosphorylation and mTORC1 signaling, IL-6 emerges as a strong candidate through which Akt may be regulating Th17 differentiation.

In this chapter, we use both unbiased and candidate approaches to investigate signaling pathways through which Akt2 may be informing Th17 differentiation. We performed a microarray on Akt2−/− and WT cells cultured in Th17 polarizing conditions to reveal potential signaling pathways that are differentially regulated in Akt2−/− versus WT cells. We also interrogated the role of mTORC1, a well described target of Akt, in Th17 differentiation based on its demonstrated role in T helper cell differentiation. However, neither of these approaches identified a single mechanism by which Akt2 regulates Th17 differentiation. Thus, the role of Akt2 in Th17 differentiation is likely multifactorial, as it does not appear to be exclusively dependent on any single common signaling pathway or cellular process downstream of Akt. However, Akt2 loss does result in modestly elevated expression of Gfi1, an known inhibitor of Th17 differentiation, and modestly reduced expression of miR-155, a miRNA important for Th17 differentiation.

4.2. Methods

Microarray

MACS isolated naïve CD4 T cells were cultured in Th17 polarizing conditions for 36 hours and RNA was isolated using RNeasy Mini Kit (QIAgen) as per manufacturer’s
instructions. Samples were hybridized to Affymetrix Mouse Gene Array 2.0ST. For data analysis, a fold change >.25, false discovery rate (FDR) <0.2 (Benjamini-Hochberg) using 3-way ANOVA followed by post-test comparison was used to generate a list of differentially expressed genes (DEG). This list was used for canonical pathway analysis module with p <0.01 by Fischer exact test. DEG list was also used for upstream analysis pathway module after performing Fischer exact test, p < 0.05, considering only cellular proteins and excluding drugs, miRNAs, and synthetic molecules. Significant Z-activation score was < -2 or > 2.

Filipin staining

Filipin III from Streptomyces filipinensis was purchased from Sigma (cat# F4767-1MG) and reconstituted at 12.5 mg/ml in dimethyl sulfoxide (DMSO). Less than 5X10^5 cells were cultured in Th17 polarizing conditions and washed three times with DPBS-/- and fixed in 4% PFA in DPBS-/- on ice for 12 minutes. Cells were then washed 3 times with DPBS-/- and incubated in 100μl of Filipin diluted at 1:50 in FACS buffer and sealed quickly with parafilm and placed at 37°C in the dark for 1 hour. Cells were then washed twice with FACS buffer and analyzed on BD LSRII Flow Cytometer. Filipin was read in the 450/50 channel using the violet laser on the flow cytometer.

Statin/FTI/GGTI experiments

Simvastatin was purchased from Sigma (product #S6196-5MG) and reconstituted in DMSO at 10mM). FTI-276 and GGTI-298 were purchased from Calbiochem (cat #344550 and 345883 respectively) and reconstituted at 5mM in DMSO.

For simvastatin experiments, FACS sorted Naïve CD4 T cells were cultured in Th17 polarizing in the presence of simvastatin (1μM) or vehicle control (DMSO at 1:10,000).
For FTI and GGTI experiments, sorted naïve CD4 T cells were cultured as above but in FTI (5µM), GGTI (5µM), or vehicle control (DMSO at 1:1000).

**Rapamycin and LyS6K2 experiments**

FACS sorted Naïve CD4 T cells were cultured on plate-bound anti-CD3 (1µg/ml) and anti-CD28 (5µg/ml) for 18 hours then cultured in the presence of Th17 polarizing conditions and Rapamycin (25ng/ml, Calbiochem cat # 553210), LyS6K (3µg/ml, generous gift from Dr. Birnbaum), or vehicle control (DMSO at 1:500) for 2.5 days before cytokine analysis by intracellular flow cytometry.

**Phosphoflow**

Cells were harvested, immediately pelleted then resuspended in 200ul BD Phosflow lyse/fix buffer (BD Pharmingen, cat # 558049) pre-warmed to 37°C and placed at 37°C for 10 minutes. Cells were then washed twice with FACS buffer and incubated in surface stains as described above. Cells were then washed and permeabilized in BD permwash (BD Pharmingen, cat # 554723) for 30 min. Next, cells were incubated in anti-pS6 antibody (Cell Signaling #4856) at 1:100 in BD permwash for 1hour followed by 1hour incubation with anti-rabbit secondary.

**Western**

Cells (5x10^5) were lysed in 1% NP40 containing 1mM NaFl, 1mM NaVanadate, 1mM PMSF, 1µM NaPyrophosphate, protease inhibitor cocktail (1:100; Sigma product # P8340) and run on NuPage Novex 4-12% Bis-Tris gel and transferred to hybond membrane. Anti-pSTAT3 (1:1000, Cell Signaling #9145) and anti-β-Tubulin (1:1000, Cell Signaling #2146) were used with donkey anti-rabbit IgG conjugated with IRDYE
680RD (LiCor, cat # 926-68073) secondary antibody. Western blotting was performed using the Odyssey System and band intensities analyzed using LiCor software. Anti-Socs3 (1:500, Cell Signaling #2932) and anti-β-Actin (1:10,000 Sigma A5441) were used with HRP-conjugated goat anti-mouse IgG (Bio-Rad, cat # 170-6516) and goat anti-rabbit IgG (Bio-Rad, cat # 170-6515) secondary antibodies. Western blotting was performed using Thermo ECL substrate and imaged on film. Band intensities were analyzed using ImageJ software.

Real-time PCR

The following TaqMan Gene Expression Assays were used and purchased from Life Technologies: Mm00468869_m1 (Hif1a), Mm00413385_m1 (Hk2), Mm00441480_m1 (Glut1), Mm01612132_g1 (Ldha), Mm00607939_s1 (Actb).

miRNA Real-time PCR

miRNA was isolated using miRNeasy Kit (QIAGen, cat # 217084) and cDNA synthesized using TaqMan MicroRNA Reverse Transcription Kit using MultiScribe Reverse Transcriptase (Life Technologies, cat # 4366956) as per manufacturer’s instructions. Real-time PCR was performed using TaqMan Fast Gene Expression Universal PCR Master Mix was used. Relative quantity was calculated using the comparative CT method (ΔΔCT). Samples were normalized to U6 snRNA and then set relative to average WT values within each experiment. The following TaqMan Gene Expression Assays were used and purchased from Life Technologies for both cDNA synthesis and real-time PCR: 001806 (mir155), 001973 (U6 snRNA control).
4.3. Results

4.3.1. Transcriptional profile of Akt2-/- Th17 cells

To better understand the consequences of Akt2 deficiency in the setting of \textit{in vitro} Th17 differentiation, we used microarray analysis to make unbiased comparisons of the transcriptional landscape in Akt2-/- and WT cells that had been cultured in Th17 polarizing conditions. RNA extracted from naïve CD4 T cells that had been cultured in Th17 polarizing conditions for 36 hours was used for the microarray to capture the effects of signals that initiate Th17 differentiation. Using the Canonical Pathway Analysis module in Ingenuity Pathway Analysis (IPA) we found that a number of pathways important for cholesterol biosynthesis were differentially affected by culture in Th17 polarizing conditions in Akt2-/- cells compared with WT. In particular, multiple pathways that involve geranylgeranyldiphosphate and mevalonate, both intermediate metabolites of cholesterol synthesis, were among those affected (Table 4.1). Consistent with perturbation of this pathway, SREBF1 and SREBF2 (transcription factors that mediate cholesterol biosynthesis) were predicted to be inhibited as indicated by Upstream Analysis (IPA). Together, these data suggested that in Th17 cells, Akt2 might be important in regulating cholesterol biosynthesis, a process previously implicated in Th17 and Treg cell differentiation (See section 4.1.1)

Upstream Analysis also predicted that IL-6 signaling, a cytokine signaling candidate important for Th17 differentiation, was globally suppressed in Akt2-/- cells compared with WT (Table 4.2), suggesting that Akt2-/- CD4 cells may have a diminished response to IL-6 signaling, resulting in impaired Th17 differentiation. In contrast, TGFβ signaling did not appear to be globally suppressed as per upstream analysis. Lastly, Upstream Analysis also predicted that the transcriptional response to HIF1α might be suppressed in Akt2-/- Th17 cells, indicating that Akt2 deficiency could either diminish the
expression of this transcription factor or interfere with its activity. Given the established role for HIF1α in promoting Th17 differentiation, this suggests that Akt2 may promote Th17 differentiation through the transcriptional activity of HIF1α.

Table 4.1: Canonical pathway analysis

<table>
<thead>
<tr>
<th>Ingenuity Canonical Pathways</th>
<th>-log(p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)</td>
<td>5.43E+00</td>
</tr>
<tr>
<td>Superpathway of Cholesterol Biosynthesis</td>
<td>3.73E+00</td>
</tr>
<tr>
<td>Geranylgeranyldiphosphate Biosynthesis</td>
<td>3.41E+00</td>
</tr>
<tr>
<td>Trans, trans-farnesyl Diphosphate Biosynthesis</td>
<td>3.41E+00</td>
</tr>
<tr>
<td>Mevalonate Pathway I</td>
<td>2.76E+00</td>
</tr>
<tr>
<td>T Helper Cell Differentiation</td>
<td>2.65E+00</td>
</tr>
<tr>
<td>EIF2 Signaling</td>
<td>2.50E+00</td>
</tr>
<tr>
<td>Tec Kinase Signaling</td>
<td>2.49E+00</td>
</tr>
<tr>
<td>Wnt/b-catenin Signaling</td>
<td>2.37E+00</td>
</tr>
<tr>
<td>Role of Lipids/Lipid Rafts in the Pathogenesis of Influenza</td>
<td>2.16E+00</td>
</tr>
<tr>
<td>GM-CSF Signaling</td>
<td>2.10E+00</td>
</tr>
<tr>
<td>Glycogen Degradation III</td>
<td>2.02E+00</td>
</tr>
</tbody>
</table>

Table 4.2: Upstream pathway analysis

<table>
<thead>
<tr>
<th>Inhibited Upstream Regulator</th>
<th>p-value of overlap</th>
<th>Activation z-score</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT4</td>
<td>3.33E-09</td>
<td>-4.524</td>
</tr>
<tr>
<td>IL6</td>
<td>3.17E-02</td>
<td>-3.587</td>
</tr>
<tr>
<td>ATF4</td>
<td>1.69E-03</td>
<td>-3.506</td>
</tr>
<tr>
<td>HIF1A</td>
<td>3.98E-04</td>
<td>-3.47</td>
</tr>
<tr>
<td>IL1B</td>
<td>4.36E-03</td>
<td>-3.276</td>
</tr>
<tr>
<td>IFNA2</td>
<td>3.49E-02</td>
<td>-2.776</td>
</tr>
<tr>
<td>FOXO1</td>
<td>8.48E-03</td>
<td>-2.692</td>
</tr>
<tr>
<td>SREBF1</td>
<td>5.73E-04</td>
<td>-2.632</td>
</tr>
<tr>
<td>MYCN</td>
<td>8.70E-05</td>
<td>-2.61</td>
</tr>
<tr>
<td>SREBF2</td>
<td>1.47E-02</td>
<td>-2.588</td>
</tr>
<tr>
<td>IL11</td>
<td>1.13E-02</td>
<td>-2.415</td>
</tr>
<tr>
<td>HGF</td>
<td>1.04E-02</td>
<td>-2.373</td>
</tr>
<tr>
<td>TNF</td>
<td>2.93E-04</td>
<td>-2.352</td>
</tr>
<tr>
<td>CD40LG</td>
<td>2.36E-06</td>
<td>-2.325</td>
</tr>
<tr>
<td>BRCA1</td>
<td>5.59E-04</td>
<td>-2.321</td>
</tr>
<tr>
<td>ATP7B</td>
<td>3.39E-02</td>
<td>-2.236</td>
</tr>
<tr>
<td>VEGFA</td>
<td>1.05E-02</td>
<td>-2.233</td>
</tr>
<tr>
<td>DTX1</td>
<td>2.38E-03</td>
<td>-2.213</td>
</tr>
<tr>
<td>PPARC1B</td>
<td>2.43E-06</td>
<td>-2.209</td>
</tr>
<tr>
<td>CD38</td>
<td>2.24E-05</td>
<td>-2.083</td>
</tr>
<tr>
<td>SIRT2</td>
<td>3.24E-03</td>
<td>-2</td>
</tr>
<tr>
<td>HBEGF</td>
<td>4.43E-02</td>
<td>-2</td>
</tr>
<tr>
<td>EIF2AK2</td>
<td>4.72E-02</td>
<td>-2</td>
</tr>
<tr>
<td>MNT</td>
<td>1.04E-02</td>
<td>2</td>
</tr>
</tbody>
</table>
4.3.2. Akt2 does not mediate Th17 differentiation using cholesterol biosynthetic pathway

Since cholesterol biosynthesis seemed to be negatively affected by Akt2 deletion in Th17 cells and the mTORC1-S6K axis can stimulate cholesterol biosynthesis through activation of SREBPs that regulate genes important for cholesterol and lipid metabolism, we interrogated this pathway in both WT and Akt2-/- Th17 cells (Owen et al, 2012). Statins are a class of cholesterol-lowering drugs that inhibit the enzyme that catalyzes the rate-limiting step in cholesterol synthesis, HMG-CoA reductase. Simvastatin has been shown to impair Th17 differentiation in human CD4+ lymphocytes as well as inhibit secretion of Th17 polarizing cytokines in dendritic cells from multiple sclerosis patients (Zhang et al., 2008). Given the identified role of simvastatin in Th17 differentiation in human CD4 T cells, its regulation by mTORC1 activity (Owen et al., 2012), as well as the suppressed cholesterol biosynthetic transcriptional program identified in our microarray of Akt2-/- Th17 cells, we investigated if there was a gross defect in cholesterol synthesis in Akt2-/- Th17 cells. After culture in Th17 polarizing conditions, WT and Akt2-/- Th17 cells were stained with filipin, a fluorescent polyene macrolide antibiotic that specifically binds unesterified (free) cholesterol found in cell membranes previously used to identify membrane cholesterol in lymphocytes (Muller et al., 1984; Karnell, 2005). WT and Akt2-/- Th17 cells had similar levels of filipin binding (Fig. 4.1A) as measured by flow cytometry. Although filipin staining suggested similar membrane cholesterol content in WT and Akt2-/- cells, not all cholesterol species are bound by filipin, resulting in some limitations of filipin staining that reduce its sensitivity as a measure of cholesterol metabolism. First, esterified cholesterol does not form a complex with filipin, and thus excess stored cholesterol is not detected by this method (Muller et al., 1984). Second, it has been reported that not all free cholesterol is susceptible to filipin binding, as filipin
cannot access cholesterol in clatherin coated vesicles. (Steer et al., 1984, Mobius et al., 2002). Thus, depending on cholesterol distribution, subtle but important differences in cholesterol synthesis may not be detected by filipin staining. To more rigorously assess if Akt2 regulates Th17 differentiation through cholesterol synthesis, we cultured WT and Akt2-/- CD4 T cells in Th17 polarizing conditions in the presence of simvastatin or vehicle control (DMSO). We reasoned that if Akt2 regulates Th17 differentiation through the cholesterol biosynthetic pathway, inhibiting cholesterol synthesis in Akt2-/- CD4 T cells would not enhance the defect in IL-17A production. However, both WT and Akt2-/- CD4 T cells were equally sensitive to HMG-CoA reductase inhibition and produced less IL-17A with a concomitant increase in Foxp3+ cells when cultured in the presence of simvastatin compared with DMSO (Fig. 4.1B).

Despite seemingly intact HMG-CoA reductase activity, the more distal portion of the cholesterol biosynthetic pathway, involving geranylgeranyl diphosphate as indicated in the microarray, could still be perturbed in Akt2-/- Th17 cells. The distal portion the cholesterol biosynthetic pathway could be important for the generation not just of cholesterol precursors but also byproducts that contribute to other cellular processes. Cholesterol synthesis results in production of geranylgeranyl diphosphate and farnesyl diphosphate, which can generate geranylgeranyl and farnesyl groups through geranylgeranyl transferase (GGT) and farnesyl transferase (FT) activity, respectively. Geranylgeranyl and farnesyl groups can then post-translationally modify proteins. Importantly, geranylgeranyl transferase and protein geranylgeranylation has been reported to be important for Th17 differentiation in CD4 T cells (Kagami et al., 2009). Although FT activity was suggested to be dispensable for Th17 differentiation, our microarray analysis predicted that trans,trans farnesyl diphosphate synthesis would be differentially regulated in Akt2-/- and WT cells. As such, we were also interested in
assessing its role downstream of Akt2 in Th17 differentiation (Kagami et al, 2009). To
test the possibility that Akt2 impacts these pathways, sorted naïve CD4 T cells from WT
and Akt2-/- mice were cultured in Th17 polarizing conditions in the presence of a GGT
inhibitor (GGTI), FT inhibitor (FTI), or vehicle control (DMSO). Again, we hypothesized
that if Akt2 regulates Th17 differentiation through geranylgeranyldiphosphate or farnesyl
diphosphate biosynthesis or protein modifications involving these byproducts of
cholesterol synthesis, inhibition of these processes would not further impair Th17
differentiation in Akt2-/- Th17 cells. To test the possibility that Akt2 may be important for
the generation of these cholesterol byproducts, sorted naïve CD4 T cells from WT and
Akt2-/- mice were cultured in Th17 polarizing conditions in the presence of a
geranylgeranyl transferase inhibitor (GGTI), farnesyl transferase inhibitor (FTI), or
vehicle control (DMSO). Again, we hypothesized that if Akt2 regulates Th17
differentiation through geranylgeranyldiphosphate or farnesyl diphosphate biosynthesis
or use of these molecules as precursors for protein modification, inhibition of these
processes would not further impair Th17 differentiation in Akt2-/- Th17 cells. WT and
Akt2-/- CD4 T cells were equally susceptible to geranylgeranyl transferase inhibition but
resistant to farnesyl transferase inhibition (Fig 4.1C). Thus, while these data support the
finding that geranylgeranyl transferase activity, and not farnesyl transferase activity, is
important for Th17 differentiation, these pathways do not appear to be dependent on
Akt2 function(Kagami et al., 2009). Taken together, these data indicate that although
cholesterol synthesis and the byproduct generating geranyl groups for protein
modification is important for Th17 differentiation, this pathway does not appear to be the
primary pathway by which Akt2 contributes to Th17 differentiation. However, given that
the pathway analysis indicated changes in these pathways in Akt2-/- cells, it is possible
that, while not responsible for the entire effect, modulation of this pathway may be partially responsible for the Th17 phenotype we observed.

Figure 4.1: Akt2 does not regulate Th17 differentiation through the cholesterol biosynthetic pathway

(A) Representative flow cytometry analysis of WT and Akt2-/- naïve CD4 T cells which were sorted and cultured in Th17 polarizing conditions for 3.5 days then stained with filipin to detect unesterified free cholesterol. Cells gated on live lymphocytes (n = 3 independent experiments).

(B) Left panel shows representative flow cytometry of sorted WT and Akt2-/- naïve CD4 T cells
sorted and cultured in Th17 polarizing conditions in the presence of simvastatin or DMSO vehicle control for 3.5 days, gated on live CD4+ lymphocytes (n = 3 independent experiments). Compilation of 3 independent experiments on right showing mean ±SEM. Genotype and simvastatin treatment *p <0.05; interaction p = 0.4442 (2-way ANOVA with matching) (C) Left panel shows representative flow cytometry analysis of sorted WT and Akt2/-/- naïve CD4 T cells cultured in Th17 polarizing conditions in the presence of GGTI, FTI, or DMSO vehicle control for 3.5 days, gated on live CD4+ lymphocytes. Compilation of 3 independent experiments on right showing mean ± SEM. Genotype p = 0.0546, FTI treatment p = 0.1426, interaction p = 0.3363 (2-way ANOVA with matching of FTI vs DMSO). Genotype p = 0.0377, GGTI treatment p = 0.0078, interaction p = 0.0706 (2-way ANOVA with matching of GGTI vs DMSO).

4.3.3. Akt2 does not promote Th17 differentiation through regulation of the IL-6 signaling pathway

Since IL-6 is an important cytokine for the induction of Th17 differentiation and genes associated with IL-6 signal transduction appeared to be globally suppressed in Akt2/-/- differentiating Th17 cells compared with WT, we assessed expression and activation of molecular components in IL-6 signal transduction. We first measured IL-6 receptor levels on the surface of naïve CD4 T cells from WT and Akt2/-/- mice and found comparable levels of surface IL-6Rα (Fig. 4.2A). Although the receptor is present, we considered the possibility that Akt2/-/- could affect the strength of IL-6 signaling, so we examined IL-6 signaling in cells of both genotypes.

IL-6 signal transduction is mediated by phosphorylation and activation of STAT3 and is negatively regulated by SOCS3, which inhibits the phosphorylation of STAT3. A potential link between Akt and SOCS3 has been suggested as mTORC1 activation has been shown to suppress SOCS3 expression, resulting in maintained phosphorylation of STAT3 (Delgoffe et al., 2011). We therefore examined both phosphorylation of STAT3 and expression of SOCS3 in WT and Akt2/-/- CD4 T cells that had been cultured in Th17 polarizing conditions for 3.5 days. We chose to perform these experiments after culture in Th17 polarizing conditions to more closely mimic the cytokine milieu in which Th17 cells differentiate as opposed to only brief exposure to IL-6. Surprisingly, WT and Akt2/-/- Th17 cells had comparable levels of phosphorylated STAT3 and SOCS3 protein.
expression (Fig. 4.2B and 4.2C). Although these results suggest that the expression of important mediators of IL-6 signaling are similar between WT and Akt2/-/- cells throughout Th17 differentiation, there is still a possibility that phosphorylation of STAT3 was reduced in Akt2/-/- cells earlier in the differentiation process. We hypothesized that perhaps Akt2/-/- up-regulates SOCS3 expression soon after exposure to IL-6, which could dampen the strength of IL-6 signal transduction, measured by phosphorylation of STAT3, impairing Th17 differentiation in these cells.

To address this hypothesis we assessed expression of pSTAT3 and SOCS3 in naïve CD4 T cells from WT and Akt2/-/- mice cells after overnight stimulation with TCR and co-stimulatory signals as well as IL-6. Of note, this experiment was only performed once, but we found that STAT3 was robustly phosphorylated to a similar degree in Akt2/-/- and WT cells (Fig. 4.2D). SOCS3 protein was undetectable in both Akt2/-/- and WT CD4 T cells. This suggests that even after short exposure to IL-6, there is no defect in IL-6 signaling in Akt2/-/- CD4 T cells.

To more carefully track STAT3 phosphorylation over the duration of Th17 differentiation, we measured levels of phosphorylated STAT3 in WT and Akt2/-/- CD4 T cells after, 24, 36, and 48 hours in Th17 polarizing conditions. This experiment was performed twice at 24 and 48 hours and once at 36 hours. Consistent with our overnight data, expression of phosphorylated STAT3 was similar in Akt2/-/- and WT cells at all time points (Fig. 4.2E). These preliminary experiments suggest that phosphorylation of STAT3 occurs in Akt2/-/- Th17 cells similar to WT cells throughout Th17 differentiation. These conclusions are consistent with the fact that Rorc expression, which is induced by STAT3, is not reduced in Akt2/-/- Th17 cells (See Fig. 3.4A).

Taken together, these results show that although the pathway analysis we performed on transcripts from WT versus Akt2/-/- cells pointed towards IL-6 signaling as
being different in the two genotypes, direct analysis of components of the IL-6 signaling pathway did not support this as an explanation for defective IL-17 production in Akt2-/- cells. As described above, one explanation to reconcile these is that findings from the transcriptional analysis may reflect the consequence of altered Th17 differentiation, rather than the cause of impaired Th17 differentiation seen in Akt2-/- CD4 T cells.

Figure 4.2: IL-6 signaling is molecularly intact in Akt2-/- Th17 cells

(A) Representative flow cytometry analysis of surface IL-6R on naïve CD4 T cells from WT and Akt2-/- mice. Cells gated on live CD4+ CD62Lhi CD44lo lymphocytes (n = 3 independent experiments). (B) Representative western blot of pSTAT3 protein in sorted WT and Akt2-/- naïve
CD4 T cells cultured in Th17 polarizing conditions for 3 days. Band intensities were normalized to β-tubulin loading control (n = 3 independent experiments). (C) Representative western blot of SOCS3 protein in sorted WT and Akt2-/- naive CD4 T cells cultured in Th17 polarizing conditions for 3 days. Band intensities were normalized to β-actin loading control (n = 3 independent experiments). (D) Left panel: Western blot showing pSTAT3, SOCS3, and β-tubulin loading control from naive CD4 T cells from WT and Akt2-/- mice after 18 hours in the presence of anti-CD3, anti-CD28, and IL-6. Right panel: quantification normalized to β-tubulin loading control. (E) Western blot showing pSTAT3 and β-actin after 24, 36, or 48 hours in Th17 polarizing conditions; quantification normalized to β-actin on bottom.

4.3.4. Akt2-/- Th17 cells have a trend towards reduced expression of HIF1α targets

From our microarray analysis, activity of the transcription factor, HIF1α, was also predicted to be reduced in Akt2-/- Th17 cells compared with WT. Hif1a mRNA is increased in Th17 cells compared with other Th subets in response to both STAT3 and mTORC1 signaling. HIF1α has been described to regulate Th17 differentiation in a number of ways, involving increasing RORγt expression and enhancing its activity. Although Rorc message is not reduced in Akt2-/- Th17 cells, the regulation of its transcription is multifactorial, and perhaps Akt2 deficiency is not sufficient to impair its transcription. As the transcriptome of HIF1α was predicted to be globally suppressed in the microarray analysis, and HIF1α is an important positive regulator of Th17 differentiation, we assessed Hif1a mRNA expression in Akt2-/- and WT Th17 cells. We found that Akt2-/- and WT Th17 cells express comparable levels of Hif1a mRNA (Fig. 4.3A). However, HIF1α is often regulated at the protein level and this had been described in some cell types to be mTORC1-dependent. In response to insulin stimulation, epithelial cells up-regulate HIF1α in Rapamycin-sensitive manner, and inhibition of the PI3K/mTORC1 pathway in endothelial cells reduces protein levels of HIF1α (S. H. Park et al., 2014; Treins, 2002). Therefore, we compared HIF1α protein levels in Akt2-/- and WT Th17 cells by flow cytometry and in several experiments found less HIF1α in the Akt2-/- Th17 cultures (data not shown). It should be noted, however, that the HIF1α antibody is difficult to use and the quality of staining was variable, making
me uncomfortable concluding that HIF1α protein levels are sufficiently different between the genotypes to explain the cytokine production phenotype. As HIF1α exerts its functions in the nucleus, future experiments comparing nuclear HIF1α protein in WT and Akt2-/- Th17 cells would provide greater insights into this question.

Given the difficulties in measuring HIF1α protein, we turned to the analysis of transcriptional targets of Hf1α as readouts of HIF1α function in Th17 cells. Though not directly measuring HIF1α protein, we used this as a surrogate for functional HIF1α protein that could be more physiologically relevant. HIF1α is important for cellular metabolic processes, especially glycolysis, which is known to be important for CD4 lineage commitment. For example, HIF1α directly increases the expression of Hexokinase 2, the enzyme that catalyzes the rate-limiting in glycolysis. HIF1α is also responsible for up-regulating genes such as Glut1 and Ldha, also important molecules in the glycolytic pathway. Importantly, these targets are expressed in Th17 cells.

Measuring the relative expression of these genes in Akt2-/- and WT Th17 cells, we found that there was a trend towards reduced expression of these HIF1α targets in Akt2-/- cells, however, this did not reach statistical significance (Fig. 4.3B).

Given these data, the role of HIF1α in Akt2-/- Th17 cells remains unclear. However, HIF1α is clearly expressed in Akt2-/- Th17 cells. Thus, perhaps the level of HIF1α expressed in Akt2-/- Th17 cells is sufficient to maintain Rorc expression, but not enough to maintain Th17 cytokine expression. Taken in context, the possible reduction in expression of HIF1α targets in Akt2-/- Th17 cells renders the relationship between HIF1α function and Akt2 an important area for future study.
Figure 4.3: Akt2−/− Th17 cells have a trend towards reduced levels of HIF1α target expression

Relative (A) Hif1α, or (B) Glut1, Hk2, and Ldha mRNA from WT and Akt2−/− Th17 cells cultured in Th17 polarizing conditions for 3 days measured by RT-PCR. CT’s were normalized to β-actin then set relative to WT (RQ = 1, dotted line in (B)), *p ≤ 0.05. Showing mean ± SEM.

4.3.5. Akt2−/− Th17 cells are sensitive to mTORC1 inhibition by Rapamycin and S6K inhibition by LyS6K2

Although strongly predicted by microarray analysis, our analysis of the cholesterol biosynthetic and IL-6 signaling pathways did not reveal that these pathways were the major targets of Akt2 in Th17 differentiation, therefore we looked directly at conventional downstream mediators of Akt signaling. mTORC1 is one such important downstream mediator of Akt signaling that is also important for regulating the Th17/Treg axis (J. Chang et al., 2013; Delgoffe et al., 2009; Delgoffe et al., 2011). Therefore, we cultured WT and Akt2−/− CD4 T cells in Th17 polarizing conditions in the presence of the mTORC1 inhibitor Rapamycin or vehicle control (DMSO) reasoning that if Akt2 regulates Th17 differentiation through mTORC1, Rapamycin should not further impair IL-17A production in Akt2−/− Th17 cells. As expected, Th17 differentiation was inhibited in WT cells treated with Rapamycin (Fig. 4.4A). However, Akt2−/− cells were equally sensitive to Rapamycin treatment, which augmented the defect in IL-17A production seen in these
cells. Notably, a concurrent increase in Foxp3+ cells occurred in both WT and Akt2-/- Th17 cells. It is important to consider that chronic exposure to Rapamycin can result in inhibition of both mTORC1 and mTORC2, thus, the effect of Rapamycin on Th17 differentiation may be compounded by effects of mTORC2 inhibition. Therefore, to more specifically interrogate mTORC1 signaling, we evaluated the role of ribosomal protein S6 kinase (S6K), a downstream target of mTORC1, in Th17 differentiation. Inhibition of S6K with the pan-S6K inhibitor LyS6K2 moderately impaired IL-17A production similarly in both WT and Akt2-/- Th17 cells (Fig. 4.4B). Importantly, both Rapamycin and LyS6K2 treatment appropriately inhibited ribosomal protein S6 (S6) phosphorylation in both WT and Akt2-/- Th17 cells compared with DMSO control, demonstrating that an effective amount of each drug was used (Fig. 4.4C). Given the similar effect of Rapamycin and LyS6K2 on WT compared to Akt2-/- Th17 differentiation it is unlikely that Akt2 is regulating Th17 differentiation solely via an mTORC1 dependent pathway. In agreement with these observations, WT and Akt2-/- Th17 cells have comparable S6 phosphorylation, indicating that S6K activity is largely intact in these cells (Fig. 4.4D).
Figure 4.4: Akt2 does not regulate Th17 differentiation solely through the mTORC1 pathway
4.3.6. Akt2−/− CD4 T cells have moderately elevated expression of Gfi1, a negative regulator of Th17 differentiation

Others have shown that expression of the transcription factor Gfi1, a negative regulator of Th17 differentiation, is suppressed through activation of the mTORC1-S6K axis (Kurebayashi et al., 2012). We first measured the kinetics of Gfi1 expression in WT Th17 cells, as it should be only transiently induced after TCR stimulation in Th17 polarizing conditions. As expected, Gfi1 expression was induced in WT cells 12 hours after culture in Th17 polarizing conditions (which also requires TCR stimulation) and then rapidly fell such that Gfi1 expression after 48 hours in Th17 polarizing conditions was similar to naïve CD4 T cells at 0 hours (Fig. 4.5A). We then assessed if Gfi1 levels were elevated in Akt2−/− cells after culture in Th17 polarizing conditions for 0, 12, 24, and 48 hours. We observed that Gfi1 expression followed similar kinetics in Akt2−/− and WT Th17 cells with highest expression after 12 hours in Th17 polarizing conditions.

However, we also found that despite comparable S6 phosphorylation (See Fig 4.4C), Akt2−/− Th17 cells had a modest elevation of Gfi1 mRNA compared to WT at the peak of
its induction at 12 hours in Th17 polarizing conditions (Fig. 4.5B). Differences in Gfi1 mRNA between WT and Akt2-/- Th17 cells were not as apparent at later time points. These data suggest Akt2-dependent but S6K-independent pathways may contribute to Gfi1 regulation during early Th17 differentiation and point to Gfi-1 as a potential Akt2 target during Th17 differentiation.

4.3.7. Akt2-/- CD4 T cells have moderately elevated expression of mir155 during Th17 differentiation

Similar to other biologic systems, there is increasing interest in the role of microRNAs in the regulation of immune responses. Considerable evidence has pointed to miR-155 as one such modulator. We were intrigued by this, and by the finding that recently, miR-155 has also been described as a downstream target of the Akt2 isoform of Akt. A study exploring macrophage polarization described an isoform specific role of Akt2 in promoting the polarization of inflammatory macrophages (Arranz et al., 2012). Macrophages can take on two functionally antagonistic phenotypes: pro-inflammatory M1 macrophages, or anti-inflammatory M2 macrophages. Akt2-/- macrophages polarized towards an M2 phenotype, which was attributed to reduced levels of miR-155 (Arranz et al., 2012). miR-155 is also important for Th17 differentiation, as miR1-55/- CD4 T cells have impaired Th17 differentiation marked by reduced Il17A, Il17f, Il23r, and Il22 expression but near normal levels of Rorc expression compared with miR-155 sufficient controls (Hu et al., 2013). Interestingly, mir-155 deficiency results in increased expression of Th2-associated genes, namely Il4, Il5, and Il13 (Hu et al., 2013; Rodriguez et al., 2007; Thai et al., 2007). Furthermore, adoptive transfer of MOG-specific miR-155-/- CD4 T cells resulted in attenuated EAE compared with WT cells (Hu et al., 2013).
Given this constellation of findings, we evaluated the expression of miR-155 in Akt2-/- Th17 cells compared with WT.

Since the kinetics of miR-155 had not yet been reported during Th17 cell differentiation, we first evaluated the temporal profile of mir155 expression in WT CD4 T cells cultured in Th17 polarizing conditions. mir155 is minimally expressed in naïve CD4 T cells (0h), but by 12 hours there is a sustained induction of mir155 expression, suggesting that it may be important throughout Th17 differentiation (Fig. 4.5C). We then assessed the relative expression of mir155 in naïve Akt2-/- CD4 T cells compared with WT cells and after 12, 24, 36, 48 hours in Th17 polarizing conditions. Although Akt2-/- naïve CD4 T cells (indicated by 0h) expressed significantly more mir155 than their WT counterparts these levels were still low compared to cells undergoing Th17 differentiation. Despite the levels present in naïve T cells, Akt2-/- cells expressed less mir155 compared with WT cells after induction and throughout Th17 differentiation (Fig. 4.5D). These data confirm that miR-155 is induced in Th17 cells and therefore may suppress important molecules that interfere with Th17 differentiation. These results also suggest that Akt2-/- CD4 T cells may have impaired miR-155 induction in addition to impaired Th17 associated cytokine production. Whether or not these findings are correlative or causative warrants further investigation.
Figure 4.5: Akt2-/- CD4 T cells have a modest elevation in Gfi1 mRNA and modest reduction in mir155 mRNA expression

(A) Relative Gfi1 mRNA in WT CD4 T cells after 0 (naïve), 12, 24, 36, 48 hours in Th17 polarizing conditions measured by RT-PCR, CT’s normalized to β-actin then set relative to 0h (naïve CD4 T cell). (B) Relative Gfi1 mRNA in Akt2-/- CD4 T cells after 12, 24, 36, and 48 hours in Th17 polarizing conditions, CT’s normalized to β-actin and then set relative to WT (i.e. dotted line represents WT level of mRNA, RQ = 1) (C) Relative mir155 mRNA in WT CD4 T cells after 0 (naïve), 12, 24, 36, 48 hours in Th17 polarizing conditions measured by RT-PCR, CT’s normalized to β-actin then set relative to 0h (naïve CD4 T cell) (D) Relative mir155 mRNA in Akt2-/- CD4 T cells after 12, 24, 36, and 48 hours in Th17 polarizing conditions, CT’s normalized to U6 snRNA and then set relative to WT (i.e. dotted line represents WT level of mRNA, RQ = 1).

4.4. Discussion

In the experiments described in this chapter, we investigated the mechanism by which Akt2 regulates IL-17A production. We found that while IL-17A production in Akt2-/-
T cells was still sensitive to inhibition of the mTORC1-S6K pathway, Th17 differentiation in these cells was associated with elevated Gfi1 mRNA, which encodes for Gfi-1, a known inhibitor of Th17 differentiation, as well as reduced mir155 expression, a miRNA that promotes Th17 differentiation.

mTORC1 is a well-known Akt target and an important signaling molecule in T helper cell differentiation. As such, we hypothesized that Akt2 could be acting through mTORC1 to support Th17 differentiation. In this study, we interrogated the role of mTORC1 downstream of Akt2 in Th17 differentiation using the well-characterized mTORC1 inhibitor, Rapamycin. We found that, similar to wild type cells, Akt2/-/- cells exhibited sensitivity to Rapamycin treatment indicating that mTORC1 is not the primary pathway through which Akt2 regulates Th17 differentiation. However, this finding does not exclude a role for mTORC1 downstream of Akt2 in Th17 cells. Akt2 may be one of many inputs upstream of mTORC1, such that treatment with Rapamycin may have a broader effect than specifically targeting Akt2-regulated mTORC1 activity. Supporting this notion is the observation that CD4 T cells deficient in Rheb, a required functional component of mTORC1, have a block in mTORC1 signaling that results in impaired Th17 differentiation including decreased levels of RORγt. In contrast, Akt2/-/- Th17 cells have normal levels of RORγt in the face of diminished Th17 differentiation. Thus, if Akt2 does contribute to mTORC1-dependent Th17 differentiation, an Akt2-independent mechanism downstream of mTORC1 must be sufficient to regulate RORγt mRNA expression. This might explain the broader defect seen in Rheb deficient cells as well as the enhanced IL-17A defect observed in Rapamycin treated Akt2/-/- Th17 cells compared with untreated.

In an attempt to isolate Akt2-specific functions of mTORC1, we also used a pan-S6K inhibitor, as S6K1 and S6K2 are directly downstream of mTORC1. Using this
approach, we hoped to identify an Akt2-specific contribution to mTORC1 signaling in Th17 cells, as recently Kurebayashi et al. reported that the Akt-mTORC1-S6K axis is important for Th17 differentiation. Furthermore, it was postulated that lack of signaling through S6K downstream of mTORC1 impaired RORγt function in the setting of normal if not elevated RORγt levels (Kurebayashi et al., 2012). However, similar to Rapamycin treatment, we found a comparable defect in Th17 differentiation in Akt2−/− and WT Th17 cells when we inhibited signaling through S6K. Furthermore, our results evaluating the activity of S6K in Akt2−/− Th17 cells are not consistent with a dominant role for this signaling axis in Th17 differentiation, as there was no difference in phosphorylation of the S6K target, S6, in Akt2−/− Th17 cells compared with WT. These results suggest that Akt2 deficiency does not affect S6K activity despite impairing Th17 differentiation.

Kurebayashi et al. also describe a role for S6K in suppressing Gfi1, which antagonizes Th17 differentiation. Interestingly, we also saw a modest increase in Gfi1 at the peak of its induction in vitro in Akt2−/− Th17 cultures. This increase is consistent with an antagonistic role for Gfi1 in Th17 differentiation, but is not contingent on a defect in S6K signaling.

However, S6K is not the only target of mTORC1 that could be regulated by Akt2. Challenging the notion that Rapamycin inhibits all mTORC1 activity, recent work has established that this drug does not effectively inhibit the phosphorylation of 4EB-P1. This branch of mTORC1 signaling that regulates mRNA translation is largely unscathed in the presence of Rapamycin treatment (Thoreen et al., 2009). As such, the possibility remains that Akt2 acts through the Rapamycin-resistant 4EB-P1 branch of mTORC1 signaling, affecting the translation of transcribed mRNAs important for Th17 differentiation. Consistent with this hypothesis, while we observed a trend towards
decreased *Il17a* mRNA in Akt2-/- Th17 cells compared with WT, we saw a significantly more pronounced defect when we measured IL-17A protein in these cultures.

Another consequence of defective signaling through 4EB-P1 could be related to the potential defect in HIF1α activity. Defective Th17 differentiation in the setting of normal levels of RORγt in Akt2-/- CD4 T cells suggests that there is a co-factor that cooperates with RORγt to up-regulate Th17 cytokines during differentiation. One potential candidate is HIF1α, which has been identified as an important cofactor enhancing RORγt function (Dang et al., 2011). HIF1α’s transcriptional activity is regulated in an mTORC1-dependent manner and although this activity has been shown to be Rapamycin-sensitive, there is also evidence that HIF1α is translationally regulated by mTORC1 signaling making it an attractive potential downstream mediator of Akt2 signaling (Land & Tee, 2007; Treins et al., 2002). As mTORC1 regulates translation of mRNA through the Rapamycin-resistant phosphorylation of 4EB-P1, perhaps there is an Akt2-dependent requirement of sufficient HIF1α translation in order to appropriately induce Th17-associated cytokine expression. Our data addressing the role of HIF1α in Th17 differentiation, are inconclusive and do not exclude HIF1α as a mediator of Akt2-dependent Th17 differentiation.

Akt signaling informs many cellular processes that are both mTORC1-dependent and -independent (reviewed in Manning & Cantley, 2007). Indeed, the transcriptional profile of Akt2-/- Th17 cells suggested that both cholesterol biosynthetic and IL-6 signaling pathways were suppressed compared to WT. While both of these pathways are important for Th17 differentiation, based on our molecular readouts, neither pathway emerged as the single downstream signaling mechanism through which Akt2 promotes Th17 differentiation.
The role of Akt2 in Th17 differentiation thus appears to be complex, such that no single pathway surfaces as the primary downstream mediator of Akt2 signaling in these cells. Perhaps it is the integration of many affected pathways downstream of Akt2 that ultimately foster Th17 differentiation making it difficult to elucidate their independent contributions. However, future studies following up on the relationship of HIF1α and Akt2 in Th17 cells and the expression of both Gfi1 and mir155 in Th17 cells may add insight into how these pathways may induce Th17 differentiation.
Chapter V: Akt2 loss dampens peripheral immune response to MOG immunization *in vivo*

5.1. Introduction

The experiments described in Chapters 3 and 4 focused on the detrimental impact of Akt2 loss on *in vitro* differentiation of Th17 cells. However, Akt2 deficiency may still have significant consequences *in vivo* and the availability of Akt2-/- mice allows us to investigate that possibility. This is important as understanding how the observed *in vitro* deficiencies correlate with peripheral immune responses *in vivo* is an important extension to establish their biological significance. Akt2 deficiency does not completely abrogate Th17 differentiation, but it may impair peripheral immune responses sufficiently to modulate Th17 responses *in vivo*. To interrogate the *in vivo* effects of Akt2-/-, we used the experimental autoimmune encephalomyelitis (EAE) murine model of multiple sclerosis (MS), as it induces a strong inflammatory immune response with robust Th17 differentiation and ultimately causes a paralytic disease that mimics many symptoms of human multiple sclerosis. Classically, EAE develops as an ascending paralysis with histologic changes illustrating the infiltration of inflammatory cells such as macrophages and T cells in spinal cord tissue. However, there is an alternative presentation of EAE referred to as atypical EAE. Atypical EAE is characterized by ataxia and disequilibrium, with mice also developing spasticity and proprioceptive defects. Histopathology in atypical EAE is defined by inflammatory infiltrate in the brain, especially the cerebellum (the part of the brain responsible for coordination and precision of movement).

Adoptive transfer of myelin-specific CD4 T cells from immunized donor mice after *in vitro* restimulation with myelin peptide is sufficient to induce EAE in host mice of certain genotypes. Additionally, immunization of such mice with MHC class II restricted
myelin peptides that specifically induce CD4 T cell response can also give rise to EAE. The most commonly used peptide for EAE induction in C57BL/6 mice is comprised of amino acids 35-55 of myelin oligodendrocyte glycoprotein (MOG). MOG immunization in the context of complete Freund’s adjuvant (CFA) and administration of Pertussis Toxin results in the recruitment and activation of macrophages, neutrophils, and T cells to tissues of the central nervous system, such as the spinal cord and brain. This immunization regime also results in the generation of Th1 and Th17 CD4 T cell responses, characterized by the production of inflammatory cytokines both in the spinal cord and brain, as well as peripheral organs such as draining lymph nodes and the spleen. Specifically, production of IFNγ (a Th1 associated cytokine) as well as IL-17A and GM-CSF (Th17 associated cytokines) are elevated in both peripheral and CNS lymphocytes in mice with EAE. The absence of IL-12p40, a subunit of IL-12 shared by both IL-12 and IL-23, renders mice completely resistant to the induction of EAE (Cua et al., 2003). Confirming a role of Th17 cells in EAE disease, IL-17-/− mice have attenuated EAE characterized by mild clinical scores and delayed disease onset (Komiyama et al., 2006). Thus, CD4 T cells are important mediators of EAE such that this model is frequently used to interrogate in vivo consequences of Th17 differentiation.

Interestingly, there is some evidence for Akt influencing the severity of EAE as Akt3 deficient mice demonstrate more severe disease characterized by an increase in transcription of inflammatory cytokines as well as a reduction of Foxp3+ cells in the spinal cord of mice immunized with MOG (Tsiperson et al., 2013). In vitro differentiated Th17 and Th1 cells from these mice are more resistant to Treg suppression than those from WT (Tsiperson et al., 2013). These findings describe an anti-inflammatory role for Akt3 in EAE, which is counter to what we hypothesize for the contribution of Akt2 in this system. However, they underscore the importance of evaluating the contribution of Akt
isoforms in immune regulation, supporting the hypothesis that Akt may inform the immunopathology underlying this disease.

This chapter examines the hypothesis that Akt2 deficient mice have a diminished response to MOG immunization and attenuated EAE compared to WT mice. In our experiments, we found that the peripheral immune response of Akt2^{-/-} mice to MOG immunization was, in fact, diminished compared to WT mice. However, this dampened immune response did not correlate with complete resistance to disease. Interestingly, we found evidence for a cell-extrinsic component to the requirement of Akt2 in the peripheral immune response to MOG immunization, as mice lacking Akt2 only in the T cell compartment had a much milder defect in inflammatory cytokine production after MOG immunization compared with Akt2^{-/-} mice.

5.2. Methods

Mice
Akt2^{fl/fl} mice (generous gift from Dr. Birnbaum) were bred to CD4cre mice in our colony to generate Akt2^{fl/fl} CD4cre+ mice. All experiments were performed in accordance with guidelines provided by the University of Pennsylvania Institutional Animal Care and Use Committee under the supervision of the University Laboratory Animal Resources.

MOG_{35-55} immunization
MOG_{35-55} immunization was performed as previously described (Xu et al., 2003). Briefly, MOG_{35-55} peptide was reconstituted in sterile DPBS (without Ca^{2+} or Mg^{2+}) at 3mg/ml and combined with equal volume of CFA containing 5mg/ml mycobacterium tuberculosis H37RA. The mixture was then emulsified before injection. 200µl of emulsion (300µg MOG) was administered subcutaneously in two separate injections of 100µl each into
the right and left flanks of the mouse. 100-400ng of Pertussis Toxin in 100µl of DBPS-/- was injected intravenously using retro-orbital venipuncture. Two days later a second injection of 100-400ng Pertussis Toxin was administered intravenously.

CellTrace Violet labeling

CellTrace Violet labeling kit was purchased from Invitrogen Life Sciences, and CellTrace Violet dye was reconstituted in 18ul sterile DMSO. CellTrace media was prepared by adding CellTrace Violet dye at 1:500 to serum free IMDM. Lymphocytes were harvested from the spleen and lymph nodes and then washed three times with serum free IMDM media after red blood cell lysis. Cells were then resuspended at 2 x 10^7 cells/ml in serum free IMDM, then an equal volume of CellTrace media was added in the dark. Cells were incubated at room temperature and then one-half the volume of FBS was added to quench the dye such that the total volume was then 3x the initial volume resuspended in. Cells were then washed twice with TCM and resuspended at 7.5 x 10^6 cells/ml. Cells were added to a 48-well plate at 200µl of cells per well. Media containing MOG peptide was then added to each well such that final concentrations of MOG peptide were 0, 25, 50, and 100µg/ml. 72 hours later, GolgiStop was added at 2.6µl/ml to each well for 5 hours and then cells were harvested and stained for intracellular IL-17A, IFNγ, and Foxp3 and analyzed on an LSRII and then using FlowJo.

ELISA

To assess peripheral cytokine production in vivo, supernatants from CellTrace Violet labeled cultures (described above) were collected 48 or 72 hours after in vitro re-stimulation with MOG peptide. ELISAs were performed using Ready-Set-Go ELISA kit
(eBiosciences) as per manufacturer’s instructions. Samples were read on Spectramax M2e Plate Reader and analyzed using SoftMax Pro Software

5.3. Results

5.3.1. Akt2-/-mice have a dampened peripheral response to MOG immunization.

To determine whether the defect in Th17 differentiation observed in Akt2-/- cells affected peripheral immune responses, we turned to in vivo immunization of mice with myelin oligodendrocyte glycoprotein peptide (MOG). The advantages of this model are two fold: MOG immunization induces a robust Th17-mediated peripheral inflammatory response and MOG immunization leads to the development of EAE with high incidence. To evaluate the peripheral immune response, WT and Akt2-/- mice were immunized with MOG peptide and splenocytes were harvested 7 days later. The splenocytes were then cultured in the presence of MOG peptide for 48-72 hours and assessed for cytokine production. As expected from our in vitro studies described in Chapter 2, Akt2 deficient cells were defective in production of IL-17A and GM-CSF compared to their wild type counterparts, although the defect was much greater in vivo than was observed in vitro (Fig. 5.1A and 5.1B). However, in contrast with our in vitro polarization findings that Akt2-/- CD4 T cells could efficiently polarize towards IFNγ-producing Th1 cells, Akt2-/- splenocytes produced less IFNγ after MOG immunization than did WT cells (Fig. 5.1C.) Interestingly, despite the defects in IL-17A, GM-CSF, and IFNγ production by Akt2-/- splenocytes, the amount of IL-4 produced was similar between WT and Akt2-/- cultures, suggesting that there is an imbalance in T helper cell differentiation after MOG restimulation (Fig. 5.1D). Importantly, cytokine production was MOG-specific, as cytokine was not detected when splenocytes were cultured in the absence of MOG peptide (data not shown).
Figure 5.1: Splenocytes from MOG immunized Akt2/- mice have defective cytokine production

Splenocytes from WT (black bar) and Akt2/- (white bar) mice (n = 3-4 mice per genotype) were harvested 7 days after MOG immunization and labeled with CellTrace Violet before in vivo culture in the presence of 50-100μg/ml of MOG. (A) On the left, representative ELISA quantifying IL-17A production in WT (closed squares) and Akt2/- (open circles) splenocytes after 72 hours in culture. Compilation of 5 independent experiments depicted on the right as percent of WT IL-17A production showing mean ± SEM. (B) On the left, representative ELISA quantifying GMCSF production in WT (closed squares) and Akt2/- (open circles) splenocytes after 72 hour in culture. Compilation of 3 independent experiments depicted on the right as percent of WT GMCSF production showing mean ± SEM. (C) On the left, representative ELISA quantifying IFNγ production in WT (closed squares) and Akt2/- (open circles) splenocytes after 72 hour in culture. Compilation of 4 independent experiments shown on the right as percent of WT IFNγ production showing mean ± SEM. (D) On the left, representative ELISA quantifying IL-4 production in WT (closed squares) and Akt2/- (open circles) splenocytes from MOG immunized mice after 48 hours in culture. Compilation of 3 independent experiments shown on the right as percent of WT IL-4 production showing mean ± SEM. Statistical analysis was performed using Student’s T test. For compiled ELISA data, statistical analysis was performed using one-sample T test comparing Akt2/- to theoretical mean of 100.

Since the number of cells can influence the amount of cytokine produced in cellular cultures, we labeled the isolated cells with CellTrace Violet before the in vitro restimulation. Akt2/- splenocytes were hypo-responsive to MOG restimulation as evidenced by less CellTrace dilution compared with WT (Fig. 5.2A). Interestingly, of the
dividing CD4 T cells, Foxp3+ cells constitute a greater proportion of this population in Akt2-/– versus WT cultures (Fig. 5.2B). Consistent with this observation, more non-regulatory cells proliferated in WT compared with Akt2-/– cultures. This finding suggests that there may be a perturbation of the Th17/Treg axis in vivo, which we did not observe in vitro (See Chapter 3). Taken together, these data indicate that Akt2-/– splenocytes are hypoproliferative to in vitro MOG restimulation, which may result from the relative greater expansion of Foxp3+ cells in these cultures.

Figure 5.2: Akt2-/– splenocytes are hyporesponsive to MOG immunization with preferential expansion of Foxp3+ cells

(A) Histogram comparing CellTrace violet dilution in WT and Akt2-/– cells cultured and gated as above. Solid grey line = WT and dotted black line = Akt2-/–. (B) Top panel: representative flow cytometry analysis of Foxp3+ splenocytes among those with CellTrace dilution. Bottom panel: compilation of 4 independent experiments as percent Foxp3+ of cells with diluted CellTrace Violet. WT = black squares, Akt2-/– = white circlesCells were gated on live CD4+ TCRβ+ NK1.1-γδTCR- lymphocytes (n = 4 independent experiments). Statistical analysis was performed using Student’s T test.
5.3.2. Akt2-/- mice are not resistant to the development of EAE

Given the dampened peripheral response to MOG immunization, we monitored immunized WT and Akt2-/- mice for the development of EAE disease, hypothesizing that Akt2-/- mice would be resistant to the development of EAE due to their diminished peripheral immune response to this antigen. We immunized Akt2-/- and WT mice with MOG peptide and then began evaluating the animals for clinical signs of neurological dysfunction 7 days later. Each mouse was given a score from 0-5 (Table 5.1), with 0 being no clinical signs of disease and 5, death. Five independent experiments were performed in three different groups. Akt2-/- mice were observed to have reduced incidence of disease compared with WT for the first two groups of experiments (n = 3 independent experiments, 13 WT mice and 11 Akt2-/- mice). A compilation of clinical score over time for these three experiments is represented in Figure 5.3A, and suggests that indeed Akt2-/- mice are more resistant to EAE. However, a subsequent set of studies consisting of two independent experiments produced conflicting results. Akt2-/- mice had equal or higher incidence of disease compared with WT mice in both experiments. Table 5.2 lists the individual peak scores of the mice in these experiments. When all experiments are taken into consideration, we conclude that despite a dampened peripheral immune response, Akt2-/- mice are not consistently resistant to clinical symptoms of EAE. Interestingly, however, although difficult to quantify, the disease that developed in Akt2-/- animals appeared to have more features of atypical EAE than WT mice, as indicated by the star next to the peak score in Table 5.2. Taking all experiments into account, the overall incidence of EAE in WT and Akt2-/- mice was 95% and 72%, respectively (Table 5.3). Collectively, these data suggest that Akt2 is
important for the early peripheral response to MOG immunization, but Akt2 loss may not be sufficient to protect from subsequent CNS disease.

Table 5.1: EAE clinical score rubric

<table>
<thead>
<tr>
<th>Clinical Score</th>
<th>Observed signs of neurological dysfunction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Healthy mouse, neurologically intact</td>
</tr>
<tr>
<td>1</td>
<td>Limp tail; no other signs of disease</td>
</tr>
<tr>
<td>2</td>
<td>Hind limb paresis with waddling gait; voluntary movement of front limbs preserved</td>
</tr>
<tr>
<td>3</td>
<td>Paralyzed hind limbs with one or more dragging; front limb paresis</td>
</tr>
<tr>
<td>4</td>
<td>Paralyzed hind limbs and paralyzed front limbs</td>
</tr>
<tr>
<td>5</td>
<td>Moribund</td>
</tr>
</tbody>
</table>

**Figure 5.3:** Akt2⁻/⁻ mice have attenuated EAE

Mean clinical EAE score of WT (black squares) and Akt2⁻/⁻ (open circles) immunized with MOG peptide starting day 9 post immunization. Number of animals per group indicated above graph in grey, as total number of animals at a specific time point changes due to length of experiment or animal death. Statistical analysis performed using 2-way ANOVA with repeated measures, *** p < 0.0001.
5.3.3. Akt2 cKO results in modestly impaired in vitro Th17 differentiation but intact peripheral response to MOG immunization in vivo

Although Akt2-/- mice were not protected from developing EAE, we saw a more pronounced defect in the peripheral immune response to MOG immunization in Akt2-/- mice compared with WT than would have been predicted from our in vitro observations. Specifically, the reduced IL-17A and GM-CSF production in Akt2-/- splenocytes after MOG immunization was more severe than would have been expected based on the defect in cytokine expression observed in vitro in Akt2-/- Th17 cells. Additionally, given the comparable polarization toward Th1 cells in both WT and Akt2-/- cells, we would not
have predicted the defect in IFNγ production observed in Akt2-/- splenocytes after MOG immunization.

One possible explanation for the fact that our *in vivo* findings did not completely recapitulate what we observed *in vitro* is that there are cell-extrinsic contributions to the immune response generated after MOG immunization. Akt2-/- mice do not express Akt2 in any cell type, and perhaps Akt2 is required for the activation or cytokine production from another cell that may be essential for CD4 T cell activation. As such, we investigated if our *in vivo* observations were a T-cell intrinsic phenomenon. To test this possibility, we analyzed mice specifically lacking Akt2 in the T cell compartment. For these studies we generated Akt2^{fl/fl}CD4cre+ mice (Akt2 cKO). We first confirmed that these mice indeed did not express Akt2 in their T cells. We evaluated by flow cytometry the Akt2 expression in naïve CD4 T cells from Akt2 cKO mice, Akt2-/- mice, and WT mice cultured in Th17 polarizing conditions for 3.5 days. We found that whereas WT cells express Akt2, both Akt2 cKO and Akt2-/- mice failed to express this Akt isoform (Fig. 5.4A).

We then asked whether Akt2 cKO mice and Akt2-/- had a similar defect in Th17 differentiation, as our previous results suggested that T cell specific Akt2 deficiency during *in vitro* Th17 differentiation would cause a diminution in IL-17A production. IL-17A production was measured in Akt2 cKO, Akt2-/- and WT sorted naïve CD4 T cells cultured in Th17-polarizing conditions. As expected, Akt2-/- Th17 cells produced less IL-17A than WT cells to a similar degree as our previous observations. Surprisingly, Akt2 cKO Th17 cells had an intermediate decrease in IL-17A expression compared with WT mice (Fig. 5.4B). This result suggests that there is a cell-intrinsic component to the role of Akt2 in promoting Th17 differentiation, as Akt2 cKO mice were still defective in IL-17A
production. However, the intermediate defect in Akt2 cKO Th17 cells suggests that there is also a cell-extrinsic component that acts on naïve CD4 T cells.

Based on this finding, we hypothesized that Akt2 cKO mice would have an intermediate defect in their peripheral immune response to MOG immunization *in vivo* compared with WT mice. To test this, WT and Akt2 cKO mice were immunized with MOG peptide and splenocytes harvested 7 days later. Splenocytes were then cultured for 48-72 hours in the presence of MOG peptide before evaluation of cytokine production. We found that while there was a trend towards reduced levels of IL-17A, GM-CSF, and IFNγ production, none of these trends reached statistical significance (Fig. 5.4C). Similar to previous experiments, Akt2 cKO and WT splenocytes were labeled with CellTrace Violet before *in vitro* restimulation with MOG peptide. Consistent with the cytokine data, there was no statistically significant difference in the proportion of Foxp3+ dividing cells in Akt2 cKO compared with WT cultures (Fig. 5.4D). Collectively, these data suggest that specific deletion of Akt2 in T cells does not diminish the peripheral response to MOG immunization as severely as a germline deletion of Akt2 and highlight a T-cell extrinsic role of Akt2 in regulating peripheral immune responses. Thus, the defect in Th17 differentiation observed in Akt2-/- CD4 T cells is both cell-intrinsic and cell-extrinsic, and the accentuated defect in peripheral CD4 T cell differentiation observed *in vivo* in response to MOG immunization is largely due to cell-extrinsic factors that support peripheral CD4 T cell differentiation.
Figure 5.4: Akt2 cKO mice intermediate defect in IL-17A production *in vitro* but are responsive to MOG immunization

(A) Representative histogram of Akt2 expression in WT (grey histogram), Akt2-/- (solid black line), and Akt2 cKO (dotted black line) Th17 cells. (B) %IL-17A expression in WT (black square), Akt2 cKO (grey circle), and Akt2-/- (white circle) Th17 cells, n = 3 experiments. Splenocytes from WT (black bar) and Akt2 cKO (grey bar) mice (n = 3-4 mice per genotype) were harvested 7 days after MOG immunization and labeled with CellTrace Violet before *in vivo* culture in the presence of 50-100ug/ml of MOG. (C) ELISA quantifying IL-17A, GM-CSF, and IFNγ production in WT and Akt2 cKO splenocytes after 72 hours in culture. Compilation of 3 independent experiments as percent of WT IL-17A production showing mean ± SEM. (D) Compiled of flow cytometry analysis of Foxp3+ splenocytes among those with CellTrace dilution. Cells were gated on live CD4+ TCRβ+ NK1.1- γδTCR- lymphocytes (n = 3 independent experiments). Statistical analysis was performed using Student’s T test.

5.4. Discussion

Here we illustrate an important role for Akt2 in the peripheral immune response to immunization with MOG peptide. We saw a striking impact of germline deletion of Akt2 *in vivo*, characterized by a hypoproliferative response to *in vitro* restimulation of splenocytes from MOG immunized mice and reduced production of inflammatory cytokines. However, this dampened immune response did not protect mice from clinical EAE disease. Importantly, these defects in proliferation and cytokine production were
less striking in mice lacking Akt2 only in T cells. These findings suggest an important role for Akt2 in regulating peripheral immune responses in vivo in both a T cell intrinsic and extrinsic manner.

As predicted from our in vitro polarization data, splenocytes from Akt2-/− animals immunized with MOG produced less IL-17A and GM-CSF than WT. However, unlike our in vitro findings that Akt2-/− CD4 T cells do not have a defect in Th1 polarization, Akt2-/− splenocytes from MOG immunized animals had decreased IFNγ production. In addition, Akt2-/− CD4 T cells were hypoproliferative in response to MOG peptide re-stimulation compared with WT cells, which does not correspond with the in vitro finding that Akt2-/− CD4 T cells do not have a defect in proliferation. It is possible that the decrease in Th1 and Th17 associated cytokines from Akt2-/− splenocytes were due to decreased proliferation in these cultures. However, impaired proliferation does not completely explain this finding, as WT and Akt2-/− splenocytes made a comparable amount of IL-4. These data suggest that an imbalance in T helper cell differentiation may be established in AKT2-/− mice in response to immunization. Specifically, IL-4 production in the face of decreased proliferation of Akt2-/− splenocytes may be due to relatively increased Th2 differentiation. The possibility that Akt2 deficiency could support Th2 differentiation becomes intriguing given the transcriptional program that promotes Th2 differentiation concomitantly antagonizes differentiation into other T helper subsets (Zhu et al., 2009). One such mechanism for suppressing Th17 differentiation during Th2 polarization involves upregulation of the transcription factor Gfi1, which interferes with RORγt activity and was modestly elevated in Akt2-/− Th17 cells (see Fig. 4.5B)(Ichiyama et al., 2009). The balance in T helper cell differentiation in Akt2 versus WT mice warrants further investigation, especially in light of our observation that Akt2-/− mice developed features of EAE that were different from the classical signs of EAE seen WT mice, and that
enhanced Th2 responses have been reported in mice with atypical EAE (Delgoffe et al., 2011). Furthermore, myelin basic protein-specific IL-4 producing CD4 T cells induce atypical EAE upon adoptive transfer into Rag1-/- mice in the setting of IFNγ deficiency (Wensky et al., 2001; 2005).

Even more striking was the observation that Tregs from Akt2-/- mice immunized with MOG made up a higher percent of proliferating cells after in vitro restimulation than they did in WT. This is consistent with our in vitro data suggesting enhanced survival of Akt2-/- Tregs compared with WT. However, combined with the more severe defect in Th17 lineage cytokine production in Akt2-/- splenocytes, these data also suggest a perturbation of the Th17/Treg axis, which was not observed in vitro. In the setting of MOG immunization, it is possible that Akt2 deficiency results in the preferential generation of Tregs at the expense of Th17 differentiation. Considered together with the defect in IFNγ production observed in vivo but not in vitro, a distortion of the normal Th17/Treg axis may point to the extrinsic factors impacting peripheral helper cell differentiation in Akt2-/- mice. Indeed, we found that Akt cKO mice do not have as severely dampened immune responses to MOG immunization as germline deletion of Akt2.

While the pathophysiology of EAE is often attributed to CD4 T cells, myeloid cells are essential to activation and recruitment of these T cells to the CNS. In response to the local cytokine environment, macrophages polarize towards the inflammatory M1 phenotype or anti-inflammatory M2-like phenotype. Suppressor of cytokine signaling 3 (SOCS3) deficiency in the myeloid lineage results in macrophages with sustained signal transducer and activator of transcription 3 (STAT3) phosphorylation, an important activating signaling molecule in macrophages as well as T cells, and M1-polarization (Qin et al., 2012). Macrophages from these mice produced increased levels of IL-16 and
IL-12, required for Th17 and Th1 differentiation respectively. Consistent with this observation, SOCS3 deficiency in macrophages resulted in an enhancement of Th1 and Th17 cells in the CNS after immunization of MOG peptide. Recently, IFNγ production from NK cells was shown to be crucial for M1 polarization of macrophages, recruitment of T cells, and inflammatory cytokine production in the setting of EAE (Dungan, McGuinness, Boon, Lynch, & Mills, 2014). Therefore, non-T cells are also important mediators of EAE pathogenesis as CD4 T cells, and make a significant contribution to the inflammatory cytokine milieu supporting neuroinflammation.

Given the established role of non-T cells such as macrophages in the development of EAE, it is possible that Akt2 exerts important effects in these cells affecting their contribution to the cytokine milieu. Akt2 deficiency in non-T cells could influence the local inflammatory cytokines necessary to efficiently promote CD4 T helper cell differentiation. Consistent with this hypothesis is our finding that naïve CD4 T cells from Akt2 cKO mice produce more IL-17A than Akt2/-/- mice after in vitro polarization, suggesting that Akt2 deficient naïve CD4 T cells from an Akt2/-/- environment are less able to polarize towards Th17 cells than Akt2 deficient naïve CD4 T cells from an Akt2 sufficient environment. Importantly, there is evidence that Akt2 is important in non-T cells, as Akt2 deficient macrophages take on more anti-inflammatory M2-like character than Akt2 sufficient macrophages. Taken together, it is likely that Akt2 deficiency in non-T cells has an impact on the inflammatory environment in the setting of EAE. Changes to the microenvironment informing CD4 T cell fate could explain some of the inconsistencies between the in vitro and in vivo alterations in CD4 T cell differentiation in Akt2/-/- cells as well as the difference noted between the consequence of germline Akt2 deficiency and T-cell specific Ak2 deficiency in vivo. Of note, it appears that changes in Akt2/-/- non-T cells have long term effects on the CD4 T cells in these mice inasmuch as
they display defective cytokine production in vitro in the presence of MOG peptide even after being removed from host antigen presenting cells. This observation emphasizes the importance influence the host environment has on peripheral CD4 T cell responses.
Chapter VI: Conclusions and Future Directions

6.1. Summary of Findings

This work has defined a critical role for the serine threonine kinase, Akt, in the regulation of peripheral CD4 T cell differentiation. Specifically, we identify Akt2 as an important positive regulator of Th17 differentiation \textit{in vitro}. Compared with WT cells, \textit{in vitro} differentiated Akt2\textsuperscript{-/-} Th17 cells have impaired production of IL-17A, reduced expression of IL-23R, and reduced expression of the Th17 cytokines IL-17F and GM-CSF. Surprisingly, despite the altered cytokine profile in these cells, Ak2\textsuperscript{-/-} Th17 cells have normal if not elevated expression of key transcription factors essential for Th17 differentiation, including the lineage specific ROR\textgreek{y}t. The defect in CD4 T cell differentiation was specific to Th17 cells, as Akt2\textsuperscript{-/-} CD4 T cells polarized towards Th1 and Tregs as efficiently as WT cells but also had enhanced survival in these conditions compared with WT. Interestingly, Akt2\textsuperscript{-/-} CD4 T cells expressed similar if not elevated levels of the Th2 cytokine IL-4 and Th2 lineage specific transcription factor GATA3. Thus, Akt2 deficiency results in a specific defect in Th17 differentiation marked by decreased expression of important Th17 associated cytokines and IL-23R.

The mechanism by which Akt2 regulates Th17 differentiation is likely multifactorial, as it does not appear to be uniquely dependent on common signaling pathways and cellular processes downstream of Akt, such as the mTORC1/S6K axis. Naïve CD4 T cells from Akt2\textsuperscript{-/-} mice expressed comparable levels of IL-6 receptor as WT mice, and had molecularly intact IL-6 signal transduction. Furthermore, Th17 differentiation in Akt2\textsuperscript{-/-} cells was sensitive to perturbations in cholesterol biosynthesis, suggesting that Akt2 does not regulate Th17 differentiation through this pathway. However, Akt2\textsuperscript{-/-} Th17 cells had modestly elevated expression of Gfi-1, a molecule that
antagonizes Th17 differentiation by interfering with RORγt and promotes Th2 expansion in response to IL-4 stimulation. Furthermore, Akt2-/- Th17 cells had a modest decrease in miR-155 expression, a microRNA that facilitates Th17 differentiation and EAE induction (Hu et al., 2013). Interestingly, miR-155 deficiency has been described to skew peripheral CD4 T cell differentiation towards a Th2 phenotype resulting in an increase in expression of Th2 associated cytokines (Hu et al., 2013; Rodriguez et al., 2007; Thai et al., 2007).

Although the precise mechanism by which Ak2 drives Th17 differentiation remains elusive, we saw a striking impact of Akt2 deficiency in vivo. Akt2 deficiency resulted in a dampened immune response to MOG immunization characterized by more pronounced decreases in IL-17A and GM-CSF production than observed from in vitro polarization studies and a defect in IFNγ production not appreciated in in vitro Th1 polarization experiments. Akt2-/- splenocytes were also hypoproliferative in response to in vitro MOG restimulation compared with WT cells. Surprisingly, in the face of decreased proliferation, splenocytes from Akt2-/- mice produced comparable amounts of IL-4 compared to those from WT mice, suggesting an imbalance in the cytokine milieu in Akt2-/- splenocytes compared with WT. Despite a more severe defect in IL-17A and GM-CSF production in vivo than would have been predicted from our in vitro data, this dampened immune response did not protect Akt2-/- mice from developing clinical signs of EAE. One possible explanation for the enhanced defect in cytokine production in vivo in Akt2-/- splenocytes is the contribution of cell-extrinsic effects on peripheral T cell activation. To test this notion, we turned to mice deficient in Ak2 specifically in the T cell compartment (Akt2fl/fl CD4cre+ mice, referred to as Akt2 cKO). Interestingly, although Ak2 cKO CD4 T cells still had a defect (albeit milder than Akt2-/- cells) in in vitro Th17 differentiation, Akt2 cKO mice had only a trend towards decreased IL-17A, GM-CSF,
and IFNγ production after MOG immunization. These findings suggest both cell-intrinsic and cell-extrinsic components contribute to the requirement of Akt2 in the peripheral immune response to MOG immunization. Collectively, this work establishes Akt2 an important signaling molecule not just in promoting Th17 differentiation, but also regulating peripheral immune responses to antigen stimulation in vivo.

6.2. The role of Akt2 in CD4 T cell differentiation

Although in vitro differentiation studies clearly define a cell-intrinsic role for Akt2 in Th17 differentiation, they raise questions about how Akt2 may regulate other peripheral CD4 T helper cells. The enhanced survival in Akt2−/− Th1 and Tregs is of particular interest, as typically Akt2 is thought of as anti-apoptotic, promoting cell survival. In that context, one would expect Akt2 loss to render cells more prone to death or apoptosis. Is increased survival in Akt2−/− Th1 and Treg cells due to a reduction in apoptosis? Does increased survival translate into enhanced effector or suppressive function? Does increased survival hold true in vivo? Characterization of the enhanced survival in these cells, including staining for apoptotic markers, measuring proliferation by CFSE labeling, and functional assays such as Treg suppression assays would shed more light on the impact of Akt2 deficiency in these cells in vitro.

Differences in survival or proliferation of specific T helper cells over others could also have significant impacts in vivo, as they could skew the balance of peripheral CD4 T cell derived cytokines and thus immune responses. Preferential survival of Tregs could prevent autoimmunity, but similarly promote a hypo-responsive immune system incapable of adequate host defense from infection and pathogen clearance. In contrast, enhanced survival of Th1 cells could result in Th1-mediated autoimmune diseases such as inflammatory bowel disease or psoriasis but facilitate clearance of intracellular
pathogens such as viruses. Thus, the effect of Akt2 deficiency in other T helper cells is an important avenue for future work, especially in the context of experimentally induced infection and immune-mediated disease that invoke peripheral CD4 T cell responses but also allow the evaluation of a clinical outcome giving a functional correlate. It is particularly important to pursue this line of investigation as inhibitors of PI3K/Akt signaling are already being investigated in humans as anti-cancer agents, which makes understanding the impact of inhibiting this pathway in an immune compromised setting even more critical.

6.3. Regulation of Th2 differentiation by Akt2

A subtle but recurring theme throughout this work is that Akt2-/- CD4 T cells may have an increased propensity towards Th2 polarization. Akt2-/- naïve CD4 T cells have a trend towards increased IL-4 and GATA3 expression after culture in Th2 polarizing conditions. On a per cell basis, splenocytes from Akt2-/- mice likely produce more IL-4 than those from WT mice after MOG immunization. Akt2-/- Th17 cells express more Gfi-1, which promotes Th2 differentiation while inhibiting Th17 differentiation (Ichiyama et al., 2009; Zhu et al., 2009). Collectively, these findings allude to the possibility that Akt2 inhibition may reduce Th17 differentiation, but enhance Th2 differentiation.

If this hypothesis holds true, it would provide insight into the cross-regulation of peripheral CD4 T cell differentiation, which is important for lineage commitment. Often differentiation into one Th subset involves simultaneous silencing of others. The shift away from Th17 differentiation towards Th2 differentiation in the setting of Akt2 deficiency may be an example the breakdown of one of these mechanisms. Gfi-1 is only transiently induced after TCR stimulation, which means its expression must be suppressed soon after stimulation unless sustained by IL-4 signaling. Given that Gfi-1
promotes Th2 proliferation and expansion and is elevated in Akt2 deficient Th17 cells. Akt2 may be important for inducing a factor that suppresses Gfi-1 after TCR stimulation, and thus Th2 polarization, in Th17 cells (Zhu et al., 2002). As such, perhaps the mechanism through which Akt2 promotes Th17 differentiation is by suppressing Th2 differentiation.

Though correlative, these observations highlight the importance of fully characterizing the “Th17/Th2 axis” in the setting of Akt2 deficiency. The possibility that Akt2 reciprocally regulates Th17 and Th2 cells renders the role of Akt2 deficiency in different in vivo immune-mediated disease models more important. In particular, characterizing the role of Akt2 deficiency in airway hyper-responsive (AHR) disease would be of great interest, as there is evidence of both Th17 and Th2 involvement in development of disease. The Th2-associated cytokine, IL-13, is necessary and sufficient to induce AHR, and conditioned media from in vitro Th2-polarized cells is similarly sufficient to induce AHR (Rajeev Venkayya, 2002; Walter, McIntire, Berry, McKenzie, Donaldson, DeKruyff, & Umetsu, 2001b). The role for Th17 cells in AHR is more controversial. Th17 cells are associated with recruitment of neutrophils to airways and Th17 associated cytokines have been detected in lung tissue of severe asthmatics (BSc et al., 2009; Hellings et al., 2003). However, treatment of mice with anti-IL-17A has also been associated with a reciprocal increase in Th2-mediated airway eosinophilia and bronchial IL-5 (Hellings et al., 2003; Schnyder-Candrian et al., 2006). Furthermore, blocking IL-17A in the effector phase of AHR after initial induction of airway inflammation enhances AHR in mice. Consistent with this finding, IL-4Rα deficiency resulted in increased IL-17 and subsequent decrease in eosinophil recruitment and AHR. Thus, the AHR model provides not only a tool for examining the Th17/Th2 axis in vivo, but reveals the potential dangers of unopposed inhibition of Th17 differentiation, as tempering one
immune response could fuel another. This is an important consideration, as Akt2 is a plausible therapeutic target and could prove useful in modification of Th17-mediated immunopathology.

6.4. The role of Akt2 in promoting Th17 differentiation: cell intrinsic versus cell extrinsic mechanisms

In dissecting the in vivo consequences of Akt2 deficiency, a cell extrinsic component of Akt2-mediated regulation of peripheral CD4 T cell differentiation was revealed. Both Akt2 cKO and Akt2-/- Th17 cells produced less Il-17A than WT after culture in in vitro Th17-polarizing conditions (suggesting a cell-intrinsic component to Akt2’s regulation of Th17 differentiation); the fact that the defect was milder in Akt2 cKO suggests that cell-extrinsic effects before T cell activation and antigen exposure inform peripheral CD4 T cell fate in these mice. Indeed, splenocytes from MOG immunized Akt2 cKO mice had only a trend towards reduced production of IL-17A, GM-CSF, and IFNγ while a more striking defect was appreciated in Akt2-/- splenocytes. The enhanced defect uniquely in Akt2-/- (compared to Akt2 cKO) cells suggests that non-T cells have a significant contribution to the peripheral immune response, especially in regards to inflammatory cytokine production. Identification of this cell is important to understanding not only of the pathophysiology of EAE, but also Akt2-dependent factors in non-T cells that contribute to in vivo peripheral CD4 T cell differentiation. Generation of lineage-specific deletions of Akt2 in non-T cells known to influence CD4 T cell differentiation, such as dendritic cells using Akt2^{fl/fl} CD11cCre+ mice or macrophages using Akt2^{fl/fl} LysMCre+ mice, would help identify Akt2-dependent contributions by these cells to peripheral CD4 T cell differentiation. Again, using the MOG immunization model, one could similarly assess cytokine production in these mice as in Akt2-/- and Akt cKO mice.
A significant defect in IL-17A, GM-CSF, or IFNγ production in Akt2^fl/fl^ CD11cCre+ mice after MOG immunization, for example, would suggest that Akt2 expression in dendritic cells is necessary for them to induce the differentiation of IL-17A, GM-CSF, or IFNγ producing CD4 T cells.

Since the use of the MOG immunization model gave insight into *in vivo* consequences of Akt2 deficiency in the setting of an immune response directed at a persistent self-antigen, it also provides incentive to look at other *in vivo* models of Th17 mediated immunity. Specifically, it would be interesting to interrogate the role of Akt2 in both T cells and non-T cells in host defense against pathogens whose clearance is known to be Th17-dependent. One such example is the *in vivo* bacterial infection model testing Th17-mediated immune responses in pulmonary infection with *Klebsiella pneumonia*. In this model, the absence of a Th17-mediated immune response as a result of IL-23 deficiency resulted in increased mortality, which was mitigated by administration of recombinant IL-17A (Happel et al., 2005). Importantly, pulmonary dendritic cells and alveolar macrophages were shown to be significant sources of IL-23, which is crucial for terminal differentiation of Th17 cells. Given the established role for both CD4 T cells (producing IL-17A) and non-T cells (producing IL-23), it would be interesting to assess the lineage-specific requirement of Akt2 in this setting. This model could answer the question of whether or not the amount of IL-17A produced by Akt2 cKO Th17 cells sufficient to mount a protective immune response (at least in this model). It could also pinpoint the role of Akt2 in IL-23 production, using Akt2^fl/fl^ CD11c Cre+ and Akt2^fl/fl^ LysM Cre+ mice. If Akt2 is important in IL-23 production in these cells, it could discern which cell type is the major source of IL-23 in the setting of bacterial pulmonary infection.
Another bacterial infection that could provide insights into the impact of Akt2 deficiency \textit{in vivo} is intestinal infection with \textit{Citrobacter rodentium}. In this model, IL-23 is required for Th17 mediated bacterial clearance, but this is independent of initial IL-17A production and Th17 differentiation. One would predict that Akt2 deficiency in non-T cells would have devastating effects on the ability of mice to survive \textit{Citrobacter rodentium} infection, but perhaps the cell-intrinsic defect in Th17 differentiation and IL-17A production in Akt2 cKO T cells may be less important. Thus, future work using \textit{in vivo} models of infection to analyze the role of Akt2 in protective immunity could shed light on the contributions of T cell intrinsic and T cell extrinsic Akt2 signaling in mounting an effective immune response.

\textbf{6.5. Conclusions}

In this thesis, I describe a critical role for Akt2 in Th17 differentiation \textit{in vitro} paralleled with a dampened peripheral response to MOG immunization in the setting of Akt2 deficiency. Despite the complex nature of Akt2’s contribution to Th17 differentiation, it is clear that it is an important signaling molecule in peripheral T cell responses. Although Akt2 has a more significant effect in Th17 differentiation compared with other Th subsets, our findings have important implications for therapies targeting effector Th responses that contribute to immune-mediated diseases, underscoring the potential complications associated with attempts to modulate a specific Th response. Although future study is required, this work offers a foundation for beginning to identify targetable signaling requirements for Th17 differentiation increasing our understanding of Th17 biology.
References


Brezski, R. J., & Monroe, J. G. (2007). B Cell Antigen Receptor-Induced Rac1 Activation and Rac1-Dependent Spreading Are Impaired in Transitional Immature B Cells Due to Levels of Membrane Cholesterol. Journal of Immunology, 179, 4464-4472.


BSc, W. A.-R., MSc, D. P., MSc, F. C., James G Martin MD, D., MD, R. O., MD, C. L., & Qutayba Hamid MD, P. (2009). TH17-associated cytokines (IL-17A and IL-17F) in severe asthma. Journal of Allergy and Clinical Immunology, 123(5), 1185–1187. doi:10.1016/j.jaci.2009.02.024


Chang, J., Burkett, P. R., Borges, C. M., Kuchroo, V. K., Turka, L. A., & Chang, C. H. (2013a). MyD88 is essential to sustain mTOR activation necessary to promote T
helper 17 cell proliferation by linking IL-1 and IL-23 signaling. *Proceedings of the National Academy of Sciences*, 110(6), 2270–2275. doi:10.1073/pnas.1206048110


Fontenot, J. D., Gavin, M. A., & Rudensky, A. Y. (2003). Foxp3 programs the
development and function of CD4+CD25+ regulatory T cells. *Nature Immunology, 4*(4), 330-336


Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M., & Weaver, C. T. (2005). Interleukin 17–producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology, 6*(11), 1123–1132. doi:10.1038/ni1254


HELLER, F., FLORIAN, P., BOJARSKI, C., RICHTER, J., CHRIST, M., HILLENBRAND, 106


Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., et al. (2000). Novel p19 Protein Engages IL-12p40 to Form a Cytokine, IL-23, with Biological Activities Similar as Well as Distinct from IL-12. *Immunity, 13*, 715-725


PI3K/mTOR/4E-BP1 pathways. *Cellular Signalling*, 26(7), 1506–1513. doi:10.1016/j.cellsig.2014.03.017


Zhou, L., Ivanov, I. I., Spolski, R., Min, R., Shenderov, K., Egawa, T., et al. (2007). IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21
and IL-23 pathways. Nature Immunology, 8(9), 967–974. doi:10.1038/ni1488

