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Evaluation of a conditional knockout of Ikaros in peripheral T-cell differentiation into helper T-cell subsets

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**EVALUATION OF A CONDITIONAL KNOCKOUT OF IKAROS IN
PERIPHERAL T-CELL DIFFERENTIATION INTO HELPER T-CELL
SUBSETS**

by

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B.S., Florida Gulf Coast University, FL 2013

Submitted in partial fulfillment of the
requirements for the degree of
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DEDICATION

I would like to dedicate this work to my parents Nico and Coral. When I moved away from home to pursue my education, we never thought I would take such a long and unconventional path, but you have followed me through it and supported me in every way without question. Thank you for being my most valuable teachers and my most important encouragement and inspiration. You have taught me to never give up when doors close and to look for other ways instead. I will always work hard and give my one thousand percent to make you proud. Thank you to my brothers Ricardo and Roberto, my grandmother, my aunt, and the rest of my friends and family. I have been blessed with unconditional support, love and encouragement from them, and I would not be where and who I am without it.

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**EVALUATION OF A CONDITIONAL KNOCKOUT OF IKAROS IN
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ABSTRACT

CD4 T helper (Th) cells differentiate into distinct effector or regulatory subsets as needed during the course of an infection. Ikaros is a transcription factor that is necessary for proper thymic T cell development. In order to study the role of Ikaros in peripheral CD4 T-cell differentiation and function, a novel Ikaros conditional knockout mouse in which Ikaros is deleted in mature T-cells (CKO mice) was developed. In this thesis, this model is characterized and used to evaluate how absence of Ikaros affects lymphocyte and myeloid populations *in vivo*, and CD4 T-cell differentiation into T helper 17 (Th17) and inducible regulatory T cell (iTreg) subsets *in vitro*. CKO mice had normal thymocyte development and normal percentages of T-cells and B-cells in the spleen. However, they had increased percentages of myeloid cells, and an abnormal population of "naive-like" CD4 T-cells that expressed low levels of CD62L and CD44, markers that identify naive and memory T cell populations. CKO CD4 T-cells cultured under Th17 polarizing conditions showed normal expression of the Th17 factors, ROR γ t and IL-17A, but overexpressed the pro-inflammatory factors T-bet, IFN γ and GM-CSF. CKO CD4 T-cells had a decreased ability to become iTregs as shown by significantly less Foxp3⁺ CD4⁺ T-cells in polarizing cultures, and overexpress T-bet, IFN γ and GM-CSF. Therefore, T-cells that lack Ikaros do not properly differentiate into either Th17 or iTreg lineages, but instead become cells with

altered pro-inflammatory characteristics. In conclusion, the data highlights new roles of Ikaros in maintaining proper CD4 T-cell populations in the periphery and in suppressing abnormal pro-inflammatory responses.

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LIST OF ABBREVIATIONS

| | |
|---------------|--------------------------------------------------|
| APC | Allophycocyanin |
| CD | Cluster of Differentiation |
| CKO | Conditional Knockout |
| DNA | Deoxyribonucleic Acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FITC | Fluorescein Isothiocyanate |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |
| ICS | Intracellular Staining |
| IFN- γ | Interferon- γ |
| IK | Ikaros |
| IL | Interleukin |
| iTreg | Inducible Regulatory T-cell |
| mg | Milligram |
| mL | Milliliter |
| mRNA | Messenger RNA |
| NK | Natural Killer |
| nTreg | Natural Regulatory T-cell |
| PCR | Polymerase Chain Reaction |
| PE | Phycoerythrin |
| PMA | Phorbol 12-Myristate |
| RBC | Red Blood Cells |

| | |
|--------------|---------------------------------------|
| RNA | Ribonucleic Acid |
| RNA | Ribonucleic Aci |
| RPMI..... | Roswell Park Memorial Institute Media |
| RT-PCR | Real Time-Polymerase Chain Reaction |
| Sp | <i>Streptococcus Pneumoniae</i> |
| Tcm..... | Central Memory T-cell |
| TCR..... | T-cell Receptor |
| Tem | Effector Memory T-cell |
| TGF | Transforming Growth Factor |
| Th | Helper T-cell |
| Treg..... | Regulatory T-cell |
| μg | Microgram |
| μL..... | Microliter |

CHAPTER 1: INTRODUCTION

CD4 T-cells carry out imperative roles in the adaptive immune system for pathogen clearance, regulation of the response and self-tolerance. They mature through an immature double positive CD8⁺ CD4⁺ T cell receptor (TCR) $\alpha\beta$ ⁺ stage in the thymus and migrate to secondary lymphoid organs after becoming single positive CD4 or CD8 T-cells (Germain, 2002). When the TCR of CD4 T-cells is engaged by antigen presented by antigen presenting cells (APCs) in the context of major histocompatibility complex class II (MHC II), they become activated and differentiate into effector or regulatory subsets. The type of subset is determined by the cytokines present in the microenvironment of the response. After the response ceases, a large portion of T-cells undergo apoptosis, while a small population differentiates into memory cells that reside in tissues and provide faster responses upon secondary exposures to the same antigen (Sallusto et al, 2004).

In response to IL-12 present in the environment, T-cells differentiate into the Th1 subset by upregulating expression of their characteristic transcription factor T-bet, which promotes expression of Th1-lineage genes (Figure 1). Th1 cells secrete IFN γ and IL-2, and function to destroy intracellular microbes. These cytokines help to promote CD8 T-cell responses, activate macrophages, stimulate IgG production by B-cells, and recruit and activate inflammatory leukocytes, while inducing tissue injury and inflammation. Differentiation of the Th2 subset occurs in presence of IL-4, which activates expression of the hallmark transcription factor GATA-3. Th2 cells secrete IL-4, IL-5, IL-13 and IL-10 (Figure 1). They induce B-cell production of IgE, recruit eosinophils, clear extracellular pathogens, and inhibit acute and chronic inflammation. Th1 and Th2 subsets were

characterized as the main effector subsets of CD4 T-cells until Th17 cells were identified. CD4 T-cells differentiate into Th17 cells when they receive signals delivered by TGF- β and IL-6, which activate expression of the transcription factor ROR γ t. Th17 cells secrete IL-17, IL-21 and IL-22. They are important players in mucosal surface immunity, and contribute to the killing of extracellular bacteria and fungi (Abbas et al, 1996; Boyman and Sprent, 2012; Zhou et al, 2009; Zhu et al, 2012).

Unlike effector subsets, regulatory T-cells (Tregs) do not play roles in protecting against infection. These cells work to maintain the homeostasis of the immune response and prevent autoimmunity by suppressing self-reactive T-cells and the responses of effector T-cells. There are two subsets of Tregs: natural regulatory T-cells (nTregs) that are thymus derived, and inducible (iT-regs) that differentiate from naïve CD4 T-cells in response to TGF- β and IL-2. Their hallmark transcription factor is Foxp3 (Bilate and Lafaille, 2012; Zhu et al, 2012) and they secrete IL-10 and TGF- β as part of their suppressive responses. IL-10 is important in inhibiting T-cell expansion, suppressing helper subset responses, and provide protection to autoimmune diseases (OGarra et al, 2004; Chaudhry et al, 2011; Littman and Rudensky, 2010), while TGF- β supports the survival and homeostasis of Tregs (Zhu et al, 2010).

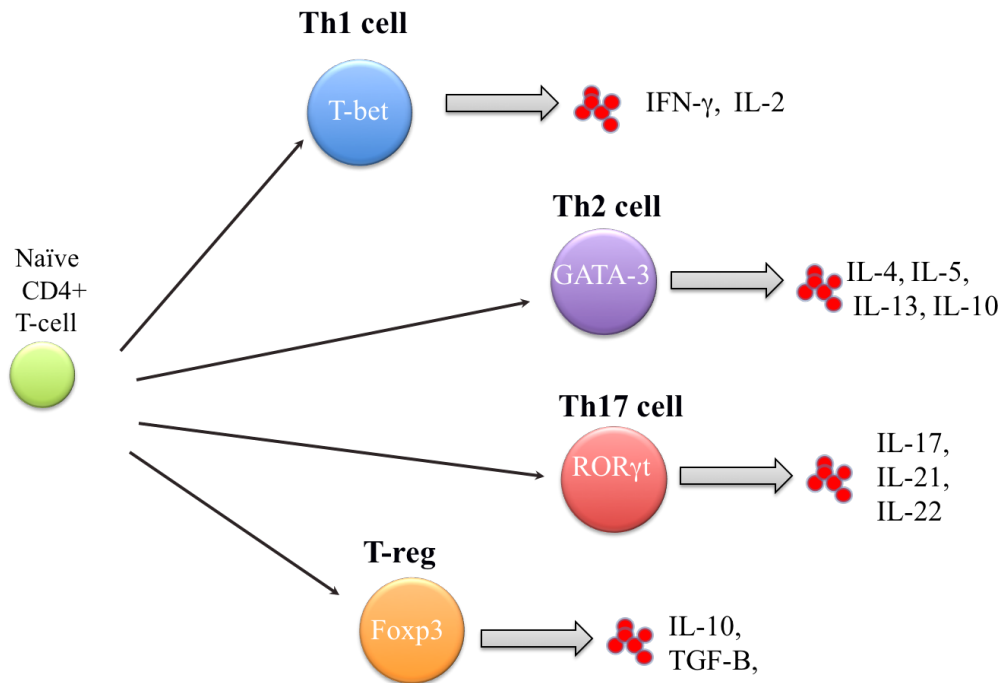


Figure 1: Naïve CD4 T-cells differentiate into different subsets that express distinct transcription factors and cytokines.

Naïve CD4 T-cells that differentiate into the Th1 subset express the transcription factor T-bet and secrete IFN γ and IL-2. Th2 cells express GATA-3 and secrete IL-4, IL-5, IL-13 and IL-10. Th17 cells express ROR γ T and secrete IL-17, IL-21 and IL-22. Regulatory T-cells express their hallmark transcription factor Foxp3, and secrete IL-10 and TGF- β .

Ikaros is an important protein in the maintenance of the adaptive arm of the immune system. It is a transcription factor highly expressed in lymphocytes that has shown to be important in development and maintenance of T-cells (Georgopoulos et al, 1997). In mice, deletions of Ikaros cause major immune cell disparities and leukemia (Georgopoulos et al, 1992; Winandy et al, 1995; Wang et al, 1996). In humans, mutations or deletions of Ikaros have been associated with bad prognosis and faster relapses in BCR-ABL acute lymphocytic leukemia (ALL) (Martinelly et al, 2009), and development of adult B-cell

ALL, myelodysplastic syndrome, acute myelogenous leukemia and blast crisis in chronic myelogenous leukemia patients (Payne and Dovat, 2011).

The aim of this thesis is to investigate how a conditional deletion of Ikaros exclusively from mature T-cells will affect the differentiation of CD4 T-cells into effector and regulatory subsets. Our results demonstrate that our model of conditional deletion of Ikaros has no effect in the development of thymic populations and B-cells. However, in the periphery, it resulted in an increase of myeloid cells and an abnormal population of naïve-like T-cells with a pattern of surface markers not previously described in literature. We also define a new role of Ikaros in suppressing inflammatory responses since lack of Ikaros results in greatly increased expression of T-bet, IFN γ and GM-CSF when CD4 T cells were differentiated in vitro into Th17 and iTreg subsets. Our data suggest that it will be important to continue to investigate this function of Ikaros and understand if it has important roles in suppression of autoimmune or chronic inflammatory diseases, and also how this role is important in regulation of the immune response.

CHAPTER 2: METHODS

2.1 Mice

The Ik^{flox} mouse was generated by Dr. Susan Winandy in conjunction with the Mouse Biology Project at UC Davis. Exon 7 (the last translated exon) and the 3'UTR of the Ikaros gene were flanked with loxP sites. The Ikaros floxed mouse was bred with an Lck distal promoter Cre transgenic mouse (obtained from The Jackson Laboratory). All mice were on the C57BL/6 inbred genetic background. Genotypes were determined by PCR analyses. All animal procedures were approved by the Boston University Institutional Animal Care and Use Committee.

2.2 Thymus and Spleen Extraction

Thymuses or spleens were dissected using aseptic techniques and placed in a petri dish on ice with complete RPMI [RPMI 1640 medium (GE Healthcare HyClone) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 50 μ M β -mercaptoethanol, 4mM L-glutamine, 50U/ml of penicillin and 50 μ g/ml streptomycin]. They were then ground between two glass slides and red cells were lysed with RBC lysis buffer (Santa Cruz). After two series of washes with complete RPMI, cells were counted on a hemocytometer using trypan blue exclusion to identify live cells.

2.3 CD4 T-cell Purification

Splenocytes were counted and resuspended in isolation buffer [500mL Phosphate buffered saline (Fisher), 0.5g bovine serum albumin (Fisher), 3g sodium citrate (EM Science)]. They were then pre-blocked with 2 μ g/ml Fc block (anti-CD32/anti-CD16, BioLegend) and rat serum (Sigma-Aldrich) for 15 minutes. Following this, CD4 cells were

purified using the Dynabeads[®] FlowComp[™] isolation kit (Invitrogen). Briefly, anti-CD4-biotin was added, followed by incubation with magnetic streptavidin-labeled beads. A magnet was used to isolate the bead-bound cells. Then the bead-bound cells were incubated in a release buffer to remove the magnetic beads from their surface. The purified CD4+ cells were counted on a hemocytometer using trypan blue.

2.4 Cell Culture

Whole splenocytes or purified CD4+ cells were plated in tissue culture dishes with 2-3 $\mu\text{g}/\text{mL}$ plate-bound anti-CD3 (Bio-X-Cell) and 5 $\mu\text{g}/\text{mL}$ soluble anti-CD28 (Bio-X-Cell) in complete RPMI.

Th17 differentiation: Cells were plated with 1 ng/mL TGF- β (R&D Systems), 5 $\mu\text{g}/\text{ml}$ IL-6 (Peprotech), 5 $\mu\text{g}/\text{ml}$ anti-IFN γ (Biolegend) and 5 $\mu\text{g}/\text{ml}$ anti-IL-4 (eBioscience). After 3 days, cells were harvested for RNA isolation or re-stimulated with 10 $\mu\text{g}/\text{ml}$ plate-bound anti-CD3 or 20 ng/mL phorbol myristate acetate (PMA) and 400 ng/mL ionomycin. When cells were used for intracellular cytokine staining, they were also incubated with 3 $\mu\text{g}/\text{mL}$ Brefeldin A (GolgiPlug, eBioscience) for 2-4 hours.

iTreg differentiation: Cells were plated with 2 ng/ml TGF- β , 50 U/ml IL-2 (Peprotech), 5 $\mu\text{g}/\text{ml}$ anti-IFN γ and 5 $\mu\text{g}/\text{ml}$ anti-IL-4 for 48 hours. Then cells were removed from anti-CD3 stimulation for 3-4 days after which they were re-stimulated with 10 $\mu\text{g}/\text{ml}$ plate-bound anti-CD3. In some cases, cells were continuously on anti-CD3 stimulation until harvest for analysis on day 5. Cells were used for intracellular cytokine staining were also incubated with 3 $\mu\text{g}/\text{mL}$ Brefeldin A (GolgiPlug, eBioscience) for 2-4 hours.

2.5 Flow Cytometry

Surface marker staining: 5.0E+05 to 1.0E+06 cells were blocked with 2 µg/mL Fc block (anti-CD32/anti-CD16) and rat serum. Cells were then incubated with fluorochrome-conjugated antibodies against the cell surface marker.

Intracellular cytokine staining: Cells were fixed and permeabilized using the Intracellular Fixation & Permeabilization Buffer Set (eBioscience), followed by staining with fluorochrome-conjugated antibodies.

Nuclear protein staining: Cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), followed by staining with fluorochrome-conjugated antibodies.

In all cases, cells were analyzed on a FACSCalibur (BD Biosciences) flow cytometer. Analyses were performed using Flow Jo software.

Table 1: Table of antibodies used for Flow Cytometry

| Antibody | Clone | Fluorophore | Company |
|----------------|----------|---------------|-------------|
| CD4 | RM4-5 | PE, APC | eBioscience |
| CD8 | 53-6.7 | PE, APC, FITC | eBioscience |
| CD11b | M1/70 | PeCy5 | eBioscience |
| CD44 | IM7 | FITC | Biologend |
| CD45R (B220) | RA3-6B2 | PE | eBioscience |
| CD62L | MEL-14 | PE | Biologend |
| Foxp3 | FJK-16s | PE | eBioscience |
| T-bet | eBio4B10 | eFluor, PE | eBioscience |
| GATA3 | TWAJ | PE | eBioscience |
| ROR γ t | B2D | APC | eBioscience |
| GM-CSF | MP1-22E9 | PE | Biologend |
| IFN γ | XMG1.2 | APC | eBioscience |
| GR-1 | RB6-8C5 | FITC | eBioscience |
| IL-17A | EBio17B7 | FITC | eBioscience |
| IL-2 | FFA21 | PE | eBioscience |

2.6 RNA Isolation and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated from cells using the SV Total RNA Isolation System (Promega). cDNA was generated with Superscript III or IV Reverse Transcriptase (Invitrogen). qRT-PCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) or SSO-Advanced Universal SYBR Green Supermix (Bio-Rad) on a BioRad MyiQ Real-Time PCR machine. Raw data obtained was analyzed using the Pfaffl method to normalize target gene data to that obtained with primers for the reference gene, *HPRT*. All primers used were generated by IDT DNA technologies.

Table 2: Table of RT-PCR Primers

| Primer | Sequence |
|-------------------------|-----------------------------|
| Foxp3 forward | GGCCCTTCTCCAGGACAGA |
| Foxp3 reverse | GCTGATCATGGCTGGGTTGT |
| GM-CSF forward | ATGCCTGTCACGTTGAATGAAG |
| GM-CSF reverse | GCGGGTCTGCACACATGTTA |
| HPRT forward | GGATATGCCCTTGACTATATTGAG |
| HPRT reverse | GCCACAGGACTAGAACACC |
| IFN γ forward | ACAATGAACGCTACACATTGC |
| IFN γ reverse | CTTCCACATCTATGCCACTTGAG |
| Ikaros (exon-7) forward | TCTACCTAACCAACCACATCAAC |
| Ikaros (exon-7) reverse | TGCTGACCACACGGAAGG |
| IL-2 forward | TGAGCAGGATGGAGAATTACAGG |
| IL-2 reverse | GTCCAAGTTCATCTTCTAGGCAC |
| IL-17A forward | CTCCAGAAGGCCCTCAGACTAC |
| IL-17A reverse | GGGTCTTCATTGCGGTGG |
| Rorc forward | ACCTCCACTGCCAGCTGTGTGCTGTC |
| Rorc reverse | TCATTTCTGCACTTCTGCATGTAGACT |

2.8 Statistics

Statistical analyses were performed using paired two-tailed Student *t* tests.

CHAPTER 3: CHARACTERIZATION OF A NOVEL IKAROS CONDITIONAL KNOCKOUT MOUSE MODEL

3.1 Introduction:

The *Ikzf1* gene, encodes for the Ikaros protein, a protein with five zinc finger motifs that is expressed at highest levels in T-cells and their progenitors (Georgopolous et al, 1992). The Ikaros protein is made of seven exons and contains a DNA binding motif, an activation domain and a dimerization domain, the last two of which are located in exon 7 (Georgopolous et al, 1997). Deletion of sequences encoding the DNA binding motif within the Ikaros gene resulted in a dominant negative Ikaros mouse model. Homozygote mice with this mutation had a complete deficiency of lymphocyte development and severe abnormalities in myeloid and erythroid populations (Georgopolous et al, 1997). Heterozygote mice developed T-cell leukemia and lymphomas within months (Winandy et al, 1995). These early studies of Ikaros suggest its imperative role in lymphocyte development and identify it as a tumor suppressor.

Other models of Ikaros deletion targeted the sequences encoding the last exon of the protein, exon 7, which resulted in an Ikaros-null (IK-null) mouse. Early studies showed that IK-null mice had abnormal thymocyte populations with a higher proportion of CD4 T-cells and lower proportions of double positive T-cells, lacked lymph nodes, B-cells, NK-cells, and had fewer $\gamma\delta$ T-cells (Wang et al, 1996; Georgopolous et al, 1997). T-cells of IK-null mice proliferated at higher rates upon TCR engagement, when compared to wild type cells (Wang et al, 1996, Georgopolous et al, 1997), contributing to the conclusions that

Ikaros deficiency allows for leukemogenic behavior in T-cells and supports the role of Ikaros as a tumor suppressor.

Subsequent experiments of IK-null mice studied the effect of the mutation on CD4 T-cells and their differentiation abilities. When IK-null CD4 T-cells were cultured in vitro they showed tendencies to become Th1-like cells, producing high amounts of IFN γ even under Th2 polarizing conditions (Quirion et al, 2009). They also showed inability to produce normal levels of IL-10 and IL-4, and increased IL-2 secretion (Umetsu & Winandy, 2009). These studies suggested that Ikaros also has important roles in regulating cytokine expression profiles and helper T-cell differentiation.

A new model for Ikaros studies, an Ikaros conditional knockout mouse, was developed by Dr. Susan Winandy in which Ikaros is deleted in peripheral T-cells. In this model, loxP sites were placed on either side of exon 7 of *Ikaros* to induce the exon's deletion upon expression of Cre-recombinase. This mouse was bred to an Lck-distal promoter-Cre-recombinase transgenic mouse. Because the distal promoter of the gene encoding Lck is only highly active in mature T-cells, it drives expression of Cre-recombinase efficiently only in these cells (Wildin et al, 1991). Lack of exon 7 in Ikaros mRNA results in an unstable message that cannot be translated to protein and is degraded. This results in absence of Ikaros exclusively in mature, single positive T-cells.

The conditional knockout of Ikaros in T cells allows us to study the roles of Ikaros in development of normal peripheral T-cell populations and their differentiation into helper subsets. This model reduces any possible effects that would result from the abnormal maturation of T-cells that occurs in the absence of Ikaros and allows for proper

comparisons between Ikaros mutant and wild type cells. Throughout the thesis, this mouse model will be referred to as “CKO”. CKO mice that have the Lck-distal promoter-Cre-recombinase transgene and therefore have Ikaros-deficient T cells are referred to as CRE+. CKO mice that do not express CRE and therefore have T cells with normal levels Ikaros protein are referred to as CRE-.

3.2 Ikaros CKO Cre+ mice show no effects on early T-cell development in the thymus.

Previous studies with IK-null mouse showed abnormal development of T-cells, B-cells, and myeloid cells accompanied by a leukemogenic phenotype. Using our novel Ikaros conditional knockout model, it is expected that thymocyte development will occur normally, and that B-cell and myeloid cell development will be unaffected. To ensure that this is the case, we evaluated thymus and spleen cell populations from three young mice from 5-8 weeks of age of each genotype, CRE+ and CRE-.

The thymuses of two mice revealed equivalent percentages of CD4+ and CD8+ single positive and double positive thymocytes in the CRE+ and CRE- mice (Figure 2). Unlike the IK-null mice that showed abnormal thymocyte development, CRE+ thymus showed no abnormalities. This suggests that the conditional deletion of Ikaros is not affecting early T-cell development and supports this model for studies of peripheral T-cell differentiation and function.

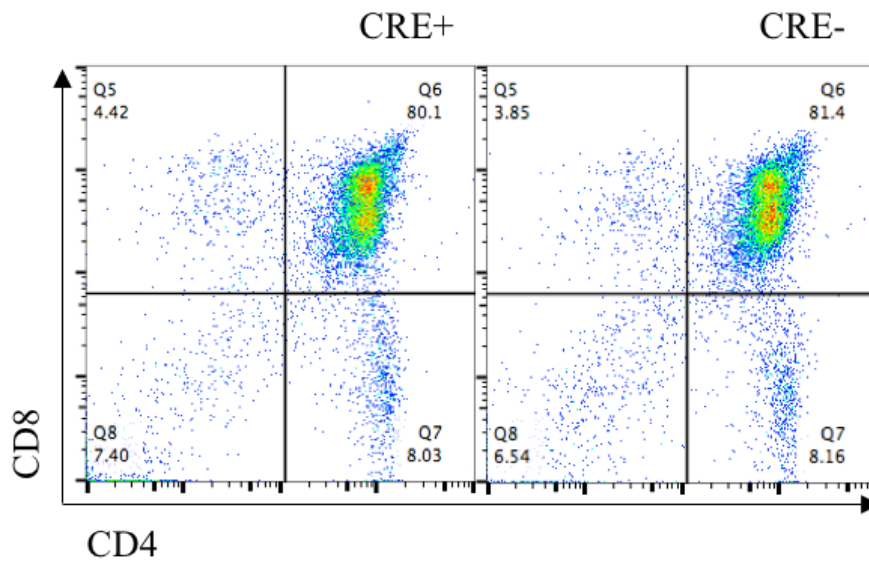


Figure 2: Thymus of CRE+ mouse is similar to thymus of CRE- mouse.

Thymuses from CRE+ and CRE- mice were processed and analyzed using flow cytometry for expression of surface markers of CD4 and CD8. Representative of 2 independent experiments.

3.3: Ikaros CRE⁺ mice show minimal alterations in the peripheral T-cell compartment.

After thymocytes mature to single positive CD4 or CD8 lineages, they migrate from the thymus towards secondary lymphoid organs, which include the spleen. Peripheral CD4 T-cells now have the ability of becoming T helper (Th) subsets. To evaluate the peripheral lymphocyte populations when Ikaros is deleted from T-cells after thymic maturation, spleens from CRE⁺ and CRE⁻ mice were analyzed.

Cellularity of spleens from CRE⁺ was equivalent to that of CRE⁻ spleens (Figure 3d). There was an increase in the proportion of CD4 T-cells (Figure 3a, e), but the difference was not statistically significant. Unlike IK-null mice, spleens from CRE⁺ mice did not show deficiencies in B-cell populations (Figure 3b, f). They also had normal lymph node development (data not shown). Myeloid cells were identified based on their surface marker expression of CD11b⁺ and macrophages based on CD11b⁺GR1⁻ (Hickstein et al, 1989; Hestdal et al, 1991). The percentage of macrophages and total myeloid cells were increased in CRE⁺ (Figure 3c). Average percentage of total myeloid cells (CD11b⁺) and macrophage cells (CD11b⁺GR1⁻) were significantly increased in CRE⁺ (Figure 3g) from three independent experiments. Overall, the cell populations in the spleens of CRE⁺ mice showed normal development of lymphoid lineages, with increases in percentages of myeloid cells. Unlike previous Ikaros deficient models, the Ikaros conditional knockout does not result in aberrancies in lymphoid lineage development.

To obtain further insight into splenic CRE⁺ T-cell populations, expression of transcription factors that identify CD4⁺ T helper lineages and regulatory T cell populations

were analyzed. The percentages of CD4+FOXP3+ T-cells were equivalent in CRE+ and CRE- spleens (Figure 4a, b). Percentages of T cells identified as Th1, Th2 and Th17 helper subsets also were not affected, as the percentages of cells expressing their corresponding transcription factors T-bet, GATA-3 and ROR γ T were equivalent in CRE+ and CRE- spleens (Figure 4). Therefore, these data suggest that T cells in the spleens of CRE+ mice are similar to those in CRE-spleens.

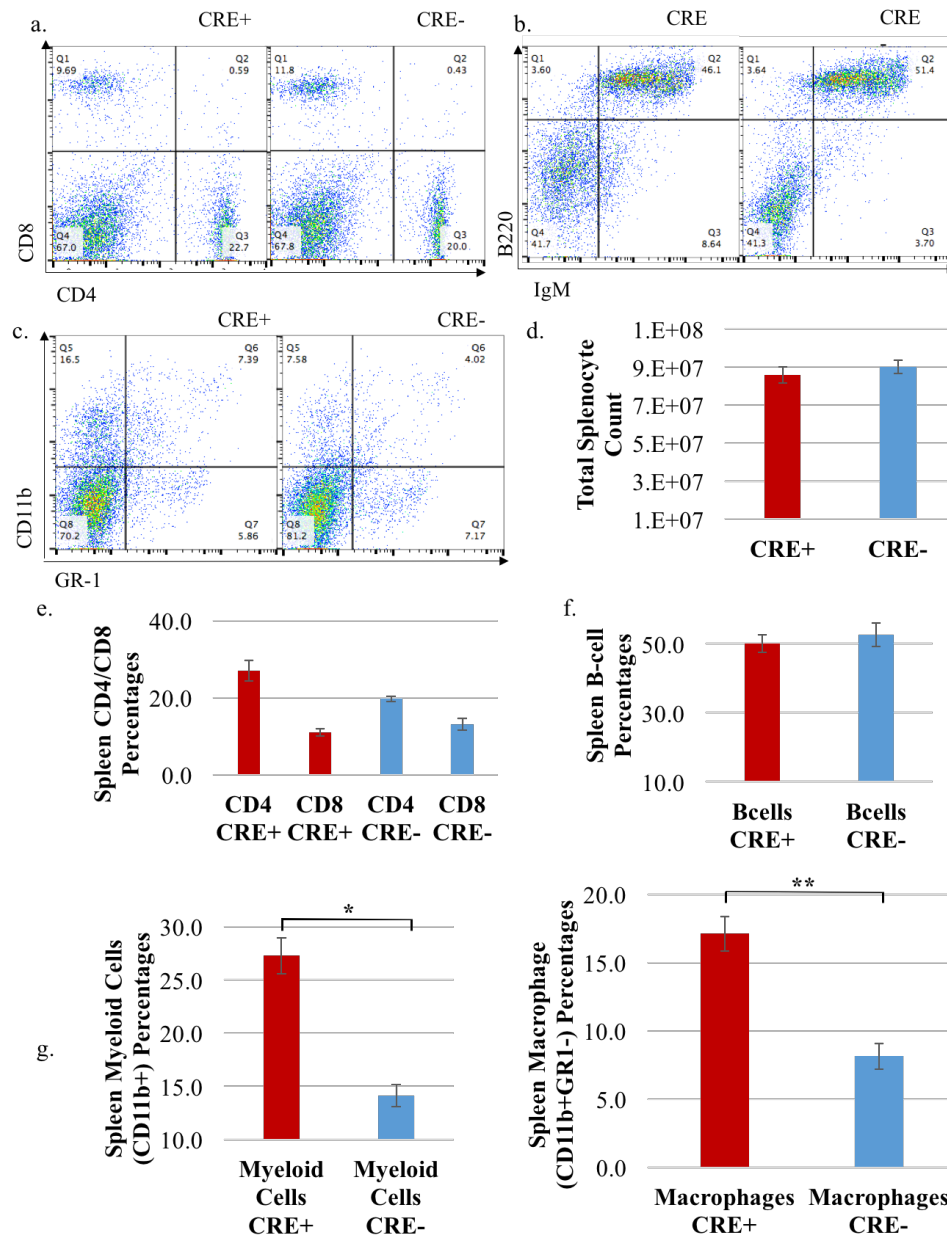


Figure 3: Lymphocyte populations in the spleens of CRE+ mice not affected, but there is an increase in myeloid cells.

Spleens of CRE+ and CRE- mice were analyzed for a) T-cells (CD4, CD8), b) B-cells (B220, IgM) c) Myeloid cells (GR-1+) and macrophages (CD11b+GR1-). Flow plots representative of 3 independent experiments d) Total splenocytes e) Percentages of CD4 and CD8 T-cells. f) Percentages of B-cells. g) Percentages of myeloid cells (CD11b+) and macrophages (CD11b+GR1-). Averages of 3 independent experiments. Help with these experiments was provided by Ksenia Arakcheeva. *p=0.0023, n=3. **p=0.0048, n=3.

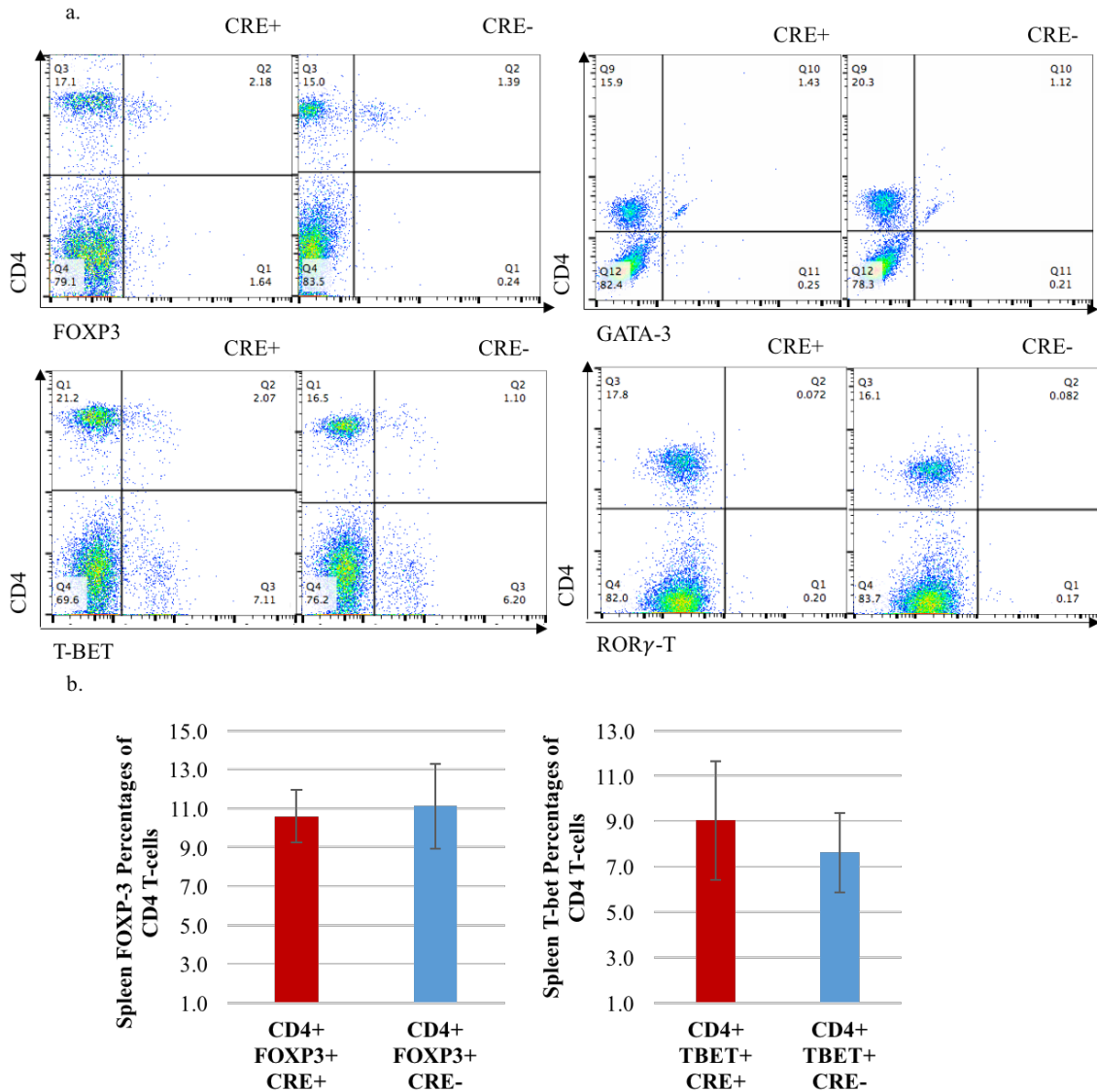


Figure 4: Transcription factor expression in splenic CD4 T-cells.

Spleens of CRE⁺ and CRE⁻ mice were processed and stained for surface markers, followed by ICS. a) Flow cytometry plots show expression of CD4 and Foxp3 and T-bet (representative of 3 independent experiments), GATA-3 and ROR γ t (representative of 2 independent experiments). b) Bar graphs of average Foxp3 and T-bet + percentages of total CD4 T-cells of three independent experiments. Help with these experiments was provided by Ksenia Arakcheeva.

3.4: An abnormal “naïve-like” T cell population develops in absence of Ikaros.

CD4 T-cells that populate the periphery survive as naïve populations until they encounter antigen, which allows them to become effector cells. Following the cessation of immune responses, T-cells may become memory cells or undergo apoptosis. Memory T-cells that remain produce rapid and robust secondary responses when they encounter antigen. Within memory populations, central memory (T_{cm}) and effector memory (T_{em}) cells are two different subsets. T_{cm} provide reactive memory, in which cells have greatly reduced effector function, but can proliferate and differentiate to effector cells in response to antigen, whereas T_{em} provide protective memory with immediate effector responses (Sallusto et al, 2004).

Naïve and memory T cell populations can be differentiated based on their surface marker expression. CD62L is expressed in antigen inexperienced cells and is removed from the surface after T-cell activation (Chao et al, 1997), while CD44 is expressed after TCR signaling and it never gets down-regulated (Budd et al, 1987; DeGrendele et al 1997). Patterns of expression of these markers can be used to profile T-cells based on their activation and antigen-experienced status. CD62L high/CD44⁻ identifies antigen inexperienced “naïve” T-cells; CD44 high/CD62L high identifies central memory T-cells (T_{cm}), and CD44 high/CD62L low and CD44 high/CD62L⁻ identifies effector memory T-cells (T_{em}) (Beura et al, 2016).

These markers were used to analyze T cell populations in CRE⁺ spleens to determine if proportions of naïve and antigen-experienced populations of T cells were altered in the absence of Ikaros. Using flow cytometry, CD4⁺ T-cells were gated and

evaluated for expression of CD62L and CD44 markers. The CRE⁻ mice showed mostly naïve cells with small populations of T_{cm} and T_{em} (Figure 5a), this is representative of spleens of normal “young” mouse housed in a pathogen-free barrier facility. Unlike the CRE⁻ mice, CRE⁺ had far less naïve cells and developed an abnormal CD62L low CD44⁻ population, but they had small memory cell populations like the CRE⁻. The proportion of central and effector memory cells was equivalent in the CRE⁺ and CRE⁻ mice (Figure 5b), implying that there is not an expansion of antigen-experienced cells in CRE⁺ spleens. However, the CRE⁺ mice had a significant decrease in the population of CD44^{high}/CD62L^{high} naïve cells (Fig 5c). This decrease can be accounted for in the development of the CD62L low and CD44⁻ population (Fig 5d). T-cells with that phenotype have not been previously defined in literature, although we do also see them in CRE⁻ spleens. It is possible that cells in this category represent another population of naïve cells that never become effector cells since they do not express the activation marker CD44. It is also possible that these cells receive antigen but are unable to express CD44 on the surface. Further experiments are necessary to determine if this expanded population of CD62L low CD44⁻ cells has any other characteristics of antigen inexperienced cells, or if they will have implications in the development of a normal immune cell repertoire and in the immune response.

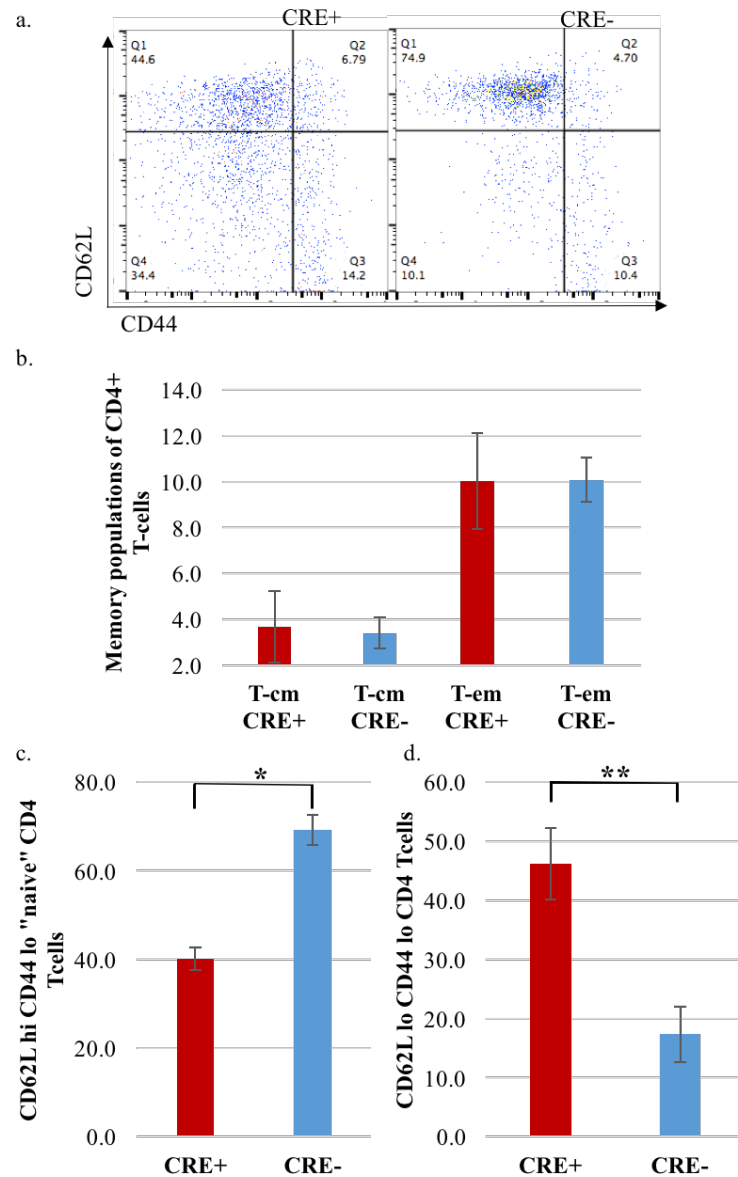


Figure 5: CRE+ spleens have normal memory CD4 T-cell populations but less naïve T-cells.

Spleens of CRE+ and CRE- mice were analyzed with flow cytometry for surface markers. CD62L and CD44 staining identifies naïve cells (CD62L+CD44-), central memory cells (CD62L+CD44+) and effector memory cells (CD62L-CD44+) within CD4+ gated T-cells

a) Flow cytometry plots representative of 3 independent experiments. b) Percentage of memory populations of CD4+ T-cells, T-cm and T-em. c) Percentage of CD62L high CD44 low “naïve” CD4 T-cells. d) Percentage of CD62L low CD44 low CD4+ T-cells. Bar graphs are averages of three independent experiments. Help with these experiments was provided by Ksenia Arakcheeva. *p=0.0023, n=3. **p=0.02, n=3

In conclusion, CRE⁺ mice have few differences with CRE⁻ mice, and they appear overall healthy. Unlike IK-null mice, they have normal development of T-cells, B-cells, and lymph nodes, and normal thymic populations. Their spleens are fairly normal with only enhanced numbers of myeloid cells and an expanded CD62L low CD44⁻ CD4 T-cell population. Importantly, thymocyte development in CRE⁺ mice appears normal, supporting the use of this Ikaros conditional knockout model to investigate the role of Ikaros in mature, peripheral T-cells. The next chapter will evaluate the abilities of CRE⁺ T-cells to differentiate into subsets of helper CD4 T-cells in the absence of Ikaros.

CHAPTER 4: CONDITIONAL KNOCKOUT OF IKAROS AFFECTS NORMAL DIFFERENTIATION TOWARDS T HELPER SUBSETS IN VITRO

4.1 Introduction:

CD4 T-cells differentiate into T helper subsets after antigenic stimulation through TCR. The cytokines present in the micro-environment are the major determinants in their decision to commit to a specific lineage, and lineage-specific transcription factors become activated to drive differentiation of specific T helper subsets (Zhu et al, 2010). Previous reports have suggested that Ikaros has important roles in T helper subset differentiation, specifically in repressing Th1 responses during Th2 differentiation and in inducing Th17 differentiation (Quirion et al, 2009; Wong et al, 2013). These studies were performed in T cells from mice with germline mutations in Ikaros in which thymic T cell development is abnormal. In this chapter, the effects of conditional knockout of Ikaros in mature T cells on differentiation of Th17 and regulatory T (T-reg) cell lineages is evaluated in order to understand the function that Ikaros plays in proper peripheral T-cell responses.

4.2: Differentiation of T-cells towards Th17 lineage is affected by conditional deletion of Ikaros.

Th17 helper T-cells are crucial players in the adaptive arm of the immune system. They are the major subtype of CD4⁺ T-cells residing in mucosal surfaces, and they function to clear extracellular bacteria and fungi that survive innate immune responses. T-cell differentiation towards a Th17 subtype is dependent on the functions of the transcription factor STAT-3 to enhance expression of ROR γ t, their hallmark transcription factor, which initiates differentiation (McGeachy and Cua, 2008). Their differentiation from a naïve

CD4⁺ T-cell occurs in response to TGF- β and IL-6 along with receptor signals delivered by TCR interactions with an antigen presenting cell (Torchinsky, 2009). Following their differentiation, Th17 cells secrete the cytokines IL-17A, IL-17F IL-22 and IL-21, which combat the infection.

IL-17A and IL-17F are responsible for stimulating and mobilizing neutrophils to the infection site by inducing tissue cells to release chemokines and colony stimulating factors (Kolls and Linden, 2004; Aujla et al, 2008). It has been reported that IL-17A can also increase the bactericidal activity of neutrophils by inducing increased expression of myeloperoxidase and elastase in vivo (Kolls and Linden, 2004). In cooperation with IL-17, IL-22 enhances tissue cell secretion of antimicrobial peptides for host defense (Liang et al, 2006). IL-21 has important roles in maintaining the Th17 phenotype, suppressing regulatory T-cell phenotypes, and promoting B-cell responses (McGeachy and Cua, 2008; Korn et al, 2007) After elucidating the role of Th17 cells, it became clear that they are crucial players in the maintenance of mucosal immunity and protect these exposed surfaces from pathogens by strengthening the inflammatory response. Using our Ikaros conditional knockout mouse model, this study aims to investigate if lack of Ikaros in peripheral T-cells will have an effect on the differentiation and functions of Th17 cells.

In order to confirm deletion of Ikaros from peripheral T-cells, expression of Ikaros mRNA was quantified using qRT-PCR. CD4⁺ T-cells from spleens of CRE⁺ mice showed lower levels of Ikaros mRNA compared to CRE⁻ cells (Figure 6a), confirming Ikaros deletion. This analysis was also done on purified CD4⁺ T-cells cultured with or without Th17 polarizing cytokines for 2-3 days in the presence of anti-CD3/anti-CD28, to mimic

TCR stimulation. In the case of the culture without polarization, referred to as “Th0”, Ikaros message was decreased in the CRE⁺ relative to the CRE⁻ culture. In Th17 cultures, the levels of Ikaros were also decreased significantly in CRE⁺ cultures (Figure 6b). These data demonstrate that the deletion was successful and that there is no overgrowth of Ikaros-sufficient cells in the CRE⁺ cultures.

The main transcription factor that characterizes Th17 cells is ROR γ t (McGeachy and Cua, 2008). We therefore assessed differentiation by measuring ROR γ t expression within CD4⁺ T cells in Th0 cultures plated with anti-CD3/anti-CD28 alone, and Th17 cultures, which included polarizing cytokines. Cultures of both CRE⁺ and CRE⁻ splenocytes had minimal percentages of CD4⁺ROR γ t⁺ T-cells in Th0 cultures, which were similarly increased with Th17 polarization (Figure 7a). Isolated CD4⁺ T-cells from CRE⁺ and CRE⁻ spleens were also cultured under Th17 polarizing conditions. Histograms of ROR γ t expression as measured by flow cytometry showed similar expression levels in CRE⁺ and CRE⁻ cultures (Figure 7b). Levels of ROR γ t gene expression measured by qRT-PCR were slightly higher in CRE⁺ cultures (Fig 7c), but the increases were not statistically significant. Taken together, these results suggest that Ikaros deficiency has no effect on the differentiation of Th17 cells.

Although differentiation towards the Th17 lineage appears to occur normally in the absence of Ikaros, there was abnormally high expression of the transcription factor T-bet in both splenocytes (Figure 8a) and purified CD4 T-cell CRE⁺ cultures (Figure 8b). This resulted in a significant population of T-bet⁺ ROR γ t⁺ cells. T-bet can be co-expressed with ROR γ T in hybrid subsets often seen in detrimental pro-inflammatory conditions in both

mice and humans (Harbour et al, 2004; Evans and Jenner, 2013; Annunziato et al, 2007). These data, together with previous literature reporting increased Th1 like phenotypes in the absence of Ikaros (Quirion et al, 2009), suggest that Ikaros may be necessary to shut down T-bet expression during Th17 differentiation to prevent development of pathological T-bet+ ROR γ t+ hybrid cells.

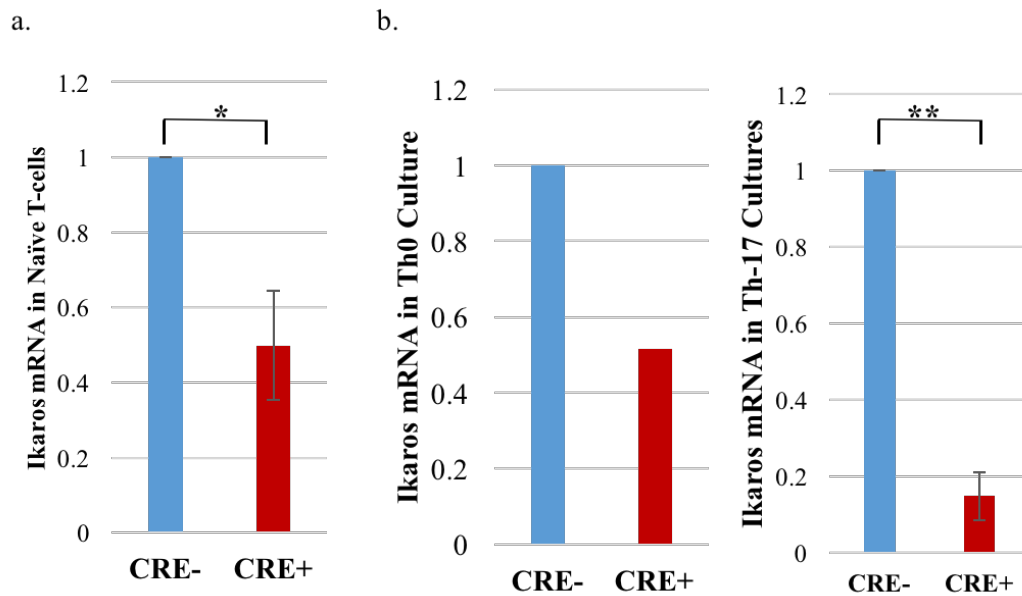


Figure 6: Ikaros mRNA was lowered in CRE+ cells.

a) CD4⁺ purified T-cells from CRE⁺ and CRE⁻ mice were processed for RNA isolation. Ikaros mRNA was measured with qRT-PCR. b) Purified CD4⁺ T-cells were cultured under Th0 or Th17 conditions and RNA was isolated. Ikaros was measured with qRT-PCR. Results from three independent Th17 experiments and one Th0 experiment are shown. For Th17 cultures. Results shown are relative to values for HPRT expression and the value in the CRE⁻ cultures was set to 1. Help with these experiments was provided by Ksenia Arakcheeva. *p=0.026, n=3. **p=0.0002, n=3.

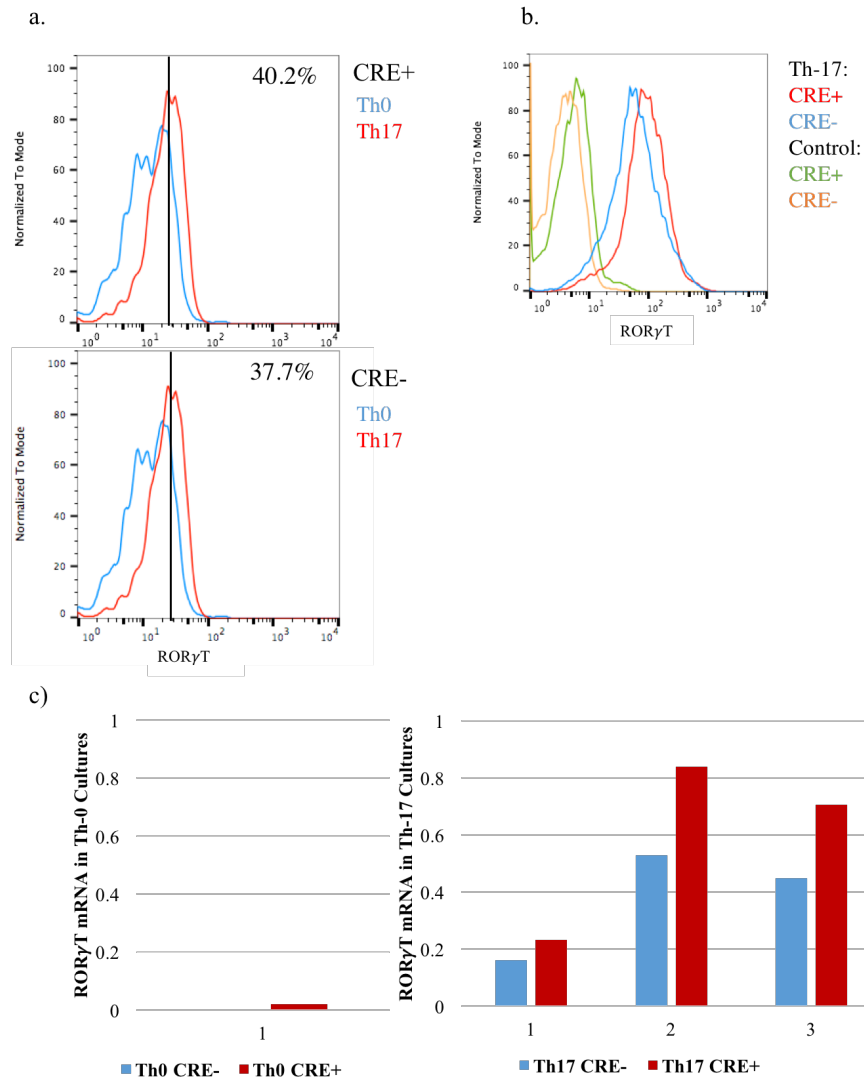


Figure 7: RORγt expression is normal in CRE+ Th17 polarization cultures.

a) Splenocytes from CRE+ and CRE- mice were cultured under Th0 and Th17 polarizing conditions and restimulated followed by ICS for RORγt and surface staining with CD4. Histograms are overlays of levels of RORγt staining, gated on CD4+ T-cells. Percentages represent CD4+ RORγt+ cells in the Th17 cultures. Vertical lines inside histograms mark RORγt+ (to right) and negative (to left) populations. b) CD4+ purified T-cells were cultured and stained as described above. Histogram is an overlay of RORγt staining and isotype controls. c) RORγt mRNA was measured with qRT-PCR from purified CD4+ T-cells treated as described above. Results from three Th17 experiments and one Th0 experiment are shown. Results shown are relative to values for *HPRT* expression.

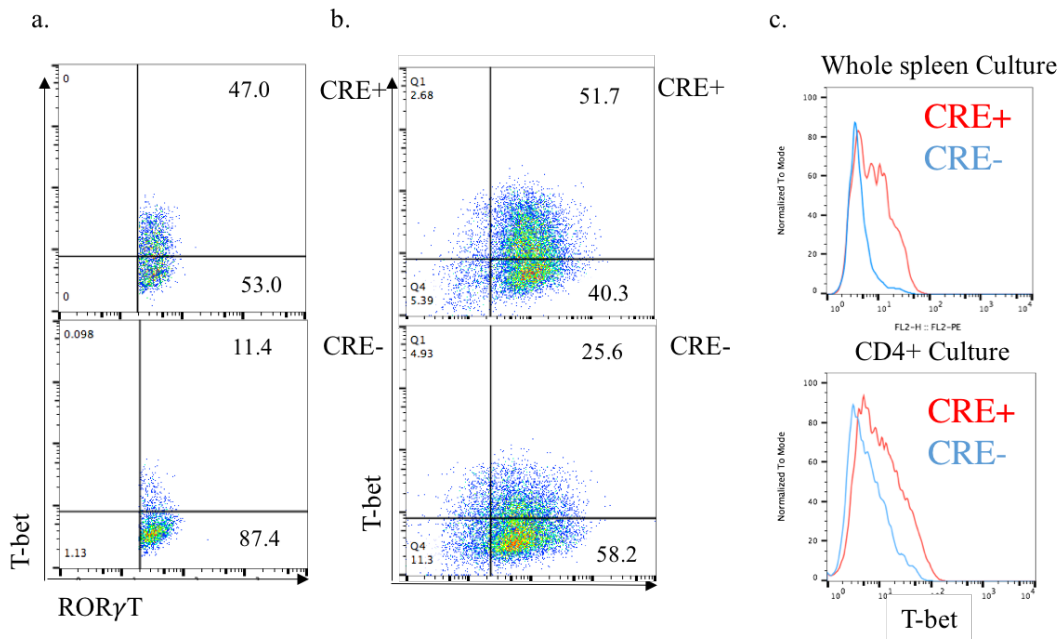


Figure 8: Higher levels of T-bet expression in CRE+ Th17 cultures.

a) Splenocytes from CRE+ and CRE- mice were cultured under Th0 and Th17 polarizing conditions and followed by ICS for ROR γ t and T-bet and surface staining with CD4. Plots show data gated on CD4+ T-cells only. b) CD4+ purified T-cells were cultured and stained as described above c) Histograms show T-bet expression in CRE+ and CRE- splenocyte and purified CD4+ T-cell Th17 cultures.

Due to the abnormal expression of the Th1 transcription factor in CRE⁺ Th17 cultures, it was expected that these T-cells would not function normally. To assess functionality of the Th17 cultures of CRE⁺ mice, cytokines were measured. When splenocytes were cultured under Th17 polarization, IL-17A was present in both CRE⁺ and CRE⁻, and IFN γ was increased in CRE⁺ cultures of both Th0 and Th17 (Figure 9a). These results were replicated in purified CD4⁺ T-cell cultures (Figure 9b). In addition, qRT-PCR analyses showed similar levels of IL-17A mRNA in CRE⁺ and CRE⁻ Th17 cultures, and increased IFN γ mRNA in both Th0 and Th17 CRE⁺ cultures (Figure 9c, d). Unlike previous studies with IK-null CD4 T cells, IL-17 secretion was not decreased in Th17 cells in CKO CRE⁺ mice, suggesting the role of Ikaros in commitment to Th17 lineages might be less definitive. However, the increase in expression of IFN γ in these cultures indicates that Ikaros may regulate Th17 function by repressing Th1 cytokines.

IL-2 expression was also analyzed in these cultures. IL-2 is secreted by T-cells, and it promotes their survival and proliferation, induces differentiation to Th1 and iTreg lineages, and suppresses Th17 cell differentiation (Liao et al, 2011; Laurence et al, 2007). In Th17 cultures of whole spleens and purified CD4 T-cells, IL-2 was increased in CRE⁺ cultures at both the protein and mRNA levels (Figure 10a, b). Previous studies with IK-null mice also have shown increased IL-2 secretion by T-cells in absence of Ikaros (Umetsu & Winandy, 2009). Therefore, it is possible that Ikaros may suppress IL-2 during differentiation to helper subsets. Further analysis is required to determine if long-term presence of IL-2 in these cultures would cause notable suppression to the Th17 phenotype overtime.

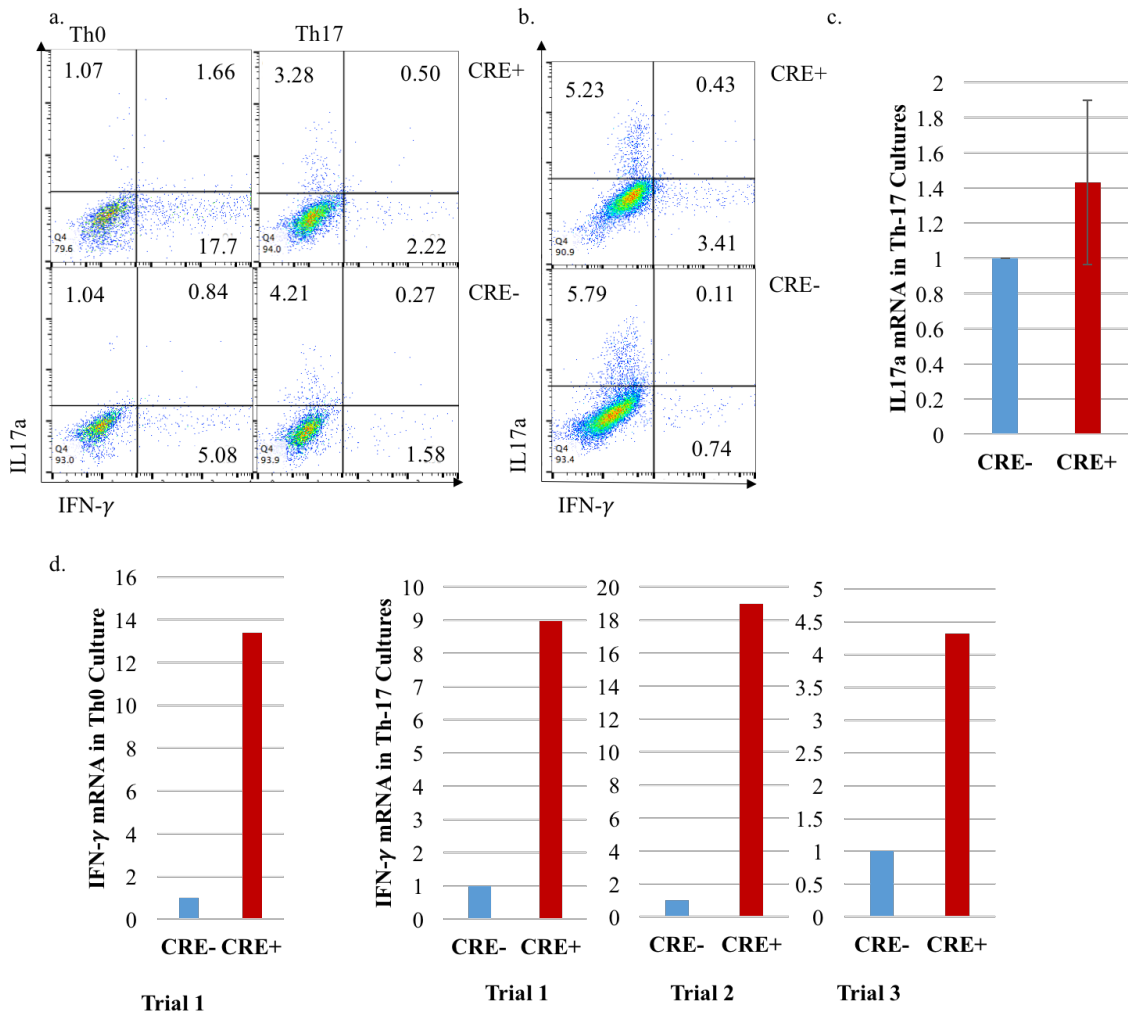


Figure 9: IL-17 and IFN γ expression is unchanged and increased, respectively, in CRE+ Th17cultures.

a) Splenocytes from CRE+ and CRE- mice were cultured under Th0 and Th17 conditions and followed by ICS for IL-17 and IFN γ and surface staining for CD4. The population shown is gated on CD4+ T-cells. b) Purified CD4+ T-cells were processed and stained as described above. c) qRT-PCR analyses of IL-17A mRNA levels, from purified CD4 purified T-cells cultured under Th17 conditions. Average of three independent experiments, not statistically significant. d) qRT-PCR analyses of IFN γ mRNA levels in one Th0 and three Th17 cultures of purified CD4 T-cells. For qRT-PCR data, results shown are relative to values for *HPRT* expression and the value in the CRE- cultures was set to 1.

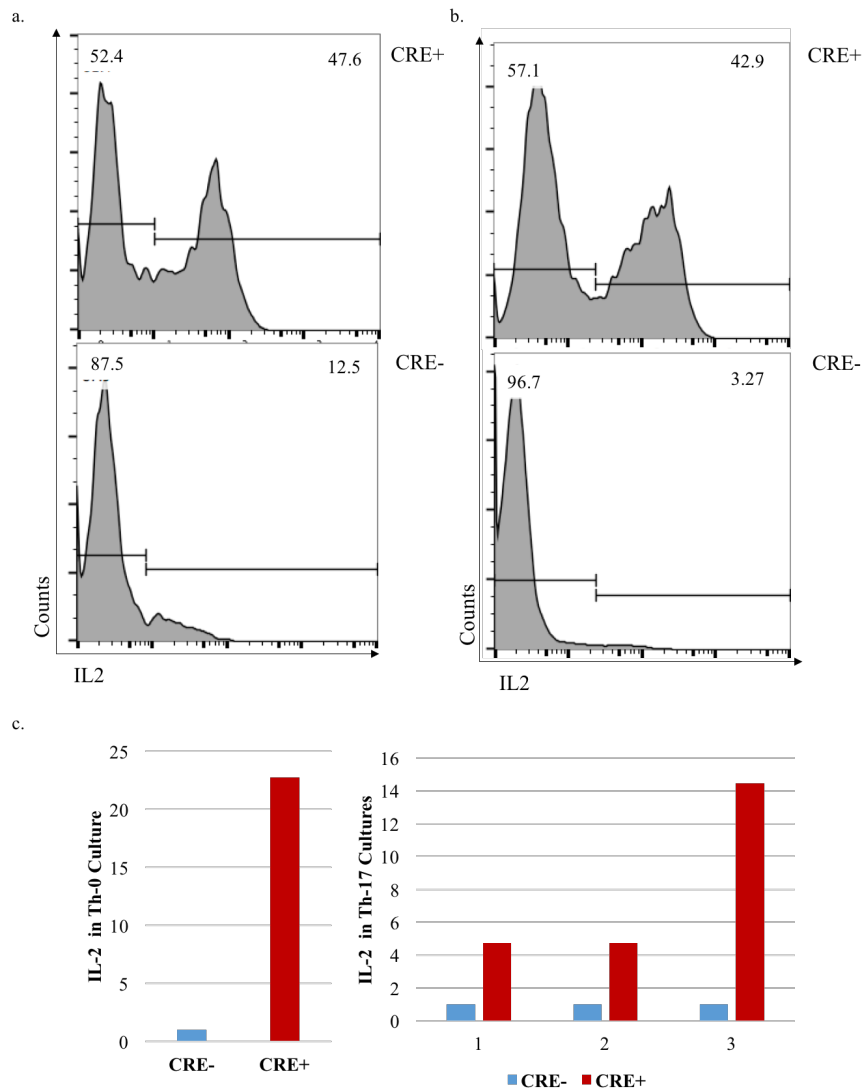


Figure 10: IL-2 expression is increased in Th0 and Th17 CRE+ cultures.

a) Splenocytes from CRE+ and CRE- mice were cultured under Th17 polarizing conditions, restimulated and followed by ICS for IL-2 and surface staining of CD4. Histograms show IL-2 expression of CD4+ gated T-cells. b) Purified CD4+ T-cells were cultured under Th17 polarization and treated as described above. Histograms show expression of IL-2 expression. Horizontal line defines cells that are positive (left side) and negative (left side) for IL-2 expression c) qRT-PCR analyses of IL-2 mRNA from purified CD4+ T-cells cultured under Th0 or Th17 conditions. Results shown are relative to values for *HPRT* expression and the value in the CRE- cultures was set to 1. Results from three Th17 experiments and one Th0 experiment are shown.

Hybrid Th17 cells that express Th1-like pro-inflammatory cytokines have been observed in autoimmune disorders and detrimental inflammatory processes (Littman and Rudensky, 2010; Evans and Jenner, 2013; Annunziatto et al, 2007). In addition, in cases where autoimmune diseases were attributed to Th17 cells, the secretion of granulocyte-macrophage colony stimulating factor (GM-CSF) by T-cells has been found to be important for the development of disease (El-Behi et al, 2001). Using flow cytometry, GM-CSF expression was evaluated in CRE⁺ and CRE⁻ CD4 T-cells cultures of Th0 and Th17 conditions. Overall expression of GM-CSF was more robust in the CRE⁺ as compared to the CRE⁻ cultures. qRT-PCR analyses using RNA prepared from purified CD4 T-cells grown in Th0 and Th17 polarization also demonstrated an increase of GM-CSF expression in CRE⁺ cultures. Further studies are necessary to investigate if the GM-CSF⁺ IL17⁻ T-cells that develop in CRE⁺ cultures represent a population of cells that express ROR γ T but are not functionally normal Th17 cells, and if this increased expression of GM-CSF in CRE⁺ cells will induce abnormal pro-inflammatory responses in vivo.

Overall, differentiation into the Th17 lineage was not compromised by absence of Ikaros, as shown by normal levels of ROR γ t and IL-17A expression, contradicting previous literature showing a role for Ikaros in Th17 lineage commitment. Nonetheless, the robust expression of GM-CSF, IL-2, IFN γ and T-bet in CRE⁺ Th17 cultures suggest that Ikaros may have important roles in suppressing pro-inflammatory programs.

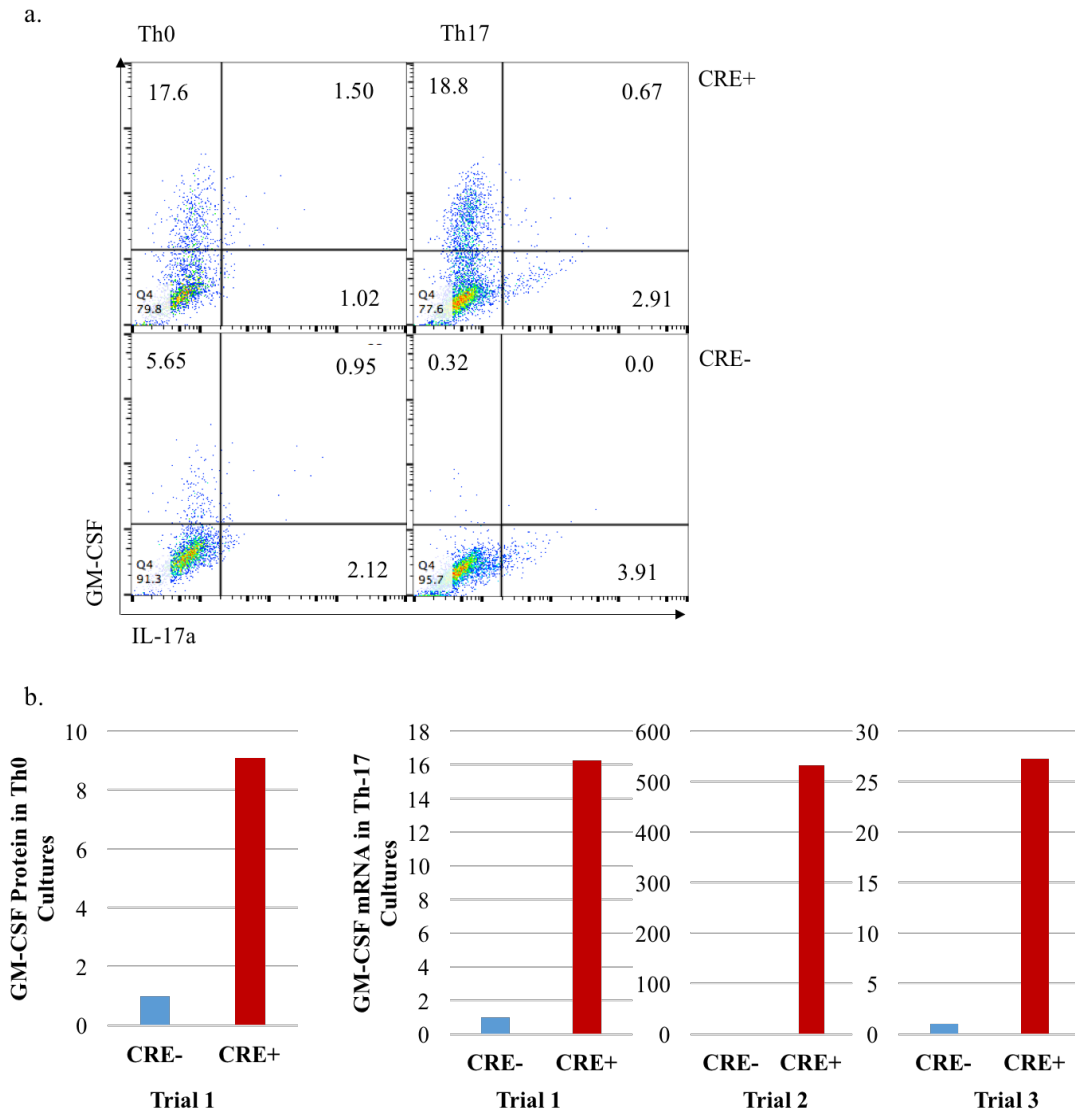


Figure 11: GM-CSF and IL-17 expression by CD4⁺ T cultured under Th0 and Th17 polarizing conditions.

a) Splenocytes of CRE⁺ and CRE⁻ mice were cultured under Th0 and Th17 conditions followed by ICS for IL17A and GM-CSF and surface staining for CD4. Flow cytometry plots show staining of CD4⁺ gated cells. b) Levels of GM-CSF mRNA were analyzed by qRT-PCR from purified CD4⁺ T-cells cultured as described above. Results from three Th17 experiments and one Th0 experiment are shown. Results shown are relative to values for *HPRT* expression and the value in the CRE⁻ cultures was set to 1.

4.3 Differentiation of T-cells towards a regulatory T cell phenotype is compromised in the absence of Ikaros.

As part of the immune system's controls, regulatory T-cells (Tregs) develop with the tasks of maintaining tolerance and suppressing the inflammatory responses of effector T-cells. Natural Tregs (nTregs) and inducible Tregs (iTregs) make up two populations that contribute to keeping immune responses in check. Cells in both of these categories are CD4⁺ T-cells that also express the surface marker CD25 (IL-2 Receptor α chain). nTregs mature in the thymus and have important roles in maintaining self tolerance through suppressing self reactive T-cells that escape the thymus, while iTregs develop in the periphery in response to antigen and down-regulate the inflammatory responses of effector T-cells (Bilate and Lataille, 2012; Itoh et al, 1999; Zhu et al, 2010; Littman and Rudenski, 2010). This group of cells is imperative for proper immune system suppression and for prevention of chronic inflammatory diseases.

Foxp3 is the defining transcription factor for Treg cells. It controls differentiation and functionality of Tregs (Hori et al, 2003) and it suppresses factors important for other T helper subsets. A good example is Foxp3 binding to RUNX1, which suppresses secretion of IL-2 and IFN γ , required for Th1 functions (Ono et al, 2007). The importance of Foxp3 for Treg functionality was evidenced by the development of overwhelming inflammatory responses and severe or fatal autoimmune diseases when the gene encoding Foxp3 was deleted or mutated (Hori et al, 2003; Littman and Rudensky, 2010). Naïve CD4 T-cells in the periphery have the ability to differentiate into Foxp3⁺ iTreg cells after TCR activation in response to TGF- β and IL-2. Among many functions of IL-2, in Tregs is necessary to

induce STAT5 expression, which in turn helps activate the gene encoding Foxp3, while TGF- β is key for their functionality and maintenance (Zhu et al, 2010). In order to study if Ikaros has a role in iTreg differentiation, the Ikaros conditional knockout model was used.

Due to the pro-inflammatory phenotype observed in CRE⁺ Th17 cultures, it is important to observe if lack of Ikaros will result in such abnormal responses in iTreg cultures as well. For these experiments, CD4⁺ T-cells were purified from spleens of CRE⁺ and CRE⁻ CKO mice and grown under Th0 or iTreg polarizing conditions as described in chapter 2. Levels of Ikaros deletion were analyzed by quantifying Ikaros message with qRT-PCR after isolation of RNA from Th0 or iTreg cultures of purified CD4 T cells. In both conditions, Ikaros mRNA was significantly decreased in the CRE⁺ cultures (Figure 12). These data indicate that there was no overgrowth of Ikaros-sufficient cells in the CRE⁺ cultures.

To evaluate the role of Ikaros in differentiation of CD4 T cells into the iTreg subset, levels of Foxp3 expression were measured from iTreg differentiation cultures. There were higher percentages of Foxp3⁺ cells in both CRE⁺ and CRE⁻ iTreg cultures when compared to Th0 cultures, indicating that iTreg differentiation occurred. However, the CRE⁺ cultures had significantly lower percentages of Foxp3⁺ cells than CRE⁻ cultures, and most cells were Foxp3⁻ (Figure 13a, b). Levels of Foxp3 mRNA also showed a statistically significant decrease of Foxp3 that supported the flow cytometry (Figure 13c). These findings suggest deficiencies in iTreg differentiation in the absence of Ikaros.

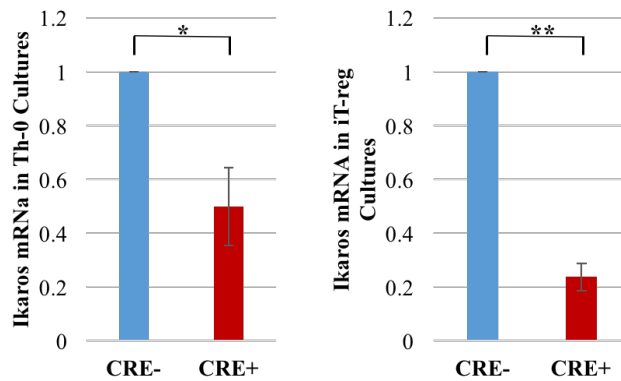


Figure 12: Levels of Ikaros expression are decreased in Th0 and iTreg CRE+ cultures.

Purified CD4+ T-cells from CRE+ and CRE- mice were cultured under Th0 or iTreg conditions and harvested for RNA isolation. Ikaros mRNA was measured with qRT-PCR. Results shown are relative to values for HPRT expression and the value in the CRE- cultures was set to 1. *p=0.026, n=3. **p=1.2E-04, n=3.

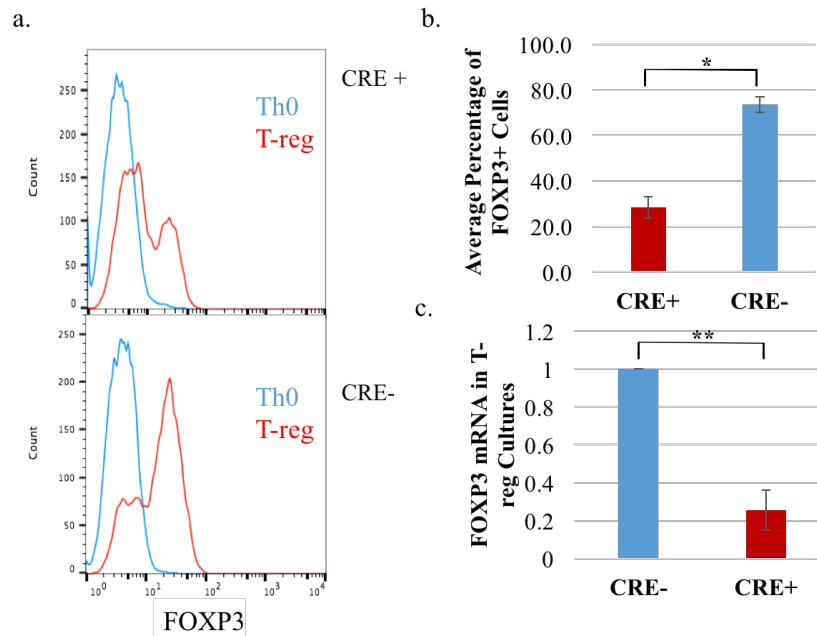


Figure 13: Levels of iTreg differentiation are decreased in CRE+ cultures.

a) Purified CD4+ T-cells from CRE+ and CRE- mice were cultured under Th0 and iTreg conditions followed by ICS for Foxp3. Histograms show Foxp3 levels as determined by flow cytometry analyses. Representative of 3 experiments. b) Bar graph shows combined data from iTreg cultures. c) Levels of Foxp3 mRNA from purified CD4+ iTreg cultures measured by qRT-PCR; results shown are relative to values for HPRT expression and the value in the CRE- cultures was set to 1. Help for these experiments was provided by Parul Agnihotri. *p=0.002, n=3. **p=0.0022, n=3.

Since previous results in Th17 cultures showed increased T-bet expression in CRE⁺ cultures, iTreg cultures were analyzed for T-bet expression. T-bet was also over-expressed in CRE⁺ iTreg cultures (Figure 14a), and they included a higher percentage of Foxp3⁺Tbet⁺ cells. The expression levels of T-bet per cell was similar between both cultures (Figure 14b). Foxp3⁺Tbet⁺ iTregs have been described in Th1-driven inflammation (Zhu et al, 2010); nonetheless, in an in-vitro situation, high FOXP3⁺Tbet⁺ cells are an abnormal finding and.

Considering the presence of a pro-inflammatory phenotype in CRE⁺ Th17 cultures, and the propensities of IK-null CD4 T cells to develop Th1 like responses, IFN γ expression was also analyzed. Under both Th0 and iTreg polarizing conditions, CRE⁺ cultures had a higher percentage of CD4 T cells that expressed IFN γ when compared to CRE⁻ cultures (Figure 15a). Levels of IFN γ mRNA were also measured using qRT-PCR and were found to be significantly increased in CRE⁺ (Figure 15b).

In the CRE⁺ iTreg cultures, 20% of cells that expressed Foxp3 also were secreting the Th1 cytokine IFN γ , while in CRE⁻ cultures this percentage was very small (1.6%). The higher expression of IFN γ in CRE⁺ Foxp3⁺ Tregs may suggest a a role of Ikaros in the functionality of Foxp3. In Treg cells, Foxp3 binds and represses RUNX-1 (Ono et al, 2007), an important initiator of transcription of the gene encoding IFN γ . Absence of Ikaros is affecting the ability to repress the IFN γ gene in iTregs, either through a direct role with RUNX-1 or an independent role, which could result in the development of abnormal pro-inflammatory iTregs.

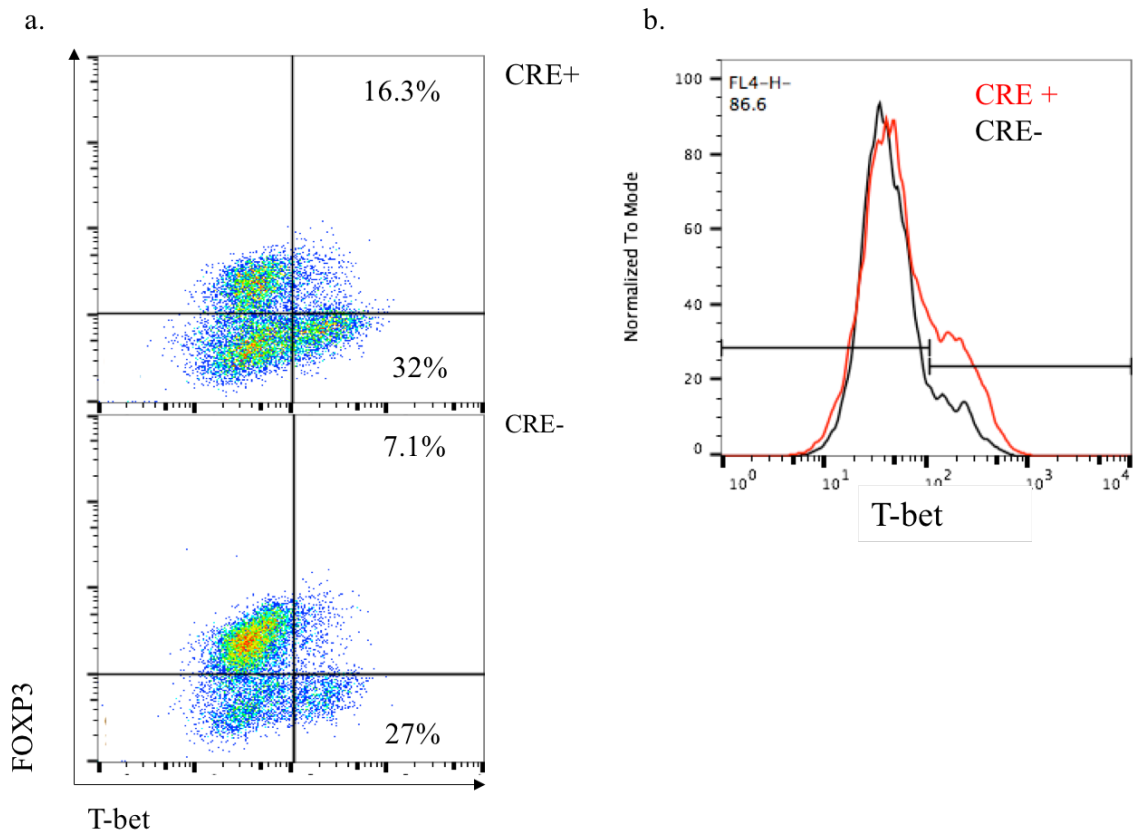


Figure 14: T-bet is over-expressed in CRE+ iTreg cultures.

Purified CD4 T-cells from CRE+ and CRE- mice were grown under iTreg polarization conditions and followed by ICS for detection of Foxp3 and T-bet. a) Flow cytometry plots of CRE+ and CRE- cultures showing percentage of cells in each quadrant. b) Histogram showing levels of T-bet expression per cell in CRE+ and CRE- cultures.

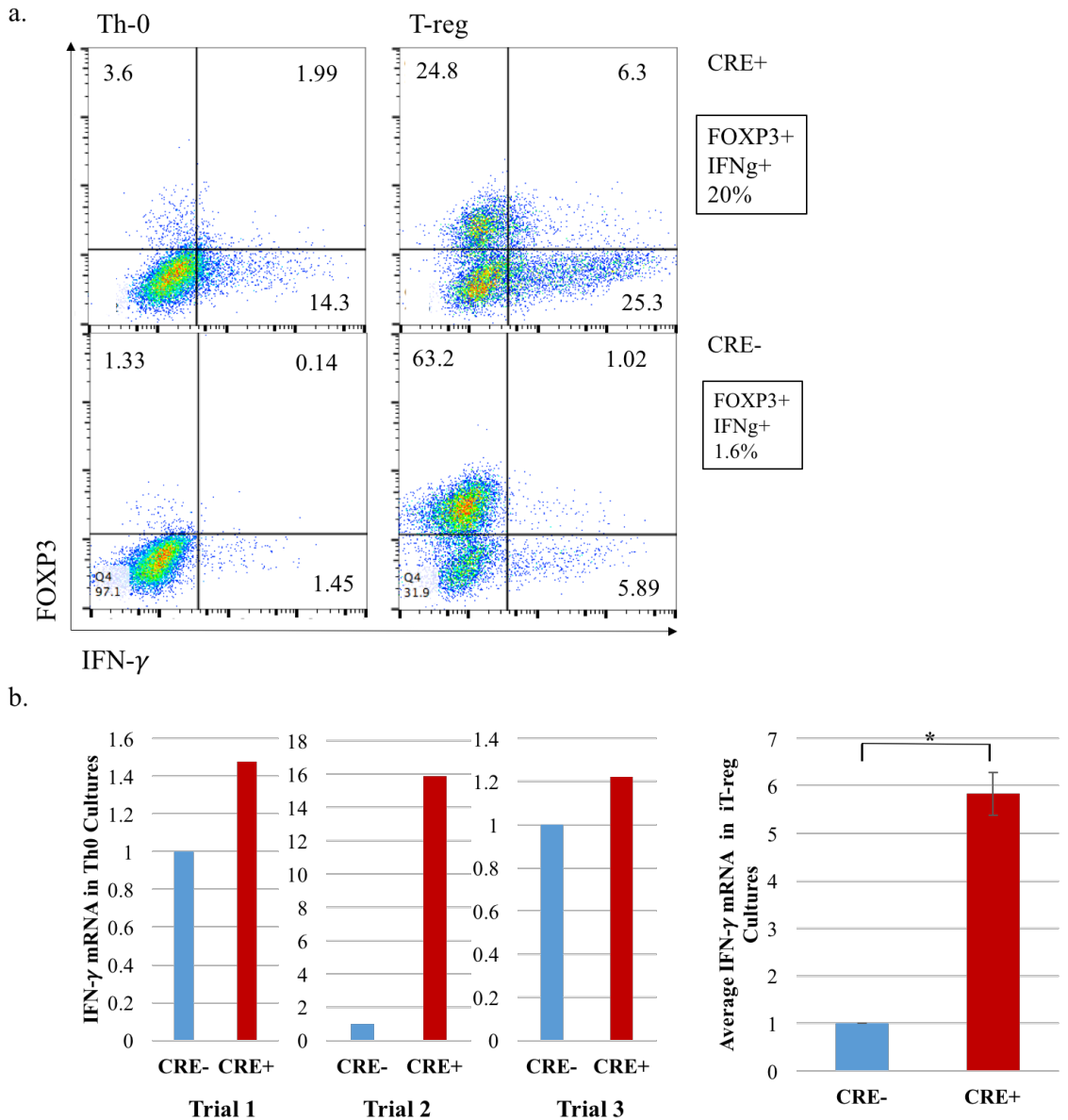


Figure 15: IFN γ is over-expressed in CRE+ cells cultured under Th0 and iTreg polarizing conditions.

a) Purified CD4 T-cells from CRE+ and CRE- mice were grown under Th0 and iTreg polarizing and followed by ICS. Flow cytometry plots show co-staining for Foxp3 and IFN γ . Boxed numbers represent percentage of FOXP3+ CD4 T-cells that stained for IFN γ .

b) Levels of IFN γ mRNA were measured by qRT-PCR in Th0 and iTreg cultures of purified CD4 T-cells. Results from three independent experiments for each condition are shown. qRT-PCR results shown are relative to values for HPRT expression and the value in the CRE- cultures was set to 1. *p=0.0004, n=3.

Expression of GM-CSF was also analyzed in the iTreg cultures. GM-CSF expression was highest in Th0 CRE⁺ cultures. However, CRE⁺ iTreg cultures also had increased GM-CSF expression relative to CRE⁻ iTreg cultures with the most predominant population representing polyfunctional cells expressing both IFN γ and GM-CSF (Figure 15a). Analyses of mRNA from purified CD4 T cell Th0 and iTreg cultures also showed increased expression of GM-CSF in CRE⁺ cultures (Figure 15b). Taken together, these results suggest the development of an abnormal pro-inflammatory cell population that arises under iTreg differentiation conditions when Ikaros is not present. Co-staining of CRE⁺ cells in iTreg cultures for Foxp3⁺, GM-CSF and IFN γ should be done to determine if these polyfunctional cells are Foxp3⁺ iTregs, or if they are part of the population of cells that cannot express Foxp3 appropriately. While published studies have shown a role for Foxp3 in blocking transcription of the gene encoding IFN γ a relationship between Foxp3 and GM-CSF expression in CD4 T-cells has not been reported. Therefore, it is possible that Ikaros is required for its repression.

In summary, in absence of Ikaros, CD4 T cells do not differentiate normally towards the iTreg lineage. Ikaros may be affecting the ability of these cells to induce expression of the FOXP3 gene after they receive differentiation signals. Also, in the absence of Ikaros, CD4 T cells in iTreg cultures expressed Th1-lineage proteins, such as T-bet and IFN γ , a surprising finding considering the roles of Foxp3 in blocking Th1 differentiation. These results agree with those observed in CRE⁺ Th17 cultures and demonstrate that Ikaros may be necessary to prevent development of inflammatory phenotypes that oppose the immunosuppressive functions of Tregs.

To date, no obvious development of autoimmunity in the CKO mice has been observed, suggesting natural regulatory T-cell (n-reg) populations in vivo may not be affected. It is also probable that the development of autoimmunity normally develops in mice of old age that have not become available in the laboratory for analysis.

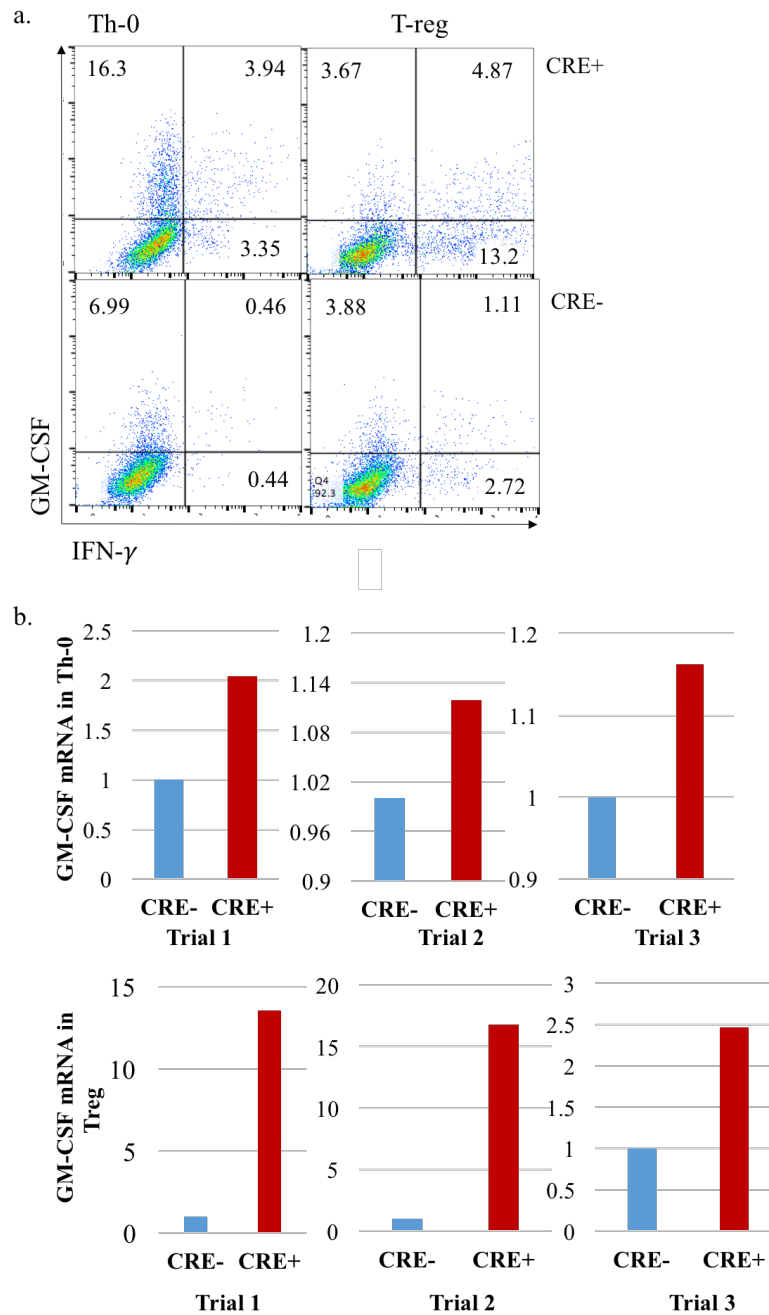


Figure 16: GM-CSF expression is increased in CRE+ Th0 and iTreg cultures.

Purified CD4 T-cells from CRE+ and CRE- mice were cultured under Th0 and iTreg polarization conditions and followed by ICS. a) Flow cytometry plots of CRE+ and CRE- cultures co-stained for GM-CSF and IFN γ . b) Levels of GM-CSF mRNA as measured by qRT-PCR in Th0 and iTreg cultures. Results from three independent experiments are shown. qRT-PCR results shown are relative to values for HPRT expression and the value in the CRE- cultures was set to 1.

CHAPTER 5: DISCUSSION

It has long been known that Ikaros is a major factor for normal development and function of T-cells. CD4 T-cells require Ikaros for their normal development and regulation of their functions (Georgopolous et al, 1997; Umetsu and Winandy, 2009; Wong et al, 2013). Studies presented in this thesis introduce a new role of Ikaros in normal differentiation of CD4 T-cells into Treg and Th17 subsets and in suppression of pro-inflammatory responses.

In vitro Th17 differentiation of Ikaros conditional knockout (CKO) CD4 T-cells demonstrated normal up-regulation of Th17 factors and cytokines, but an abnormal expression of T-bet, IFN γ and GM-CSF, in the absence of Ikaros. A population of Th17 cells with such phenotypes have often been associated with autoimmune diseases in mice and humans (Duhon et al, 2013; Korn et al, 2007; Annunziato et al, 2007). Although Ikaros has not been characterized as a suppressor of autoimmunity, this study suggests it might may have such function.

To continue deciphering this unique Th17 phenotype, it is necessary to investigate pathways that may be involved. IL-23 has been identified as a necessary cytokine for stabilization of the Th17 lineage, and has been implicated, along with IL-21, in development of Th17 cells that secrete GM-CSF in autoimmune diseases (Littman and Rudensky, 2010; El-Behi et al, 2011; Korn et al, 2007). Intracellular pathways activated by these cytokines can be assessed to evaluate if they are highly activated in absence of Ikaros.

It may also be useful to observe if Ikaros-deficient Th17 T-cells could induce autoimmune responses in mice. Such experiment can be done by following old CKO CRE⁺ mice and observing if they have any indications of such diseases. More importantly, it has yet to be proven what these abnormal Th17 cells mean to the ability of the immune system to clear extracellular bacteria and fungi. An in vivo study to induce activation of a Th17 response would be a good method to assess their functionality. Th17 cells are important in clearing extracellular bacteria like *Streptococcus pneumoniae* and *Citrobacter rodentium* (McGeachy and Cua, 2008; Curtis and Way, 2008). Infection of Ikaros CKO mice by one of these to activate adaptive immune responses, could be used to evaluate the ability of Ikaros-deficient CD4 T-cells to induce a proper response, clear bacteria, and properly down-regulate the response afterwards.

Unlike the Th17 experiments, in vitro iTreg differentiation experiments showed that CD4 T-cells had difficulties in becoming iTreg cells when Ikaros was not present. The CRE⁺ cultures had lower percentages of Foxp3⁺ T-cells, and the majority of cells did not differentiate into the iTreg lineage. In addition, a higher percentage of cells co-expressed T-bet and Foxp3, and co-secreted IFN γ and GM-CSF. Since the Foxp3 transcription factor is a transcriptional repressor of IFN γ genes (Ono et al, 2007), it would be important to study if lack of Ikaros is affecting the proper functions of Foxp3. However, it cannot be assumed that the Foxp3⁺ T-cells were the polyfunctional cells; co-staining with FOXP3, IFN γ and GM-CSF should also be done to address such assumption.

IL-10 secretion should also be analyzed in CRE⁺ iTreg cultures. This cytokine, secreted by Tregs, has important roles in suppressing helper T-cell-mediated inflammatory

responses (Chaudhry et al, 2011; Littman & Rudensky, 2010). In studies using IK-null cells, it was demonstrated that Ikaros is necessary for IL-10 secretion by CD4 T-cells when they were grown under Th2 polarizing conditions (Umetsu and Winandy, 2009). Because Ikaros binds to regulatory regions of the gene encoding IL-10, it is probable that secretion of this cytokine will be affected in Tregs as well. Flow cytometry, qRT-PCR and ELISA would be useful methods to analyze IL-10 expression in culture. With flow cytometry, co-staining of Foxp3 and IL-10 would be helpful to analyze the function of the few iTregs present in CRE⁺ cultures.

Although iTreg differentiation was abnormal, preliminary *in vivo* experiments of the conditional knockout mice have shown no abnormalities in numbers of CD4⁺CD25⁺ (data not shown) and CD4⁺Foxp3⁺ (Figure 4) T-cells in the spleens of CRE⁺ mice. This reveals that nTreg populations might not rely on Ikaros for their development; and lack of Ikaros may only result in deficiencies during peripheral CD4 T-cell differentiation into iTregs. Another observation from the CRE⁺ CKO mice that support normal nTreg development is the lack of autoimmune disease in mice observed to date. Nonetheless, more careful *in vivo* experiments that assess nTreg and iTreg development and function are necessary to strengthen this conclusion.

In CRE⁺ cultures, abnormal IL-2 secreting Th17 cells as well as low percentages of Foxp3⁺ cells raise the question of whether IL-2 receptor (IL-2R) is functional in cells that lack Ikaros. IL-2 signaling through IL-2R (CD25) initiates downstream activation of the AKT, RAS and STAT5 pathways that are important for T-cell survival, proliferation and differentiation (Boyman and Sprent, 2012). In Th17 cells, STAT5 suppresses Th17

proteins like IL-6 receptor (IL-6R) and induces expression of IL-12 receptor and T-bet mRNA, important in Th1 differentiation (Liao et al, 2011; Zhu, 2010), thereby constraining their development. On the contrary, IL-2 is required for Treg differentiation. STAT5 was found to bind to the Foxp3 gene in CD4⁺CD25⁺ T-cells, and deletion of STAT5 was enough to reduce Treg populations in vivo (Burchill et al, 2007; Zhu, 2010; Littman and Rudensky, 2010). In the CRE⁺ Th17 cultures from Ikaros CKO T-cells, where IL-2 was found to be present, Th1-lineage proteins such as T-bet and IFN γ were over-expressed along with Th17-lineage proteins, suggesting STAT5 might not be working properly to repress the Th17 differentiation in these cultures. Similarly, in CRE⁺ iTreg cultures, percentages of Foxp3⁺ cells were decreased, also indicating that STAT5 binding to the Foxp3 gene could be affected. Flow cytometry to measure expression of CD25, as well as western blots of STAT5 or phosphorylated-STAT5, the active form, could be used to evaluate expression and proper activation of this pathway in CRE⁺ cells.

We have already conducted initial in vivo experiments to assess Th1 and Th17 responses in Ikaros conditional knockout mice. In collaboration with Dr. Joseph Mizgerd, we are using a model of pneumococcal pneumonia in order to assess memory Th17 responses, and the ability of Th17 cells that lack Ikaros to clear an infection and control inflammation. In the model, mice receive three intranasal infections with *Streptococcus pneumoniae*. The first two infections are with a low virulence strain (Sp19F) that the mice can clear, followed by a third infection with a high virulence strain (Sp3). Infection with Sp19F induces memory Th17 responses in the lungs that allows survival of Sp3 infection. Early results have demonstrated that lungs of CRE⁺ CKO mice had Th17 cells that

expressed T-bet, high levels of IFN γ and GM-CSF. Subsequent stages of this study will evaluate if these abnormal Th17 cells can appropriately clear the bacteria from lungs as effectively as their CRE- Ikaros-sufficient counterparts. Although the in vivo experiment has only been conducted once, it replicates the results from the in vitro cultures shown in this thesis. This suggests that, in absence of Ikaros, Th17 cells generated in vivo also express high levels of pro-inflammatory cytokines and Th1-lineage factors.

We also performed a second in vivo experiment that aimed to study development of memory T-cells in the spleen and responses to a continuous exposure to pathogen. For this experiment, CKO mice were housed in a "dirty" room within the mouse facility, where they continuously received dirty bedding from sentinel cages historically infected with norovirus for a period of 8 weeks. During analyses for this experiment, we observed that Ikaros-deficient CRE⁺ T-cells from mice that did not receive pathogen exposure, since they were housed in the specific pathogen-free barrier, already presented with higher expression of T-bet, IFN γ and GM-CSF than CRE⁻ T cells. Such expression became exacerbated in CRE⁺ T-cells after exposure to norovirus. These results suggest that lack of Ikaros in T cells could have underlying roles in maintaining the naive T cell pool and suppressing development of pro-inflammatory responses, even in the absence of pathogen.

The novel model of Ikaros conditional knockout in mature T-cells introduced in this thesis has revealed new roles for Ikaros in naive CD4 T-cell differentiation into Th17 and iTreg subsets. More importantly, we have shown data that Ikaros is necessary to suppress pro-inflammatory responses in culture. Preliminary studies not shown in this thesis support that this is the case in vivo as well. This new role of Ikaros should continue

to be unraveled, as it could introduce new explanations of chronic inflammatory responses caused by T-cells.

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CURRICULUM VITAE

