UNIVERSITY OF CALIFORNIA SAN DIEGO

A Pathogen Independent T-cell Intrinsic role for IRF3

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Biology

by

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TABLE OF CONTENTS

LIST OF FIGURES

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ABSTRACT OF THE THESIS

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Professor Michael David, Chair

IRF3 has been recognized as a crucial transcription factor for the activation of innate immune responses. Recent studies have implicated IRF3 in regulating IL17A production of the CD4+ subset, Th17. Previous results using in vivo models of autoimmune disorders suggest this regulation is necessary for optimal disease induction. Using in vitro culture methods, we further demonstrated the relevance of IRF3 to mouse Th17 cells by co-culturing murine Antigen Presenting cells with purified murine naïve CD4 T cells under Th17 polarizing conditions. We show that IRF3 is required in both Antigen Presenting Cell and CD4 T cells to elicit proper Th17 differentiation independently of antigen presentation. Lastly, we validated an existing Th17 culture method that facilitated Th17 differentiation to better correlate IRF3 with IL17A

production in Th17 subsets and provide a robust cytokine cocktail formulation to pinpoint differences in Th17 cell yields in WT vs. IRF3KO cells. Our results demonstrate the multi-faceted role of IRF3 in regulating the immune response within cell subsets and further establish its T-cell intrinsic role.

METHODS

Mice: IRF3$^{-/-}$ mice have been previously described (Sato et al., 2000, Honda et al., 2005). All other mice were purchased from Jackson Laboratories and bred in specific pathogen-free conditions in the animal facility at University of California San Diego in accordance with the UCSD animal care facility.

Cell Isolation: Inguinal, brachial, cervical, and mesenteric lymph nodes and spleen were procured from each mouse on day 0 of culture. Single cell suspensions were prepared via mechanical disruption of tissue in sterile-filtered DPBS containing 2% FBS and 1 mM EDTA using a syringe plunger and passed through 70 um filter. Cells were counted and resuspended to appropriate volumes for negative selection of cells of interest. Naïve CD4 T cells were obtained with the EasySep Mouse Naïve CD4 T Cell Isolation Kit (StemCell). Feeder cells were obtained via depletion of Thy1.2 expressing cells using Mouse Pan T (Thy1.2) Dynabeads. B cells were purified using the EasySep Mouse B Cell Isolation Kit (StemCell). Feeder cells and B cells were treated with 30ug/mL Mitomycin C (ToCris) in cell media for 30 minutes at 37 C and washed 3 times with fresh media prior to plating.

Cell Media: RPMI 1640 from LifeSciences was supplemented with 10% FBS (OMEGA), 0.5% 2-mercaptoethanol, sodium pyruvate, non-essential amino acids, and penicillin/streptomycin/glutamate. IMDM was obtained from LifeSciences and supplemented with 5% FBS (OMEGA), 0.5% 2-mercaptoethanol, and penicillin/streptomycin/glutamate.

Reagents and Cytokines: Functional grade mouse anti-CD3e antibody was purchased from eBioscience and plated at a concentration of 5 ug/mL for plate-bound cultures or added at a concentration of 1 ug/mL for co-culture experiments, as per the protocol used by the Lu Lab at

UCSD. Functional grade mouse anti-CD28 (eBioscience) was used at a concentration of 2 ug/mL for plate-bound assays. IL-6, TGF-B, and IL-1B, were all purchased from PeproTech and reconstituted according to the manufacturer's instructions. FICZ was purchased from R&D Systems and reconstituted according to manufacturer's instructions. Cytokines were added at day 0 to plated cells at the following concentrations: IL-6 at 10 ng/mL for co-culture and 50 ng/mL for plate-bound anti-CD3e, TGF-B at 2 ng/mL for co-culture and 1 ng/mL for plate-bound anti-CD3e, IL-1B at 10 ng/mL, FICZ at 300 nM.

T Cell Polarizations: All polarization assays were conducted for 4 days post-stimulation. Th0 conditions were prepared with addition of anti-CD3, and either anti-CD28 or feeder cell co-culture as appropriate. Th17 conditions were prepare with IL-6, TGF-B, FICZ, and IL-1B where appropriate.

Flow Cytometry: Flow cytometry was conducted on a BD LSRII Fortessa and all analysis was done in FlowJo. 4 hours prior to staining, cells were re-stimulated with PMA and Ionomycin (Sigma-Aldrich, St. Louis, MO) at 500 ng/mL, and treated with Brefeldin A (eBioscience) and incubated at 37 C. Cells were resuspended in FACS buffer (2% FBS, 2mM EDTA, 0.02% sodium azide) prior to surface staining in the dark and on ice. Cells were stained for surface markers with anti-CD3 FITC, anti-MHCII FITC, anti-CD4 PE, anti-B220 APC, and intracellularly with anti-IL17A PE/Cy7 from BioLegend. Intracellular permeabilization was carried out using the Intracellular Fixation & Permeabilization Buffer Set from eBioscience.

INTRODUCTION

Interferon is a major immunomodulatory cytokine with a host of isoforms and functions. It is regulated by the IRF (Interferon-Regulatory Factor) family of transcription factors, comprised of 9 distinct proteins that control interferons transcription. These IRFs are thus crucial to the immune response, and they are capable of being expressed in almost all nucleated cells (Mourik, B. C., et al., 2017). There are three major types of interferon to note, classified as Type I, Type II, and Type II. The focus is on type I and type II interferon due to their dual roles in the innate and adaptive immune systems (González-Navajas et al., 2012), following activation of Pattern recognition receptors (PRRs) that mediate the initial response. Activation of a PRR via PAMP (pathogen associated molecular pattern) leads to a signaling cascade involving a host of signaling and adaptor molecules that begin the immune response. The most significant cascade occurs with TRIF or MyD88, adaptor molecules that lead to activation of transcription factors including NF-kB, AP-1, and members of the IRF family (Bo et al, 2006).

IRF induction, usually via Toll-like Receptor (TLRs), a type of PRR, induces interferon production. Interferon production is commonly associated with the antiviral immune response, but studies show that TLR3,4,7, and 9, which sense bacterial pathogens, induce type I interferon as well. Furthermore, interferon production may also be induced in a TLR-independent manner following stimulation of cytoplasmic sensors RIG-I (retinoic-acid-inducible gene I) and MDA5 (melanoma differentiation-associated gene 5) with RNA and DNA ligands (González-Navajas et al., 2012).

This type of interferon production is coordinated mainly by IRF3 and IRF7 in most nucleated cells. They form a functional complex when IRF7 dimerizes with IRF3 and further associates with NF-kB. This complex binds the *Ifnb* promoter region and initiation of interferon beta transcription, a type I interferon (González-Navajas et al., 2012). However, IRF3 and IRF7 have distinct signaling pathways that suggest individual roles for each in interferon production. For example, IRF3 is crucial in the initial mounted response, but IRF7 is essential for the positive feedback loop that amplifies interferon production (Ning, S., et al., 2011).

Concurrent with this interferon response is antigen presentation, the process by which invading pathogens are recognized by the immune system. Antigen presentation is done via MHC (major histocompatibility complex) molecules. These MHCs are found within all nucleated cells and normally display host-associated epitopes, preventing the presenting cell from being targeted by the immune response. MHC molecules are split into two classes, MHC-I and MHC-II. MHC-I molecules display intracellular epitopes and are thus the primary markers of virus-infected cells. Upon viral infection, an infected cell will display virus-associated peptides, which engages a CD8+ T cell and facilitates destruction of that cell.

MHC-II molecules are found on antigen presenting cells (APCs) and display epitopes from phagocytosed pathogens. APCs will activate upon PRR stimulation and phagocytose invading extracellular pathogens. This phagocytosis leads to proteolysis of the invading pathogen, resulting in the production of peptide epitopes that are presented on the surface of APCs via MHC-II. (Belicha-Villanueva, A., et al., 2010). The recognition of an MHC associated epitope by a T cell receptor allows for a attenuated activation of that cell, signifying a transition to the adaptive immune response (Guermonprez et al, 2002).

The adaptive immune response is heavily studied as it forms the basis for long-lasting protection from pathogens. The key advantage of the adaptive immune response is the ability to prevent re-infection by the same type of pathogen. This is accomplished through the unique receptors present on cells of the adaptive immune system. These receptors are primed to respond to specific epitopes, generated de novo via genetic editing of the receptor-coding locus (Alcover, A., et al., 2018). The result is an array of "naïve" cells that await stimulation of their molecularly unique receptors with appropriate costimulatory signals. Within T-cells, the receptor complex is comprised of a TCR, and a complex of CD3 molecules comprised of multiple subunits and heterodimers. During antigen presentation, MHC molecules on APCs will bind to the TCR-CD3 complex and induce a signaling cascade via the cytoplasmic domains of CD3. Furthermore, T-cells require co-stimulation of both the TCR complex and an accessory receptor, usually CD28. CD28 is typically recognized by the ligands CD80 or CD86 on APCs and helps prime the T-cell response (Smith-Garvin, J. E., et al., 2009).

T cells are further specified via expression of either CD4 or CD8 co-receptor, which identifies them as either "helper" T-cells or cytotoxic "killer" T cells respectively. CD8+ T cells (cytotoxic T cells) are important due to their role as "killers". Through secretion of granzyme B, chemokines, and pro-inflammatory cytokines, they mediate effect killing and removal of cancerous or virus-infected cells (Demers, K. R., et al., 2013). The CD4+ T cell group encompasses a wide variety of "helper T-cell", or Th cell subsets, that assist other effector cell types in clearing pathogen and establishing immunity. These subsets are further divided based on their distinct transcription factors and secreted cytokines.

The first two helper T cell subsets discovered, Th1 and Th2, have been widely implicated in various immune responses. Th1 is known for its secretion of type II interferons and its

assistance against viral pathogens. Th2 has been implicated in the extracellular pathogenic response via secretion of proinflammatory cytokine IL4. In more recent years, new helper T cell subsets have been discovered with further specialized roles. An immunomodulatory helper subset marked by production of anti-inflammatory cytokine IL-10 and transcription factor FOXP3 was identified and named the T-reg subset, responsible for preventing the immune response from excessively damaging the host. Another subset, the Th17 lineage, is gaining significance due to its role in autoimmune disorders. Th17 cells are categorized by the secretion of proinflammatory cytokines IL17A, IL17F, and IL-21, as well as the expression of transcription factor RORyt. They cells have been identified in the past decade as key mediators in the immune response against extracellular pathogens. Significant findings have been made that implicate Th17 cells in pathogenic responses, especially autoimmune conditions such as colitis and multiple sclerosis (Zhu, J. and W. E. Paul, 2008).

As Th17 research advances, transcription factors (TFs) associated with other cell types have been demonstrated to impact Th17 cells. TFs of note are the IRFs, as studies have shown an interplay between Type I interferon and Th17 responses within tuberculosis infections (Mourik, B. C., et al., 2017). This finding suggests a link between IRFs and Th17 cells that may reveal functions for both outside their classically defined roles. Other studies have shown direct associations between IRF8 and the IL17A promoter and indicate IRF8 is a negative regulator of the Th17 response (Ouyang, Xinshou, et al, 2011). This finding suggests a much broader role for IRFs within adaptive immune subsets and shows direct impact of IRF regulation on Th17 cells. Based on the previous findings, we sought to elucidate the role of IRF3 specifically within T-cell subsets. Other studies affirm existing data implicating IRF3 as a key molecule needed for cellular cross-talk between Antigen Presenting Cells (APCs) and Th17 cells. One study

specifically reported that IRF3 deficient T-cells were unable to polarize to the Th17 cell subset in the presence of antigen specific DCs (Guinn et al, 2016).

It has been found that naïve CD4+ T cell differentiation to Th17 lineages is driven by exposure to IL-6 and TGFB. This leads to upregulation of IL23R, which recognized APC-secreted IL-23 and promotes induction of RORyt, the hallmark transcription factor of Th17 cells. TGF-B presents an issue for proper differentiation as it is also used in the induction or Regulatory helper T cells (iTregs) that reduce T-cell proliferation and differentiation (Caza, T. and S. Landas, 2015). Th17 cells also have added complexity in that they exhibit a high degree of cytokine plasticity. Recent findings reveal that CD4+ T cells polarized to Th17 cells can assume an intermediate Th1-Th17 phenotype expressing IL-17A and IFNg (Shi, G., et al., 2008), in addition to a Treg17 phenotype expressing the immunosuppressive cytokine IL-10, as well as the T-reg associated FOXP3 (Singh, B., et al., 2013).This sets Th17 cells apart from other effector helper T-cell subsets, as they can assume intermediate phenotypes and express proteins characteristic of pathogenic and suppressive helper subsets depending on the microenvironment.

Our lab had previously demonstrated in vitro that IRF3 translocates to the nucleus within naïve CD4+ T-cells activated with agonistic CD3/CD28 antibody. Furthermore, our lab demonstrated that colitis models using adoptive transfer of IRF3KO T-cells into RAGKO recipient mice induced milder inflammation and damage in recipient colons, compared to WT transfers. The use of RAGKO mice as recipients allows for a T and B cell free environment, as RAG deficiency prevents maturation of the adaptive immune system. Parallel experiments done with IRF7 deficient mice did not display the same phenotype, suggesting a T-cell intrinsic and pathogen independent role for IRF3 in Th17 cells, hallmarked by a lack of IL17A production (Fig 1A). The phenotype obtained with CD3/CD28 agonistic antibody stimulations further

suggest this role may be pathogen-independent as MHC-mediated activation of the TCR complex is bypassed in this model. As work with IRF3 deficient mice showed specific differences in Th17 cells, we began investigating the role of IRF3 within this cell subset.

RESULTS

Preliminary in vitro analysis of IRF3 within CD4+ cell subsets involved profiling cytokine and transcription factor expression within IRF3 deficient Th1 (IFNg + Tbet+), Th2 (IL4+), Th17 (IL17A+ RORyt+), and iTreg (FOXP3+) cells.

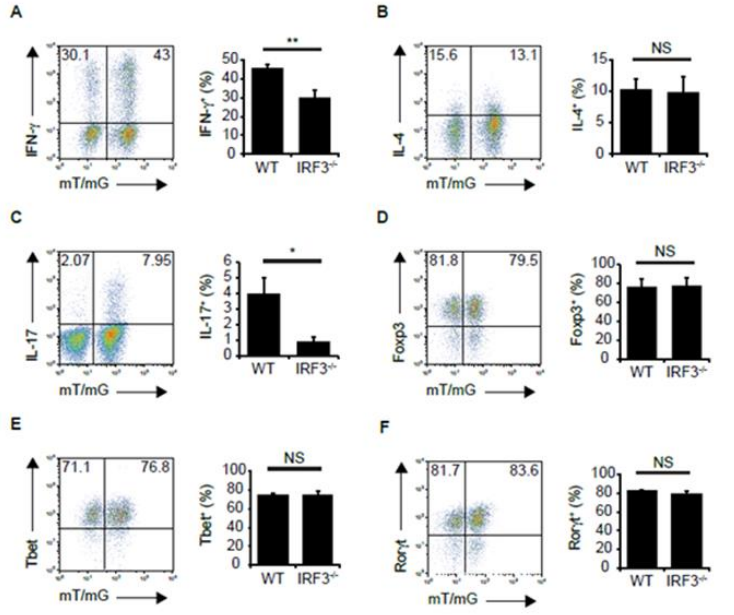

Figure 1A. **Impaired in vitro IFN-y and IL-17 production in IRF3-/- T cells**. Naive CD4+ T cells from wild type (mT/mG) and IRF3-/- mice were co-cultured under TH1-, TH2, TH17-, and Treg-polarizing conditions. Cytokine production and transcription factor expression were assessed after 96 hours. Results were pooled from 6 (A,C), 4 (B,D) or 2 (E,F) independent experiments. Numbers in flow plots represent the percentage of positive cells within each population. Bar graphs represent mean ± SEM. Statistical significance was determined by Student's t-test (*P≤.05, **P≤.01). Figures and captions provided by Nancy Fares-Frederickson.

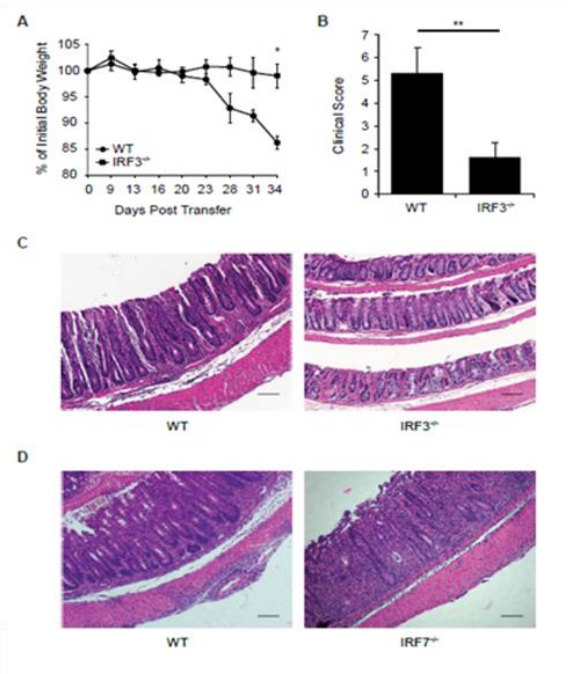

Figure 1B. **IRF3-/- CD4+ T cells do not induce colitis.** A-C) 4x105 naive CD4+ T cells (CD45RBhiCD25-) from wild type or IRF3-/- mice were injected (i.p.) into RAG-/- hosts. Mice were monitored for weight loss (mean ± SEM) and colon histology was assessed by H&E staining at the indicated time (n=4-5 mice/group, representative of 4 independent experiments). D) 4x105 naive CD4+ T cells from wild type or IRF7-/- mice were injected (i.p.) into RAG-/- hosts. Colon histology was assessed by H&E staining 5 weeks post T cell transfer (n=4 mice/group). Statistical significance was determined by Student's t-test (*P≤.05, **P≤.01). Scale bars = 0.1 mm. Figures and captions provided by Nancy Fares-Frederickson.

Flow cytometry analysis of IRF3KO cells revealed a specific decrease in number of CD4+ IL17A producing cells, independent RORyt staining (Fig 1A). Additionally, adoptive transfer of Naïve CD4+ T cells from WT, IRF3KO, or IRF7KO (Fig 1B) sources into RAGKO hosts revealed a lower disease severity specifically with IRF3KO transfers. Based on these results, we next investigated in vitro differences between WT and IRF3KO Th17 cells. Initial attempts to generate significant amounts of Th17 cells in vitro with CD3 and CD28 agonistic antibodies were unsuccessful.

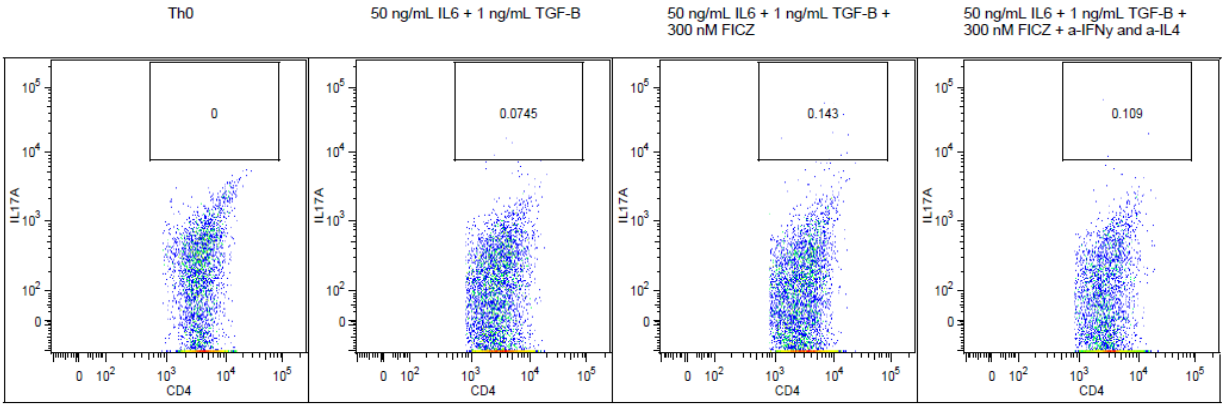

Figure 2. **RPMI does not induce Th17 populations efficiently.** Naïve CD4+ T cells were isolated via negative selection and plated at 200,000 cells/well in 96 well plates treated with 5 ug/mL plate-bound CD3 antibody. RPMI media containing CD28 antibody along with specified cytokines/reagents was added. Cultures were incubated at 37 C and harvested 4 days post plating to be prepared for intracellular staining.

Standard CD4+ T-cell culture methods are insufficient to elicit Th17 polarizations. Our group previously showed IL17A production in Th17 cell subsets is dependent on IRF3 via a colitis model. We attempted to generate an in vitro model of Th17 differentiation to further elicit signaling pathways and transcription factor interactions with promoter sequences. However, standard culture methods using RPMI media with plate-bound anti-CD3 + soluble anti-CD28 stimulations with added IL-6 and TGF-B were insufficient in inducing sufficient Th17 differentiation. Changing amounts and ratios of cytokines did not improve differentiation rates (Fig 2).

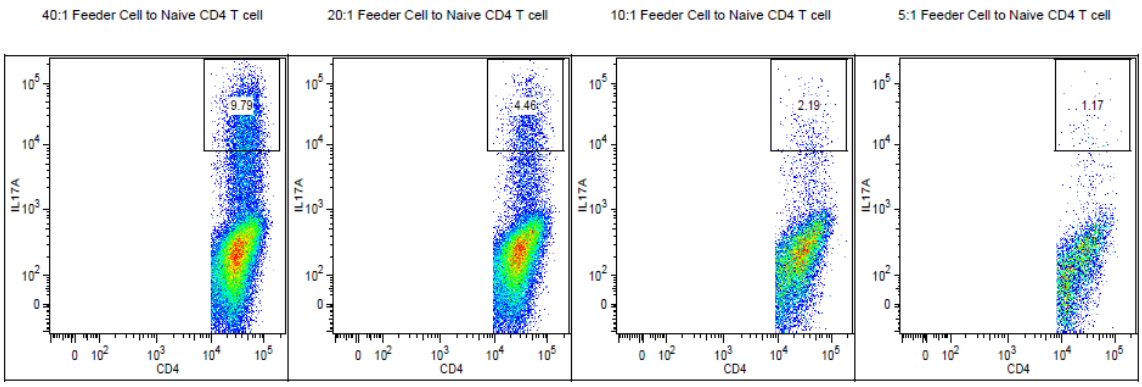

Figure 3. **Co-culture of Naïve CD4+ T cells with APCs induces better Th17 polarizations.** Naïve CD4+ T cells from wild type and IRF3KO mice were harvested via negative selection and plated at 50,000 cells/well with 2 million mitomycin treated feeder cells/well of wild type or IRF3KO origin. A cytokine cocktail was prepared per condition specified, with addition of either 10 ng/mL IL-1B, 300 nM FICZ, or both.

Feeder cell induced Th17 differentiation is more successful than plate-bound anti-CD3 stimulation. To further optimize differentiation, we used a feeder cell co-culture method of stimulation, with a protocol obtained from the Lu lab at UCSD. In brief, mitomycin treated Thy1.2^{-} cells are plated with Naïve CD4+ T cells, and soluble anti-CD3 and appropriate cytokine stimulations are added. This technique was further optimized by adjusting the ratio of feeder cells to naïve T cells (Fig 3). Based on these results, the optimal ratio of feeder cells to naïve T cells was 40:1.

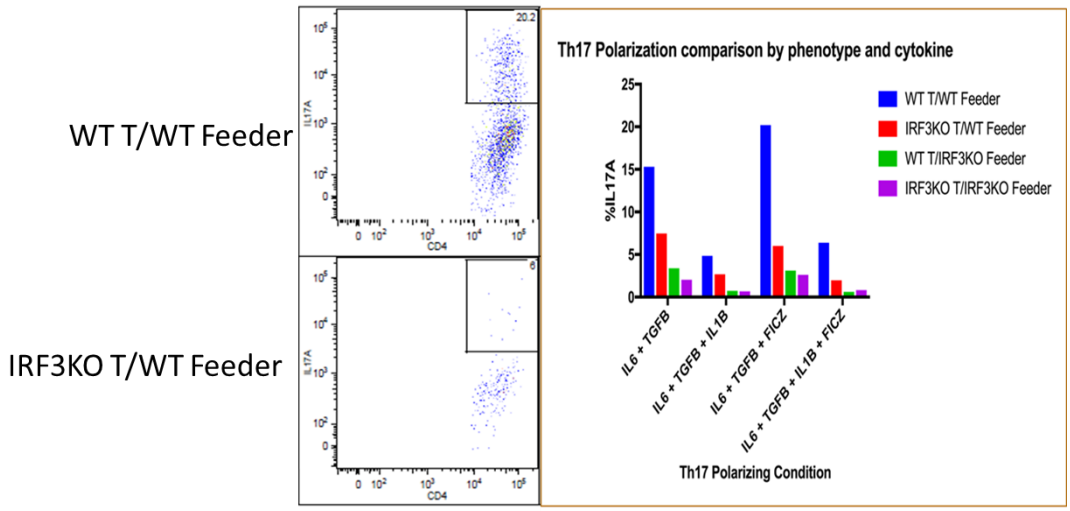

Figure 4. **IRF3KO CD4+ T-cells do not differentiate into Th17 cells in presence of APCs.** Naïve CD4+ T cells were plated at 50,000 cells/well in a 96 well plate along with the proportionate amount of mitomycin treated feeder cells as specified above. The numbers of feeder cells were: 2 million, 1 million, 500,000, and 250,000, corresponding to ratios of 40:1, 20:1. 10:1, and 5:1 respectively. A cytokine cocktail containing 1 ug/mL CD3 antibody, 10 ng/mL IL-6, 2 ng/mL TGF-B, and 300 nM FICZ was added to the culture. Samples were incubated at 37 C and harvested 4 days post-plating for intracellular staining.

Differences in Th17 differentiation with IRF3KO and WT feeder cells. To examine IRF3 dependent cellular interactions between feeder cells and naïve T cells, we assessed IL17A production in cells obtained from IRF3KO and WT mice. We additionally assessed the impact of pro-inflammatory cytokine IL-1B and AhR agonist FICZ based on literature reporting improved Th17 differentiation rates with these two reagents (Veldoehn et al, 2009). Four combinations of co-cultured samples were grown under Th17 inducing conditions with addition of FICZ: WT Feeder with WT Tn, WT Feeder with IRF3KO Tn, IRF3KO Feeder with WT Tn, and IRF3KO Feeder with IRF3KO Tn (Fig 4). Our results further support previous data that indicated IRF3 is involved in IL17A production and the possibility that IRF3 is involved in interactions between

feeder cells and T cells, as previously reported (Guinn et al., 2016).

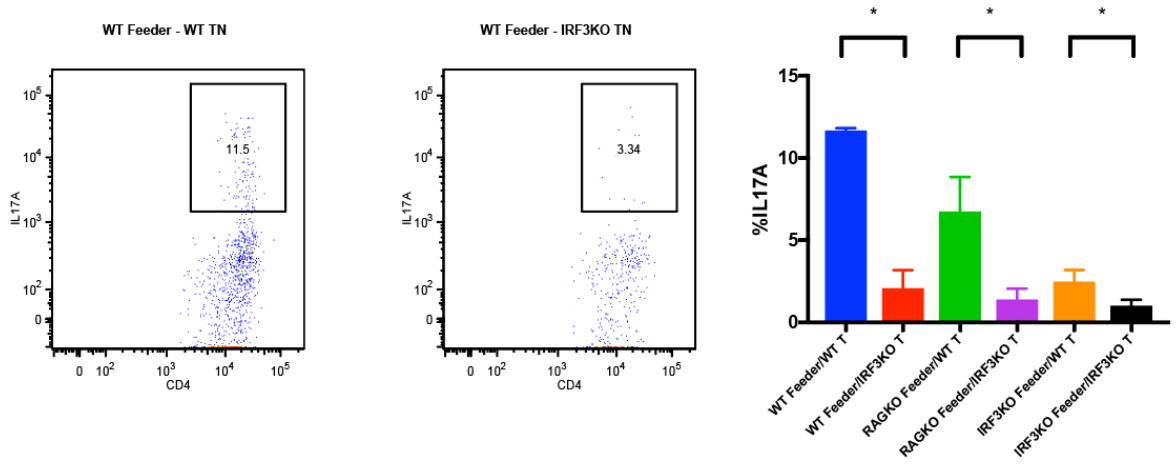

Figure 5. **IRF3 is required on both APCs and CD4+ T cells to induce IL17A production.** Naïve CD4+ T cells from wild type and IRF3KO mice were harvested via negative selection and plated at 50,000 cells/well with 2 million mitomycin treated feeder cells/well of wild type or IRF3KO origin. A cytokine cocktail containing 1 ug/mL CD3 antibody, 10 ng/mL IL-6, 2 ng/mL TGF-B, and 300 nM FICZ. Samples were incubated at 37 C and harvested 4 days post-plating for intracellular staining.

RAGKO Feeder cells induce fewer IL17A+ T cells. To further study feeder cell induced Th17 differentiation, we cultured feeder cells obtained from RAGKO mice in the presence of WT or IRF3KO naïve T-cells under Th17 polarizing conditions (Fig 5). The use of RAGKO mice allowed us to study Th17 differentiation in a B-cell free environment and suggested that IL17A production is lowered in the absence of RAG+ feeder cells.

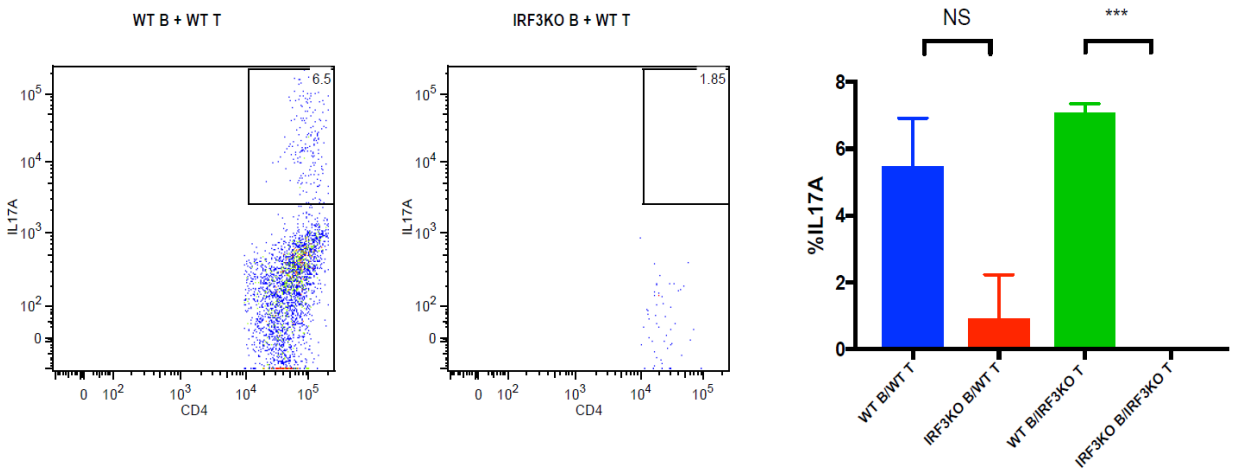

Figure 6. **IRF3KO B-cells are less efficient at polarizing Th17 cells.** Naïve CD4+ T cells and Total B cells from wild type and IRF3KO mice were harvested via negative selection and plated at 50,000 cells/well with 2 million mitomycin treated B cells/well of wild type or IRF3KO origin. A cytokine cocktail containing 1 ug/mL CD3 antibody, 10 ng/mL IL-6, 2 ng/mL TGF-B, and 300 nM FICZ was added to samples, except the Th0 control. Samples were incubated at 37 C and harvested 4 days post-plating for intracellular staining.

B-cell involvement in Th17 differentiation. To further confirm the role of B-cells in Th17 differentiation, we obtained B-cells via negative selection from WT and IRF3KO mice and performed co-culture experiments with WT and IRF3KO Tn cells. All four possible combinations were cultured according to the previously used feeder cell protocol. The results do not indicate a significant role for IRF3 in B cells in IL17A production from Th17 cells (Fig 6).

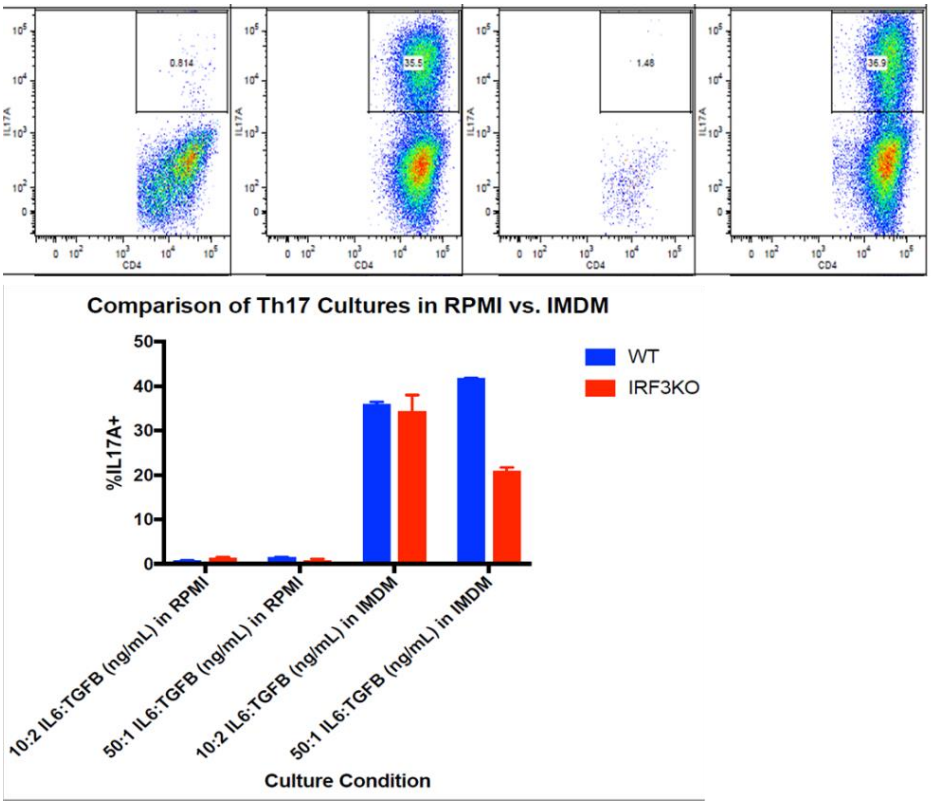

Figure 7. **Th17 cells polarize efficiently in IMDM media.** Naïve CD4+ T cells were isolated via negative selection and plated at 200,000 cells/well in 96 well plates treated with 5 ug/mL plate-bound CD3 antibody. IMDM media containing CD28 antibody along with specified cytokines/reagents was added. Cultures were incubated at 37 C and harvested 4 days post plating to be prepared for intracellular staining. A graph of 3 replicated experiments with standard deviation is shown.

IMDM media greatly enhances Th17 differentiation. Due to the need for pure Th17 harvests for mechanistic and chromatin studies, a further change was made to the original protocol involving the substitution of RPMI media with IMDM media. The culture was assessed using agonistic CD3/CD28 antibody stimulations in lieu of feeder cells to simplify downstream cell purification. Two separate Th17 culture conditions, corresponding to the different cytokine ratios used in prior studies with agonistic antibodies vs. feeder cell co-cultures, were assessed. The percentage of WT IL17A producing T cells was greatly enhanced in either condition with the use of IMDM media, and remained consistently poor in the corresponding RPMI conditions.

Based on the results, a 50:1 ratio of IL-6 to TGF-B appeared to improve the percentage of IL17A+ cells better than the 10:2 ratio, with the opposite trend appearing in IRF3KO cells (Fig 7).

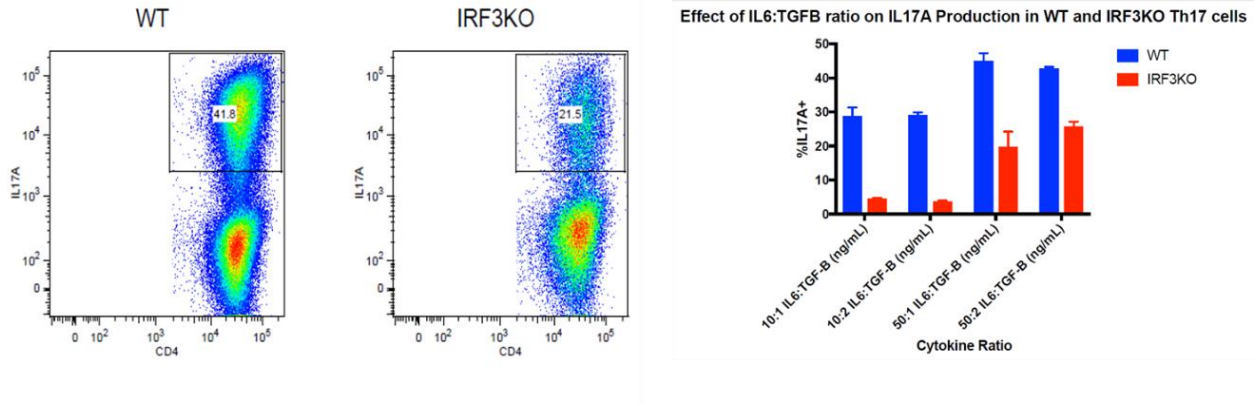

Figure 8. **IRF3KO cells require greater amounts of IL-6 to polarize to Th17 cells.** Naïve CD4+ T cells were isolated via negative selection and plated at 200,000 cells/well in 96 well plates treated with 5 ug/mL plate-bound CD3 antibody. IMDM media containing CD28 antibody along with specified cytokines/reagents was added. Cultures were incubated at 37 C and harvested 4 days post plating to be prepared for intracellular staining. A graph of 3 replicated experiments with standard deviation is given.

Differential %IL17A production in IRF3KO and WT cells. We further assessed differentiation rates in a more comprehensive culture condition panel. Prior to this experiment, the ratio of IL-6 to TGF-B employed in cultures was either 50:1 for agonistic antibody stimulation, or 10:2 for feeder cell co-culture stimulations. This was expanded to make 4 possible culture conditions, with two new ratios being 10:1 and 50:2 tested with agonistic antibody stimulation. We observed an increase in IRF3KO %IL17A+ cells with 50 ng/mL IL-6, compared to 10 ng/mL (Fig 8).

DISCUSSION

Our results indicate a clear role for IRF3 in the production of Th17 pro-inflammatory cytokines, primarily IL17A. Here, we confirm a better optimized method for Th17 differentiation and further support previous findings that report a link between IRF3 and the Th17 subset. We also further elucidated the role of IRF3 in cell-cell interactions during Th17 differentiation.

Initial studies in the lab (Fig 1) suggested a possible Th17 phenotype associated with IRF. However, we needed to generate a more robust model for Th17 differentiation to support this correlation. Traditional T-cell culture methods, mainly conducted in RPMI, are insufficient to produce Th17 cells in large quantities. The reason is the unique expression of AhR (aryl-hydrocarbon receptor), on Th17 surfaces. RPMI contains insufficient metabolites and AhR ligands to stimulate Th17 differentiation. Substituting this media for one containing more aromatic amino acids (particularly tryptophan) greatly enhances differentiation by greater stimulation of AhR. Initially, we surmised that using the AhR ligand FICZ would be sufficient to restore Th17 polarization within RPMI media, but this was only partially successful. Further literature reports that IL-2 is an inhibitor of Th17 differentiation, and only with IL-2 neutralizing antibody were Th17 cultures in RPMI comparable to IMDM cultures (Veldoehn et al, 2009).

In light of this data, we re-optimized the protocol and were able to generate a robust Th17 differentiation protocol and report a reduction in IL17A production, but not RORyt expression, within Th17 cells (Fig 1A). This finding supports the initials studies and other published literature that implicates IRF3 in disease severity for colitis and EAE models (Fig 1B, Fitzgerald, D. C., et al., 2014). The in vitro studies reported here specifically highlight IRF3 dependent regulation of IL17A producing cells within CD3+ CD4+ cell subsets.

Concurrently, we also wished to investigate IRF3 dependent interactions between APCs and CD4+ T cells. Previous studies have shown that IRF3 regulates cytokine production in APCs and as a result, limits Th1 and Th17 differentiation (Koshiba et al. 2013, Molle et al, 2007). However, our data suggests an interplay between APCs and Tn cells during Th17 differentiation, given that IRF3 deficiency on either cell type reduces the percentage of CD4+ IL17A+ cells. This confirms a previous finding that reports that IRF3 is essential in both APCs and T-cells for for optimal IFNy and IL17 production using Poly I:C stimulated cultures (Guinn, Z., et al., 2016). Our results further expand this interaction to include Th17 differentiation specifically, as our cultures did not contain antigen specific T-cells or APCs and used Th17 differentiating cytokines only.

This finding demonstrates a pathogen independent role and sheds light on the broad significance of IRF3 in multiple cell subsets. Normally, naive CD4+ T cells are exposed to IL6 and TGFB, which induces an upregulation of IL23R and polarization towards the Th17 subset. Dendritic cells will then secrete IL-23, which then binds IL23R on Th17 cells and promotes Th17 survival. Our findings suggest a possible role for IRF3 within this interaction, as a previous study reports APCs express TRIM21, which modulates IRF3 expression via ubiquitination. Specifically, this study found that knockdown of TRIM21 in monocytes resulted in decrease IL-6 and IL-1B expression, which contribute to Th17 differentiation. Furthermore, TRIM21 has been shown to decrease IRF8 and disruption of this balance leads to an increase Th17 response, mediated by IL17 (Ahn, Y., et al., 2017).

Our results indicate that the role of IRF3 in the TH17 response goes beyond transcription control of polarizing cytokines, as all experiments were conducted in the presence of exogenous IL-6 and IL-1B, suggesting that Th17 polarization is TRIM21 independent. As a previously

mentioned study revealed silencing of IRF8 enhances TH17 responses (Ouyang, X et al., 2016), there seems to be evidence to support a "balancing act" involving IRFs and Th17 differentiation given our research into IRF3. It is possible that IRF3 and IRF8 interact in a method analogous to IRF3-IRF7 mediated interferon transcription in an antiviral response.

Previous studies have revealed that IRF3 is important in the balance between Th1 and Th17 cells in the context of EAE models of autoimmunity. Parallel to our own previous studies with colitis models, these findings suggest adoptive transfer of IRF3 deficient CD4+ T cells cannot induce EAE in recipient mice and report a decrease in IL17A production within IRF3KO Th17 cells. Moreover, they find that an IRF3 deficiency leads to an increased proportion of Th1 effector subsets as measured by IFNg (Fitzgerald, D. C., et al., 2014). This raises an intriguing question as to the role of the previously reported intermediate Th1-Th17 phenotype with dual IL17 and IFNg secretion (Shi, G., et al., 2008), since IRF3KO has now been reported to decrease %IL17A+ cells in multiple settings, and there is evidence to suggest that %IFNg+ cells increase in tandem in an in vivo EAE model (Fitzgerald, D. C., et al., 2014). Thus, the role for IRF3 in the transcriptional regulation of an intermediate phenotype that expresses both cytokines would most likely inform the field of Th17 biology with regards to the regulation of their own plasticity.

Lastly, the findings from Fig 7 and Fig 8 raise issues with regards to the consistency of IRF3KO Th17 polarizations. Fig 7 indicates a clear difference between the two described culture conditions, with the 10 ng/mL IL6 + 2 ng/mL TGF-B producing near comparable polarizations to the WT samples. However, this trend does not repeat in the expanded dosage experiment shown in Fig 8. There is a consistent decrease in IL17A production in all IRF3KO samples. One possibility is due to the age difference in mice, as the IRF3KO mice used in experiments for Figure 8 were 6 months old, compared to the 2-month old mice used in previous experiments.

This may warrant investigation, but first requires more repeats to ensure reproducibility, as both experiments maintain relatively consistent %IL17A+ populations across replicates.

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