The Role of Peroxisome Proliferator-activated Receptor Alpha in the Regulation of Sex Differences in T Helper 1 Immunity

by

Monan Zhang

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Immunology
University of Toronto

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2015

Abstract

It is reported that female CD4+ T cells are biased towards T helper (Th) 1 cytokine production, whereas male CD4+ T cells are biased towards Th2. It has been suggested that the female bias in Th1 immunity is the reason why females not only generate enhanced anti-viral and anti-tumour responses, but also are more likely to develop certain autoimmune diseases than males. Our group previously reported that the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα) has a sex-specific role in negatively regulating Th1 immunity in mice. The objective of the thesis is to further explore the roles of PPARα in the regulation of sex differences in Th immunity.

We first investigated sex differences in Th cytokine production in SJL mice and human primary CD4+ T cells. We observed that female CD4+ T cells of both species produced higher levels of interferon (IFN)-γ and lower levels of IL-17 than male counterparts. Furthermore, we showed that this sex bias in Th immunity was controlled by androgens. The effects of androgens in suppressing Th1 immunity were shown to be mediated through PPARα. Moreover, we showed that PPARα activity negatively correlated with IFN-γ production by CD4+ T cells. Subsequent studies in mice identified IFN-γ as the gene repressed by PPARα. We showed that this repression was mediated through the recruitment of PPARα and the nuclear receptor co-repressor 1(NCOR)-containing co-repressor complex to certain conserved non-coding sequences
(CNS) at the *Ifng* locus in male T cells. Conversely, repressing PPARα activity with the antagonist NXT-1120 lead to global derepression at the *Ifng* locus and increased IFN-γ production. This control of IFN-γ production by PPARα in male T cells also extended to natural killer (NK) T cells and CD8⁺ T cells.

On the other hand, we found that the sex difference in Th17 immunity was regulated by PPARγ. Androgens decreased PPARγ mRNAs in murine and human CD4⁺ T cells. Furthermore, knocking down PPARγ expression using siRNAs was associated with higher IL-17 production by female CD4⁺ T cells. Taken together, these findings have furthered the understanding of sex differences in Th immunity.
Acknowledgments

I would like to thank my supervisor Dr. Shannon Dunn for all her unwavering help and support for the past 5+ years. I will never forget all the years we worked side-by-side setting up the lab. You have taught me so much in many aspects of my scientific and personal life that include how to design and conduct good experiments, write abstracts and manuscripts, present data at seminars and meetings, and handle the ups and downs in life in general. I still have a long way to go in building my career but you have provided me with an excellent start.

I would also like to acknowledge and thank my committee members Drs. Eleanor Fish, Dana Philpott, and Juan-Carlos Zuniga-Pflucker. Thank you for always challenging me during my committee meetings. I always learn so much from each meeting and come out of it with new ideas. I would like to thank all past and current members of the Dunn Laboratory, with special thanks to Marina Moshkova, Linda Zhao, Jennifer Ahn, Rainer Akkermann and Paulina Drohomrecky, who have provided me with scientific discussions and much-needed friendship.

I would also like to highlight certain collaborators/co-authors that contributed for my projects. I would like to give special thanks to Dorothy Rego for the help with recruiting human subjects and setting up human immune assays. I would like to give special thanks to Drs. Thierry Mallevaey and Thirumahal Selvanantham for help with the NK and NKT cell experiments, and Dr. Dana Philpott for her expertise in the infection model of *Listeria monocytogenes*. I would also like to thank Drs. Peppi Prasit and David Spaner for providing us with the small molecule PPARα antagonist.

I would like to thank my parents for being so patient with me throughout my PhD. I would like to give special thanks to my best friend and significant other, Jason Fraser, who took a chance, moved to Toronto with me to help further my career and stood by me throughout my PhD. I would also like to thank my many wonderful friends and colleagues in the Department of Immunology for all their professional and personal support. I will never forget all the scientific and non-scientific discussions we have had at all the department outings.

Finally I would like to thank the department for giving me this opportunity to be a part of this family. The journey has been incredible.
Table of Contents

Abstract ii-iii
Acknowledgements iv
Table of Contents v-ix
List of Figures x-xii
List of Tables xiii
List of Abbreviations xiv-xviii
List of Publications xix

Chapter 1: Introduction 1

1.1. T Helper (Th) Cells 2-3
   1.1.1. Th Differentiation 3-4
   1.1.2. Transcription Factor and Cytokine Cues 4
      1.1.2.1. Th1 Cells 4-7
      1.1.2.2. Th2 Cells 7
      1.1.2.3. Th17 Cells 7-9
      1.1.2.4. T Regulatory (Treg) Cells 9-11
   1.1.3. Cross-regulation amongst the Different Th Lineages 11-12
   1.1.4. Overview of T Cell Receptor (TCR) Signaling 12-16
      1.1.4.1. TCR Signal Strength and Th Differentiation 16-17

1.2. Interferon (IFN)-γ 17
   1.2.1. IFN-γ and IFN-γ Signaling 17-18
   1.2.2. Effects of Deficiencies in IFN-γ Signaling in Mice 18-19
   1.2.3. Effects of Deficiencies and Polymorphisms in IFN-γ Signaling in Humans 19-20
   1.2.4. Kinetics of IFN-γ Production During an Immune Response 20-21
   1.2.5. The Importance of IFN-γ to the Clearance of Listeria Monocytogenes v
1.2.6. The Regulatory Mechanisms of IFN-γ Expression in CD4+ T cells 22-24
1.2.7. General Epigenetic Regulation in Eukaryotes 24-26
1.2.8. Epigenetic Regulation at the Ifng Locus in CD4+ T cells 26-28
1.2.9. Trans-acting Molecules at the Ifng locus in CD4+ T cells 30-31
1.2.10. Histone Modifications at the Ifng locus in Other Cell Types 31-32

1.3. Sex Differences in Adaptive Immunity 32
   1.3.1. Immune Pathogenesis of Multiple Sclerosis (MS) and Autoimmune Experimental Encephalomyelitis (EAE) 33-34
   1.3.2. Sex Differences in MS and EAE 34
   1.3.3. Sex Differences in Adaptive Immune Responses 35
      1.3.3.1. Sex Differences in Th Cell Numbers and Proliferation 35
      1.3.3.2. Sex Differences in Th Cytokine Production 36
      1.3.3.3. Sex Differences in Treg Cells 37
      1.3.3.4. Sex Differences in B Cells 37-38
      1.3.3.5. Sex Differences in CD8+ T Cells 38
      1.3.3.6. Sex Differences in Natural Killer (NK) and NKT Cells 38-39
   1.3.4. Effects of Sex Hormones on Immune Responses 39-40
      1.3.4.1. Effects of Androgens on Adaptive Immunity 40-41
      1.3.4.2. Effects of Estrogens on Adaptive Immunity 41-43
   1.3.5. Candidate Molecules that Mediate Androgen Effects on Th1 Immune Responses 43-44

1.4. Peroxisome Proliferation-activated Receptors (PPARs) 44
   1.4.1. The General Structure and Function of PPAR Family Members 44-48
   1.4.2. Transactivation 48-50
   1.4.3. The Roles of PPARs in Immune Cells 50-52
   1.4.4. PPAR-dependent Trans-repression 52-55

1.5. Thesis Rationale and Objectives 55-56

Chapter 2: Peroxisome Proliferator-Activated Receptor (PPAR)-α and -γ Regulate IFNγ and IL-17A Production by Human T Cells in a Sex-specific Way 57
2.1. Abstract 58
2.2. Introduction 59-60
2.3. Materials and Methods 60
   2.3.1. Human Subjects and Blood Collection 60
   2.3.2. Isolation and Culture of Human CD4+ T cells 60-61
   2.3.3. Hormone Assays 61-62
   2.3.4. Mice and Surgeries 62
   2.3.5. EAE Induction, Recall Assays, and Treg Staining 62
   2.3.6. Proliferation and Cytokine Measurements 63
   2.3.7. In Vitro Mouse T Cell Culture 63-64
   2.3.8. Real-Time PCR Detection of Human and Murine PPARα and PPARγ mRNAs 64
   2.3.9. Chromatin immunoprecipitation (ChIP) of PPARα promoter and Ifng (CNS-6 and CNS-22 Regions) 64-66
   2.3.10. siRNA Transfection 67
   2.3.11. Statistical Analyses 67-68
2.4. Results 68
   2.4.1. Sex Bias in CD4+ T-Cell Expansion and Th Cytokine Production 68-71
   2.4.2. Androgen Dependence of Sex Difference in Th Cytokine Production 71-72
   2.4.3. Cellular Basis of T-Cell Expansion and Cytokine Production 73
   2.4.4. PPARα Is an Androgen-Sensitive Regulator of Th Cytokine Production 73-75
   2.4.5. Human Female CD4+ T Cells are Th1-Prone 75-80
   2.4.6. Regulation of Human PPARα by Androgens and TCR Stimulation 81-83
   2.4.7. PPARα Represses IFN-γ Production by Human T Cells 84
   2.4.8. Mechanism of Repression of Human IFN-γ by PPARα 84-88
   2.4.9. PPARγ is a Potential Sex-Dependent Regulator of Th17 88-91
2.5. Discussion 92-94

Chapter 3: Antagonizing Peroxisome Proliferator Activated Receptor-α (PPARα) Abrogates Sex Differences in IFN-γ Production 95
3.1. Abstract

3.2. Introduction

3.3. Materials and Methods
   3.3.1. Mice
   3.3.2. In Vitro Assay of APC Function
   3.3.3. Treg Suppression Assay
   3.3.4. In Vitro T Cell Stimulations
   3.3.5. Cytokine Measurements and Intranuclear Staining
   3.3.6. Measurement of mRNA gene expression
   3.3.7. ChIP
   3.3.8. Protein electrophoresis and western blot analysis
   3.3.9. Experimental infection with L. Monocytogenes (EGD strain)
   3.3.10. Statistics

3.4. Results
   3.4.1. PPARα Acts within Effector CD4+ T Cells to Limit Th1 Cytokine Production
   3.4.2. PPARα Inhibits IFN-γ mRNA Expression
   3.4.3. PPARα Regulates Histone Acetylation at the Mouse IFN-γ Locus
   3.4.4. PPARα also Limits IFN-γ Production by Male NKT and CD8+ T Cells
   3.4.5. Treatment with NXT-1120 Protects against L. Monocytogenes Infection Exclusively in Males

3.5. Discussion

Chapter 4: Summary and Future Directions

4.1. Further Investigation of Sex-specific Th Immune Responses
   4.1.1. Does the Th1 Bias Underlie the Higher Susceptibility of Females to Develop Central Nervous System Autoimmunity?
   4.1.2. What are the Implications of the Sex Bias in Th1 and Th17 on Immunity and Health of the Organism?
4.1.3. What is the Evolutionary Advantage of Having a More Robust Immune Response in Females and Why are PPARs Involved in This Regulation?  
141-143

4.2. Further Investigation of the Regulation of \textit{Ifng} by PPAR\textalpha  
143-144

4.2.1. What is the Role of NF-\kappa B in the PPAR\textalpha-dependent Regulation of \textit{Ifng} Expression?  
144-145

4.2.2. Does PPAR\textalpha Regulate IFN-\gamma Expression through Other Means?  
145-146

4.3. Metabolic Functions of PPAR\textalpha  
146-147

4.3.1. Does Metabolism Factor into the Regulation of IFN-\gamma Production by PPAR\textalpha?  
147-149

4.3.2. Does Metabolism Factor into the Control of T Cell Proliferation Play by PPAR\textalpha?  
149-150

4.4. Can a PPAR\textalpha Antagonist Be Used to Boost Male-specific IFN-\gamma Production in an Antitumour Mouse Model?  
150-151

4.5. Conclusions  
151-152

\textbf{Chapter 5: References}  
153-200
List of Figures

Chapter 1

Figure 1.1: Th differentiation 5

Figure 1.2: Major molecules involved in the TCR signaling pathway 13

Figure 1.3: The organization of the Ifng locus 29

Figure 1.4: Basic structure of PPARs and mechanism of trans-activation 46

Figure 1.5: Trans-repression mechanism of PPARγ in LPS-stimulated macrophages 54

Chapter 2

Figure 2.1: PLP p139-151-reactive CD4+ from female SJL mice produce more IFNγ and proliferate more than male CD4+ cells 69

Figure 2.2: Frequency of T regs in the periphery and frequencies of CNS-infiltrating Th1 and Th17 cells during EAE. 70

Figure 2.3: Sex difference in cytokine production associates with hormone status in male mice 72

Figure 2.4: Sex of T cell determines Th cytokine production 74

Figure 2.5: Preliminary human T-cell experiments 76

Figure 2.6: CD4+ T cells from women are more prone to produce IFNγ and less IL-17A than male T cells 78

Figure 2.7: Male T cells are more Th17-prone when co-cultured with monocytes 80

Figure 2.8: PPARα has a sex-specific role in inhibiting Th1 cytokine production by human T cells 82

Figure 2.9: Correlation of T-cell PPARα mRNAs with circulating testosterone and estradiol
Figure 2.10: Effect of PPARα siRNAs on murine CD4⁺ T-cell proliferation and cytokine production

Figure 2.11: Dose-dependent effect of fenofibrate on IFNγ production by murine CD4⁺ T cells

Figure 2.12: Effects of PPARα at the IFNG locus

Figure 2.13: PPARγ operates selectively in female T cells to repress IL-17A production

Chapter 3

Figure 3.1: PPARα does not function in APC or T reg cells to negatively regulate Th1 responses

Figure 3.2: PPARα regulates IFN-γ production by CD4⁺ T cells

Figure 3.3: Intracellular staining of male WT and PPARα⁻/⁻ CD4⁺ T cells under Th-polarizing conditions

Figure 3.4: Titration of PPARα antagonists and effect of NXT 1120 on CD4⁺ T cell proliferation

Figure 3.5: IFN-γ is the gene target of PPARα-dependent suppression

Figure 3.6: PPARα⁻/⁻ male CD4⁺ T cells do not exhibit a differential phosphorylation or abundance of key signaling intermediates downstream of TCR/CD28

Figure 3.7: Reducing PPARα activity enhances H4-Ac at the Ifng locus

Figure 3.8: PPARα does not regulate the basal expression of IFN-γ or H4-Ac at the Ifng locus in quiescent naïve CD4⁺ T cells

Figure 3.9: Fenofibrate induces the recruitment of PPARα to specific regulatory elements in the Ifng locus to repress H4-Ac
**Figure 3.10:** PPARα negatively regulates IFN-γ production by NKT cells in males, but not females

**Figure 3.11:** PPARα negatively regulates IFN-γ production by male, but not female, CD8⁺ T cells

**Figure 3.12:** NXT-1120 treatment enhances IFN-γ responses selectively in males during *L. monocytogenes* infection

**Chapter 4**

**Figure 4.1:** Regardless of Th status, female PLP p139-151-reactive T cells are more highly encephalitogenic than male T cells.

**Figure 4.2:** PPARα⁻/⁻ male naïve CD4⁺ T cells exhibit higher levels of both OCAR and ECAR after anti-CD3 and anti-CD28 stimulation
List of Tables

Chapter 1

Table 1.1: List of major endogenous and synthetic ligands for PPARs 47

Chapter 2

Table 2.1: Human primer sequences used for SYBR real-time PCR. 66

Table 2.2: Proliferation and cytokine production by human naïve CD4+ T cells 79

Table 2.3: Effect of PPARα siRNA on Th Cell Cytokine Production 87

Chapter 3

Table 3.1: Antibodies used for ChIP experiments 103

Table 3.2: Primer sequences used for real-time PCR and ChIP 104
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>15d-PGJ₂</td>
<td>D₂ metabolite 15-deoxy-Δ&lt;sup&gt;12,14&lt;/sup&gt; prostaglandin J₂</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ATF-2</td>
<td>activating transcription factor 2</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AU-rich</td>
<td>adenylate-uridylate-rich</td>
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<td>BCG</td>
<td>Bacillus Calmette–Guérin</td>
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<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation (e.g. CD&lt;sub&gt;4&lt;/sub&gt;⁺)</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<td>CNS</td>
<td>conserved non-coding sequences</td>
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<td>CFA</td>
<td>Complete Freund’s Adjuvant</td>
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<td>CFSE</td>
<td>carboxyfluorescein succinimidyl ester</td>
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<td>CFU</td>
<td>colony forming unit</td>
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<td>CoA</td>
<td>acyl-coenzyme A</td>
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<tr>
<td>cpm</td>
<td>counts per minute</td>
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<td>CTLA-4</td>
<td>cytotoxic T-lymphocyte-associated protein 4</td>
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<td>diacyl-glycerol</td>
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<td>deoxyribonucleic acid</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>dendritic cells</td>
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<td>DHT</td>
<td>dihydrotestosterone</td>
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<td>DNMT</td>
<td>DNA methyltransferase</td>
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<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
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<td>ECAR</td>
<td>extracellular acidification rate</td>
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<td>Eomes</td>
<td>Eomesodermin</td>
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<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>Acronym</td>
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<td>FITC</td>
<td>fluorescein</td>
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<td>forkhead box P3</td>
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<td>ETC</td>
<td>electron transport chain</td>
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<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>glucose-6-phosphate</td>
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<td>GATA-3</td>
<td>GATA-binding protein 3</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<td>H3K4-Me</td>
<td>di-/tri-methylation of histone H3 lysine 4</td>
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<td>H3K27-Me</td>
<td>tri-methylated histone 3 lysine 27</td>
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<td>H4-Ac</td>
<td>acetylated histone H4</td>
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<td>HATs</td>
<td>histone acetyltransferases</td>
</tr>
<tr>
<td>HDACs</td>
<td>histone deacetylases (e.g. HDAC3)</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HMTs</td>
<td>histone methyltransferases</td>
</tr>
<tr>
<td>HS</td>
<td>hypersensitive</td>
</tr>
<tr>
<td>HuR</td>
<td>human antigen R</td>
</tr>
<tr>
<td>HY</td>
<td>Y-linked histocompatibility</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin (e.g. IgG)</td>
</tr>
<tr>
<td>IkB</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (e.g. IkBα)</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IPEX</td>
<td>immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome</td>
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<tr>
<td>IP3</td>
<td>Inositol 1,4,5-triphosphate</td>
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<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motifs</td>
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<td>i.p.</td>
<td>intraperitoneal</td>
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<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IFNAR</td>
<td>IFN-α/β receptor</td>
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<td>IFNGR</td>
<td>IFN-γ receptor</td>
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<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun n-terminal kinase</td>
</tr>
<tr>
<td>KDMs</td>
<td>lysine demethylases</td>
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<tr>
<td>LAT</td>
<td>linker for activation of T cells</td>
</tr>
<tr>
<td>LCK</td>
<td>lymphocyte-specific tyrosine kinase</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>lethal dose, 50%</td>
</tr>
<tr>
<td>L</td>
<td>ligand</td>
</tr>
<tr>
<td>LFA</td>
<td>lymphocyte function-associated antigen</td>
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<tr>
<td>L. monocytogenes</td>
<td><em>Listeria monocytogenes</em></td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK or ERK kinases</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NcoR</td>
<td>nuclear receptor co-repressor 1</td>
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<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NOD</td>
<td>nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>OCAR</td>
<td>oxygen consumption rate</td>
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OD$_{600}$  optical density measured at a wavelength of 600 nm
OVA  ovalbumin
OXPHOS  oxidative phosphorylation
PBMCs  peripheral blood mononuclear cells
PCR  polymerase chain reaction
PD-1  programmed cell death protein 1
PDK4  pyruvate dehydrogenase kinase isoform 4
PHA  phytohemagglutinin
PI3K  phosphatidylinositol 3-kinase
PI(4,5)P$_2$  Phosphatidylinositol 4,5-bisphosphate
PKC$\theta$  Protein kinase C-theta
PLC$\gamma$1  phosphoinositide-specific phospholipase C gamma 1
PLP  proteolipid protein
PMA  phorbol myristate acetate
PPAR  peroxisome proliferator-activated receptor (e.g. PPAR$\alpha$)
PPRE  peroxisome proliferator response element
PTPN1  protein tyrosine phosphatase nonreceptor type 1
Rac  Rho-related C3 botulinum toxin substrate
Raf-1  rapidly accelerated fibrosarcoma 1
Rag  recombination activating gene
Ras  rat sarcoma
RNA  ribonucleic acid
siRNA  small interfering RNA
ROR$\gamma$T  retinoic acid receptor-related orphan nuclear receptor gamma T
ROS  reactive oxygen species
Runx  Runt-related transcription factor (e.g. Runx3)
RXR  retinoic acid receptor
SEM  standard error of the mean
s.c.  subcutaneous
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SH2</td>
<td>Src homology region 2</td>
</tr>
<tr>
<td>SHP-1</td>
<td>SH2 domain-containing protein-tyrosine phosphatase 1</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator for retinoid or thyroid-hormone receptors</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppressor of cytokine signaling</td>
</tr>
<tr>
<td>STAT</td>
<td>signaling transducer and activator of transcription</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle or the Krebs Cycle</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper</td>
</tr>
<tr>
<td>Tg</td>
<td>transgenic</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<tr>
<td>Th</td>
<td>T helper</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TR1</td>
<td>type 1 regulatory T cell</td>
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<tr>
<td>Treg</td>
<td>regulatory T cells</td>
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<tr>
<td>TYK-2</td>
<td>tyrosine kinase 2</td>
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<tr>
<td>UTR</td>
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<td>WT</td>
<td>wild type</td>
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<tr>
<td>ZAP70</td>
<td>ζ-chain-associated protein kinase 70-kDa</td>
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List of Publications


Prepared Manuscript

CHAPTER 1

Introduction

The immune system is responsible for eliminating pathogenic microorganisms while maintaining tolerance to self-antigens (1). Both innate and adaptive immune systems work in synergy to achieve the perfect balance for maintaining the health of the organism (1). To fully understand the functioning of the immune system, how sex affects immune responses needs to be taken into consideration. It has been known for over 30 years that female mice tend to develop more robust T helper (Th)-1 immune responses as compared to male mice (2). Similar sex-based differences in adaptive immune responses have been observed in humans (3, 4). These sex differences may explain why women not only generate enhanced anti-viral and anti-tumour immune responses (5, 6), but also have a higher propensity to develop certain T cell-mediated autoimmune diseases (7, 8). The first part of this thesis (Chapter 2) addresses the involvement of the nuclear receptors peroxisome proliferator-activated receptor-α (PPARα) and PPARγ in the regulation of sex differences in Th1 and Th17 differentiation, respectively. The second part (Chapter 3) investigates the molecular mechanism of how PPARα negatively regulates Th1 differentiation. This introductory chapter will provide an overview of the related background literature and will include discussions of (1) Th cells and factors that regulate Th cell fate, (2) the regulation of the expression of the Th1 cytokine IFN-γ, (3) sex differences in adaptive immunity and autoimmunity, and (4) the roles of PPARs in inflammation and metabolism.

1.1. T Helper (Th) Cells

Since Chapter 2 of this thesis contains an exploration of how members of the PPAR family regulate Th1 and Th17 differentiation, this section will provide an overview of what is known about the regulatory mechanisms of Th differentiation, to provide a context for how PPAR family members may factor into this regulation.
1.1. **T Helper (Th) Cells**

CD4⁺ T cells play many critical roles in the immune system, such as providing help to B cells in generating antibody responses, activating and maintaining CD8⁺ T cell function, and regulating macrophage activities (1). As a result, CD4⁺ T cells are able to orchestrate an array of immune responses against pathogenic microorganisms, control autoimmune inflammatory responses, and regulate tumour growth and survival (1). In order to elicit these important functions, naïve CD4⁺ T cells first respond to cognate antigens presented on antigen presenting cells (APC) and differentiate into specialized effector Th cells (1). This section will provide an overview of what is known about the transcription factors, cytokines, and T cell receptor (TCR) signals that specify Th cell fate. It will also discuss the cross-regulatory mechanisms that operate amongst the different Th lineages to determine cell fate.

1.1.1. **Th Differentiation**

Since the initial discovery of Th1 and Th2 cells by Mosmann and Coffman in 1986 (9), new subsets of differentiated Th lineages have been identified including Th17 cells (10–12) and regulatory T cells (Treg) (13–16). A fifth subset, called the T follicular helper (Tfh) cell, has been found to play an important role in providing help to B cells in germinal centers, and is largely thought to belong to a developmentally distinct Th lineage (17–21). There have also been reports of transforming growth factor-beta (TGF-β)-producing Th2 cells, interleukin (IL)-9-producing Th9 cells, and IL-10-producing type 1 regulatory (TR1) cells (22–25). Given that Treg produce TGF-β, all Th cells produce IL-10 under certain conditions, and Th2 cells produce IL-9, the general consensus in the literature is that these newly identified Th cell types simply
represent variations within existing Th populations (19). This following discussion will focus on the major Th subsets: Th1, Th2, Th17, and Treg cells.

Th1 cells, defined by the production of IFN-γ, are important in the generation of cellular immunity against intracellular pathogens including viruses and bacteria such as *Listeria monocytogenes* (26–28). Th2 cells are defined by the production of IL-4, and are important in clearing extracellular pathogens such as helminthes and parasites (29–32). Th17 cells produce distinct cytokines (i.e., IL-17A, IL-17F, IL-21, IL-22) (10–12, 33, 34) and are important in clearing extracellular bacteria and fungi, though they also contribute to autoimmune inflammation (10, 35, 36). CD4⁺ CD25⁺ forkhead box P3 (Foxp3)+ Treg produce immune-suppressive cytokines such as TGF-β and IL-10, and control inflammatory immune responses to prevent the development of autoimmunity (13, 14, 16, 37).

1.1.2. Transcription Factors and Cytokines Cues

Upon TCR signaling in the presence of cytokine cues, naïve CD4⁺ T cells proliferate and differentiate into distinct Th subsets. Each Th subset is defined by its own master regulatory transcription factor and signature cytokine: T-bet and IFN-γ for Th1, GATA-binding protein 3 (GATA-3) and IL-4 for Th2, retinoic acid receptor (RAR)-related orphan nuclear receptor gamma T (RORγT) and IL-17A for Th17 cells, and Foxp3 and TGF-β for Treg (12, 14–16, 38–40). All the major transcription factors and cytokines involved in each Th differentiation pathway are summarized in Figure 1.1.

1.1.2.1. Th1 Cells
Fig. 1.1 Th differentiation. The major cytokines, receptors, STAT family members, and transcription factors are shown during the differentiation of Th1, Th2, Th17 and Treg cells. This figure is modified from Figure 1 in Zhu et al. (19)
Differentiation towards the Th1 lineage is promoted by the cytokines IL-12 and IFN-γ (27, 41, 42) (Fig. 1.1). The subsequent expression of the master transcription regulator T-bet stabilizes the Th1 lineage (39) (Fig. 1.1). The importance of T-bet in Th1 differentiation was demonstrated by studies that showed that ectopic expression of T-bet in undifferentiated naïve CD4+ T cells promotes Th1 differentiation; conversely, T-bet-deficient CD4+ T cells are unable to fully develop into Th1 cells (39, 43). T-bet promotes Th1 differentiation by inducing its own expression and the expression of the IL-12 receptor subunit, IL-12Rβ2 (44), and by binding to cis elements at the Ifng locus to activate IFN-γ transcription (39, 45, 46). T-bet has also been shown to cooperate with Runt-related transcription factor 3 (Runx3) to activate IFN-γ expression at the Ifng locus (47). On the other hand, IFN-γ promotes Th1 development by inducing T-bet expression through IFN-γ receptor/signaling transducer and activator of transcription (STAT)-1 signaling and by inducing IL-12 production from activated macrophages (48, 49). The related T-box family member Eomesodermin (Eomes), which is considered to be a master regulator of IFN-γ production in CD8+ T cells (50), is required for optimal IFN-γ production by Th1 cells (51, 52). Eomes enhances IFN-γ production by countering the IL-21-mediated suppression of this cytokine (51, 52).

Though naïve CD4+ T cells quickly produce both IFN-γ and IL-4 in response to the TCR signals, STAT family members STAT4 and STAT1 are required to sustain Th1 lineage commitment (Fig. 1.1). These signaling molecules enhance IFN-γ transcription by inducing the optimal expression of T-bet (44, 53). STAT1 is activated by IFN-γ receptor ligation, and in turn induces T-bet expression to further amplify IFN-γ production (44). On the other hand, IL-12 released by APC acts through the IL-12R and the JAK2/TYK2 pathway to induce the tyrosine phosphorylation and activation of STAT4, which is recruited to cis-regulatory elements at the
Ifng locus to induce IFN-γ expression (54, 55). STAT4 can also induce some T-bet expression that is independent of the IFN-γ/STAT1 pathway (56). The importance of STAT4 in the Th1 differentiation pathway is underscored by the finding that mice deficient in STAT4 exhibit severe impairment in IL-12-mediated IFN-γ production (57).

1.1.2.2. Th2 Cells

Th2 cells are differentiated in vitro in the presence of IL-4 and IL-2, and in turn produce the signature cytokines IL-4, IL-5 and IL-13. These cytokine genes are linked and controlled by the Th2 locus control region on mouse chromosome 11 and human chromosome 5q (30–32, 58, 59). GATA-3 is the master transcriptional regulator for Th2 cells (40, 60). The expression of GATA-3 is up-regulated by IL-4/STAT6 signaling (Fig. 1.1); GATA-3, in turn, trans-activates the expression of Il4, Il5, and Il13 (58, 61–63). Though IL-2 acts as a pleiotropic cytokine that is important for the regulation of the growth and maintenance of various Th cell types (64), IL-2 also promotes Th2 differentiation in a STAT5-dependent way by upregulating IL-4Rα expression and by increasing the accessibility of the Il4 locus (65–67) (Fig. 1.1). STAT-6 plays an analogous role as STAT4 does in IL-12-mediated IFN-γ production and is responsible for IL-4-mediated Th2 differentiation and expansion (68). The expression of STAT6 is necessary and sufficient for inducing high expression levels of GATA3 (69) and chromatin remodelling at the Il4 locus in developing Th2 cells (70).

1.1.2.3. Th17 Cells

The IL-17-producing Th17 subset was originally discovered through studies conducted in autoimmune mouse models. When experimental autoimmune encephalomyelitis (EAE) was still
considered to be a Th1-mediated autoimmune disease, it was observed that blocking the activity of IL-12p40, a subunit of IL-12 (p35 and p40), protected mice from developing this disease (71, 72). The beneficial effect of anti-IL-12p40 was originally attributed to the neutralization of IL-12 activity (71, 72). However, the related cytokine IL-23 shares the p40 subunit with IL-12 and also contains a distinct subunit, p19. To differentiate between the effects of IL-12 and IL-23 in autoimmune inflammation, Cua et al. compared the development of EAE in mice that were deficient in either p19, p40, or p35 (10, 73). It was found that mice lacking IL-12p19 or IL-12p40 were protected from EAE, whereas mice lacking IL-12p35 still succumbed to the disease, hinting at an important pathogenic role for IL-23 in autoimmune inflammation (10). The same group made similar observations in a murine model of collagen-induced arthritis and demonstrated that IL-23 promotes the expansion of a new Th subset that produced IL-17 (73).

Subsequent studies further elucidated the details of the transcriptional programming of this new Th subset (11, 12, 74). TGF-β and IL-6 were identified to be important factors for the generation of Th17 cells, whereas TGF-β alone was shown to promote differentiation towards the Treg lineage (25, 75, 76) (Fig. 1.1). In addition, IL-21, which is induced by IL-6, plays a crucial role in the induction of IL-17 production by acting as an autocrine cytokine (Fig.1.1); IL-21 up-regulates its own expression and the expression of many other key Th17-related genes such as RORγT and IL-23R (77). IL-23 also up-regulates IL-23R expression on CD4+ T cells and enhances the accessibility of Il17 and Rorc gene loci (78). Though IL-23 was originally identified to be a key factor in the identity of Th17 cells, further studies showed that this cytokine is more important in the maintenance, as opposed to the induction, of the Th17 lineage (12, 25, 75) (Fig. 1.1). IL-23 along with IL-6 and IL-1β also produce a more pathogenic type of Th17 cells that causes more severe autoimmune inflammation in mice (78). In combination with
IL-23, IL-1β was shown to play a unique role in driving human naïve CD4⁺ T cells towards the Th17 lineage (79) (Fig. 1.1).

Once differentiated, Th17 cells produce an array of cytokines including IL-17A, IL-17F, IL-21, IL-22, and granulocyte macrophage colony-stimulating factor (GM-CSF), with IL-17A being the primary effector Th17 cytokine (80, 81). RORγT is considered to be the master regulator of Th17 cells: overexpression promotes the transcriptional programming of Th17 cells, whereas deficiency in RORγT leads to impaired Th17 development (82). An additional orphan nuclear receptor RORα was also found to be induced by TGF-β and IL-6, and to work synergistically with RORγT to drive Th17 differentiation (83). The STAT family member STAT3 that acts downstream of the IL-6R and the IL-21R plays an important role in Th17 differentiation (77, 84) (Fig. 1.1). Naïve CD4⁺ T cells that are deficient in STAT3 cannot produce IL-17 in the presence of TGF-β, IL-21, and IL-23, and instead express high levels of Foxp3 (77).

1.1.2.4. Tregulatory (Treg) Cells

There are two main types of FoxP3⁺ Treg, natural Treg (nTreg) and induced Treg (iTreg) (85). nTreg develop in the thymus, whereas iTreg differentiate in the periphery from conventional CD4⁺ T cells in response to environmental antigens and extrathymic signals (85). Upon TCR and CD28 stimulation, CD4⁺ CD25⁺ Foxp3⁺ iTreg can differentiate from CD4⁺ CD25⁻ Foxp3⁻ naïve T cell precursors in the presence of TGF-β (13–15, 86). In addition to TGF-β, IL-2 also plays a non-redundant role in inducing Foxp3 expression in Treg cells (86) (Fig. 1.1). The requirement for IL-2 in iTreg development may relate to the downstream activation of STAT-5, which is essential for inducing Foxp3 expression (87, 88) (Fig. 1.1).
The physiological role of Foxp3 has been dissected by studying the phenotype of scurfy mice; these mice spontaneously acquired an X-linked recessive mutation that results in lethality in hemizygous males within 25 days after birth (89). Studies that attempted to pinpoint the exact gene that is responsible for this scurfy phenotype identified Foxp3 to be the gene mutated in these mice (90). Besides exhibiting premature death, scurfy mice have abnormal skin (scaly and ruffled), larger spleens and lymph nodes (91), more CD4+ T cells (91), and elevated levels of the pro-inflammatory cytokines IL-2, IFN-γ and TNFα (92). These autoimmune inflammatory symptoms have similarly been observed in humans with the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), which is caused by a mutation in Foxp3 (93). Ectopic expression of Foxp3 in CD4+ CD25− naïve T cells results in conventional T cells acquiring a more Treg phenotype, producing lower levels of all cytokines including IFN-γ (94). Differentiated Treg cells further function to suppress antigen-specific expansion and cytokine production by CD4+ T cells in vivo (13). These immunosuppressive effects of Treg have been demonstrated in murine autoimmune models including EAE, where depletion of CD4+ CD25+ Treg cells with anti-CD25 in vivo prior to immunization with myelin peptide resulted in a reduced expansion of autoreactive cells and a lower incidence of this disease (95).

Treg cells can elicit immune suppression through both contact-dependent and – independent mechanisms (96). Contact-dependent mechanisms include, but are not limited to, the engagement and down-regulation of CD80 and CD86 on APCs (96), whereas contact-independent mechanisms include the production of immunosuppressive cytokines such as IL-10 IL-35, and TGF-β (97). The importance of IL-10 in Treg-dependent regulation has been illustrated by the finding that selective ablation of IL-10 in Foxp3-expressing Treg cells leads to impaired inflammatory control at environmental interfaces such as the gut and colon in vivo (98).
Similarly, Treg cells lacking the IL-10 receptor have been found to be unable to suppress Th17-mediated inflammation in a colitis model in vivo (99).

1.1.3. Cross-regulation amongst the Different Th Lineages

Differentiation towards one Th lineage is dependent upon the suppression of other Th lineages. Cross-regulatory pathways work in synergy to maintain Th lineage commitment (19). These cross-regulatory pathways operate by regulating the expression of cytokine genes and master transcription factors, and through direct protein-protein interactions amongst the master transcriptional regulators.

T-bet cross-inhibits the production of Th2-type cytokines (IL-4, IL-5 and IL-13) by suppressing the expression of GATA-3 (56). Furthermore, upon being phosphorylated by the nuclear tyrosine kinase, T-bet can directly interact with GATA-3 to inhibit its transcriptional activity (100). T-bet also suppresses the Th17 lineage commitment by interacting directly with Runx1 to suppress the Runx1-mediated transactivation of the RORγT gene (101). IFN-γ can also inhibit Th17 differentiation. For instance, neutralizing IFN-γ during Th17 differentiation leads to the enhanced development of Th17 cells, while the addition of exogenous IFN-γ reverses this effect regardless of the presence of IL-12 (11). Consistent with these findings, mice deficient in IFN-γ exhibit an enhanced production of Th17-type cytokines during mycobacterial infection and collagen-induced arthritis (102–104).

In terms of the cross-regulatory mechanism driven by Th2 regulators, the overexpression of GATA-3 negatively regulates the expression of IFN-γ and STAT4 (105). IL-4 also inhibits Th1 differentiation by dampening IL-12Rβ2 expression, although the loss of IL-12 production can be reversed by adding exogenous IFN-γ (49). Thus, antagonizing IL-4 in vitro skews CD4+ T cells
towards a Th1 phenotype (31, 32), whereas the addition of exogenous IFN-γ alone cannot reverse the Th2 programming (30).

Th17 cells can also cross-regulate the development of other Th lineages. IL-6 and STAT3 promote Th17 differentiation and dampen Treg cell development by down-regulating Foxp3 expression (106). IL-21 also induces Th17 differentiation through STAT3 signaling (34), while suppressing Th1 programming and IFN-γ production through Eomes (51).

Conversely, the master regulator of Treg cells, Foxp3, can suppress IL-17 production by directly modulating the transcriptional activity of RORγT (107). TGF-β can inhibit RORγT expression by up-regulating Foxp3 expression in a dose-dependent manner: low concentrations of TGF-β favour the development of Th17 cells by upregulating IL-23R expression on CD4+ T cells, whereas high concentrations of TGF-β skew CD4+ T cells towards a Treg phenotype by repressing IL-23R expression (107). Moreover, TGF-β inhibits Th1 differentiation and IFN-γ production by suppressing the expression of T-bet and STAT4 (108, 109). TGF-β also inhibits the Th2 programming by interfering with GATA-3 expression, whereas ectopic expression of GATA-3 can effectively restore IL-4 and IL-5 production (110). Hence, a large body of evidence has established that the development of one Th lineage is accompanied by the cross-inhibition of other Th lineages.

1.1.4. Overview of T Cell Receptor (TCR) Signaling

TCR signal strength also factors into Th lineage commitment. Hence, this section will provide an overview of the major TCR signaling pathways as well as how signal strength relates to Th differentiation. The major molecules involved in the TCR signaling pathway are reviewed in this section and summarized in Figure 1.2. TCR signaling is initiated upon recognition of the
Fig. 1.2 Major molecules involved in the TCR signaling pathway. Shown are phosphorylation/dephosphorylation events related to the nuclear translocation of transcription factors (phosphorylation shown in green). This figure is modified from an image on the Cell Signalling website (http://www.cellsignal.com/contents/science-pathway-research-immunology-and-inflammation/t-cell-receptor-signalling-pathway/pathways-tcell).
cognate peptide: major histocompatibility complex (MHC) complex (111). Along with TCR, CD3 is important in transducing the antigen signal (112). The co-receptor CD4 enhances TCR signaling by binding to MHCII residues to facilitate the recognition of peptide: MHCII by the TCR, and by recruiting downstream signaling elements to further propagate TCR signaling (113). CD28 on the CD4+ T cell also provides an important co-stimulatory signal following the engagement of CD80/CD86 on APC (1).

Upon TCR engagement, the SRC family kinases LCK (114) and FYN (115) become phosphorylated, bringing LCK in close proximity to CD3 γ, δ and ε chains (116). This event leads to the LCK-dependent phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) in the CD3ζ chain (117), and the subsequent recruitment and phosphorylation of ζ-chain-associated protein kinase 70-kDa (ZAP-70) (118) via the Src homology region 2 (SH2) domain (119). This clustering and phosphorylation leads to a conformational change in ZAP70, which in turn phosphorylates linker for activation of T cells (LAT) (120). The phosphorylation of LAT leads to the convergence of multiple signaling molecules such as phosphoinositide-specific phospholipase C (PLC)γ1 and Vav that lead to the downstream activation of a number of pathways (116).

The three major pathways that are activated downstream of TCR and CD28 include the mitogen-activated protein kinase (MAPK) pathway, the nuclear factor Kappa B (NF-κB) pathway, and the Ca2+/Calcineurin pathway (121). The first two pathways operate downstream of PLCγ1, which hydrolyzes the membrane lipid PI(4,5)P₂ to produce secondary messengers inositol trisphosphate (IP₃) and diacyl-glycerol (DAG) (121). DAG in turn induces two major pathways: Ras/MAPK and PKCθ/NF-κB. Ras initiates the MAPK phosphorylation cascade by activating Raf-1, a MAPK kinase kinase, which subsequently activates the dual specificity
MAPK or ERK kinases (MEK)1 and MEK2 (122). MEK1/2 then activates MAP kinases, extracellular signal-regulated kinase (ERK)1 and (ERK)2, by phosphorylating tyrosine and threonine residues on these proteins (123, 124). ERK in turn promotes the transcription of c-fos (125) and directly phosphorylates the transactivation domains of this protein (126). On the other hand, JNK and p38 are activated by a parallel MAPK cascade that operates downstream of the Rho family of GTPase Rac and Vav-1, the guanine nucleotide exchange factor for Rac (127). JNK phosphorylates c-Jun, which complexes with c-fos to form the activator protein 1 (AP-1) transcription complex (128), while both JNK and p38 can phosphorylate activating transcription factor (ATF)-2 (129). Both AP-1 and ATF-2 are in turn important for trans-activating multiple cytokine genes including IL-2 and IFN-γ (130).

The canonical NF-κB pathway is initiated in response to PKCθ activation downstream of DAG (121). PKCθ associates with the cytoplasmic tail of CD28 through the intermediate LCK, and in turn regulates IκB kinase (IKK)- IκBα to control downstream NF-κB activity (131). In quiescent cells, NF-κB, which is comprised of a heterodimer of p50 (NF-κB1) with p65 (RelA), associates with the inhibitor IκBα and is sequestered in the cytosol (132). Upon T cell activation, IκBα becomes phosphorylated at serine residues by the IKK complex, and undergoes ubiquitin-mediated degradation, allowing NF-κB to translocate into the nucleus to promote the expression of genes that are involved in regulating T cell survival, growth and cytokine production (132). For instance, IFN-γ production is impaired in transgenic mice that express IκB that is phosphorylated but not degraded, partly due to the inhibition of T-bet and STAT4 expression (133).

The third major pathway activated downstream of the TCR is the Ca^{2+}-calcineurin pathway. This pathway is activated downstream of IP$_3$ (121) that triggers the release of Ca^{2+}
from the endoplasmic reticulum. This increase in intracellular Ca\(^{2+}\) levels results in the activation of the phosphatase calcineurin, and the subsequent dephosphorylation and the nuclear translocation of nuclear factor of activated T cells (NFAT) (121). In the nucleus, NFAT forms a transcriptional complex with other transcription factors. The most studied interaction is between NFAT and AP-1 that leads to IL-2 production (134); however, this interaction can be disrupted by Foxp3, leading to the formation of a NFAT/Foxp3 complex and the induction of a Treg-like programming (135).

Mechanisms of negative regulation are also in place to dampen TCR signaling. For example, the phosphatase CD45 dephosphorylates LCK and limits its activity (121). The SH2 domain-containing protein-tyrosine phosphatase (SHP)-1 dephosphorylates LCK to inhibit TCR signaling in the presence of weak TCR signals (121). Other inhibitors of TCR signaling include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) (121).

### 1.1.4.1. TCR Signal Strength and Th Differentiation

In addition to cytokine signals, TCR signaling strength is an important determinant of Th fate. While an initial aggregation of signaling molecules occurs within seconds to minutes after TCR engagement, sustained signaling for several hours is required to support T effector lineage commitment and T cell proliferation (136). In general, it has been found that a weak TCR signal favours Th2 differentiation while a strong TCR signal favours Th1 differentiation (137, 138). For instance, stimulating moth cytochrome c-specific TCR transgenic CD4\(^{+}\) T cells with a high affinity peptide leads to the strong activation of ERK and promotion of the Th1 lineage, whereas stimulation with a low affinity peptide leads to the transient activation of ERK and promotes Th2
differentiation (137). This preference for Th2 differentiation after stimulation with a low affinity peptide was shown to correlate with the formation of the Jun-Jun homodimer that is more appropriate for IL-4 gene transcription (137). On the other hand, a strong TCR signal was observed to lead to the formation of the Jun-c-fos heterodimer (137). In addition, another study showed that while a weaker TCR signal supports the expression of Foxp3 and the development of Treg, a stronger TCR signal prevents Foxp3 up-regulation and favours Th17 differentiation (139). This inhibition of Treg development by strong TCR stimulation is in part due to NF-κB-dependent inhibition of TGF-β/IL-2-mediated signals (140).

1.2. Interferon (IFN)-γ

Since Chapter 3 of this thesis focuses on the molecular regulation of the Th1 cytokine IFN-γ by PPARα, this section will provide an overview of the roles of IFN-γ in the immune system, and the molecular regulation of IFN-γ signaling and expression in multiple cell subsets including CD4+ T cells.

1.2.1. IFNs and IFN-γ Signaling

IFNs are classified into two types (I and II) based on their structures and functions. Type I IFNs are comprised of multiple family members (141). IFN-α and IFN-β are the most broadly expressed and best characterized, although others including IFN-κ, IFN-δ, and IFN-ω are present in specific species (e.g. humans) (141). All type 1 IFNs bind to a common receptor called the IFN-α/β receptor (IFNAR) (142). On the other hand, IFN-γ is the only type II IFN. It differs structurally from type I IFNs (143) and binds a distinct receptor, the IFN-γ receptor (IFNGR) (142). IFNGR is comprised of the ligand-binding IFNGR1 chain and the signal-transducing
IFNGR2 chain (142). Neither chain has intrinsic kinase or phosphatase capabilities. IFNGR signaling is accomplished through the Janus kinase (JAK)/STAT1 pathway (142). The binding of IFN-γ to IFNGR leads to the activation of receptor-associated JAK1 and JAK2, which in turn phosphorylate a receptor tyrosine residue Y440 that acts as a docking site for STAT1 (142). STAT1, which normally resides in the cytoplasm, becomes activated after being phosphorylated at Y701 and S727, and translocates into the nucleus to activate gene transcription (144). STAT1 signaling ceases about one hour after IFN-γ treatment, indicating the presence of negative feedback mechanisms in this pathway (145). One negative feedback mechanism is the internalization and dissociation of IFN-γ from IFNGR1 (142, 146, 147), whereas a second mechanism is the action of suppressor of cytokine signaling 1 (SOCS-1), which associates and interferes with JAK1/2 (142).

1.2.2. Effect of Deficiencies in IFN-γ Signaling in Mice

The development of mice deficient in IFN-γ, the IFN-γ receptor, and STAT1 has aided in the understanding of the role of IFN-γ signaling in both innate and adaptive immunity (26, 148, 149). Although IFN-γ−/− and IFNGR1−/− mice develop normally, they exhibit severe impairment in mounting IFN-γ responses against a number of intracellular pathogens including Listeria monocytogenes (L. monocytogenes) and Mycobacterium tuberculosis, and cannot control the replication of these pathogens (26, 150, 151). Similarly, STAT1−/− mice produce lower levels of IFN-γ against vesicular stomatitis virus, and exhibit lethality upon infection with L. monocytogenes to the same degree as wild type mice given an anti-IFN-γ monoclonal antibody (148). Studies of T cell-specific or myeloid cell-specific STAT1 knockout mice further implicated a functional role for STAT1 in IFN-γ production in response to L. monocytogenes infection (152).
IFN-γ also acts as a pro-inflammatory cytokine in the context of autoimmune diseases (153). IFN-γ plays a crucial role in the initiation of experimental autoimmune encephalomyelitis (EAE), the mouse model of multiple sclerosis (MS) (154). This has been similarly demonstrated in humans where administering IFN-γ to MS patients exacerbated their disease (155). Surprisingly, both IFN-γ−/− (156) and IFN-γR−/− (157) mice have been reported to develop more severe EAE compared to wild type mice, which was later shown to be a result of the compensatory upregulation of pro-inflammatory Th17 cells. IFN-γ has been shown to play a pathogenic role in systemic lupus erythematosus (SLE): the peripheral blood mononuclear cells from SLE patients produce higher levels of IFN-γ compared to those from healthy people upon anti-CD3 and anti-CD28 stimulation (158). Most recently, mice were generated to have a deletion of the 3’ untranslated region (UTR) in the IFN-γ gene, and were shown to exhibit increased IFN-γ mRNA stability (159). These mice display chronic IFN-γ production by T lymphocytes, NK and NKT cells, and exhibit autoimmune tendencies, such as elevated levels of anti-nuclear antibodies (159).

1.2.3. Effect of Deficiencies and Polymorphisms in IFN-γ Signaling in Humans

Deficiencies in IFN-γ or IFNGR are also reported for humans. Similar to findings for mice, complete loss of function mutations in either IFN-γ or IFNGR are associated with severe susceptibility to mycobacteria infection, and a poor prognosis for one in nine affected surviving children (160–162). On the other hand, case reports revealed that partial deficiency in either IFNGR was associated with impaired IFN-γ responses to in vitro stimulation as compared to healthy children (163, 164). These children also showed a predisposition to mild infections with salmonella and tuberculosis (163, 164). Although infants that are deficient in IFN-γ production were shown to have normal levels of blood neutrophils, T lymphocytes, monocytes, and
immunoglobulin, the functions of key immune cells were severely impaired compared to those from healthy children: neutrophils exhibited much lower motility, NK cells did not exhibit any cytolytic activities in a chromium release assay, and blood mononuclear cells did not produce any IFN-\(\gamma\) in response to phytohemagglutinin (PHA) stimulation (161).

In addition to mutations, several single nucleotide polymorphisms (SNP) in IFN-\(\gamma\) have been reported in humans including those that lie in the introns and the 3’ UTR of \(IFNG\). For instance, the T-to-A polymorphism located at the +874 position in intron 1 of the IFN-\(\gamma\) gene has been shown to directly influence the level of IFN-\(\gamma\) production (165). The three possible genotypes at position +874 (A/A, T/A, and T/T) are associated with low, intermediate, and high level production of IFN-\(\gamma\), respectively (166, 167). The T/T polymorphism in IFN-\(\gamma\) gene is associated with cirrhosis in patients with chronic hepatitis C (CHC) (165), whereas a A/A polymorphism is associated with lower IFN-\(\gamma\) serum levels and susceptibility to atopic diseases (168). Altogether, studies in mice and humans have demonstrated the importance of proper IFN-\(\gamma\) production in maintaining health.

1.2.4. Kinetics of IFN-\(\gamma\) Production During an Immune Response

CD4\(^+\) T, CD8\(^+\) T, gamma delta (\(\gamma\delta\)) T, NK and NKT cells are the major producer of IFN-\(\gamma\) (1, 169). These cells produce IFN-\(\gamma\) with different kinetics during an immune response. For instance, it takes CD4\(^+\) and CD8\(^+\) T cells several days to mount an effective IFN-\(\gamma\) response during a natural infection (1, 169), while NK and NKT cells constitutively express IFN-\(\gamma\) mRNA and produce IFN-\(\gamma\) within hours of infection (1, 169).

During infection, NK, NKT (1) and \(\gamma\delta\) T cells (170) are an early source of IFN-\(\gamma\) and provide the first line of defense against intracellular pathogens. These microbes stimulate
macrophages to produce IL-12, which activates NK cells to produce IFN-γ (171–174); the production of IFN-γ further stimulates the production of IL-12 by these myeloid cells (48). Early IFN-γ production leads to the up-regulation of MHC I, which alerts CD8+ T cells to the presence of intracellular pathogens (142). IFN-γ also promotes the expression of MHC II on B cells, DCs, and macrophages, and thereby enhances the antigen presentation ability of these cells to CD4+ T cells (1). As a result, early IFN-γ production by NK and NKT cells helps skew CD4+ T cells towards the Th1 phenotype by enhancing T-bet expression through IFN-γ/STAT1 signalling and by further augmenting IL-12 production (1, 44). The activated CD4+ T cells provide help to cytotoxic CD8+ T cell effectors by activating APC via CD40–CD40L interactions (175).

1.2.5. The Importance of IFN-γ to the Clearance of *Listeria Monocytogenes*

Since the model of *L. Monocytogenes* will be utilized in Chapter 3 to investigate the function of PPARα in regulating Th1 responses, we will review in detail the importance of IFN-γ production in the control of an infection.

Experimental infection of mice with *L. monocytogenes* has been an instrumental model for deciphering the importance of IFN-γ in host immune responses (176, 177). *L. monocytogenes* is an opportunistic, gram-positive bacterium that causes food-borne illness in immune-compromised people (178). Infection in mice can be induced through intravenous, intraperitoneal, or oral administration, leading to the rapid dissemination and subsequent proliferation of bacteria in the spleen and liver, where they are internalized by resident macrophages (179). In the case of infection with a sub-lethal dosage of *L. monocytogenes*, bacteria replicate until they are controlled by innate immune cells. Neutrophils are important for the initial control of bacterial growth by generating reactive nitrogen and oxygen intermediates
upon bacterial engulfment (180, 181). Macrophages are also found at sites of bacterial replication and contribute to the initial control of the bacteria by secreting cytokines such as TNFα and IL-12 (27, 171, 182), both of which induce NK cells to produce IFN-γ in order to enhance the activation and killing capacity of these macrophages. Additionally, dendritic cells play a crucial role in bridging innate and adaptive responses. When dendritic cells are infected with live L. monocytogenes, the activation of toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD)-like receptor pathways leads to the upregulation of co-stimulatory molecules (CD40, CD80 and CD86) on the surface of APC (177, 183, 184).

While innate responses are important for the initial control of the pathogen, L. monocytogenes-specific T cell responses are required for bacterial clearance and the generation of memory responses to protect against re-infection (185, 186). Upon encountering antigens presented by APC in the presence of IL-12 and IFN-γ, CD4+ T cells proliferate and differentiation towards a Th1 phenotype and secrete IFN-γ, which activates macrophages for bacterial killing (187). CD8+ T cells elicit anti-listerial immunity not only by secreting perforin and granzymes to lyse the infected cells, but also by producing IFN-γ to further activate macrophages (177). This primary T cell response is followed by a contraction phase which produces a smaller stable population of memory T cells (188). Compared to the T effector cells involved in a primary response, memory T cells are present in greater numbers, can be activated with a lower stimulation threshold, and quickly mobilize to the site of infection to elicit antigen-specific effector responses (189, 190). Hence, the L. monocytogenes infection model has been a useful tool to study the importance of IFN-γ production by both innate and adaptive immune subsets in the control of intracellular pathogens.

1.2.6. Regulatory Mechanisms of IFN-γ Expression in CD4+ T Cells
The expression of the IFN-γ gene is regulated at the transcriptional, post-transcriptional, translational and post-translational levels (191). Post-transcriptional regulation of IFN-γ includes the regulation of mRNA stability. This is regulated at the adenylate uridylate (AU)-rich element (ARE) in the 3’ UTR, since the removal of this region leads to abnormally stable IFN-γ mRNA (159). Activating CD4+ T cells with certain stimuli has been shown to increase the stability of IFN-γ mRNA, and thereby enhance the translation of IFN-γ (191). Examples of stimuli include anti-CD3 + anti-CD28 in T cells (192), anti-CD3 + anti-LFA-1 in Jurkat cells (193), IL-2 + IL-12 in NK cells (194), phytohaemagglutinin (PHA) + IL-12 + IL-2 in T cells (195), and IL-12 + IL-18 in human peripheral blood mononuclear cells (196). p38 MAPK signaling was shown to regulate the IFN-γ mRNA stability through the 3’ UTR, since the treatment of HeLa cells with a p38 MAPK-specific inhibitor caused rapid IFN-γ mRNA degradation (196). Additionally, RNA-binding proteins are thought to interact with the 3’ UTR region to regulate mRNA stability of IFN-γ. Human antigen R (HuR), a protein known to bind to the 3’ UTR of other cytokine mRNAs, was shown to stabilize IFN-γ mRNAs in response to anti-CD3 and anti-LFA1 stimulation, although a direct binding of HuR to IFN-γ mRNAs was not demonstrated (193). In contrast, a second RNA-binding protein, tristetraprolin (TTP), was shown to interact with the AUE at the 3’ UTR of the IFN-γ promoter to destabilize IFN-γ mRNAs, which became more stable upon T cell activation in TTP knockout mice (197). A third RNA-binding protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was recently shown to directly bind the 3’ UTR of the IFN-γ transcript to inhibit the translation of IFN-γ mRNAs under glucose-free metabolic conditions (198). Additional post-transcriptional controls include the formation of a secondary structure called a “pseudoknot” at the 5’ UTR that interferes with IFN-γ mRNA translation as demonstrated in a cell-free translation system using rabbit reticulocyte lysate (199).
IFN-γ expression is also regulated at the post-translational level through the control of protein folding (200). It has been demonstrated in Jurkat cells that in response to PHA, phorbol myristate acetate (PMA) and ionomycin, IFN-γ forms a complex with the chaperone protein calreticulin, which protects it from thermal inactivation at high temperatures (e.g. 43°C) (200). This thermal protective nature of this post-translational control may have implications during inflammatory processes associated with fever.

IFN-γ expression can also be regulated by newly identified regulatory RNAs called long intergenic noncoding RNA (201). These long intergenic noncoding RNA can upregulate or downregulate the expression of neighboring target genes (201). For instance, TMEVPG1, located at 170 kb from the IFNG coding region, was found to contribute to the active transcription of the IFN-γ gene (201). TMEVPG1 expression occurs under Th1-polarizing condition and is dependent on the expression of T-bet and STAT-4 (201).

In addition to these post-transcriptional and post-translational mechanisms, IFN-γ expression is controlled at the transcriptional level. This transcriptional regulation encompasses both epigenetic changes that alter the accessibility of the Ifng locus and trans-acting transcription factors that bind at the cis regulatory elements at Ifng to facilitate transcriptional activation. The following sections will describe the general mechanisms of epigenetic regulation in eukaryotes, how these mechanisms factor in the regulation of Ifng, and transcription factors that regulate IFN-γ expression.

1.2.7. General Epigenetic Regulation in Eukaryotes

Eukaryotes face the challenge of storing a massive amount of genetic information, and fitting several billion nucleotides into a nucleus that is only a micron in diameter (202). This
effective packaging is achieved by wrapping DNA around nucleosomes, which are comprised of histone protein octamers (containing two sets each of H2A, H2B, H3 and H4) (202). A number of epigenetic modifications regulate the extent of this DNA wrapping and the accessibility of gene loci to transcription factors. One mechanism is CpG methylation that involves the conversion of cytosine bases to 5-methylcytosine by DNA methyltransferase (DNMT) in eukaryotes (203), and is commonly associated with gene silencing (203).

The second mechanism is the post-translational modification of the amino terminal tails of core histones (204). Post-translational histone modifications are mediated by various enzymes that have been described as either “writers” such as histone acetyltransferases (HATs) and histone methyltransferases (HMTs), or “erasers” such as histone deacetylases (HDACs) and lysine demethylases (KDMs) (204). Earlier literature oftentimes describes a direct relationship between histone hyper-acetylation and transcriptional activation; however, this view is now deemed overly simplistic (204). For example, it was observed that as many genes are repressed as there are activated in leukemia cell lines that were treated with a HDAC inhibitor (204, 205). The current model of transcriptional activation depicts a multifaceted interplay of different epigenetic modifications to regulate gene expression in the form of multi-protein complexes (204).

Studies have employed two main methods to locate the regulatory elements that are involved in gene regulation. The first method is to identify the DNase I hypersensitive (HS) genomic regions (206). The second method is to locate regions of conserved non-coding sequences (CNS) amongst different species, since important regulatory genomic elements tend to be conserved amongst species and can be identified by performing cross-species sequence comparisons using the web-based global sequence alignment tool VISTA (45, 206). The
identification of these CNS sites is followed by further functional studies using in vitro reporter
expression assays, transgenic mice that express IFN-γ reporter, or knockout mice that are
deficient in key CNS elements (206). Since important sites of epigenetic modification usually
coincide with the locations of key regulatory elements, early studies focused on identifying and
characterizing the cis-regulatory elements of genes (207, 208).

1.2.8. Epigenetic Regulation at the Ifng Locus in CD4+ T cells

In 1985, Dr. Howard Young and colleagues were the first to correlate changes in the DNA
structure of IFN-γ with cytokine gene expression in mice (209). They demonstrated that
prevention of DNA methylation leads to the long-term, stable production of IFN-γ by a mouse
cell line that normally loses the ability to produce IFN-γ over time (209). The authors
subsequently identified a unique single CpG site located at the position of -52 base pairs in both
human and mouse IFN-γ promoter (210). The methylation state of this CpG site correlates with
the transcription of the IFN-γ gene: both Th0 and Th1 cells exhibit complete or partial
hypomethylation whereas this site is over 98% methylated in Th2 cells (210, 211). The proximal
promoter region surrounding this central CpG site, between -73 to -48 base pairs from the
transcriptional start site, was further shown to be important for the binding of c-Jun or c-
Jun/ATF-2 heterodimer to mediate T cell activation (130, 212). Chromatin modification at
various cytokine gene loci were further dissected and found to be associated with specific Th
differentiation and cytokine production (70): DNase I HS sites were identified near the promoter
and enhancer regions within the Ifng locus that associated with the commitment to the Th1
lineage (70). Using molecular tools such as histone deacetylase (HDAC) inhibitors and a DNA
methylation inhibitor, it was confirmed that chromatin remodeling at the Ifng locus plays an
important role in Th fate determination (70).
Subsequent detailed mapping of the cis-regulatory elements in the *Ifng* and *IFNG* loci identified intronic DNase I HS sites within an 8.6 kilobases (kb) of human *IFNG* gene (213, 214). Despite the finding that these regions could act as enhancers *in vitro*; they were insufficient to generate Th1/Tc1-specific expression in a mouse transgenic model (215, 216). This lack of lineage-specific, activation-driven expression was similarly demonstrated in another transgenic mouse model that expressed a 3.4 kb fragment of the *Ifng* promoter (217). Mice were then constructed that carried a longer fragment of *IFNG* that included 90 kb of the upstream and downstream regions of *IFNG*; the inclusions of these distally-located cis-regulatory elements were able to generate lineage-specific expression in T cells (215). Using techniques of conventional DNase I and CNS mapping and chromatin immunoprecipitation (ChIP), researchers subsequently identified 5 key CNS sites in mice that associated with regulations related with Th1 lineage commitment, including CNS1 (later CNS-6), CNS2 (later CNS+17), CNS-22, CNS-34, and CNS-55 (45, 46, 218), though additional CNSs have been identified in the literature. The relative positions of histone marks and key transcription factors that operate at the *Ifng* locus are summarized in Figure 1.3. More recently, studies have coupled these techniques with high-throughput parallel sequencing (ChIP-seq and DNase-seq) or microarray techniques (ChIP-ChIP and Dnase-ChIP) to generate a more unbiased and comprehensive mapping of the *Ifng* gene regulatory sites (219–221).

In naïve murine CD4+ T cells, CNS-22 and CNS-34 along with the *ifng* promoter are marked by di-/tri-methylation of histone H3 lysine 4 (H3K4-Me) (222) (Fig. 1.3). The finding of this permissive mark at these sites has led to the hypothesis that CNS-22 and CNS-34 may already be active in naïve CD4+ T cells and can act as pioneering elements within the *Ifng* locus (222). As a first step to test this hypothesis, Weaver and colleagues recently generated mice with a CNS-22 deletion at the *Ifng* locus so that all cells, including T cells, lack CNS-22 at *ifng* (223).
CD4+ T cells that were isolated from CNS-22-deficient mice exhibit a reduced abundance of the H3K4-Me at CNS-34 upon anti-CD3 and anti-CD28 stimulation as compared to those isolated from wild type mice, confirming the role of CNS-22 in early remodelling of the *Ifng* locus not only at CNS-22 but also in the vicinity of this CNS site (223).

A number of studies have further investigated how histone marks change across the *Ifng* locus in murine CD4+ T cells under both Th1- and Th2-polarizing conditions. Upon Th1 differentiation, the abundance of the permissive mark, H3K4-Me and acetylated histone H4 (H4-Ac) increase across the entire *Ifng* locus, signaling a global de-repression at the locus (45, 55) (Fig. 1.3). Conversely, repressive histone marks such as tri-methylated histone 3 lysine 27 (H3K27-Me) and di/tri-methylation on histone 3 lysine 9 (H3K9-Me) are generally present at low levels at the *Ifng* locus under Th0 conditions and minimally under Th1 conditions, but become more prominent during Th2 polarization (224, 225). The *Il4/Il13/Il5* locus also gains histone modifications that are consistent with repression during Th1 differentiation (336, 341). A recent study also investigated histone modifications at the *Ifng* locus in CD4+ T cells that were polarized under Th17-skewing conditions and showed that the pattern of histone marks was surprisingly similar to that found in Th1 cells, raising the possibility that Th17 cells acquire a Th1-like phenotype during development (228).
Fig. 1.3 The organization of the *Ifng* locus. This cartoon shows the location of key conserved non-coding sequence (CNS) sites (black oblong shapes) and the promoter (blue box) of the *Ifng* gene. The region between -73 and -48 base pairs from the transcriptional start site in the proximal region of the *Ifng* promoter contains an AP-1 (c-Jun)-binding site, while -52 contains a unique CpG site that is partially or completely hypomethylated under Th0 and Th1 conditions (130, 210). Round symbols indicate sites that show Dnase I hypersensitivity (HS) and/or contain permissive histone modifications (i.e. H4-Ac and H3K4-Me). The binding of T-bet, NF-κB (p50 or RelA), Runx3, and STAT4 to these sites is best characterized under Th1 conditions. The amounts of the repressive histone mark H3K27-Me are minimal under Th0 condition and absent under Th1 condition (not shown). This figure is modified from Figure 2 in Balasubramani *et al.* (222). The direct binding of Eomes at the IFN-γ gene has not been reported.
1.2.9. Trans-acting Molecules at the Ifng Locus in CD4+ T Cells

Many transcription factors have been reported to associate with CNS regulatory elements during IFN-γ transcription; four of them have been described at length in the literature and are key to this process: STAT4, T-bet, NF-κB, and Runx3 (Fig. 1.3). These transcription factors are co-recruited to many of the same CNS sites in the Ifng locus, suggesting a possible role for multi-protein complexes in the regulation of IFN-γ gene expression (45, 47, 229, 230) (Fig. 1.3). STAT4 is activated downstream of IL-12 signals and is recruited to many CNS sites across the Ifng locus including CNS-34, CNS-22, CNS+30, CNS+40, CNS+46, CNS+54 and the promoter (55) (Fig. 1.3). T-bet expression is induced downstream of IFNGR/STAT1 signaling and is recruited to the same sites as STAT4 in activated Th1 cells in addition to CNS-6 and CNS+17 (45, 46, 55, 218) (Fig. 1.3). This recruitment of T-bet has been linked with the displacement of HDAC-associated complexes at CNS sites across the locus, and correlates with enhanced histone acetylation and transcriptional activation of Ifng (226).

The NF-κB family member RelA is activated downstream of both TCR and CD28 stimulation as well as downstream of IL-12 and IL-18 stimulation (231, 232). The role of this transcription factor in the regulation of IFN-γ expression was initially recognized through the identification of RelA binding sites in the Ifng promoter (233, 234). It was later shown that upon re-stimulation of Th1 cells with IL-12 and IL-18 or anti-CD3 and anti-CD28, RelA is recruited to a number of CNS regulatory elements (CNS-54, −34, −22, +40, +46 and +54) as well as the promoter to drive IFN-γ transcription (55) (Fig. 1.3). IL-12 signaling was found to be necessary for the optimal recruitment of RelA to NF-κB target elements in the Ifng locus, indicating a possible mechanism of co-recruitment of STAT4 with RelA (55). Another NF-κB family
member p50 was found to also bind at CNS-22 in both Th0 and Th1 cells, and is thought to function as either a homodimer or a p50-RelA heterodimer (55) (Fig. 1.3).

Less is known about the recruitment of Runx3 to the *Ifng* locus. Runx3 was recently reported to be up-regulated in a T-bet-dependent way during Th1 differentiation, and functions together with T-bet to generate the optimal IFN-γ production (47). Since a ChIP-grade antibody for Runx3 was lacking at the time of the report, the recruitment of its partner core binding factor (CBF)-β at the *Ifng* locus was investigated (230). Consistent with earlier observations that Runx3 cooperates with T-bet (47), it was found that CBFβ is recruited to the same CNS sites as T-bet, with the exception of CNS+17 and CNS+46 (230) (Fig. 1.3). These studies together indicate that CNS-6, CNS+17, CNS-22, CNS-34, CNS-55 and the promoter are the key regulatory sites for the congregation of multiple transcription factors and histone modifications that regulate the transcription of *Ifng*.

**1.2.10. Histone Modifications at the *Ifng* Locus in Other Cell Types**

A number of studies have investigated the transcriptional regulation of *Ifng* expression in other IFN-γ-producing cells and discovered similar mechanisms of regulation as seen in CD4+ T cells. For example, deleting CNS-22 effectively abolishes the IFN-γ-producing ability of CD8+ T cells and NK cells (45), suggesting that CNS-22 is also important in regulating IFN-γ production in these cell types. In addition, NKT cells have been found to share roughly the same CNS regulation region (between -92 to -18 kb) with CD4+ T cells at the *IFNG* locus (235).

On the other hand, certain cell type-specific epigenetic regulatory mechanisms have been described. For example, RelA, but not T-bet, is important for the early induction of IFN-γ transcription in cytotoxic CD8+ T cells (55). In addition, resting NK cells, unlike other cell types,
show hyper-acetylation at the Ifng locus even prior to cell activation (229, 236). There also exist differences in certain regulatory regions amongst different cell types. For instance, deleting CNS-34 leads to impaired IFN-γ expression in CD4+ T but not NK cells (235). Although not required for CD4+ T cells, CNS-6 and CNS+22 are needed for IFN-γ expression in NK cells (237).

1.3. Sex Differences in Adaptive Immunity

Females are known to exhibit more robust adaptive immune responses than males (2, 8). This was illustrated in early vaccination studies in mice, in that both primary and secondary antibody responses to vaccination were enhanced in females as compared to males (238). It was then demonstrated that the more robust adaptive immune response to vaccination in females was due to effects of sex on both T cells and APC (239, 240). It is also important to note that the male T cell phenotype can be made “feminine” through castration, implicating a role for sex hormones in this immune regulation (240). Similar sex differences in adaptive immune responses have been observed in humans (3, 4). These sex-based differences in immune responses are thought to explain why women generate elevated anti-viral and anti-tumour immune responses (5, 6), but also have a higher propensity to develop certain T cell-mediated autoimmune diseases (7, 8).

This section will overview what drives sex differences in adaptive immune responses and the role of sex hormones in this regulation. Since many of the studies that are reviewed here were conducted in the EAE model of MS, there will be a discussion about the basic immunology of EAE and MS, and a detailed review of the EAE models that have been used to study sex differences in Th immunity.
1.3.1. Immune Pathogenesis of MS and EAE

Genome-wide association studies in MS recently have identified SNP beyond those in the human leukocyte antigen (HLA) region that confer susceptibility to this disease (241). Many of these SNP situate close to immune genes such as those involved in Th cell activation and Th1/Th17 cytokine production (241), supporting the notion that MS is an autoimmune disease that is driven by Th1 and Th17 cells. EAE is an MS-like, Th-mediated disease that is induced in rodents by vaccination with protein components of the myelin sheath mixed with Complete Freunds’ Adjuvant (CFA) (active EAE) (242). EAE is most commonly induced in mice and, with the exception of SJL, most strains require injection of pertussis toxin to break tolerance. Pertussis toxin is an adjuvant that can enhance T cell responses and facilitates the crossing of T cells through the blood-brain barrier (BBB) into the central nervous system (243, 244). EAE can also be induced by adoptively transferring activated myelin-reactive Th cells from mice with EAE into healthy recipient mice (245), or can occur spontaneously in mice that are engineered to express transgenic TCRs for specific myelin antigens (246, 247).

How Th1 and Th17 cells mediate inflammation in MS has been largely surmised from studies in the EAE model. Normally, peripheral immune tolerance mechanisms including the action of Treg keep self-reactive Th cells in check. However, when myelin-reactive CD4+ T cells recognize myelin in the context of a microbial stimulus such as CFA, tolerance is broken and Th cells start to expand and differentiate into pathogenic Th1 and Th17 cells (248). Myelin-activated Th1 and Th17 cells then traffic across the BBB into the central nervous system, where they re-encounter myelin antigens presented by resident APC such as microglia; these events trigger the secretion of pro-inflammatory cytokines and chemokines, which promote the recruitment of other immune cells including B cells, CD8+ T cells, monocytes, and neutrophils (248–250). The action
of these immune cells and their immune products leads to myelin and axon damage, resulting in the formation of the acute EAE lesion (248–250).

1.3.2. Sex differences in MS and EAE

Women develop MS three times more often than men (251). This sex bias in disease incidence is present after, not before puberty, implicating sex hormones as opposed to sex chromosome influences on disease development (252). Similar to findings in MS, some rodent strains exhibit a female bias in EAE development. For instance, EAE induced in the Lewis rat with guinea pig spinal cord homogenate and CFA manifests as a monophasic disease in males and a chronic, relapsing-remitting disease in females (253). A female-biased disease is also observed in ASW and NZW mouse strains as well as the highly susceptible SJL mouse strain, which has since become the preferred model for studying sex differences in central nervous system autoimmunity (254). Depending on the mode of vaccination or EAE induction, young adult female SJL mice can develop either a higher incidence of EAE (254, 255), or a higher propensity to relapse (256) than the male counterparts. Furthermore, it has been shown that the adoptive transfer of female myelin-reactive SJL T cell lines into female SJL recipients induces more severe EAE as compared to the transfer of the male counterparts, indicating that Th cells are major drivers of this sex difference (257, 258). Notably, a sex bias in EAE is not observed in all mouse strains. Some commonly used inbred strains such as C57BL/6 and B10.Pl exhibit either no sex difference or slightly more severe EAE in males (254). It has been speculated that these strain differences are related to either a higher susceptibility of male mice to pertussis toxin, or inherent genetic differences between strains (254). Thus, a number of rodent strains exhibit a female bias in EAE development and are useful models for the study the underlying biology of the more robust autoimmune attacks observed in females.
1.3.3. Sex Differences in Adaptive Immune Responses

This section will review what is known about the sex differences in the functions of various immune cells involved in innate and adaptive immune responses including Th, Treg, B, CD8+ T, NK and NKT cells.

1.3.3.1. Sex Differences in Th Cell Numbers and Proliferation

Women have a higher number of CD4+ T cells in the peripheral blood as compared to men (259), and this higher number likely relates to the higher thymic T cell output in females (260). Studies in mice have indicated that this sex difference in thymic output may be due to a suppressive effect of androgens on thymocyte development (238). Female T cells are also known to expand more robustly than male counterparts upon antigenic stimulation (261). This distinguishing feature of female T cells has been observed both in the context of EAE in mice (262, 263) and in humans after vaccination with the herpes simplex virus vaccine (264). The precise cellular mechanism of the more robust proliferation of female T cells is unknown; however, studies in mice suggest that it relates to sex differences in both the intrinsic activation potential of T cells (263) and APC functioning (239, 261, 263). Indeed, mouse female CD4+ T cells proliferate more robustly than male CD4+ T cells when cultured in isolation with sub-maximal amounts of anti-CD3 and anti-CD28 (263). In addition, several groups have reported that female but not male mouse APC have a stimulatory effect on Th cell proliferation in co-cultures (239, 261, 263). It is reported that female macrophages produce higher levels of IL-12 and lower levels of IL-10 as compared to male APC: IL-12 supports the growth of Th1 cells, which are also more abundant in females, whereas IL-10 inhibits T cell growth and Th differentiation by downregulating MHC Class II and co-stimulatory molecule expression on APC (239).
1.3.3.2. Sex Difference in Th Cytokine Production

In addition to expanding more robustly, CD4$^+$ T cells from females are more biased towards Th1 cytokine production as compared to male CD4$^+$ T cells. The higher propensity of females to produce the Th1 cytokine IFN$\gamma$ was first reported in 1984 in a study that examined cytokine responses in mice in response to Bacillus Calmette–Guérin (BCG) vaccination (2). This sex difference was later observed in the context of EAE: a higher production of IFN$\gamma$ by myelin-reactive T cells in the periphery strongly correlated with the development of more severe active or adoptive transfer EAE in the female sex (255, 256). A female Th1 bias in cytokine production was also observed in MS patients in studies by Pelfrey and colleagues (3, 4). Using ELISPOT assays, they investigated cytokine production by peripheral blood mononuclear cells (PBMC) isolated from MS patients and healthy controls after stimulation with myelin antigens, vaccine-relevant antigens (e.g. tetanus toxoid, diptheria toxoid), or polyclonal stimuli such as anti-CD3 (3, 4). They found that female MS patients have a higher frequency of cells that secreted the Th1-cytokine IFN$\gamma$ and a lower frequency of cells that secreted the Th2-cytokine IL-5 in the peripheral blood as compared to male MS patients, when PBMCs were cultured with certain proteolipid protein (PLP) peptides and vaccination-related antigens (3, 4). Sex differences were not observed in the production of the pro-inflammatory cytokine TNF$\alpha$ or the anti-inflammatory cytokine IL-10, whereas IL-17A production was not assessed (4). The notion of a Th1 bias in women was also supported by a microarray study that compared the expression of immune genes between female and male PBMCs after $ex$ $vivo$ activation (265). Taken together, these studies provide human and mouse evidence that auto-reactive Th cells are biased towards a Th1 cytokine profile in females and towards a Th2 cytokine profile in males. Whether a sex difference exists in Th17 responses is
not known, since the majority of studies that assessed Th cytokine responses in males and females predate the discovery of the Th17 subset.

1.3.3.3. Sex Differences in Treg Cells

In a healthy human population, males have a higher number of Treg cells than females in the peripheral blood (266). A similar trend for higher Treg cell number in males has been reported for SJL mice in the spleen (267). Multiple groups have compared the suppressive capacities of male and female murine CD4+ CD25+ Treg in in vitro assays. Although one study reported a higher production of IL-10 by CD4+ CD25+ cells in male SJL mice (267), this study and other reports did not observe sex differences in Treg function in in vitro suppressor assays (95, 267). Although FoxP3 is X-linked, it does not escape X-inactivation and thus is expressed at the same gene dosage in males and females (268). Therefore, there is no strong evidence to suggest that sex differences in Treg functioning account for the sex differences in adaptive immune responses.

1.3.3.4. Sex Differences in B Cells

There have been clear indications from both human and mouse vaccination studies that females exhibit more robust humoral responses than males (238, 269). Women have higher circulating levels of immunoglobulin than men in the steady state (270) and display more robust antibody responses to vaccination against a number of infectious agents (influenza, yellow fever, rubella, etc.) compared to men (269). Similarly, female mice have been shown to develop stronger and longer lasting antibody responses to vaccination compared to male mice to a variety of antigens (238). This greater humoral response is also thought to underlie why autoantibody driven disorders such as SLE predominate in women (271). While this greater humoral immune response
in females may be partly linked to the more robust Th cell responses, the female sex hormones estradiol and prolactin have direct actions on B cells by inhibiting B cell tolerance and promoting antibody production (271). Whether sex differences also exist in other B cell properties such as APC function, cytokine production, or B regulatory activity has not been studied.

1.3.3.5. Sex Differences in CD8+ T Cells

Naïve CD8+ T cells can be programmed to differentiate into cytotoxic effectors that produce IFNγ, also referred to as Tc1 cells (1). These cells are crucial for the control of infection with viruses and intracellular pathogens, and for the clearance of transformed host cells in the context of cancer (1). It has been observed that CD8+ T cells from female naïve mice produce higher levels of IFNγ compared to those from the male counterparts upon polyclonal stimulation (272). When immunized with a recombinant vaccinia virus expressing a human immunodeficiency virus (HIV)-1 viral epitope, female mice also exhibit enhanced CD8+ T cell activation in both the colon and the mucosal sites compared to male mice (273). Similarly in humans, women infected with HIV-1 have been shown to have higher levels of CD8+ T cell activation ex vivo compared to men (274). Thus, similar to CD4+ T cell responses, CD8+ T cell activation and effector function are more robust in females.

1.3.3.6. Sex Differences in Natural Killer (NK) and NKT Cells

NK cells are important innate immune cells; these cells along with NKT cells and γδ T cells form the first line of defense against invading pathogens via the rapid secretion of cytokines and cytolytic activity (1). NK cell activity is relevant to the sex differences in adaptive immune mechanisms, since these cells prime Th1 immune responses through IFN-γ secretion. Indeed, it
has been shown that female NK cells in the mouse spleen produce higher levels of IFNγ than male NK cells during the first 16 hours of infection with the D Variant Encephalomyocarditis Virus (275). Similarly, compared to healthy men, women exhibit a higher expression of the NK activation marker NKp46, correlating with enhanced anti-viral killing and cytotoxicity in vitro (276). Conversely, although SJL mice naturally have NK cells that are lower responders (277), adult SJL male mice have been shown to have a higher number of NK cells that produce IL-10 when stimulated with PMA and ionomycin compared to their female counterparts (278). Thus, it seems that NK cells in females maybe be more able to support Th1 responses and to elicit enhanced anti-microbial killing compared to those in males.

NKT cells also produce higher levels of IFN-γ in the serum of female mice compared with male mice upon in vivo challenge with the lipid ligand alpha-GalCer (279). In addition, healthy women have been shown to have higher numbers of NKT cells in the circulation compared to men (280), and the number of NKT may decrease at a more rapid rate with age in men versus women (281). Thus, the initial evidence seems to suggest that NKT cell effector function may also be more robust in females.

1.3.4. Effects of Sex Hormones on Immune Responses

Epidemiological studies in humans have shown that, with pubertal onset, there is a marked increase in both the incidence and the female preponderance of autoimmune immune diseases such as MS and SLE, suggesting that the hormonal changes that occur with puberty have a major influence on the immune system (252, 282). Much of what we know about sex hormones and immune regulation is derived from studies in rodents, since sex hormone levels can be manipulated by performing gonadectomy surgeries or by a treatment with exogenous hormones. These studies
have implicated a role for both androgens and estrogens in the regulation of the adaptive immune responses. Due to the copious nature of the literature on sex hormones, this section is limited to the effects of androgens and estrogens on adaptive immune responses and on central nervous system autoimmune disease.

1.3.4.1. Effects of Androgens on Adaptive Immunity

Androgen receptor (AR) ligands, testosterone and its metabolite dihydrotestosterone (DHT), have suppressive effects on adaptive immune responses. Studies of the effects of gonadectomy in mice revealed that sex differences in androgen levels account for the majority of the sex differences in Th immunity (240). For example, castration of males, but not ovariectomy of females, increases the severity of EAE that correlates with a shift towards a “feminine” profile of more robust T cell expansion and Th1 cytokine production (240, 257, 263, 283), and a more pro-inflammatory macrophage profile (239). On the other hand, treatment with testosterone or DHT inhibits EAE induction in female SJL mice (284) and androgen-treated myelin-reactive T cell lines are less able to transfer EAE (285). In both active and adoptive transfer EAE, the androgen-related protection correlates with a lower production of IFNγ and higher production of IL-10 by myelin-reactive CD4+ T cells (284, 285). The treatment of female T cells with androgens also reduces cell proliferation upon stimulation with anti-CD3 (286, 287).

In addition to these effects in mice, testosterone treatment has been shown to reduce the production of pro-inflammatory cytokines (TNFα and IL-1β) by human macrophages (288). In addition, in a small trial in relapsing-remitting MS, daily testosterone treatment (100 mg/day, Androgel) decreased delayed-type hypersensitivity responses to tetanus toxoid, reduced the frequency of CD4+ T cells in the blood, and lowered the production of IL-2 by PBMCs (289).
These immunosuppressive functions of androgens were shown to occur in mice mediated by effects on macrophages and T cells, both of which express AR (285). How testosterone may affect other Th-subsets such as the Th17 cell population has not been addressed.

1.3.4.2. Effects of Estrogens on Adaptive Immunity

How female sex hormones regulate Th immune responses is more controversial. Some studies suggest that the ovarian hormone estradiol is immunosuppressive and does not contribute to the robust Th immune responses in females (240, 290, 291), whereas other studies have suggested that the effect of estradiol on T cells is dose-dependent, being immunostimulatory at low concentrations and immunosuppressive at higher concentrations (7, 292, 293). On the other hand, estrogens have been shown to have clear stimulatory effects on antibody production by B cells (294–296).

The key evidence that demonstrated the immunosuppressive nature of estradiol is that treating mice with hormone pellets that gradually release estradiol protect against EAE development (240, 297, 298). Estradiol has been delivered to mice at doses that recapitulate diestrus (i.e. follicular phase in humans), metestrum (i.e. luteal phase in humans), or pregnancy levels; all of these doses have been shown to reduce EAE development in mice (297). These immune suppressive effects of estrogens on EAE were shown to be mediated through estrogen receptor alpha (298) and correlate with a reduced expansion and CNS infiltration of pro-inflammatory Th1 and Th17 cells (298, 299), an increased frequency of FoxP3+ Treg (300), and a shift of dendritic cells towards a more “tolerogenic” phenotype characterized by higher PD-L1 expression and IL-10 production (301, 302). In a pilot study in RRMS patients, oral therapy with estriol, a form of estrogen that circulates at high levels during pregnancy, was shown to decrease
TNFα and increase IL-5 production by circulating T cells, as well as elevate IL-10 production by monocytes in women (303).

Estradiol has also been shown to enhance T cell adaptive immunity when administered at doses that recapitulate the lowest end of the physiological range (10^{-11} to 10^{-9} M). For example, treatment of splenocytes in vitro with a low dose of estradiol enhances IFNγ mRNA expression and production upon ConA or LPS stimulation (292, 293), and this appears to occur through direct effects of estradiol on IFNγ promoter activity (293). Stimulatory effects of estrogens on dendritic cell maturation have also been noted: a low dose of estradiol increases the yields of bone marrow-derived dendritic cells and enhances the ability of anti-CD40 treated dendritic cells to prime responding T cells in culture (304). Finally, the delivery of low levels of estradiol to female mice that are first ovariectomized enhances the recall T cell responses to vaccination relative to levels in placebo control counterparts (305). Together, these findings suggest that estradiol enhances Th responses at low levels yet becomes immune-suppressive at higher levels.

To add to the complexity of the effects of estrogens on immune responses, both low and high doses (i.e. pregnancy levels) of estradiol promote humoral immune responses. Estradiol stimulates antibody production by PBMCs (294) and enhances circulating antibody levels in murine models of lupus (295, 296). Estradiol has been shown to stimulate humoral immunity by enhancing B cell survival through the anti-apoptotic molecule B-cell lymphoma 2 (Bcl-2) and by enhancing the threshold of B cell activation, thus protecting B cells from apoptosis (271). In addition, estradiol promotes high-affinity IgG antibodies through deaminase-mediated class switching and somatic hypermutation (271). The stimulatory effects of pregnancy-level estrogens on B cell autoantibody production are in sharp contrast with the profound suppressive effects of estrogens on T cell immunity, and are thought to be the reason why autoantibody disorders such as lupus become more
severe with pregnancy (306), while MS improves in the third trimester (307).

1.3.5. Candidate Molecules that Mediate Androgen Effects on Th1 Immune Responses

Given the strong link between androgen and the inhibition of Th cell activation, there has been interest in identifying genes that are sensitive to androgens and have modulatory effects on Th1 immune responses. To date, studies have indicated that androgens exert suppressive effects at three key control points in Th1 differentiation pathways: 1) IL-12 production by APCs (239), 2) JAK2-tyrosine kinase 2 (TYK2) and STAT4 phosphorylation downstream of IL-12 (308, 309), and 3) IFNγ gene expression downstream of the TCR and CD28 signals (283). Until recently, the molecular players involved in this androgen-dependent regulation were unknown.

Protein tyrosine phosphatase nonreceptor type 1 (PTPN1) was recently found to be expressed at lower levels in CD4+ T cells that are isolated from patients undergoing androgen deprivation therapy compared to those from untreated patients (308). PTPN1 operates within the second arm of this androgen-mediated IFNγ suppression by interfering with the phosphorylation of JAK2/TYK2 downstream of IL-12 receptor signaling (308). Another candidate molecule, PPARα, is the focus of the thesis and was identified to operate in this third arm of regulation of IFNγ production by male CD4+ T cells (283). Our group previously reported that PPARα-deficient (PPARα−/−) male mice develop more severe EAE compared to their wild type (PPARα+/+) male counterparts, correlating with elevated IFNγ production (283); this difference in Th1 cytokine production was not evident in the female mice. The sex difference in PPARα activity may be related to the finding that PPARα is expressed at higher levels in male versus female T cells (283). It was further shown that this expression in male T cells correlated with
circulating androgen levels in the male mice (283). However, whether PPARα is a mediator of androgen effects in suppressing Th1 immunity was not addressed.

1.4. Peroxisome Proliferator-activated Receptors (PPARs)

This section will overview what is known about the PPAR family of transcription factors: their tissue distribution and expression, the mechanism of trans-activation, and regulation of metabolic and inflammatory processes. Given that this thesis comprises an investigation of the immune functioning of PPARα and PPARγ, this section will mainly focus on these two PPAR family members.

1.4.1. The General Structure and Function of PPAR Family Members

PPARs are members of a nuclear hormone receptor superfamily that includes 9-cis-retinoic acid receptor (RXR), the vitamin D receptor, the thyroid hormone receptor, and other hormone-binding receptors (310). There are three family members of PPARs: PPARα, PPARδ/β, and PPARγ (311). The first PPAR to be cloned was PPARα (310). Independent reports of the cloning of PPARα and other PPAR isoforms in Xenopus laevis (312), rat (313), and human (314) emerged around the same time. PPARα, PPARβ/δ, and PPARγ each have unique tissue distributions. PPARα is most highly abundant in the liver, but is also expressed in the heart, kidney, muscle and intestine (315). PPARγ is most highly expressed in brown and white adipose tissue, but also at lower levels in pancreatic β-cells (316). The expression of PPARδ is ubiquitous, although it is most highly expressed in the muscle and heart (317). PPARs are also expressed at modest levels in immune cells. For instance, PPARα has been shown to be expressed in T cells, B cells, and macrophages (283, 318), PPARγ is expressed in macrophages
and T cells (319, 320), while PPARδ is expressed in most immune cell types including T cells (321).

Structural analyses of PPARs have revealed several functionally important domains: a DNA-binding domain that contains two zinc fingers, a hinge region, a trans-activation domain, as well as a ligand-binding domain that also serves as a site for dimerization and trans-activation (310, 312, 322) (Fig. 1.4A). The DNA-binding domain is highly conserved amongst PPARs since all three PPARs bind the same peroxisome proliferator response element (PPRE), which consists of direct repeats of an AGGTCA motif (312, 322) (Fig. 1.4B). On the other hand, the ligand-binding domain is less conserved amongst PPARs due to the fact that they bind a wide range of ligands (312, 322).

Natural ligands of PPARα include medium- and long-chain unsaturated fatty acids and compounds that are structurally similar including arachidonic acid and oleoylethanolamide (323, 324) (Table 1.1). A number of synthetic ligands have also been found to activate PPARα including members of the fibrate drug family (gemfibrozil, clofibrate, fenofibrate), Wy14643 and GW7647 (325) (Table 1.1). Physiological concentrations of fatty acids, especially polyunsaturated fatty acids, significantly activate PPARα activity to the same degree as the synthetic ligands (323). The fibrate drugs are currently used in the clinic for the treatment of metabolic syndrome to lower triglyceride levels (311). The endogenous ligands for PPARδ overlap with those of PPARα and include medium-chain unsaturated fatty acids. PPARδ can also be activated by synthetic ligands GW0742 and GW501516 (325) (Table 1.1). This shared ligand specificity between PPARα and PPARδ is also reflected by the fact that these nuclear receptors activate some of the same genes (e.g. acyl-coenzyme A synthetase) in tissues such as the heart (326).
Fig. 1.4 Basic structure of PPARs and mechanism of trans-activation. (A) This cartoon shows the major functional domains of PPARs. (B) shows the mechanism of trans-activation by PPARs. Upon binding to the ligand, PPARs heterodimerize with RXRs and together bind to the PPRE region in the target genes. These actions lead to active transcription of target genes. The blue triangle signifies the PPAR ligand while the red triangle indicates the RXR ligand. This figure has been adapted from Motojima et al. (322).
Table 1.1 List of major endogenous and synthetic ligands for PPARs. This is adapted from Straus et al. (325)

<table>
<thead>
<tr>
<th>PPARs</th>
<th>Endogenous Ligands</th>
<th>Synthetic Ligands</th>
</tr>
</thead>
</table>
| PPARα | Unsaturated fatty acids  
         | Saturated fatty acids  
         | Leukotriene B4  
         | 8-HETE  
         | Oleoylethanolamide | Fenofibrate  
         | Clofibrate  
         | Gemfibrozil  
         | Wy14643  
         | GW7647 |
| PPARγ | Nitrated unsaturated fatty acids  
         | (e.g. nitrated linoleic acid)  
         | Oxidized unsaturated fatty acids  
         | (e.g. 15d-PGJ₂) | Rosiglitazone  
         | Pioglitazone |
| PPARδ | Unsaturated fatty acids  
         | Saturated fatty acids | GW0742  
         | GW501516 |
On the other hand, PPARγ is activated by nitrated and oxidized fatty acids including the oxidized prostaglandin D2 metabolite 15-deoxy-12,14-prostaglandin J₂ (15d-PGJ₂) (327) (Table 1.1). Synthetic ligands of PPARγ include the insulin-sensitizing thiazolidinediones (rosiglitazone and pioglitazone) that are in the clinic for treating type II diabetes (325) (Table 1.1). While the two PPARγ isoforms play an important role in adipocyte differentiation and glucose metabolism, they have different levels of expression: PPARγ1 is expressed in nearly all cells including immune cells, while PPARγ2 with additional 30 amino acids is found mainly in adipose tissue (328).

1.4.2. Transactivation

PPARs function to either trans-activate or trans-repress gene expression. This section will overview the general mechanism of transactivation and will provide examples of metabolic genes that are targeted by PPARα and PPARγ in the liver and adipose tissue, respectively. It is known that in metabolic tissues, upon binding their ligands, PPARs heterodimerize with RXR and bind to peroxisome proliferator response elements (PPRE) to trans-activate gene expression (323) (Fig. 1.4B).

Since its discovery in the early 1990s, it has become clear that PPARα is the master regulator of hepatic lipid metabolism (310). The function of PPARα was first elucidated by treating mice with synthetic PPARα ligands; this induced a massive proliferation of peroxisomes in the liver of PPARα<sup>+/−</sup> but not PPARα<sup>−/−</sup> mice, leading to the namesake of this receptor family (329). Furthermore, PPARα<sup>−/−</sup> mice that were fasted for 24 hours displayed severe hypoglycemia, hypothermia, hypoketonemia and elevated plasma free fatty acids as a result of metabolic dysfunction in the liver (330). A series of subsequent studies elucidated that PPARα regulates
many metabolic processes including the transport and uptake of fatty acids, intracellular fatty acid binding and activation, peroxisomal β-oxidation, mitochondrial β-oxidation and ketogenesis, lipoprotein synthesis, and glycerol metabolism (311). One of the first genes identified to have a PPRE region is the acyl-coenzyme A (CoA) oxidase gene, which encodes a rate-limiting peroxisomal enzyme in long-chain fatty acid oxidation, thereby establishing the first link between PPARα and fatty acid catabolism (311, 331). Since the identification of the first target gene, subsequent gene expression microarray analyses revealed that between 50 and 600 genes are differentially regulated either in primary mouse hepatocytes or human hepatocytes in response to treatments with either dietary or synthetic PPARα ligands (332, 333). One example of these genes targeted by PPARα is cytosolic glycerol 3-phosphate dehydrogenase gene (cGPDH) that functions to induce hepatic gluconeogenesis (334). Another target gene is pyruvate dehydrogenase kinase isoform 4 (PDK4) that is up-regulated during starvation to inactivate pyruvate dehydrogenase complex, which in turn blocks the oxidation of pyruvate and turns on gluconeogenesis (335). Enhanced PDK4 expression may lead to increased free fatty acids that are available to activate PPARα, which further induces PDK4 expression (335).

On the other hand, the related PPAR family member PPARγ plays an indispensable role in adipose tissue by regulating adipocyte differentiation (325). Although mice that lack both copies of the PPARγ gene (PPARγ−/−) die in utero (336, 337), PPARγ+/− mice do survive and exhibit lower body weights and reduced amounts of adipose tissue as compared to PPARγ+/+ mice (336, 337). PPARγ regulates adipocyte differentiation by turning on a number of genes, the first of which identified was fatty acid-binding adipocyte protein 2 (338). Furthermore, PPARγ has been shown to regulate insulin sensitivity by controlling an additional subsets of genes including c-CBL-associating protein (339). A subsequent gene expression microarray study in rats that were treated with a PPARγ ligand revealed that close to 70 genes are differentially regulated in the
adipose tissue, liver and muscle; these genes regulate important processes in many metabolic pathways involving lipid, fatty acids, glucose and amino acids (340).

Given that the PPRE is shared amongst PPARs, it is not surprising that PPARα and PPARγ may target some of the same genes. For instance, lipoprotein lipase, which catalyzes the hydrolysis of triglycerides in lipoprotein particles into fatty acids and monoacylglycerol, is trans-activated by both PPARα and PPARγ (341). However, the transcriptional induction of the lipoprotein lipase gene by either PPARα-specific fibrates or PPARγ-specific thiazolidinediones shows a tissue-specific distribution that is reflective of where the target PPAR is expressed (i.e., PPARα in liver and PPARγ in adipose tissue) (341).

1.4.3. The Roles of PPARs in Immune Cells

PPARs are also expressed in certain immune cells and have generally been shown to exhibit anti-inflammatory functions (318, 342), which are thought to occur by a mechanism of trans-repression of target genes. Studies of the anti-inflammatory activities of PPARs followed those of the metabolic functioning of these nuclear receptors. It was first observed that activation of PPARγ with the ligands troglitazone and the prostaglandin 15d-PGJ2 led to the dose-dependent suppression of TNF, IL-6 and IL-1β in monocytes (343). The effects of 5d-PGJ2 on PPARγ in inhibiting inflammatory genes correlated with a reduction in the activity of AP-1, STAT1 and NF-κB (344). A similar anti-inflammatory function for PPARα was also demonstrated in the liver and in smooth muscle cells. For instance, treatment of human aortic smooth muscle cells with the PPARα ligand Wy14643 led to the suppression of IL-1-mediated IL-6 production by interfering with NF-κB activity (345).
More recently, it was demonstrated that PPARs may also regulate T cell function. For example, it has been shown that activating PPARγ in mouse splenocytes using ciglitazone promoted IL-4 production, but suppressed the production of IFN-γ and IL-2 (346). To further dissect the T cell-intrinsic function of PPARγ, T cell–specific PPARγ knockout mice (CD4-PPARγ knockout or KO) were generated by crossing CD4-Cre mice with mice carrying loxP sites that flank exons 1 and 2 of the PPARγ gene (320). Upon EAE induction, CD4-PPARγ KO mice developed worse symptoms as compared to the wild type counterparts (320). These exacerbated symptoms correlated with an elevated frequency of myelin-specific IL-17-producing, but not IFN-γ-producing CD4+ T cells in CD4-PPARγ KO mice (320). Pioglitazone activation of PPARγ in wild type, but not CD4-PPARγ KO, CD4+ T cells led to suppression of IL-17 production and Th17-associated genes (RORγT, IL-21, IL-23R, IL-17A and IL-17F) without affecting other Th pathways (320). This study highlighted a role for PPARγ in the suppression of Th17 differentiation.

On the other hand, PPARα has been shown to shift T helper responses from Th1 to Th2 as well as inhibit T cell proliferation. Lovett-Racke et al. showed that treating myelin basic protein (MBP) Ac1–11 TCR transgenic splenocytes with 5-200 μM of PPARα fibrate ligands elevated the production of IL-4 in response to MBP Ac1-11 stimulation (347). Similarly, in vivo treatment of mice with these fibrate drugs polarized the myelin-reactive responses towards Th2 and attenuated disease severity in the EAE model (347). This group further showed that the effects of gemfibrozil on EAE were directly mediated through IL-4, since the drug cannot attenuate EAE in IL-4−/− mice (348). However, not all fibrate drugs can enhance IL-4 production by T cells, suggesting that IL-4 induction may not be specific to PPARα functioning (346). This notion is supported by the finding that both gemfibrozil and Wy14643 still alleviate EAE symptoms in PPARα−/− mice (349). Given the off-target effects of fibrate drugs, it is difficult to discern the
specific role of PPARα in regulating T cell immune responses using PPARα ligands as the only tools.

As a result, the PPARα<sup>−/−</sup> mouse has been utilized as an additional experimental tool to study the role of this molecule in the control of inflammatory processes. PPARα<sup>−/−</sup> mice that lack PPARα protein expression were generated in 1995 by inserting a neomycin cassette into exon 8, which encodes a part of the ligand-binding domain (329). Using these mice, Jones <em>et al.</em> showed that PPARα<sup>−/−</sup> CD4<sup>+</sup> T cells express higher levels of IFN-γ and T-bet as compared to PPARα<sup>+/+</sup> CD3<sup>+</sup> T cells (350). Our group similarly observed that CD3<sup>+</sup> T cells purified from naïve PPARα<sup>−/−</sup> male mice produce elevated levels of IFN-γ and IL-2, and lower levels of IL-4 and IL-10 upon anti-CD3 and anti-CD28 stimulation; however these differences were observed only in males (283). Upon EAE induction, PPARα<sup>−/−</sup> male mice develop elevated Th1-mediated inflammation, correlating with the development of more severe symptoms in the acute phase of EAE compared to PPARα<sup>+/+</sup> male mice (283). These differences in EAE development were also only found in males (283). This study not only supported a role for PPARα in regulating Th immunity, but also highlighted a novel sex-specific role for PPARα in regulating these differences.

### 1.4.4. PPAR-dependent Trans-repression

Although it is well known that PPARα trans-activates gene expression through PPREs in the liver, the precise mechanism by which PPARα turns off the expression of inflammatory genes is just being elucidated. It was theorized early on that immune repression by PPARs may occur independently of direct PPRE-binding. This was supported in subsequent biochemical studies that PPARγ associates more strongly with co-repressor complex components such as NCoR and SMRT in solution than PPRE-containing DNA (351). A number of earlier studies that
examined the effects of PPAR ligands in immune cells and other cell types found that the ligands have suppressive effects on the binding of AP-1 and NF-κB to target sequences in inflammatory genes (343–345). Based on these studies, early models of PPAR-dependent trans-repression suggested that PPARs may suppress gene expression by either directly binding to AP-1 and NF-κB or interfering with co-activator association with these transcription factors (352). More recently, PPARs have been shown to trans-repress target inflammatory genes by interacting and stabilizing co-repressors at NF-κB-binding sites in these genes (353).

For example, Glass and colleagues recently dissected the mechanism of how PPARγ represses the transcription of the target gene inducible nitric oxide synthase (Inos) in LPS-activated macrophages (319) (Fig. 1.5). It was found that upon treatment with the ligand rosiglitazone, PPARγ is sumoylated and that this post-translational protein modification is associated with the recruitment of PPARγ to the Inos promoter, where it stabilizes a NCoR- and HDAC3-containing co-repressor complex (319). This process effectively prevented the ubiquitin-mediated degradation of the co-repressor complex by the proteasome that normally occurs upon LPS stimulation. A similar mechanism of repression was demonstrated for PPARγ in the suppression of the RORγT gene in Th17 cells (320). It was found that the treatment of Th17 cells with the PPARγ ligand pioglitazone prevented the removal of NCoR-like silencing mediator for retinoid or thyroid-hormone receptors (SMRT) from the RORγT gene, leading to the trans-repression of the RORγT gene (320).

Preliminary evidence suggests that PPARα may function via a similar mechanism of sumoylation-mediated trans-repression to inhibit the expression of target inflammatory genes. A recent study showed that treating mice with Wy14643 results in the suppression of the
Fig. 1.5 Trans-repression mechanism of PPARγ in LPS-stimulated macrophages. Upon treatment with its ligand rosiglitazone, PPARγ is sumoylated and this post-translational protein modification is associated with the recruitment of PPARγ to the Inos promoter, where it stabilizes a NCoR- and HDAC3-containing co-repressor complex. This process effectively prevents the ubiquitin-mediated degradation of the co-repressor complex by the proteases that normally occurs upon LPS stimulation. This is adapted from Pascual et al. (319).
cytochrome C enzyme oxysterol 7α-hydroxylase gene in the liver, and correlates with the sumoylation of PPARα in a mouse fibroblast cell line (354). Furthermore, it was shown that the inhibitory effects of fibrates on IL-6 production in human aortic smooth muscle cells occur via inhibition of RelA and c-jun activity (355). Consistent with this, our group reported that nuclear extracts prepared from activated male PPARα−/− T cells exhibit an enhanced binding of RelA and c-jun to target consensus elements in a DNA-binding assay compared to those from PPARα+/+ T cells (283). Given the importance of NF-κB and AP-1 in trans-activating Ifng downstream of TCR/CD28 stimulation, these data strongly suggest that PPARα may inhibit Th1 gene expression through a mechanism of trans-repression. Interestingly, Jones et al. proposed an alternative mechanism by which PPARα may regulate immune responses (318, 350). After previously demonstrating the detection of PPARα in the cytoplasm of resting CD4+ T cells using immunofluorescence, the authors showed that PPARα negatively regulates the phosphorylation of p38 in a ligand-independent manner to negatively regulate T-bet expression (318, 350). Given that p38 regulates IFN-γ gene expression (356), these data raised the possibility of an alternative pathway whereby PPARα inhibits MAPK activity to dampen Th1-mediated inflammation. Given that several aforementioned studies also demonstrated that PPARα ligand treatment induces IL-4 gene expression (347), these studies altogether highlight the need for a more detailed analysis of how PPARα may regulate Th1 immune responses and gene expression.

1.5. Thesis Rationale and Objectives

It has been established that mouse female CD4+ T cells are more Th1-biased, while mouse male CD4+ T cells produce higher levels of Th2-type cytokines (240). There is also some evidence that a similar Th bias in the immune response exists in humans (3, 4). Whether mice or humans exhibit a sex bias in Th17 responses has not been explored. A previous study by our
group suggested a possible role for PPARα in the sex-specific regulation of Th1 immune responses in mice (283); however, whether this nuclear receptor plays a similar role in human CD4+ T cells was not addressed. Furthermore, despite the finding that PPARα is more highly expressed in male CD4+ T cells and that this expression correlates with androgen levels in mice (283), how androgens regulate the expression of PPARα has not been investigated. Finally, while it is established that PPARα−/− male CD4+ T cells produce higher levels of IFN-γ compared to PPARα+/− male T cells, the molecular mechanisms of how this nuclear receptor functions to repress Th1 cytokine production was not examined.

In Chapter 2 of the thesis, we will investigate sex differences in Th1, Th2, and Th17 responses in both SJL mice and in human primary CD4+ T cells. Furthermore, we will investigate whether the effects of androgens in dampening Th1 cytokine production occur through PPARα and whether PPARα plays a similar sex-specific role in regulating Th1 immune responses in human CD4+ T cells. We plan to also explore the mechanism of how androgens regulate PPARα expression. Finally, in light of the recently described role for PPARγ in regulating Th17 immune responses (320), we will also investigate whether this molecule plays a role in regulating Th17 cytokine production by human CD4+ T cells.

The objective of Chapter 3 is to understand precisely how PPARα regulates the Th1 immunity. To this end, we will explore which gene is regulated by PPARα in T cells and will narrow in on the mechanism of this regulation. We also intend to explore whether PPARα plays a similar role in regulating IFN-γ production in IFN-γ-producing cells other than CD4+ T cells. Given that Th1 responses are critical in the clearance of intracellular pathogens, we also intend to investigate the utility of a novel PPARα-specific antagonist to boost IFN-γ production in mice during Listeria monocytogenes infection.
CHAPTER 2

Peroxisome Proliferator-Activated Receptor (PPAR)-α and -γ Regulate IFN-γ and IL-17A Production by Human T Cells in a Sex-specific Way

The data in this chapter were published in Proceedings of National Academy of Sciences:


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Dr. Shannon Dunn performed the experiments and/or analyzed the results for the experiments in Figures 2.3, 2.5, 2.6 (C-D, F-G), 2.7 (A), 2.8 (H-I), 2.9, and Table 2.2. Dr. Dorothy Rego recruited human subjects and conducted siRNA transfections in human studies. Marina Moshkova performed the gonadectomy surgeries in Figure 2.3 and contributed to culture setup in multiple figures. Rainer Akkermann contributed to PPARγ experiments in Figure 2.13. Dr. Hania Kebir performed the human cell co-culture experiments in Figure 2.7 (B-C). Dr. Frank Z. Stanczyk performed the sex hormone measurements for in Figure 2.9. HoangKim Nguyen contributed to preliminary human assay setup. Drs. Edward Engleman and Paula Colmenero provided help with preliminary human experiments. Drs. Andrzej Chruscinski, Robert Axtell, Christopher Lock, and May Han as well as the Canadian and Stanford Blood Services staff provided help with blood draws. Dr. Jennifer Gommerman provided the 5B6 TCR Tg mice of the SJL/J strain.
2.1. Abstract

Women develop certain autoimmune diseases more often than men. It has been hypothesized that this may relate to the development of more robust Th1 responses in women. To test whether women exhibit a Th1 bias, we isolated naïve CD4\(^+\) T cells from peripheral blood of healthy women and men and measured the proliferation and cytokine production by these cells in response to submaximal amounts of anti-CD3 and anti-CD28. We observed that CD4\(^+\) T cells from women produced higher levels of IFN-γ as well as tended to proliferate more than male CD4\(^+\) T cells. Intriguingly, male CD4\(^+\) T cells instead had a predilection toward IL-17A production. This sex dichotomy in Th cytokine production was found to be even more striking in the SJL mouse. Studies in mice and humans indicated that the sexual dimorphism in Th1 and Th17 cytokine production was dependent on the androgen status and the T-cell expression of PPAR\(\alpha\) and PPAR\(\gamma\). Androgens increased PPAR\(\alpha\) and decreased PPAR\(\gamma\) expression by human CD4\(^+\) T cells. PPAR\(\alpha\) siRNA-mediated knockdown had the effect of increasing IFN-γ by male CD4\(^+\) T cells, while transfection of CD4\(^+\) T cells with PPAR\(\gamma\) siRNAs increased IL-17A production uniquely by female T cells. Together, our observations indicate that human T cells exhibit a sex difference in the production of IFN-γ and IL-17A that may be driven by expressions of PPAR\(\alpha\) and PPAR\(\gamma\).
2.2. Introduction

For reasons that remain unclear, the incidence of multiple sclerosis (MS) is increasing in women (251) and, overall, women are three times more likely to be diagnosed with MS than men (251, 357). The same is true for a number of other autoimmune diseases (358). In MS, the higher female prevalence of disease emerges around puberty (252), implicating a role for sex hormones in disease risk. Results from both genome-wide association and pathological studies in MS suggest that Th1 and Th17 cells play key roles in the development of this disease (241, 359, 360). Furthermore, studies in the rodent model of MS, EAE, have provided clues that the higher female preponderance of disease is attributable to the development of more robust Th1 responses in females (255, 256, 258). Female myelin-specific T cells produce more IFN-γ and less Th2 cytokines than male T cells and are more encephalitogenic upon adoptive transfer (256, 258, 285). However, despite numerous attempts to explore this issue in humans (3, 361–364), conclusive evidence in support of a Th1 bias in women is lacking.

Castration of male mice worsens EAE (257), whereas ovariectomy does not have a major impact on disease (240, 290), suggesting that the higher androgen levels in males protects this sex from developing autoimmunity. Regarding possible mediators of these androgen effects, we previously identified that that the nuclear receptor PPARα is expressed at higher levels by male versus female T cells and appears to control T-cell proliferation and Th1 cytokine production uniquely in male mice (283). Furthermore, we observed that a PPARα ligand reduced the incidence of EAE in male, but not female mice, indicating a possible link between PPARα activity and protection from autoimmunity (283). The goal of this study was to explore whether a sex difference in Th cytokine production exists in humans and to investigate the potential role for PPARs in this regulation. Here, we report the observation that naïve CD4⁺ T cells from women
are intrinsically geared to proliferate and produce higher levels of IFN-γ and lower levels of IL-17A compared with male T cells. This sex difference in Th biology was also apparent in SJL mice. Furthermore, we found that the female Th1 bias was associated with a higher expression of PPARα by male activated T cells and higher expression of PPARγ1 by activated female T cells.

2.3. Materials and Methods

2.3.1. Human Subjects and Blood Collection

Blood samples (40–70 mL) were drawn from healthy men and women (aged 20–30 y) after obtaining informed consent with approval from the University Health Network Research Ethics Board (REB 09-0577-AE) or the Stanford University Institutional Review Board (protocol identification no. 97959). Ten milliliters of blood were collected in a clot tube for serum collection for hormone measurement. All participants were free of medications except for the use of oral contraceptives by ~50% of women. Other exclusion criteria include smoking or drug use, recent infection (<4 wk), history of cancer, thyroid problems, autoimmune, inflammatory, neurological or cardiovascular conditions, bleeding disorders, anemia, recent surgery, or obesity (body mass index, >30 kg/m²). Women provided a sample of blood during the self-reported follicular (self-reported) or luteal (21 d after the start of the last menstrual period, 28-d cycle) phase of the menstrual cycle. All blood draws were conducted in the morning hours with one man and one woman drawn per occasion.

2.3.2. Isolation and Culture of Human CD4+ T Cells
PBMCs were isolated from heparinized blood using Ficoll-Paque density gradient centrifugation (GE Healthcare). CD14+ monocytes and naïve (CD45RA+) or total CD4+ T cells were further fractionated using magnetic beads (Miltenyi). The purity of isolated CD4+ T cells was >96%, as determined using flow cytometry. Naïve CD4+ T cells (50,000/well) were cultured in serum-free X-VIVO-15 media (Lonza; supplemented with 2 mM L-glutamine and 100 U/mL penicillin) in 96-well plates with Dynabeads human T-activator anti-CD3 and anti-CD28. For hormone stimulation of CD4+ T cells, CD4+ T cells were cultured in complete RPMI (2) that contained 10% charcoal-stripped FCS (HyClone) with either 100 nM DHT (Wako; catalog no. 045-26071) or ethanol vehicle (0.01%). For T-cell and monocyte co-culture experiments (Fig. S4A), CD4+ T cells (50,000) were added, along with anti-CD3 (Clone OKT) anti-CD3 (eBioscience), to monocytes (50,000) that had been pre-activated with peptidoglycan (Fluka; 77145; 1 μg/mL) for 5 h. Memory CD4+CD45RO+ Th17 cells were expanded according to a previously published protocol (360, 365).

2.3.3. Hormone Assays

Testosterone and estradiol were quantified in serum by previously described radioimmunoassay methods (366, 367). Before the radioimmunoassay methods, steroids are extracted with hexane:ethyl acetate (3:2), and testosterone and estradiol are separated by Celite column partition chromatography (John Morris Scientific). Appropriate tritiated internal standards are added to each sample before the extraction step to determine and correct for procedural losses. A highly specific antiserum is used in conjunction with an iodinated radio-ligand in each RIA. The assay sensitivities are 1.5 ng/dL and 2 pg/mL for testosterone and estradiol, respectively. The interassay CVs for testosterone 11%, 9%, and 10% at concentrations
of 14.9 ng/dL, 32.8 ng/dL, and 105 ng/dL, respectively. For estradiol, the interassay CVs are 12%, 11%, and 10% at concentrations of 22 pg/mL, 66 pg/mL, and 183 pg/mL, respectively.

2.3.4. Mice and Surgeries

The 129S1/SvIm/J (PPARα+/+) and 129S4/SvJae-Pparatm1Gonz/J (PPARα−/−) mice were from The Jackson Laboratory. The 5B6 TCR Tg mice of the SJL/J strain were obtained from Jennifer Gommerman (University of Toronto, Toronto, Canada) through an agreement with Pfizer. Castration and sham surgeries and pellet implantations were conducted on 4–5-wk-old mice as described previously (283). Thirty-day release pellets of DHT (5 mg) or placebo were purchased from Innovative Research of America. Animal protocols were approved by the University Health Network in accordance with the guidelines of the Canadian Council on Animal Care.

2.3.5. EAE Induction, Recall Assays, and Treg Staining

Mice (8–10 wk) were vaccinated (s.c.) with an emulsion (volume ratio, 1:1) of 100 μg of PLP p139-151 (2 mg/mL in 1× PBS) mixed with CFA (4 mg/mL heat-killed Mycobacterium tuberculosis H37Ra; Difco Laboratories) as described (368). PLP p139-151 was synthesized by the Stanford Pan Facility (Stanford, CA). Spleens and lymph nodes were harvested from mice 8 d postimmunization and were dissociated into a single-cell suspension. The recall proliferation and cytokine production by cultured cells in response to PLP p139-151 were measured (368). CD4+FoxP3+CD25+ Treg cells were stained using the mouse regulatory T-cell staining kit (88-8111-40; eBioscience). Mononuclear cells were isolated from spinal cords of EAE mice as described previously (369).
2.3.6. Proliferation and Cytokine Measurements

Proliferation was measured using a $[^3]$H-thymidine incorporation assay (368). The levels of mouse and human cytokines in culture supernatants were measured using Ready-SET-Go mouse or human ELISA kits (eBioscience). Flow cytometric analysis of intracellular cytokine staining on mouse central nervous system mononuclear cells was conducted as described previously (369). For human intracellular cytokine staining, cells were stimulated with 10 ng/mL PMA (Sigma) and 750 nM ionomycin (Calbiochem) for 4 h in the presence of GolgiStop (BD Pharmingen) before staining with Cy7-APC-CD4 and LIVE/DEAD fixable aqua (Invitrogen). Cells were then fixed and permeabilized (BD reagents and protocols) before staining for human IL-4 (PE; clone 8D4-8; BD Pharmingen), human IFN-γ (APC; clone B27; BD Pharmingen), or human IL-17A (PE; eBioscience). Data were collected using the LSR II (BD) and were analyzed using Flowjo software.

2.3.7. In Vitro Mouse T Cell Culture

CD4$^+$ T cells (negative-selection) or CD11c$^+$ (positive-selection) cells were isolated from secondary lymphoid organs using magnetic beads (Miltenyi). For co-culture experiments, naïve 5B6 TCR Tg cells (50,000) were cultured with half (25,000) the number of irradiated CD11c$^+$ cells in round-bottomed, 96-well plates with indicated concentrations of PLP p139-151. Alternatively, CD4$^+$ T cells (150,000/well) were cultured in 96-well flat-bottomed plates precoated with various quantities of anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51) (eBioscience) in complete RPMI that contained either 10% (vol/vol) FCS or 1% syngeneic sera. For Th17-skewing experiments, complete media was further supplemented with 3 ng/mL TGF-β, 30 ng/mL IL-6, and 10 μg/ml of anti-IFN-γ (clone XMG1.2; eBioscience). For PPAR ligand
experiments, T cells were first preincubated with fenofibrate (Sigma; F6020; 1–10 μM), rosiglitazone (R2408; 1 μM), or DMSO vehicle (0.1%) in X-VIVO-15 (human) or X-VIVO-20 (mouse) media (Lonza) overnight before TCR and CD28 co-stimulation.

2.3.8. Real-Time PCR Detection of Human and Murine PPARα and PPARγ mRNAs

Total RNA was isolated using Absolutely RNA miniprep kit (Agilent Technologies). Transcript levels were measured either from (i) RNA using TaqMan one-step RT-PCR master mix (Applied Biosystems; 4309169) and primer probe sets; or (ii) cDNA that was reverse-transcribed from total RNA (Superscript II; Invitrogen) (2) before being amplified using QuantiTect SYBR Green PCR reagents and sequence-specific primers (3) (Table 2.1). For TaqMan, the following primer/probes were used: mouse PPARα (Mm00440939_m1), mouse PPARγ (Mm01184322_m1), human PPARα (Hs00947538_m1), mouse β-actin (Mm00607939_s1), and human 18S rRNA (Hs03003631_g1) with the following cycling conditions: 48 °C for 30 min, 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 seconds and 60 °C for 60 s. For the SYBR method, the following cycling conditions were used: 95 °C for 15 min, followed by 50 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s. All reactions were run using the Roche LightCycler480 real-time PCR machine.

2.3.9. ChIP of PPARα promoter and Ifng (CNS-6 and CNS-22 Regions)

ChIP was performed to investigate the recruitment of AR to the human PPARα promoter. PBMCs (50 × 10^6) were obtained from each of three female donors, and these cells were stimulated with Dynabeads human T-activator (half of recommended strength) in the presence or absence of 100 nM DHT or vehicle for 16–18 h. Cells were fixed, and then ChIP was performed
immediately according to the directions of the manufacturer (Cell Signaling; SimpleChIP Enzymatic Chromatin IP kit; no. 9002S) with the addition of a manual homogenization step (10 gentle, manual strokes using a tissue grinder) at the time of lysis in buffer A. The following rabbit antibodies were used for IP: anti-AR (1:30 dilution; Millipore; PG-21, 06-680) or an anti-IgG negative control antibody raised in rabbit (1:500; Cell Signaling; no. 2729). DNA template was amplified using real-time PCR (cycling conditions as described above) or traditional PCR using the following conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. PCR primer sequences are detailed in Table 2.1. In a second experiment, we investigated the abundance of acetylated histone-H4 and RelA at the CNS-6 and CNS-22 sites of the IFNG. T cells were isolated from five men for this purpose and were transfected with either PPARα siRNAs or nontargeting control siRNAs before stimulation with Dynabeads human T-activator for 8 h or 24 h. Cells from these donors were pooled (15–20 × 10⁶ T cells total) after the fixation step. Anti- acetylated H4 (1:25; Cell Signaling; no. 2594S), anti-RelA (p65 NFκB) (1:100; Cell Signaling; no. 8242S), and a negative control antibody raised in rabbit were used for IP. PCR was conducted similarly as above with the same cycling conditions as above, except with an annealing temperature set to 53 °C.
Table 2.1. Human primer sequences used for SYBR real-time PCR.

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Sequences (5’-3’)</th>
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<tr>
<td>Human PPARα FWD</td>
<td>TCGACTCAAGCTGGTG</td>
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<tr>
<td>Human PPARα REV</td>
<td>TTCCTGAGAGGATGACCC</td>
</tr>
<tr>
<td>Human PPARγ1 FWD</td>
<td>CTTGGTGCGGCTCGAG</td>
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<tr>
<td>Human PPARγ1 REV</td>
<td>CATTACGGAGAGATCCACGGA</td>
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<tr>
<td>Human β-actin FWD</td>
<td>CCTCGCCTTTGCGGA</td>
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<tr>
<td>Human β-actin REV</td>
<td>TGGTGCTGGGCG</td>
</tr>
<tr>
<td>Human CNS-5 FWD</td>
<td>CTAATAGCAGATTTTCACTGC</td>
</tr>
<tr>
<td>Human CNS-5 REV</td>
<td>CGTTTTCAGCTGTGTTCCACAA</td>
</tr>
<tr>
<td>Human CNS-22 FWD</td>
<td>GCGATTTCTTTTTCTCAGGGTG</td>
</tr>
<tr>
<td>Human CNS-22 REV</td>
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</tr>
<tr>
<td>Human PPARα promoter FWD</td>
<td>CTGACGCTACGCGGTGTC</td>
</tr>
<tr>
<td>Human PPARα promoter REV</td>
<td>CTCAGCGGCTCCACCTA</td>
</tr>
<tr>
<td>Human IFNγ FWD</td>
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</tr>
<tr>
<td>Human IFNγ REV</td>
<td>GCGTTGGACATTCAAGTCAG</td>
</tr>
<tr>
<td>Human IL-17A FWD</td>
<td>CCCCTAGACTGAGGCTTCTC</td>
</tr>
<tr>
<td>Human IL-17A REV</td>
<td>TCAGCTCCTTTCTGGGTTGT</td>
</tr>
</tbody>
</table>

FWD, forward; REV, reverse.
2.3.10. siRNA Transfection

Transfection of human (Human T Cell Nucleofector Kit) or mouse (Mouse T Cell Nucleofector Kit) CD4+ T cells was conducted according to product directions and the Nucleofector device (Amaxa). Cells were transfected with ON-TARGETplus SMART pool siRNAs (Thermo Scientific) designed against: human PPARγ (L-003436-00-0005), human PPARα (L-003434-00-0005), mouse PPARα (L-040740-01), or mouse PPARγ (L-040712-00), along with a GFP expression vector provided in the kits. As a control, CD4+ T cells were transfected with nontargeting siRNAs (ON-TARGETplus control pool; D-001810-10-05). Human CD4+ T cells were recovered from the electroporation vial and then allowed to rest overnight in Lymphocyte Growth Medium (LGM) (Lonza) supplemented with 10% FCS (HyClone), penicillin (100 U/mL), and 2 mM glutamine (both from Gibco). Mouse CD4+ T cells were recovered from the electroporation vial and then allowed to rest overnight in supplemented Mouse T Cell Nucleofector Medium (containing 5% FCS (HyClone), 2 mM glutamine (Gibco), and Medium A and B). The next day, some T cells were reserved for flow cytometric analysis of GFP expression and for PCR measurement of PPAR mRNA knockdown, whereas the rest were plated with stimuli for cytokine measurement. The level of PPAR mRNA knockdown was determined using real-time PCR or traditional RT-PCR.

2.3.11. Statistical Analyses

Data are presented as means ± SEM. When data were parametric (kurtosis and skewness of <2) and group variances were homogenous (Bartlett homogeneity test), a one-way ANOVA and Tukey post hoc test (for more than two groups) or a t test (n = 2 groups) were used to detect between-group differences. When data were nonparametric, ranks were compared among groups
using a Kruskal—Wallis test and non-parametric test for multiple comparisons (for more than two groups) or a Mann–Whitney U test (n = 2 groups). For paired human data, a paired t test was used to compare differences between males and females. A value of $P \leq 0.05$ was considered significant.

2.4. Results

2.4.1. Sex Bias in CD4+ T-Cell Expansion and Th Cytokine Production.

SJL is one of the few mouse strains that exhibits a female bias in EAE development (255, 256). We, thus, used this strain as a model system to explore the basis of the sex differences in Th cytokine production. We immunized male and female mice of this strain with PLP p139-151 in CFA and examined the recall proliferation and cytokine responses of splenic and draining lymph node cells to PLP p139-151 *ex vivo*. We detected PLP p139-151-reactive cells primarily in the spleens of males and in both the spleen and draining lymph nodes of females at 8 d post-immunization, indicating possible sex differences in the trafficking of these cells *in vivo* (Fig. 2.1A and B). However, when considered together, splenocytes and lymph node cells cultured from female mice clearly expanded more than male T cells (Fig. 2.1A and B). Treg cells (CD25+FoxP3+CD4+) also tended to expand more in female mice (Fig. 2.2A).

Consistent with previous reports (255, 256), we observed that female SJL PLP p139-151-reactive T cells produced higher levels of IFN-γ compared with male cells (Fig. 2.1A and B). However, instead of exhibiting a Th2 bias (no IL-4 was detected), male splenic PLP p139-151-reactive T cells tended to produce more IL-17A than female cells (Fig. 2.1B). We also followed several male and female mice to the acute stage of EAE and examined the cytokine production
Fig. 2.1. PLP p139-151-reactive CD4+ from female SJL mice produce more IFNγ and proliferate more than male CD4+ T cells. (A and B) Male and female SJL mice (8 wk) were immunized with PLP p139-151 in CFA. Eight days later, cells from draining lymph nodes (A) and spleens (B) were harvested, were pooled within groups, and then cultured with PLP p139-151. (C) CD4+ T cells were isolated from nonimmunized male and female mice and were activated with plate-bound anti-CD3 and anti-CD28. Shown are the proliferation and cytokine responses to the various stimuli. In A and B, proliferation values are expressed as a stimulation (Stim.) index: \(^3\)H-thymidine incorporation in peptide-stimulated wells divided by the media control wells. In C, proliferation values are expressed as cpm. In all cases, values are means ± SEM of triplicate cultures. *Significant difference (P ≤ 0.05) from males, as determined using a t test (two-tailed). Results are representative of three experiments.
Fig. 2.2. Frequency of Tregs in the periphery and frequencies of CNS-infiltrating Th1 and Th17 cells during EAE. Female and male SJL/J mice (n = 4/group) were immunized with PLP p139-151 in CFA. (A and B) Eight days later, individual spleens were harvested from mice, dissociated into a single-cell suspension, and stained using fluorochrome-conjugated antibodies (CD4-FITC, CD25-APC, and FoxP3-PE). (A) Mean ± SEM number of FoxP3+CD25+CD4+ cells in unimmunized (naïve) and immunized mice. Results are of individual mice from one experiment. (B) Frequency of FoxP3+CD25+ cells in the live CD4+ gate. *Significantly different (P ≤ 0.05), as determined by a one-way ANOVA and Tukey post hoc test, from the naive mice of the same genotype. (C) Several mice immunized with PLP p139-151 and CFA were followed until 6 d after the onset of clinical signs. At this time, male and female mice with synchronized disease (EAE score 3) were perfused with PBS, and mononuclear cells were isolated from the spinal cord. Mononuclear cells were pooled within sex and were stimulated with PMA/ionomycin in the presence of GolgiStop for 4 h. These cells were then stained for CD4-FITC, IFNγ-PE, and IL-17-APC. Shown are the frequencies of IFNγ- and IL-17A-producing cells in the live CD4+ T gate. Results in C are representative of two independent experiments.
by spinal cord-infiltrating CD4+ cells. We observed a higher frequency CD4+ cells that produced IFN-γ (mainly IFNγ- and IL-17A-coproducing CD4+ cells) in the central nervous system of female as compared with male mice (Fig. 2.1C). Thus, these results indicate that Th cells from female SJL mice have a higher propensity to expand in general and produce more IFN-γ and less IL-17A than male T cells. The discrepancy between our results and past studies that indicated a Th2 bias in male SJL mice may relate to the fact that: (i) past studies were conducted before the discovery of Th17 cells; and (ii) the levels of IL-4 reported in these studies were measured in cultures of whole lymph nodes or spleens (255) or by IL-2 expanded T-cell lines (256).

2.4.2. Androgen Dependence of Sex Difference in Th Cytokine Production

Castration of male mice worsens EAE in males (257), whereas ovarietomy does not have a major impact on this disease in female mice (240, 290). These observations suggest that it is the higher level of androgens in males that is protecting this sex from EAE development. We, thus, explored the effect of castration on Th proliferation and cytokine production. Male SJL mice (4–5wk of age) were castrated or provided a sham surgery. When these mice reached adulthood, they were immunized with PLP p139-151 and CFA, and the recall responses to antigen were measured as before. We found that castration was associated with a “feminization” of the Th profile in male mice, accompanied by an enhanced expansion of PLP p139-151-reactive T cells over sham-operated counterparts (Fig. 2.3A) and a selective outgrowth of IFNγ-versus IL-17A-producing cells (Fig. 2.3 B versus C). Castrated mice that were followed to the development of EAE symptoms were also found to exhibit a higher ratio of IFNγ- to IL-17-producing T cells in the central nervous system as compared with sham mice (Fig. 2.3D). These observations suggest that androgens shift Th cytokines from Th1 to Th17.
Fig. 2.3. Sex difference in cytokine production associates with hormone status in male mice. (A–D) Male SJL/J mice (5 wk old; n = 5/group) were subjected to castration or sham surgery. Three weeks later, mice were immunized with PLP p139-151 in CFA. (A–C) Eight days later, the spleens were collected from mice and were pooled, and cells were stimulated with PLP p139-151. *Significantly different (P ≤ 0.05) from sham using a t test (two-tailed). (D) Mononuclear cells were isolated from the spinal cords of male sham and castrated mice during the acute phase of EAE. Mice were all at score 3 and were sick for an equivalent period. (E–G) WT or PPARα−/− SV.129 female mice (5 wk of age; n = 3/group) were implanted with pellet containing DHT (5 mg) or carrier-binder (placebo). Three weeks later, CD4+ T cells were pooled and then cultured with anti-CD3 and anti-CD28 in RPMI containing with 1% autologous serum. (E) Relative abundance of PPARα mRNA (relative to β-actin) in freshly isolated CD4+ T cells. (F and G) Production of IFNγ (F) and IL-17A (G) in T-cell cultures. *Significantly different (P ≤ 0.05) from placebo counterpart, as determined using a one-way ANOVA and Tukey post hoc test. Values are means ± SEM of measurements in triplicate cultures. Results are representative of two to three experiments.
2.4.3. Cellular Basis of T-Cell Expansion and Cytokine Production

To investigate the cellular basis of the sex difference in Th proliferation and cytokine production, we took advantage of the fact that female SJL T cells do not react against the male Y-linked histocompatibility (HY) antigen (258) and conducted co-culture experiments of male or female APCs with naïve male or female PLP p139-151-specific TCR Tg T cells. We found that cultures that contained female APCs and female naïve CD4+ T cells exhibited the highest proliferation to PLP p139-151 (Fig. 2.4A). On the other hand, the production of Th cytokines more closely mapped with the sex of the T cells in these assays (Fig. 2.4A). Cultures that contained female T cells produced higher levels of IFN-γ and IL-12p40, whereas the cultures that contained male T cells instead exhibited higher levels of IL-17A at the lowest PLP p139-151 concentration tested (Fig. 2.4A). Again, the levels of IL-4 and IL-10 were low and not found to be different between groups (Fig. 2.4A). Similar results were observed when we cultured purified CD4+ T cells in isolation with anti-CD3 and anti-CD28 (Fig. 2.1C). Furthermore, the Th17 bias by male CD4+ T cells was evident regardless of the tissue source of cells (Fig. 2.4B).

Collectively, these results suggest that two mechanisms are operating to regulate the sex differences in Th proliferation and cytokine production: (i) a mechanism that regulates the expansion of T cells that is determined by the sex of the APCs and the T cell; and (ii) a mechanism that regulates Th cytokine production that is determined primarily by the sex of the CD4+ T cell. Herein, we focused on the T-cell-intrinsic mechanisms that regulate sex differences in the production of IFN-γ and IL-17A.

2.4.4. PPARα Is an Androgen-Sensitive Regulator of Th Cytokine Production
Fig. 2.4. Sex of T cell determines Th cytokine production. (A) Spleens were harvested from male or female SJL/J mice (8 wk old; n = 5/group; pooled) and were dissociated into a single-cell suspension. CD11c<sup>+</sup> cells were isolated, irradiated, and then cocultured with naïve 5B6 TCR transgenic CD4<sup>+</sup> T cells in the presence of PLP p139-151 at various concentrations. Values are means ± SEM picograms per milliliter of cytokine levels measured in triplicate cultures. Results are representative of two independent experiments. *Significantly different from cultures that contained male T cells (P ≤ 0.05); §cultures that contained female APC and female T cells were significantly different from cultures that contained male APCs and male T cells (P ≤ 0.05). These data were analyzed using a one-way ANOVA and Tukey post hoc test. (B) CD4<sup>+</sup> T cells were isolated from age-matched male and female SJL/J mice (n = 5/group; pooled) from the indicated secondary lymphoid organs and were stimulated in vitro with anti-CD3 and anti-CD28 (2 μg/mL). The productions of IL-17A and IFNγ were measured by ELISA. Shown is the ratio of production of IL-17A to IFNγ in male and female CD4<sup>+</sup>T-cell cultures.
We found previously that PPARα has a male-specific role in dampening Th1 cytokine production in mice (20). To address whether PPARα is an intermediary of androgen effects in shifting the Th cytokine production from Th1 → Th17, we “re-programmed” female WT or PPARα−/− mouse T cells in vivo with the AR agonist 5α-DHT. Three weeks later, we isolated splenic CD4+ T cells from mice and, as before, stimulated them in vitro with anti-CD3 and anti-CD28. We observed that DHT-treated WT T cells displayed higher expression of PPARα mRNAs compared with placebo control cells (Fig. 2.3E). This was associated with lowered IFN-γ and higher IL-17A production by WT T cells (Fig. 2.3F and G). Interestingly, the decrease in the potential to make IFN-γ in response to androgen treatment did not occur in PPARα−/− T cells, whereas the increased potential to make IL-17A did (Fig. 2.3 F and G). These results indicate that PPARα may be a molecular intermediary of androgen effects on IFNγ, but not IL-17A.

2.4.5. Human Female CD4+ T Cells are Th1-Prone

We next set out to investigate whether human CD4+ T cells exhibit a sex difference in Th cytokine production. The variability associated with past reports of sex differences in human Th cytokine production (3, 361–364) may be attributed to the fact that cytokine production was assayed in whole blood or in cultures of PBMCs, which are comprised of mixtures of cells that are present at varying frequencies in individuals. We, thus, investigated cytokine production by naive CD4+CD45R+ T cells in response to anti-CD3 and anti-CD28 stimulation. This stimulus was titrated to produce cytokine levels and proliferative rates that were within the dynamic range of these assays (Fig. 2.5A). Healthy men and women were recruited in a pair-wise fashion with one woman and one man donating a blood sample each day (n = 25 pairs) to control for the day-to-day variability. Furthermore, to further reduce
Fig. 2.5. Preliminary human T-cell experiments. Naïve CD4+ T cells were isolated from peripheral blood of men and women (n = 4–5/group). Women were at the follicular (A and B) or luteal (C) phase of their cycle at the time of blood draw. CD4+ T cells were then cultured in X-VIVO-15 media with various concentrations of anti-CD3- and anti-CD28-coated Dynabeads that were prepared according to the instructions of the manufacturer. Proliferation was measured by [3H]thymidine incorporation assay (cpm). IFN-γ was measured in culture supernatants by ELISA. The arrows in A indicate the concentration of Dynabeads that were chosen for assays of proliferation and cytokine measurement. In B and C, * indicates a significant difference between men and women using a t test (two-tailed, P ≤ 0.05). n.s., not significant.
noise in the system, we controlled for the time of the menstrual cycle in women (follicular phase) (Fig. 2.5B and C). Consistent with our findings in SJL mice, we observed that naïve CD4+ T cells of women secreted significantly higher levels of IFN-γ compared with men in response to anti-CD3 and anti-CD28 stimulation (Fig. 2.6A). In fact, 20 of the 25 women sampled exhibited higher production of IFN-γ than their male pair (Fig. 2.6F).

In females, we also detected a higher frequency of IFNγ-producing T cells and significantly higher T-cell expression of IFN-γ mRNAs than male counterparts (Fig. 2.6B and C and Table 2.2). A trend for higher proliferative rates by female T cells was also observed (Table 2.2). Although the level of IL-17A detected in these cultures under Th0 conditions was low and not different between the sexes (Fig. 2.6D), when IL-17A production was examined in individual pairs, we observed a tendency for higher production of this cytokine in cultures of male versus female T cells (Fig. 2.6G). A trend for higher IL-17A mRNA expression by male T cells was also observed (Fig. 2.6E). As in the SJL mouse, the productions of Th2 cytokines were low and not found to be different between the sexes (Table 2.2). To further investigate the male Th17 bias, we explored the production of IL-17A in two T culture systems associated with enhanced production of this cytokine: (i) naïve CD4+ T cells activated with anti-CD3 plus peptidoglycan-matured autologous monocytes (21); and (ii) memory CD4+ T cells that were cultured with anti-CD3 plus autologous monocytes before expansion with IL-23 (6). In both of these systems, we observed a higher propensity for IL-17A production when donor cells were derived from men compared with women (Fig. 2.7). Thus, the presence of male monocytes amplified the sex difference in IL-17A that was specified by TCR- and CD28-elicited signals. Altogether, these findings in human T cells align with our results in the murine system and suggest a Th1 bias in females and a Th17 bias in males.
Fig. 2.6. CD4+ T cells from women are more prone to produce IFNγ and less IL-17A than male T cells. Naïve (CD45RA+) CD4+ T cells were isolated from peripheral blood of healthy men and women (n = 25/group) and were cultured in X-VIVO-15 serum-free media in the presence of anti-CD3- and anti-CD28-coated beads. Supernatants were collected at 72 h post-stimulation. T cells were expanded in IL-2 containing X-VIVO-15 media for an additional 7 d before stimulation PMA/ionomycin. (A and D) Absolute IFNγ and IL-17A levels detected in culture supernatants. (B and E) IFNγ and IL-17A mRNAs (expressed relative to β-actin). (C) Frequency of IFNγ-producing T cells. Values are means ± SEM measurements from individual human subjects. (F and G) Difference in IFNγ and IL-17A production (pg/mL) in individual pairs (female minus male value). *Significantly different (P ≤ 0.05) from male, as determined using a paired t test (two-tailed).
Table 2.2. Proliferation and cytokine production by human naïve CD4\(^+\) T cells

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<th></th>
<th>Men</th>
<th>Women</th>
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<td></td>
<td>13,015 (1,098)</td>
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<td><strong>Cytokine Production</strong></td>
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<td>4,874 (671)</td>
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<tr>
<td>IL-2</td>
<td>1,232 (111)</td>
<td>1,127 (178)</td>
<td>0.47</td>
</tr>
<tr>
<td>TNF</td>
<td>751 (121)</td>
<td>722 (79)</td>
<td>0.80</td>
</tr>
<tr>
<td>IL-10</td>
<td>102 (20)</td>
<td>107 (14)</td>
<td>0.73</td>
</tr>
<tr>
<td>IL-17A</td>
<td>55 (23)</td>
<td>35 (11)</td>
<td>0.37</td>
</tr>
<tr>
<td>IL-4</td>
<td>11 (4)</td>
<td>14 (5)</td>
<td>0.80</td>
</tr>
<tr>
<td><strong>Frequency of CD4(^+) T cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN(\gamma)^+IL-17A^-</td>
<td>22.3 (2.8)</td>
<td>31.3 (2.8)</td>
<td>0.0066*</td>
</tr>
<tr>
<td>IFN(\gamma)^+IL-17A^+</td>
<td>0.8 (0.1)</td>
<td>1.1 (0.3)</td>
<td>0.37</td>
</tr>
<tr>
<td>IFN(\gamma^-IL-17A^+</td>
<td>4.4 (0.7)</td>
<td>4.1 (0.7)</td>
<td>0.71</td>
</tr>
<tr>
<td>IFN(\gamma)^+IL-4^-</td>
<td>19.4 (2.7)</td>
<td>25.3 (2.9)</td>
<td>0.019*</td>
</tr>
<tr>
<td>IFN(\gamma)^+IL-4^+</td>
<td>3.7 (0.6)</td>
<td>4.7 (0.8)</td>
<td>0.20</td>
</tr>
<tr>
<td>IFN(\gamma^-IL-4^+</td>
<td>8.7 (1.2)</td>
<td>8.4 (1.1)</td>
<td>0.88</td>
</tr>
<tr>
<td>IFN(\gamma) (total)</td>
<td>23.1 (2.8)</td>
<td>32.5 (3.0)</td>
<td>0.005*</td>
</tr>
<tr>
<td>IL17A (total)</td>
<td>5.3 (0.7)</td>
<td>5.2 (0.8)</td>
<td>0.94</td>
</tr>
<tr>
<td>IL-4 (total)</td>
<td>12.4 (1.5)</td>
<td>13.2 (1.5)</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Values of proliferation (counts per minute or cpm), cytokine production (pg/ml) and frequency of CD4\(^+\) T cells (%) are means (SE) (N=25 for proliferation and cytokine production; N=18 for intracellular cytokine analysis).

*Significantly different between men and women using a paired t test (two-tailed; \( P \leq 0.05 \)).
Fig. 2.7. Male T cells are more Th17-prone when co-cultured with monocytes. (A) Human naïve CD4+ T cells and CD11b+ cells were isolated from peripheral blood of healthy men and women (n = 10/group). These cells were then co-cultured together in X-VIVO-15 media that also contained peptidoglycan (1 μg/mL) and Dynabeads (1 bead:10 T cells). The concentration of IL-17A was measured in these cultures at 72 h of culture. *Significant (P ≤ 0.05) difference, as determined by t test (two-tailed). (B) These cells are memory CD4+ T cells that were isolated from the blood of healthy men and women according to the following protocol. T cells were cultured with autologous monocytes at a ratio of 2:1 and stimulated with anti-CD3 (2.5 μg/mL) in the presence of rhIL-23 (10 ng/mL) and neutralizing antibodies against IFNγ and against IL-4 (5 μg/mL). Cells were harvested on day 6 and analyzed by flow cytometry for IL-17 and IFNγ determination. **Significant difference (P ≤ 0.01), as determined using a t test (two-tailed).
2.4.6. Regulation of Human PPARα by Androgens and TCR Stimulation

Next, we focused on a possible role for PPARα in the Th1 bias in women. First, we investigated whether PPARα exhibits sexual dimorphic expression in human CD4+ T cells and then followed with experiments to address whether this gene is sensitive to androgen levels. We found that this gene was expressed at low levels in naïve CD4+ T cells of both sexes. However, PPARα mRNAs were strikingly up-regulated in T cells upon TCR and CD28 stimulation, particularly in males (Fig. 2.8A and B). Next, we stimulated CD4+ T cells from men and women in the presence of either 100 nM DHT or vehicle, as well as examined the relationship of PPARα mRNAs with circulating testosterone and 17-β-estradiol levels in men and women. We found that in vitro DHT treatment enhanced PPARα expression in T cells of women but did not alter the expression of this gene in T cells of men (Fig. 2.8C). In addition, we observed that PPARα mRNA expression in freshly isolated male T cells tended to associate with circulating levels of testosterone (P = 0.07) but not 17-β-estradiol levels (Fig. 2.9). The lack of sensitivity of male T cells to in vitro DHT treatment may relate to the fact that these cells were taken from an environment that already contained high levels of androgens. To address the molecular basis of androgen-sensitivity human PPARα, we used Alibaba2 prediction software (www.gene-regulation.com/pub/programs.html) to search for hormone-responsive elements in the promoter region of this gene. This search revealed the presence of an AR/glucocorticoid receptor-binding site in close proximity to an estrogen-binding site in the human and mouse promoters (Fig. 2.8D). ChIP experiments revealed that DHT and anti-CD3 and -CD28 signals act in synergy to induce the recruitment of the AR to the human PPARα promoter in primary T cells (Fig. 2.8 E and F). These studies suggest a direct effect of androgens in increasing PPARα mRNAs.
Fig. 2.8. PPARα has a sex-specific role in inhibiting Th1 cytokine production by human T cells. (A) CD4⁺ T cells were isolated from peripheral blood of healthy men and women (n = 10/group) and were either frozen down or stimulated with anti-CD3 and anti-CD28 for 31 h. PPARα mRNAs were measured using real-time PCR and were normalized to β-actin mRNAs. (B) Relative expression of PPARα mRNAs in stimulated male and female T cells. (C) PPARα mRNA expression by CD4⁺ T cells of men or women (n = 4/group) after stimulation for 72 h with 100 nM DHT or vehicle. (D) Location of probable AR (boxed) and estrogen receptor (underlined) binding sites in the human and mouse PPARα promoters. (E and F) PBMCs were isolated from women and were cultured in the presence (+) or absence (−) of anti-CD3/anti-CD28, plus (+) 100 nM DHT or vehicle (−). DNA was isolated from cells for ChIP of the PPARα promoter using an anti-AR or isotype control antibody. Representative gels (one donor) (E) and relative abundance of the PCR-amplified product of the PPARα promoter in three female donors post-ChIP (F). (G–I) CD4⁺ T cells from men and women (n = 10/group) were transfected with a GFP construct, along with either PPARα or nontargeting control siRNAs, before stimulation with anti-CD3 and anti-CD28 for 48 h. (G) Extent of PPARα knockdown. (H) Absolute IFNγ levels detected in CD4⁺ T-cell cultures after stimulation. (I) Mean percentage increase in IFNγ over nontargeting control siRNA for male (gray bar) and female (black bar) donors. (J) Percentage decrease in IFNγ in cultures after stimulation of T cells of men and women (n = 4 each) with 2.5 μM fenofibrate relative to vehicle (DMSO) control. In all cases, values are means ± SEM of individual human donors. *Significantly different (P ≤ 0.05), as determined using a t test (two-tailed) or a one-way ANOVA.
Fig. 2.9. Correlation of T-cell PPARα mRNAs with circulating testosterone and estradiol. Naïve CD4+ T cells were freshly isolated from peripheral blood of healthy men (n = 20) and women (n = 20). The blood draw was conducted in the morning hours and when women were at the follicular phase of their cycle. Total RNA was isolated from these cells and was reverse-transcribed to cDNA. cDNAs were amplified using real-time PCR using primers specific for human PPARα and human β-actin. PPARα transcript levels were normalized to β-actin. Total testosterone and 17-β-estradiol were measured in the sera of the same individuals. (A–D) Correlations (Pearson) of PPARα mRNAs with circulating testosterone (A and C) and 17-β-estradiol (B and D) in men (A and B) and women (C and D).
2.4.7. PPARα Represses IFN-γ Production by Human T Cells

To address whether PPARα functions in human T cells to repress IFNγ, we transfected primary T cells of men and women (n = 10/group) with PPARα-specific siRNAs before activating these cells with anti-CD3 and anti-CD28. Approximately half of T cells (52.5 ± 3.0% in men and 56.1 ± 3.8% in women) were transfected using our approach, resulting in a ~70–75% reduction of T-cell PPARα mRNAs in T cells of both sexes (Fig. 2.8 G). When stimulated with anti-CD3 and anti-CD28, male T cells transfected with PPARα siRNAs produced higher levels of IFN-γ compared with those transfected with the nontargeting siRNAs (Fig. 2.8 H and I). This effect was not observed in women (Fig. 2.8 H), and appeared to be specific to IFN-γ (Table 2.3). Similar results were obtained when siRNA transfection studies were conducted in SJL mice (Fig. 2.10). We also investigated whether the PPARα ligand fenofibrate would have sex-specific effects in limiting IFN-γ production. We found that fenofibrate reduced IFN-γ levels by WT but not by PPARα−/− T cells when administered at concentrations from 1 to 5 μM (Fig. 2.11 A). This same drug also had a sex-specific effect in decreasing IFN-γ production by male but not female T cells both in mice (Fig. 2.11 B) and humans (Fig. 2.8 J) when provided at a 5 μM dose. Together with siRNA findings, these results validate that PPARα has a sex-specific role in the negative regulation of T-cell IFN-γ production.

2.4.8. Mechanism of Repression of Human IFN-γ by PPARα

The induction of expression of the IFN-γ gene is regulated by both TCR- and cytokine-dependent signals acting at multiple genomic elements, located far upstream and downstream of this gene (222, 370). These genomic elements comprise a number of highly-conserved CNS sites that contain binding sites for transcription factors including T-bet and STAT4 (222, 370).
Fig. 2.10. Effect of PPARα siRNAs on murine CD4\(^+\) T cell proliferation and cytokine production. CD4\(^+\) T cells were isolated from spleens and lymph nodes of SJL/J mice and were transfected with PPARα siRNAs or nontargeting siRNAs and then left to rest overnight before being cultured (0.1 × 10\(^6\) cell/well) with plate-bound anti-CD3 and anti-CD28 (0.1, 1, or 2 μg/ml). Cytokine levels (A–D) were measured at 48 h by ELISA (shown are cytokine levels at 1 or 2 μg/mL) while proliferation (E) was measured at 64–66 h by \(^{3}H\)thymidine incorporation after the addition of \(^{3}H\)thymidine at 48 hours of cell culture (shown are cpms at 0.1 μg/mL). An aliquot of cells was reserved prior to stimulation for assessment of PPARα mRNA expression by real-time PCR (F), and the frequency of GFP\(^+\) cells was measured by flow cytometry (shown are frequencies of GFP\(^+\) cells in PPARα siRNA samples in the live CD4\(^+\) gate) (G). Values are means ± SE. Results are from one of three independent experiments. *Significantly different (P ≤ 0.05) from the nontargeting siRNA group.
Fig. 2.11. Dose-dependent effect of fenofibrate on IFNγ production by murine CD4+ T cells. (A) CD4+ T cells were isolated from spleens of WT or PPARα−/− 129 male and were stimulated with plate-bound anti-CD3 and anti-CD28 in X-VIVO-20 serum-free media after overnight pretreatment with vehicle or fenofibrate. Note that beyond 5 μM, fenofibrate also reduced IFNγ production by PPARα−/− cells. (B) CD3+ T cells were taken from male or female SJL/J mice and were cultured in X-VIVO-20 media with fenofibrate or equal volume of vehicle. The next day, cells were transferred to 96-well plates, precoated with anti-CD3 and anti-CD28. IFNγ production was measured by ELISA at 48 h (A+B). Values are means ± SEM. A is representative of three independent experiments. B is representative of two independent experiments.
Table 2.3. Effect of PPARα siRNA on Th Cell Cytokine Production.

<table>
<thead>
<tr>
<th></th>
<th>Nontargeting siRNA (pg/mL)</th>
<th>PPARα siRNA (pg/mL)</th>
<th>P value</th>
<th>P value (men vs. women)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>IFNγ</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>643 (180)</td>
<td>987 (276)</td>
<td>0.024*</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>793 (140)</td>
<td>790 (125)</td>
<td>0.970</td>
<td>0.038*</td>
</tr>
<tr>
<td><em>IL-2</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>905 (201)</td>
<td>988 (215)</td>
<td>0.061</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>958 (138)</td>
<td>954 (150)</td>
<td>0.252</td>
<td>0.170</td>
</tr>
<tr>
<td><em>TNF</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>622 (131)</td>
<td>767 (175)</td>
<td>0.059</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>1,024 (330)</td>
<td>921 (249)</td>
<td>0.309</td>
<td>0.12</td>
</tr>
<tr>
<td><em>IL-17</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>15 (4)</td>
<td>25 (9)</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>46 (26)</td>
<td>31 (17)</td>
<td>0.19</td>
<td>0.79</td>
</tr>
</tbody>
</table>

*Significant (paired t test; two-tailed; P < 0.05).
Under Th1 conditions, T-bet and STAT4 play key roles in the establishment of histone marks at these regions, thus permitting chromatin opening, transcription factor binding, and recruitment of the transcriptional activation machinery (222, 370). To gain insight into how PPARα may factor into this process, we used ChIP to assess the abundance of the activating histone mark acetylated-histone H4 at two well-characterized CNS regions, CNS-22 and CNS-6, in male T cells that were transfected with PPARα or nontargeting siRNAs (Fig. 2.12A). We found that T cells transfected with PPARα siRNAs displayed an enhanced abundance of acetylated-histone H4 at both of these CNS regions after anti-CD3 and anti-CD28 stimulation (Fig. 2.12 B and C). Thus, one way that PPARα may operate to repress IFN-γ production is through epigenetic changes in the IFN-γ locus that make it less poised for IFN-γ transcription. In light of our previous findings of a higher abundance of RelA in the nucleus of PPARα−/− T cells (283), we further investigated whether this transcription factor is recruited in increased amounts to these CNS regions. Using ChIP, we detected an enhanced abundance of RelA at these sites in T cells that were transfected with the PPARα siRNAs relative to those transfected with the non-targeting siRNAs (Fig. 2.12D). This occurred even in quiescent T cells (Fig. 2.12D). Taken together, our findings suggest that PPARα actions on the IFN-γ gene are very complex and involve effects on upstream TCR-signaling intermediates in addition to epigenetic changes at the IFNG locus.

2.4.9. PPARγ is a Potential Sex-Dependent Regulator of Th17

Our findings in mice indicated that PPARα expression is not responsible for the higher IL-17A production by male T cells. Indeed, recent reports have implicated roles for a number of other nuclear receptors in the control of IL-17A production (i.e., RORγT, vitamin D receptor,
Fig. 2.12. Effects of PPARα at the IFNG locus. (A) Alignment of the dog, human, and chimpanzee with the mouse genomic sequence upstream of the coding region of Ifng, highlighting the location of CNS-22 and CNS-6 sites. The arrow shows the start of transcription of Ifng. This alignment was conducted using Vista tools (http://genome.lbl.gov/vista/index.html). (B–D) T cells from five healthy male donors were isolated and were transfected with either nontargeting (−) or PPARα-specific siRNAs (+), after which they were left to rest (−) or were stimulated (+) with anti-CD3 and anti-CD28 for 8 or 24 h. Cells were fixed and then pooled for ChIP using antibodies specific for acetylated-histone H4 (B and C), RelA (D), or a rabbit isotype control antibody (B and D). After reversal of cross-links, immunoprecipitated chromatin was amplified using primers that spanned the CNS-22 or CNS-6 regions. Representative gels of the PCR-amplified products of CNS-22 and CNS-6 regions post-ChIP are shown for acetylated-H4 (B) and RelA (D). (C) Average relative densitometric analysis of the 8- and 24-h experiments normalized first to input DNA and then to the nonstimulated, nontargeting control.
retinoic acid receptor, liver X receptor, and PPARγ1) (371). With the knowledge that PPARγ exhibits a female preponderance in certain adipose stores (372, 373), we investigated whether the immune-expressed PPARγ variant, PPARγ1, is expressed by human CD4+ T cells in sex-specific way or is sensitive to androgen levels. In contrast to our findings for PPARα, we observed that PPARγ1 mRNAs were down-regulated more strikingly in T cells of men upon anti-CD3 and anti-CD28 stimulation (Fig. 2.13A). Furthermore, in vitro DHT treatment inhibited the T-cell expression of this gene in mice and humans, suggesting that PPARγ1 is tuned down in T cells by antigen and androgen signals (Fig. 2.13B and G). Using the approach of siRNA transfection and ligand activation, we next explored whether the control of T-cell IL-17A production by PPARγ1 is sex-dependent. As for our studies for PPARα, we isolated T cells from males and females of both species, transfected these cells with either PPARγ or nontargeting siRNAs and then stimulated cells with anti-CD3 and anti-CD28, this time in the presence of Th17-skewing cytokines. Although we did not detect a sex difference in IL-17A production by the human CD4+ T cells transfected with nontargeting siRNAs (Fig. 2.13E), we did observe this effect in mice (Fig. 2.13H). Furthermore, in both the murine and human systems, knockdown of PPARγ1 mRNAs had a more profound effect in increasing IL-17A production by T cells of females versus males (Fig. 2.13E and H). Finally, we observed that the synthetic PPARγ ligand rosiglitazone reduced IL-17A production by CD4+ T cells of women but not men (Fig. 2.13F). Altogether, these data suggest that PPARγ1 may be one molecule that may have sex-specific effects in limiting the production of IL-17A by female T cells.
Fig. 2.13. PPARγ operates selectively in female T cells to repress IL-17A production. (A) PPARγ1 mRNAs were measured in naïve or activated CD4+ T cells that were isolated from men or women (n = 10/group). (B) Mean PPARγ1 expression by CD4+ T cells of men or women after in vitro treatment (72 h) with 100 nM DHT or ethanol vehicle in media containing charcoal-stripped FCS. (C–E) CD4+ T cells were transfected with a GFP-expression construct, along with either PPARγ or nontargeting control siRNAs. The following day, transfection efficiency (GFP+ T cells) was assessed by flow cytometry, and the level of knockdown was assessed by real-time PCR. Remaining cells were stimulated with anti-CD3 and anti-CD28 for 48 h in the presence of Th17-skewing cytokines. (C) Extent of PPARγ1 knockdown, as measured using real-time PCR. (D) Percentage of GFP-positive events in the live CD4+ gate posttransfection. (E) Absolute IL-17A levels. (F) Percentage decrease in IL-17A relative to vehicle control after stimulation of CD4+ T cells of men and women in the presence of 1 μM rosiglitazone (expressed as percentage change in IL-17A relative to vehicle control). Values are means ± SEM of individual donors. (G) PPARγ1 mRNA expression relative to β-actin by freshly isolated CD4+ T cells from female 129 mice that were treated with a placebo or DHT pellet (5 mg pellet dose; n = 5/group). (H) IL-17A levels by male and female SJL/J T cells (n = 4/group) after transfection with either nontargeting or species-specific PPARγ siRNAs and stimulation with anti-CD3 and anti-CD28 in the presence of IL-6 and TGF-β. Results are representative of two experiments. *Significantly different (P ≤ 0.05), as determined using a t test (two-tailed) or a one-way ANOVA and Tukey post hoc test.
2.5. Discussion

Based upon work in mice, it has been hypothesized that the reason why women are more prone than men to develop T-cell autoimmune diseases is because they exhibit more robust Th1 adaptive immunity (7). Our finding that naïve CD4+ T cells of women produced higher levels of IFN-γ and tended to proliferate more than male T cells provides direct support for this notion. An unexpected feature of our study was the finding that male T cells produced higher levels of IL-17A, which goes against the current dogma that males have a Th2-biased immune system.

It is not yet clear why male SJL mice, in having more Th17 cells (considered by some to be pathogenic), were protected from EAE (255, 256). It is possible that the male PLP p139-151-reactive Th17 cells that we detected were highly encephalitogenic but were unable to cause severe EAE because of a limited potential for expansion or because additional regulatory mechanisms are in place in male mice that counter the pathogenicity of these cells. Although we did not observe higher IL-10 production or higher Treg frequencies in males during EAE, it has been previously reported that male SJL Treg have a greater capacity than female Treg to suppress T effector cells (95). An alternative possibility is that the Th17 cells that we detected in male SJL mice were not highly pathogenic. Indeed, not all Th17 cells are created equally (374). For instance, myelin-reactive Th17 cell lines are highly encephalitogenic if generated with IL-23, but are not when generated in the presence of TGF-β. Certainly, further work is necessary to define what feature(s) of male T cells (i.e., lower IFN-γ production, higher threshold of activation, reduced expansion, increased Treg capacity, or lesser propensity for dual IFNγ/IL-17A production) is responsible for protection from EAE.

Our findings of a Th17 bias in males is not surprising when considered in the context of what is known about the cross-regulation between Th1 and Th17 pathways (19). Indeed, IL-2
produced by Th1 cells activates STAT5, which competes with STAT3 for binding at the IL-17A promoter (375). Moreover, the Th1 lineage-determining factor T-bet inhibits Th17 differentiation by preventing the transactivation of the RORγT gene (101). Thus, the higher T-cell production of IL-17A that we observed in males may, in part, be a consequence of lowered IFN-γ production by these cells. Notwithstanding, our functional studies indicate that the lower ratio of PPARγ1 to PPARα in male T cells during T-cell activation is an additional T cell-intrinsic factor that drives this sex bias in cytokine production. We observed that TCR and CD28 signals coupled with androgen signals had a direct effect in up-regulating PPARα mRNAs in T cells of men, whereas these stimuli together extinguished PPARγ1 mRNA expression in female T cells.

In addition to defining the underlying reasons for the sexual dimorphism in PPAR expression, our experiments provided a number of molecular insights into how PPARα may exert its control over IFN-γ production in T cells. We found that knock-down of PPARα mRNAs resulted in an increased abundance of an activating histone mark, acetylated histone H4, at CNS-22 and CNS-6 enhancer regions, suggesting a role for PPARα in modulating accessibility at the IFNG locus. Normally, acetylated histone H4 marks chromatin when T-bet or IL-12/STAT4 activity is high (222, 370). Although we were unable to address T-bet or STAT4 activity in these T cells because of limited sample availability, it has been reported that T-bet is expressed at higher levels in PPARα−/− versus WT T cells (350). Thus, it is possible that these epigenetic changes that we observed are attributable to increased T-bet activity. In addition to this effect, we observed an enhanced recruitment of RelA to the CNS-22 enhancer region, thus coinciding with our previous finding of a higher abundance of this protein in the nucleus of male PPARα−/− versus WT T cells (283). Although our study did not address the mechanism of PPARγ repression of IL-17A production by human CD4+ T cells, previous elegant work in mice has indicated that ligand activation of PPARγ prevents the clearance of SMRT-containing
complexes from the RORγT promoter, resulting in reduced expression of this master regulator of Th17 differentiation (320). Taken together, these results suggest that PPARα and PPARγ, respectively, control key regulatory elements in Th1 and Th17 pathways.

Although we did observe a sexual dimorphism in IFN-γ production at the site of inflammation in the early stage of EAE, our study did not address whether women and men develop Th1- or Th17-biased forms of MS. In this regard, there is some precedent in the literature that female MS patients may be more Th1-prone. For instance, Pelfrey et al. reported that female patients with relapsing-remitting MS have a higher frequency of myelin-reactive IFNγ-producing cells in peripheral blood compared with male MS patients (3). Moreover, the frequency of IFNγ-producing CD3+ cells in peripheral blood of MS patients is reported to correlate with the severity of disease in women but not men (364). Finally, analysis of magnetic resonance imaging data from a large randomized trial of secondary progressive MS has indicated that women may be more responsive than men to IFNβ therapy (376), a treatment that may preferentially mitigate Th1 inflammation (377). If, indeed, these sex differences in Th cytokine production persist during autoimmune inflammation, it raises the prospect that women and men with MS should be treated differently.
CHAPTER 3

Antagonizing Peroxisome Proliferator Activated Receptor-α (PPARα) Abrogates Sex Differences in IFN-γ Production

Monan Angela Zhang, Jeeyoon Jennifer Ahn, Fei Linda Zhao, Thirumahal Selvanantham, Thierry Mallevaey, David Spaner, Shannon E. Dunn


Dr. Shannon Dunn performed the experiment in Figure 3.1 (C) and analyzed the results. Jeeyoon Jennifer Ahn assisted in both performing the Treg suppression and APC co-culture experiments and data analysis in Figure 3.1 (A-B), as well as performed the measurements of bacterial load in Figure 3.12 (D). Fei Linda Zhao performed the initial cell culture and RNA isolation in the experiments depicted in Figures 3.7 (E) and 3.9 (B). Drs. Thierry Mallevaey, Thirumahal Selvanantham and Dana Philpott provided reagents and aided with the set-up of the L. monocytogenes model in Figure 3.11 and 3.12. Peppi Prasit at Inception Biosciences and David Spaner developed the antagonist, determined effective doses of drugs for in vitro and in vivo use, and provided this compound to be used in multiple figures.
3.1. Abstract

Females exhibit more robust Th1 immune responses than males. Our previous work suggested that this sex disparity may be a consequence of higher activity of the androgen-induced gene PPAR-α in male CD4+ T cells. The objective of this study was to elucidate how PPARα inhibits Th1 cytokine production in males and to explore the translational potential of a novel PPARα antagonist to treat bacterial infection. We found that treatment of male CD4+ cells with the PPARα agonist fenofibrate induced the recruitment of PPARα to specific cis regulatory elements in the Ifng locus. This recruitment was associated with the enhanced presence of NCOR and reduced histone acetylation at these same sites. Conversely, treatment of male T cells with the novel small molecule PPARα antagonist NXT-1120 increased histone acetylation across the Ifng locus and enhanced IFN-γ mRNAs selectively in male T cells. Furthermore, NXT-1120 treatment abrogated the sex difference in IFN-γ production by NKT, CD4+ and CD8+ T cells and improved the survival of male, but not female mice during infection with the classic Th1-associated pathogen Listeria monocytogenes. Thus, our findings provide a novel mechanism of why IFN-γ responses are more robust in females and introduce a PPARα antagonist that can be used to overcome the sex disparity in Th1 immunity.
3.2. Introduction

Th cells can be classified into distinct subsets based on their expression of effector cytokines: IFN-γ for Th1, IL-4 for Th2, and IL-17A for Th17 cells (19, 222). Naïve CD4⁺ T cells acquire distinct Th cell fates in order to orchestrate the appropriate immune response against invading pathogens (19, 222). Th1 cells are specialized for the generation of cellular immunity against intracellular pathogens and are generated upon TCR and CD28 engagement in the presence of IL-12 (19). During Th1 differentiation, the IFN-γR-STAT1 signaling pathway converges with trans regulatory elements in the TCR signaling pathway such as NF-κB to induce expression of T-bet, the master regulator of Th1 programming and IFN-γ expression. T-bet promotes Th1 immunity by: (1) mediating epigenetic changes that make chromatin more accessible at the *Ifng* locus, (2) binding directly to cis elements to transactivate *Ifng*, and (3) increasing CD4⁺ T cell responsiveness to IL-12, which is important for stabilizing the Th1 lineage (19, 222, 378). In addition to being the major Th1 effector cytokine, IFN-γ serves as an amplification signal in the Th1 pathway, acting through IFNγR/STAT1 to enhance T-bet expression (44).

One intriguing feature of Th1 immune responses is that they are more robust in females than in males (7). Work in both mice and humans has shown that female CD4⁺ T cells produce higher levels of IFN-γ and expand more robustly upon antigen stimulation as compared to male T cells (255, 263, 283). These sex differences in Th1 immunity are thought to underly why women generate enhanced anti-viral and anti-tumour immune responses (5, 6), but also have a higher propensity to develop certain T cell-mediated autoimmune diseases (7). It has been suggested that sex differences in the Th1 response arise because of suppressive effects of androgens on three key points in Th1 differentiation pathway: 1) IL-12 production by APC
(239), 2) JAK2-TYK2 and STAT4 phosphorylation downstream of IL-12 (308), and 3) IFN-γ gene expression downstream of TCR and CD28 signals (263, 283). Until recently, the molecular players involved in this androgen-dependent regulation were unknown. Our group has identified that PPARα is an androgen-induced gene that operates in this third arm of regulation to dampen IFN-γ production by male CD4+ T cells (283). However, the exact mechanism of how PPARα suppresses Th1 immunity in male T cells is not understood. The aims of this study were to dissect the cellular and molecular mechanism of how PPARα regulates the sex difference in IFN-γ responses in mice, and to test the utility of a novel small molecule antagonist of PPARα, NXT-1120, to enhance Th1 responses in vivo in the context of experimental infection with the classic Th1-associated pathogen, Listeria monocytogenes (L. monocytogenes).

3.3. Materials and Methods

3.3.1. Mice

C57BL/6J and breeder pairs of PPARα+/+ (129S1/SvIM/J) and PPARα−/− mice (129S4/SvJae-Ppartm1Gonz/J) on the Sv129 background were from the Jackson Laboratory. Breeder pairs of PPARα+/+ (model B6) and PPARα−/− (model ppara) on the B6 background were from Taconic Farms. The latter mice were also crossed onto the T-bet−/− background (Jackson Laboratory). WT and PPARα−/− mice were crossed to generate F1 offspring and these mice were crossed to generate F2 littermates that were used for some experiments. Experimental results were similar using F2 littermate controls or offspring established from WT and PPARα−/− breeders. Mice were maintained in UHN facilities and work was conducted in accordance with the guidelines of the Canadian Council on Animal Care under animal use protocols (AUP 1747 and AUP 3213).
3.3.2. *In Vitro* Assay of APC Function

2D2 TCR transgenic CD4\(^+\) T cells that were isolated by negative magnetic bead selection (Miltenyi Biotech) were co-cultured in U-bottom 96-well plates (5 x 10\(^4\)/well) at a 2:1 ratio with irradiated splenocytes in the presence of 5-20 μg/mL of MOG p35-55 (from Stanford Pan facility, Stanford, CA).

3.3.3. Treg Suppression Assay

CD4\(^+\) T cells were enriched from the spleens of PPAR\(\alpha^{+/+}\) and PPAR\(\alpha^{-/-}\) mice via negative selection according to directions supplied in the mouse CD4\(^+\) isolation kit II (Miltenyi Biotec). Cells were then stained with the following antibodies: CD4-FITC, clone RM4-5; CD44-PE (clone 1M7) and CD25-APC (clone PC61.5). CD4\(^+\)CD25\(^{\text{high}}\) Treg and CD4\(^+\)CD44\(^{\text{low}}\)CD25\(^{-}\) populations were sorted using the Aria II FACS sorter (BD Biosciences, Flow Cytometry Facility at Hospital for Sick Children, Toronto). CD4\(^+\)CD44\(^{\text{low}}\)CD25\(^{-}\) responding T cells (2.5 x10\(^4\)/well) were then co-cultured together at the indicated ratio with CD4\(^+\)CD25\(^{\text{high}}\) Tregs (in complete RPMI) in 96-well U-bottomed plates with irradiated splenocytes (5 x10\(^4\)/well) and anti-CD3 as described previously (379). The proliferation of responding T cells was measured between 72 and 90 h of culture using a [3H]-thymidine incorporation assay (283).

3.3.4. *In Vitro* T Cell Stimulations

Naïve CD62L\(^+\) CD4\(^+\) T cells and total CD8\(^+\) T cells were isolated from spleens and lymph nodes of mice using magnetic bead selection (Miltenyi Biotec or Stem Cell Technologies) and were resuspended in RPMI 1640 media that contained 10% FCS) or X-VIVO-20 media (Lonza) that was supplemented with 2 mM, L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential
amino acids, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.5 μM 2-mercaptoethanol (from Gibco). T cells (2 x 10^5/well) were cultured in 96-well flat-bottomed plates that were pre-coated with 0.5 μg/ml of anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51). For Th polarization studies, media was also supplemented with the following cytokines and antibodies (all from eBioscience): 10 ng/ml IL-12 + 10 μg/ml anti-IL-4 (clone 11B11) for Th1 polarization; 10 ng/ml IL-4 + 10 μg/ml anti-IFN-γ (clone XMG1.2) for Th2 polarization, or 3 ng/mL TGF-β + 30 ng/mL IL-6 + 10 μg/ml of anti-IFN-γ + 10 μg/ml anti-IL-4 (clone 11B11) for Th17 polarization.

In experiments with the PPARα ligand or antagonist, X-VIVO-20 media was supplemented with 2.5 μM fenofibrate (Sigma), 100 nM NXT-592 (Inception), 100 nM NXT-1120 (Inception) or DMSO vehicle (0.1%, Sigma) with incubations initiated 2 h prior to stimulation.

3.3.5. Cytokine Measurements and Intracellular Staining

The levels of cytokines in culture supernatants or sera were measured using Ready-SET-Go! ELISA kits (eBioscience). For intracellular analysis, in vitro cultured T, NK, or NKT cells were stimulated for 4-5 h with 10 ng/mL PMA (Sigma) and 750 nM ionomycin (Calbiochem) in the presence of GolgiStop (0.66 μl stock/ml culture) (BD Pharmingen). For the in vivo L. monocytogenes infection studies, spleens were isolated from infected or non-infected mice and were either stained immediately (for assessment of NK and NKT cell responses), or were stained after re-activation ex vivo for 20-24 with preparations of heat-killed L. monocytogenes (1:1 ratio) with GolgiStop added in the last 4 h of culture (for assessment of CD4+ and CD8+ T cell responses). Cell surface and intracellular staining was conducted as described previously (369) using aqua live/dead stain (Invitrogen) and the following antibodies or tetramers: CD4-PECy5 (GK1.5), CD8-FITC (53-6.7), TCRβ-PE-Cy7 (H56-597), IFNγ-PE (XMG1.2), IL-17A-APC
(17B7), IL-4-FITC (11B11), NKp46-APC-eFluor780 (29A1.4), NKp46-AlexaFluor700 (29A1.4, BD), and PBS57/mCD1d tetramer-APC (gift from NIH Tetramer Core Facility).

Intranuclear staining of transcription factors was performed using reagents and protocols provided with the Foxp3 Transcription Factor Staining Buffer Set (eBioscience) and the following antibodies: GATA-3-PE (clone TWAJ), RORγT-PE (clone B2D), and T-bet-PE-Cy7 (clone 4B10). Data were acquired using the LSRII (BD) (BD Biosciences, Flow Cytometry Facility at Hospital for Sick Children, Toronto) and were analyzed using Flowjo software (V9+) (Flowjo). Flow cytometry antibodies were from eBioscience unless otherwise noted.

3.3.6. Measurement of mRNA gene expression

Total RNA was isolated from CD4^+ T cells using RNeasy Mini Kit (Qiagen) and was reverse transcribed to cDNA (369). The relative expression of specific mRNAs was measured using real-time PCR (Roche LightCycler480) using FastStart Universal SYBRGreen Master Mix (Roche) and specific primer pairs (see Table S1). The following amplification parameters were used: 95°C for 15 min, followed by 50 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 30 s. The relative abundance of each PCR product was determined using the standard curve method and was normalized to β-actin.

3.3.7. ChIP

T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (5 µg/ml each) for 2 h for CD8^+ T cells or 4 h for CD4^+ T cells. Th1-polarized cells were generated for these studies by first stimulating cells with anti-CD3 and anti-CD28 in the presence of IL-12 and anti-IL-4 for 4 days. ChIP was performed after stimulation using the SimpleChIP Enzymatic Chromatin IP kit
(Cell Signaling) according to kit directions. The only deviation from the protocol was the addition of a homogenization step (10 gentle, manual strokes using a dounce homogenizer on ice) prior to lysis in buffer A for 40-50 minutes on ice. DNA was immunoprecipitated using specific antibodies (Table 3.1) and was amplified using real-time PCR as described above using primer sequences that were designed to span CNS regions and the promoter of the Ifng (Table 3.2).

3.3.8. Protein electrophoresis and western blot analysis

PPARα+/+ and PPARα−/− CD4+ T cells were washed with ice-cold serum-free X-VIVO 20 (Lonza) and were resuspended at a concentration 1.4 x 10⁸ cells/ml. Cells were allowed to rest on ice for 15 minutes prior to the addition of soluble anti-CD3 antibody (10 µg/ml) and anti-CD28 (10 µg/ml). The cross-linker antibody (20 µg/ml goat anti-hamster IgG, Pierce) was subsequently added, followed by stimulation at 37ºC. At various times after stimulation, cells were lysed by adding one volume of 2 x lysis buffer (100 mM Tris, 4% SDS, 10% glycerol, 2% 2-mercaptoethanol, 10 mM EDTA, 1 x Roche protease inhibitor cocktail, 1 x Halt phosphatase inhibitor cocktail from Pierce). Cell lysates were then boiled for 15 minutes and the protein concentration was determined using the Quantit Kit (Molecular Probes). Protein electrophoresis was performed using Novex Mini-Cell system (Life Technologies) using NuPAGE 4-12% Bis-Tris Gels and MOPS 2D2 running buffer (Life Technologies). Proteins were transferred to PVDF membranes overnight at 4ºC using the Mini Trans-Blot Cell (Bio-Rad). Western blotting was conducted as described previously (368) and blots were probed overnight at 4ºC with the following primary antibodies from Cell Signaling: phospho-ZAP70 (2701), ZAP70 (3165), phospho-JNK (9251), JNK (9252), IκBα (9242), phospho-p38 (9215), p38 (9212), phospho-Erk1/2 (9101), and Erk1/2 (9102).
Table 3.1. Antibodies used for ChIP experiments

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Source (Cat#)</th>
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<tbody>
<tr>
<td>acetyl-histone H4 (K8)</td>
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<td>Cell Signaling, 2594S</td>
</tr>
<tr>
<td>tri-methyl-histone H3 (K27)</td>
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<td>Cell Signaling, 9733S</td>
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<tr>
<td>PPARα</td>
<td>1:50</td>
<td>Cayman, 101710</td>
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<tr>
<td>NCOR1</td>
<td>1:50</td>
<td>Thermo Scientific, PA1-844A</td>
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<tr>
<td>Isotype control IgG (from native rabbit sera)</td>
<td>1:500</td>
<td>Cell Signaling, 2729P</td>
</tr>
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Table 3.2. Primer sequences used for real-time PCR and ChIP

<table>
<thead>
<tr>
<th>Primer Names</th>
<th>Sequences (5'→3')</th>
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<tr>
<td>IFNγ FWD</td>
<td>TGGCTCTGCAGGATTTTCATG</td>
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<tr>
<td>IFNγ REV</td>
<td>TCAAGTGCTGATAGTTGGAAGAA</td>
</tr>
<tr>
<td>tbx21 FWD</td>
<td>TGCTTTGATGGCTCTAAA</td>
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<tr>
<td>tbx21 REV</td>
<td>TGGCCCCGCTTCTCTCTCAAACCA</td>
</tr>
<tr>
<td>Eomes FWD</td>
<td>TAGGCGGAGGGTTCTCCGCTCTAC</td>
</tr>
<tr>
<td>Eomes REV</td>
<td>GGGCCGTTGCACACAGGTAGAGCTG</td>
</tr>
<tr>
<td>IL-4 FWD</td>
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<td>IL-4 REV</td>
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<td>GATA-3 FWD</td>
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<td>GATA-3 REV</td>
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<td>IL-17A FWD</td>
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<td>CNS-1 FWD</td>
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<tr>
<td>CNS-1 REV</td>
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</tr>
<tr>
<td>Ifng promoter FWD</td>
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<tr>
<td>Ifng promoter FWD</td>
<td>GGTCAAGCCGATGGGCAACTA</td>
</tr>
<tr>
<td>CNS+17 FWD</td>
<td>AGGATGCCCGGTAGTGCTCAGC</td>
</tr>
<tr>
<td>CNS+17 REV</td>
<td>CACTGGTGAAAGTCACAGGAACCT</td>
</tr>
</tbody>
</table>

The primer sequences that encoded the *ifng* promoter and CNS sites were provided by Dr. Robin Hatton and Dr. Casey Weaver (University of Alabama Birmingham)
Blots were then probed with Horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1:5000-1:10000 #NA934V, from GE Healthcare Life Sciences) at room temperature for 1 h and were developed on Hyperfilm using ECL Plus western blotting detection reagent (GE Healthcare). The quantity of each protein product was shown as a value less of the background value and relative to the value of β-actin as assessed using software ImageJ.

3.3.9. Experimental infection with L. Monocytogenes (EGD strain)

Glycerol stocks (50% glycerol + 50% L. monocytogenes overnight culture) were streaked onto a plate that contained Brain Heart Infusion (BHI) agar (3.8% BHI broth and 1.5% agar, both from BD Biosciences). Single colonies of L. monocytogenes were picked and were grown overnight in 3 mL of BHI media in a 37°C 220-240 rpm shaking incubator. When L. monocytogenes reached the logarithmic phase of growth, the CFU associated with OD_{600} was determined by conducting a 10 fold-dilution series using BHI agar plates and by counting colonies. Bacterial load was determined at day 2 post-infection as follows. Spleens and livers were weighed and then spleens were dissociated through a 70-µm nylon cell strainer into a petri dish that contained 1x PBS. Livers were homogenized in 0.1 % Triton-X in 1x PBS with 1.5-2 mm glass beads (pre-washed with acid) in a high-speed shaking TissueLyser II (Qiagen) for 3 minutes at a frequency of 30 Hz. A 10 fold-dilution series of homogenates was prepared and was streaked onto plates containing the BHI agar. After 48 hours, CFUs were counted and the CFU/mg was calculated as the CFU on the plate/dilution factor/0.1ml plated/mg of tissue.

For survival studies, C57BL6/J mice were infected i.p. with the LD_{50} dose of the pathogen (determined to be 100,000 CFU for males and 150,000 CFU for females, see Fig. 3.13). For immune studies, a dose of 100,000 CFU was used to elicit optimal NK and NKT IFN-γ
responses, while a sublethal dose of 20,000 CFU was used for experiments that assessed CD4\(^+\) and CD8\(^+\) T lymphocyte responses. Endpoints for survival studies consisted of a 20% loss in body weight or with the development of at least two of the following clinical signs: lethargy, ruffled fur appearance, hunched appearance, labored breathing and dull/sunken eyes. For NXT-1120 treatment studies, mice were administered the drug (30 mg/kg in 0.5% methylcellulose in PBS) or vehicle by gavage, twice daily (9-10 h apart), starting on the day prior to infection. Serum was also collected from mice at 2 days post infection (with 100,000 CFU of pathogen) by cardiac puncture.

3.3.10. Statistics

Data are presented as means + SEM. When data were parametric (kurtosis and skewness <2) and group variances were homogenous (Bartlett homogeneity test), a two-tailed \( t \) test was used to detect between-group differences. When data were nonparametric, ranks were compared among groups using a Mann-Whitney \( U \) test. A difference in mouse survival between groups was determined using a log rank test. A value of \( P \leq 0.05 \) was considered significant.

3.4. Result

3.4.1. PPAR\(\alpha\) Acts within Effector CD4\(^+\) T Cells to Limit Th1 Cytokine Production

Previously, we reported that male PPAR\(\alpha^{-/-}\) mice develop a more hyper-acute form of EAE than PPAR\(\alpha^{+/+}\) (herein referred as wild type or WT) mice and display a higher production of IFN-\(\gamma\) by myelin-reactive Th cells (283). This hyper-Th1 response in PPAR\(\alpha^{-/-}\) mice occurred exclusively in males and appeared to be due to a defect in the CD4\(^+\) T cell compartment, because purified CD4\(^+\) T cells sourced from PPAR\(\alpha^{-/-}\) mice proliferated more robustly and expressed
higher levels of IFN-γ than WT CD4+ T cells upon TCR and anti-CD28 stimulation (283). To investigate whether PPARα also regulates Th1 responses through actions in APC, we cultured PPARα−/− and WT splenocytes (as a source of APC) with purified naïve myelin oligodendrocyte glycoprotein (MOG) p35-55 TCR transgenic CD4+ T cells in the presence or absence of MOG p35-55 antigen. However, varying the genotype of APC in these assays did not influence the proliferation or IFN-γ production by responding CD4+ T cells (Fig. 3.1A).

We also explored a role for PPARα in the Treg cell compartment by conducting conventional in vitro Treg suppressor assays using CD4+CD25+CD44low T effector cells and CD4+CD25− Treg that were FACS-sorted from male PPARα−/− and WT mice. As observed previously (283), naïve PPARα−/− effector CD4+ T cells proliferated more robustly than WT counterparts and produced higher levels of IFN-γ (Fig. 3.1B). However, WT and PPARα−/− Treg did not exhibit any difference in their capacity to suppress the expansion or IFN-γ production by effector CD4+ T cells (Fig. 3.1B). Consistent with this, we found that PPARα mRNAs were more highly expressed in CD4+CD25− than in CD4+CD25+ Treg cells (Fig. 3.1C). Together, these results confirm that PPARα functions within effector CD4+ T cells to negatively regulate Th1 responses.

3.4.2. PPARα Inhibits IFN-γ mRNA Expression

When a CD4+ T cell differentiates along one Th path, alternative Th fates are inhibited through cross-regulatory mechanisms (19). Indeed, we had previously observed that the enhanced Th1 cytokine production in male PPARα−/− mice during EAE occurred at the expense of the Th17 cytokine, IL-17 (283). It is also reported that certain fibrate ligands of PPARα can induce Th2 cytokine production by CD4+ T cells (346). To gain further insights into which Th
Fig. 3.1. PPARα does not function in APC or T reg cells to negatively regulate Th1 responses. (A) Splenic mononuclear cells from WT or PPARα−/− mice were irradiated and co-cultured at a 1:2 ratio with naïve MOG p35-p55 TCR transgenic (2D2) CD4+ T cells in the presence of MOG p35-55. At 48 h, cultures were pulsed with [3H]-thymidine and cells were harvested 18 h later for measurement of incorporated radioactivity in counts per minute (CPM). IFN-γ was measured in culture supernatants after 72 h of culture by ELISA assay. B, FACS-sorted CD4+ CD25−CD44low naïve T effector (Teff) cells from WT or PPARα−/− male mice were co-cultured with CD4+CD25+ T regulatory cells from WT or PPARα−/− male mice in the presence of irradiated splenocytes and 5 µg/ml anti-CD3. The proliferation (between 72 and 90 h) and cytokine production (at 96 h) was measured as described above. In A-B, values are means +/- SEM of values obtained in triplicate cultures and are representative of 2 independent experiments. (C) CD4+CD25− and CD4+ CD25+ cell subsets were enriched from spleens of male or female 129S1/SvImJ mice using a Treg enrichment kit (Miltenyi Biotec). Real-time PCR for PPARα was performed as described below. * indicates a significant (P<0.05) difference from the CD4+CD25− sex-matched population.
pathway is the target of PPARα-dependent regulation, we compared the cytokine production by male PPARα−/− and WT CD4+ T cells that were stimulated under Th0, Th1, Th17, or Th2 polarization conditions. We observed that male PPARα−/− CD4+ T cells produced IFN-γ at higher levels than WT counterparts under all conditions examined (Fig. 3.2A). This enhanced IFN-γ production by male PPARα−/− CD4+ T cells was accompanied by reductions in IL-4 or IL-17 levels (Fig. 3.2A). Similar trends in cytokine production and mRNA expression were evident by intracellular cytokine staining (Fig. 3.3) and real-time PCR analysis (Fig. 3.2B). This Th1 bias in cytokine production in male PPARα−/− CD4+ T cells was also accompanied by increases in mRNA and protein expression of the Th1 regulator T-bet and decreases in the mRNA expressions of the Th2 regulator GATA-3 and the Th17 regulator RORγT (Fig. 3.2B and C).

Genetic deficiency of PPARα in CD4+ T cells is associated with the compensatory upregulation of the related nuclear receptors PPARδ and PPARγ (283). Thus, to further address the involvement of PPARα in the regulation of Th cytokine production, we investigated effects of the PPARα ligand activator fenofibrate and the novel small molecule PPARα antagonists NXT-592 or NXT-1120 on cytokine production by CD4+ T cells. In preliminary studies, we established the concentrations of the drugs that modulate IFN-γ production by WT, but not by PPARα−/− cells (Fig. 2.11A and Fig. 3.4A and B). We found that treatment with fenofibrate reduced, whereas treatment with the antagonists enhanced IFN-γ production by CD4+ T cells (Fig. 3.2D-F). However, in contrast to the studies using PPARα−/− CD4+ T cells, these drugs altered IFN-γ production without effecting opposite changes in IL-4 or IL-17 (Fig. 3.2D-F). In fact, the antagonist NXT-1120 had the effect of slightly enhancing the production of IL-4 (Fig. 3.2F), which may relate to the stimulatory effect of this drug on T cell proliferation (Fig. 3.4C). These data thus support a mechanism whereby PPARα inhibits Th1 cytokine production directly as opposed to an indirect mechanism involving promotion of Th2 or Th17 pathways.
Fig. 3.2. PPARα regulates IFN-γ production by CD4+ T cells. (A–C) Lymph node cells (A, B) or naïve CD4+ T cells (C) were isolated from WT and PPARα−/− mice (N=5/group), were pooled, and were stimulated with anti-CD3 and anti-CD28 under Th0, Th1, Th2, or Th17-polarizing conditions. (A) Cytokine levels in culture supernatants after 60 h. (B) Relative abundance of each transcript measured in CD4+ T cells after 24 h of stimulation under the Th0 condition. Transcript levels were normalized to β-actin mRNAs and then were expressed as a fold-change relative to non-stimulated samples. (C) Intranuclear staining of T-bet, GATA-3 and RORγT in the CD4+ T cell gate after 60 h of stimulation under the Th0 condition. (D–F) WT CD4+ T cells were pre-incubated with 2.5 μM fenofibrate (D) or 100 nM of the PPARα antagonists NXT-592 (E) or NXT-1120 (F), or DMSO vehicle for 4 hours prior to stimulation with anti-CD3 and anti-CD28. Cytokine levels in culture supernatants were measured at 48 h post-stimulation. Values are means ± SEM of triplicate cultures or PCR reactions. Data are representative of 3 independent experiments. * indicates a significant difference (P<0.05) from the WT group (A–C) or the vehicle group (D–F) as determined using a two-tailed T-test.
Fig. 3.3 Intracellular staining of male WT and PPARα−− CD4+ T cells under Th-polarizing conditions. Lymph node mononuclear cells were isolated from PPARα+/+ and PPARα−− (N=5 mice/group), were pooled, and were stimulated with anti-CD3 and anti-CD28 under Th0, Th17, or Th2-polarizing conditions. Intracellular staining was performed at 4.5 days post-stimulation after PMA/ionomycin re-stimulation in the presence of Golgi Stop. Cell surface staining for anti-CD4 was performed followed by intracellular staining for IFNγ, IL-4 and IL-17. Shown is representative staining of cytokines in the CD4+ gate of cells that were cultured under Th0 (A), Th17 (B), and Th2 (C)-polarizing conditions. These data are representative of three independent experiments.
Fig. 3.4. Titration of PPARα antagonists and effect of NXT 1120 on CD4+ T cell proliferation. Total CD4+ T cells were isolated from male PPARα+/+ and PPARα−/− mice and were pre-incubated with various concentrations of the indicated PPARα antagonists or DMSO vehicle (0.1% volume) prior to stimulation with anti-CD3 and anti-CD28. IFNγ production was measured after 48 hours using an ELISA kit (A & B), while the proliferation was measured between 72-90 h by [3H]-thymidine incorporation assay (C). CPM=counts per minute. Values represented are means +/- SEM of triplicate cultures and data are representative of 2-3 independent experiments. * indicates a significant difference from the vehicle-treated counterpart by two-tailed T-test (level of significance, p<0.05).
To determine which gene in the Th1 pathway is regulated by PPARα, we focused on the three Th1-associated genes (T-bet, Eomes, and IFN-γ) that were expressed at higher levels in PPARα<sup>−/−</sup> versus WT CD4<sup>+</sup> T cells (Fig. 3.2B). IFN-γ and T-bet act in a feed forward loop to enhance each other’s expression during Th1 differentiation, while Eomes is important for optimal expression of IFN-γ in CD4<sup>+</sup> T cells and plays a redundant role in inducing the expression of IFN-γ in the absence of T-bet (19). We first conducted time-course studies to investigate the kinetics of T-bet, Eomes, and IFN-γ mRNAs upon stimulation with anti-CD3 and anti-CD28 (Fig. 3.5A). In WT CD4<sup>+</sup> T cells, the levels of IFN-γ mRNA increased gradually over the period of 24 h, while the mRNA expressions of T-bet and Eomes were enhanced as early as 4 h and peaked between 16-24 h post-stimulation (Fig. 3.5A). The expressions of all three of these genes were higher in PPARα<sup>−/−</sup> versus WT CD4<sup>+</sup> T cells at most time-points examined (Fig. 3.5A).

To investigate whether it was the higher T-bet expression in PPARα<sup>−/−</sup> CD4<sup>+</sup> T cells that was driving the enhanced IFN-γ mRNA levels, we repeated this experiment using CD4<sup>+</sup> T cells that were isolated from PPARα<sup>+/+</sup> T-bet<sup>−/−</sup> and PPARα<sup>−/−</sup> T-bet<sup>−/−</sup> male mice (Fig. 3.5B). IL-12 was also added to these cultures to help amplify IFN-γ mRNA expression. We observed that PPARα<sup>−/−</sup> T-bet<sup>−/−</sup> CD4<sup>+</sup> T cells still exhibited higher IFN-γ mRNA levels and cytokine production than PPARα<sup>+/+</sup> T-bet<sup>−/−</sup> counterparts (Fig. 3.5B), suggesting that T-bet is not absolutely required for the PPARα regulation of IFN-γ production. However, in the absence of T-bet, we did observe an early compensatory increase in the mRNA expression of Eomes at 4 h post-stimulation in the PPARα<sup>−/−</sup> CD4<sup>+</sup> T cells (Fig. 3.5B).

Since the expressions of T-bet and Eomes are both induced downstream of IFN-γR-STAT1 signals (44, 51), it remained possible that the higher expressions of these genes in PPARα<sup>−/−</sup>
Fig. 3.5. **IFN-γ is the gene target of PPARα-dependent suppression.** WT or PPARα⁻/⁻ naïve CD4⁺ T cells on the T-bet⁺/+ (A, C) or T-bet⁻/⁻ background (B) were stimulated with anti-CD3 and anti-CD28 for 24 hours in the presence (C) or absence (A, B) of an anti-IFN-γ neutralizing antibody. For the experiment using T-bet⁻/⁻ CD4⁺ T cells (in B), IL-12 was also added to the media. Total RNA was isolated at various time points post-stimulation and was reverse-transcribed to cDNA. The relative abundance of each transcript was measured using real-time PCR and was normalized to β-actin mRNAs. IFN-γ levels (pg/ml) in culture supernatants were measured at 24 h of culture using ELISA kits. Shown is the fold-change of transcript levels relative to mRNA levels at time 0 h. Values are means +/- SEM of triplicate reactions and are representative of 2-3 independent experiments. * indicates a significant difference (P<0.05) from the PPARα⁺/+ group using an independent T-test (two-tailed).
CD4⁺ T cells occurred secondary to early increases in IFN-γ production by PPARα⁻/⁻ CD4⁺ T cells. To test this, we repeated these T cell stimulations in the presence of an IFN-γ neutralizing antibody. We found that the expressions of IFN-γ mRNAs were still elevated in PPARα⁻/⁻ CD4⁺ T cells above WT control levels (Fig. 3.5C). In addition, the early peak in T-bet mRNAs triggered by early TCR or IFN-γR signals (44) was still present. However, the secondary increase in T-bet mRNAs disappeared, as did the differences in the early expressions of T-bet and Eomes between WT and PPARα⁻/⁻ CD4⁺ T cells (Fig. 3.5C). Together, these data strongly suggest that Ifng is the Th1-associated gene that is repressed by PPARα in male CD4⁺ T cells.

3.4.3. PPARα Regulates Histone Acetylation at the Mouse IFN-γ Locus

We had observed that PPARα inhibited IFN-γ expression in male CD4⁺ T cells even under Th0 conditions, which suggested that this transcription factor may be acting as a rheostat, modulating the strength of TCR or CD28 signals that lead to IFN-γ transcription. Though previous reports have suggested a role for PPARα in the negative regulation of the phosphorylation of p38 or the abundance of IκB (350, 380), we did not observe any aberrations in the expression or phosphorylation of key signaling intermediates downstream of TCR and CD28 signaling pathways in male PPARα⁻/⁻ CD4⁺ T cells (Fig. 3.6). These findings suggest that PPARα operates more distally in this pathway, possibly at the Ifng locus itself to control IFN-γ expression.

The transcription of IFN-γ is regulated by both TCR and cytokine-dependent signals and downstream transcription factors (STAT4, T-bet, RelA, etc.), which bind to a number of highly-conserved CNS sites (Fig. 3.7A) (19, 222). These CNS sites act as regulatory cis elements at the Ifng (222, 378). The binding of these transcription factors is also supported by epigenetic
Fig. 3.6. PPARα⁺⁻ male CD4⁺ T cells do not exhibit a differential phosphorylation or abundance of key signaling intermediates downstream of TCR/CD28. (A-C) Total CD4⁺ T cells isolated from male PPARα⁺⁺ and PPARα⁻⁻ mice were stimulated with anti-CD3, anti-CD28 and a cross-linking antibody for various time points. At the indicated times after stimulation, cells were lysed with 2 x lysis buffer and boiled, and protein lysates was separated by SDS-PAGE and immunoblotted with the antibodies indicated using western blotting as shown in A. β-actin was used as a loading control. (B) The quantities of phosphorylated protein relative to total protein expression of key signaling molecules over time were calculated using ImageJ from the western blot in A. (C) The quantities of IκBα relative to β-actin protein expression over time were calculated using ImageJ from the western blot in A. Data are representative of 2 experiments.
Fig. 3.7. Reducing PPARα activity enhances H4-Ac at the Ifng locus. (A) Schematic of the Ifng. (B-C) Naïve CD4+ T cells isolated from spleens of male WT or PPARα−/− mice (N=5/group) were stimulated with anti-CD3 and anti-CD28 for 4 hours (Th0, left panel) or with IL-12 + anti-IL-4 for 4.5 days (Th1, right panel) prior to re-stimulation with anti-CD3 and anti-CD28. ChIP was performed using anti-H4-Ac and the abundance of immunoprecipitated DNA was measured using real-time PCR and primers designed to span CNS sites and the Ifng promoter. Shown is the relative abundance of immunoprecipitated DNA normalized to input DNA less the minimal signal generated from the IgG control. (C) Fold increase of H4-Ac in stimulated versus non-stimulated CD4+ T cells. (D-E) Naïve CD4+ T cells were isolated from male or female WT mice (N=5/group) and were stimulated with anti-CD3 and anti-CD28 in the presence of 100 nM NXT-1120 or vehicle. ChIP was performed as described above (D) and T-bet and IFN-γ mRNAs were measured using real-time PCR (male cells only) (E). Values are means + SEM of triplicate reactions. Data are representative of 2-3 independent experiments. * Significantly different (P<0.05) from the WT (B) or the vehicle group (D, E) as determined using a two-tailed T-test.
modifications that make chromatin more accessible at the \textit{Ifng} locus (222, 378). Acetylated-histone 4 (H4-Ac) is one epigenetic mark that is permissive to transcription and accumulates across the \textit{Ifng} locus during Th1 differentiation. This acetylation event occurs as a result of the T-bet-dependent removal of a HDAC-containing co-repressor complex at the \textit{Ifng} locus (226). We therefore compared the abundance of H4-Ac at CNS sites and at the promoter region of \textit{Ifng} in male WT and PPAR\(\alpha^{-/}\) CD4\(^+\) cells using a ChIP assay. For these assays, male naïve CD4\(^+\) cells or Th1-polarized CD4\(^+\) cells were activated for 4 h with anti-CD3 and anti-CD28 and this was followed by ChIP using an H4-Ac antibody. We observed that both naïve and Th1-polarized PPAR\(\alpha^{-/}\) CD4\(^+\) T cells exhibited an enhanced abundance of H4-Ac at the \textit{Ifng} promoter and at most CNS sites examined as compared to WT cells (Fig. 3.7B). Consistent with previous findings (378), a notable induction in H4-Ac in WT CD4\(^+\) T cells was only evident in WT cells that were cultured under the Th1 polarization conditions (Fig. 3.7C). We noted no differences in the levels of IFN-\(\gamma\) mRNAs and the abundance of H4-Ac marks between quiescent naïve WT and PPAR\(\alpha^{-/}\) CD4\(^+\) T cells (Fig. 3.8A & B), indicating that the full influence of PPAR\(\alpha\) on IFN-\(\gamma\) transcriptional suppression only occurs under conditions of T cell activation.

We also compared the abundance of H3K27-Me, a repression mark that accumulates at the \textit{Ifng} locus upon Th2 differentiation (227). Though naïve CD4\(^+\) T cells show only limited H3K27-Me at the \textit{Ifng} locus, we noted that the abundance of H3K27-Me was lowered at CNS sites in PPAR\(\alpha^{-/}\) as compared to WT cells after stimulation under Th0 conditions (Fig. 3.8C), suggesting that male PPAR\(\alpha^{-/}\) CD4\(^+\) T cells are not only more poised to become Th1, but may be less poised to become Th2 cells.

Next, we investigated whether the small molecule PPAR\(\alpha\) antagonist NXT-1120 could evoke similar changes in histone acetylation at the \textit{Ifng} locus as seen PPAR\(\alpha^{-/}\) CD4\(^+\) T cells. For
Fig. 3.8. PPARα does not regulate the basal expression of IFN-γ or H4-Ac at the Ifng locus in quiescent naïve CD4+ T cells. (A) Naïve CD4+ T cells were isolated from spleens of male WT and PPARα−/− mice. RNA was isolated from these cells for real-time measurement of IFNγ mRNA expression. Shown is the abundance expressed relative to β-actin. (B) ChIP was also performed on the same cells described in (A) using an anti-H4-Ac antibody. (C) Naïve PPARα+/+ and PPARα−/− CD4+ T cells were activated for 4 h with anti-CD3 and anti-CD28 (8 µg/ml each) and ChIP was performed using anti-di/tri-methylated histone H3 (K27). In B & C, the abundance of H4-Ac and H3K27-Me at various CNS sites and the ifng promoter was determined using real-time PCR and was normalized to the total input DNA less the minimal signal generated from the isotype IgG control. Values are means ± SEM of levels obtained in triplicate PCR reactions. Values are representative of at least 2 independent experiments. * indicates a significant difference (P<0.05) from PPARα+/+ group as determined using a one-tailed paired T-test.
these experiments, male and female CD4+ T cells were stimulated with anti-CD3 and anti-CD28 for 4 h in the presence of NXT-1120. Similar to findings of male PPARα-/- CD4+ cells, treatment of male WT CD4+ T cells with the antagonist increased IFN-γ mRNAs and induced H4-Ac throughout the Ifng locus (Fig. 3.7D & E). Consistent with the broad-scale nature of these changes, NXT-1120 treatment also increased T-bet mRNAs in male T cells (Fig. 3.7E). These effects of the antagonist in increasing H4-Ac were not observed in assays conducted using female CD4+ T cells (Fig. 3.7D).

How PPARα is recruited to the Ifng locus and represses the expression of this gene is not known. Previously, Glass and colleagues unraveled the molecular mechanism of how the related nuclear receptor PPARγ represses the transcription of the inducible nitric oxide synthase gene (Inos) in macrophages (319). It was found that upon treatment with the ligand rosiglitazone, PPARγ is sumoylated and that this post-translational modification is associated with the recruitment of PPARγ to the inos promoter, where it stabilizes the presence of the nuclear receptor co-repressor 1 (NCOR)-containing HDAC3 co-repressor complex, rendering the gene refractory to lipopolysaccharide (LPS) stimulation (319). Given that PPARα can also be sumoylated upon ligand binding (354), we hypothesized that PPARα may operate by a similar mechanism to repress Ifng. We therefore tested whether treatment of male CD4+ T cells with the PPARα ligand fenofibrate would enhance the recruitment of PPARα to regulatory elements at the Ifng gene and whether this recruitment would be associated with reduced H4-Ac and enhanced NCOR at these sites. For these studies, we used Th1-polarized CD4+ T cells that exhibited a higher basal level of H4-Ac (Fig. 3.7B). Treatment of male WT Th1 cells with fenofibrate reduced the abundance of H4-Ac at two CNS sites (CNS-22 and CNS-34) and the IFN-γ promoter relative to levels in the vehicle control group (Fig. 3.9A). These changes were independent of T-bet, as fenofibrate did not reduce T-bet expression in spite of reducing IFN-γ
Fig. 3.9. Fenofibrate induces the recruitment of PPARα to specific regulatory elements in the Ifng locus to repress H4-Ac. Naïve CD4+ T cells isolated from spleens of male WT mice (N=5/group) were activated in the presence of IL-12 in the presence of 2.5 µM of the PPARα ligand (fenofibrate) or DMSO vehicle control. After 4.5 days, cells were re-stimulated with anti-CD3 and anti-CD28 for 4 hours in the presence of ligand or vehicle. ChIP was performed as described in Fig. 3.7 using anti-H4-Ac (A), anti-NCOR (C), or anti-PPARα (D) antibodies. Shown is the relative abundance of immunoprecipitated DNA normalized to total input DNA less the minimal signal generated from the IgG control. In D, the fold change represents the relative abundance of PPARα in the fenofibrate- versus DMSO-treated samples. (B) Shows the levels of T-bet and IFN-γ mRNAs in the T cells used in ChIP studies. Values are means ± SEM of levels obtained in triplicate PCR reactions. Data are representative of 2 independent experiments. * indicates a significant difference (P<0.05) from the vehicle group as determined using a two-tailed T-test.
expression within the short 4 h time frame of the experiment (Fig. 3.9B). In addition, the three cis-regulatory elements that showed reduced H4-Ac also displayed an increased presence of NCOR and PPARα as detected by ChIP (Fig. 3.9C and D). Taken together, these data are consistent with the notion that PPARα functions via a ligand-dependent mechanism to directly repress IFN-γ production by stabilizing the NCOR-containing co-repressor complex at select cis elements at the Ifng locus.

3.4.4. PPARα also Limits IFN-γ Production by Male NKT and CD8+ T Cells

IFN-γ is not only produced by CD4+ T cells, but also in large amounts by cytotoxic CD8+ T lymphocytes, NK, and NKT cells (381). It has also been reported that these cell types exhibit a higher production of IFN-γ in females than in males (272, 279, 382). To gain insights into the breadth of regulation of IFN-γ production by PPARα, we stimulated splenocytes from male and female WT and PPARα−/− mice with PMA/ionomycin and examined IFN-γ responses by NK and NKT cells by intracellular cytokine staining. We found that the frequencies of IFN-γ-producing NK cells did not differ between WT and PPARα−/− groups in either sex, though there was a tendency for a higher frequency of IFN-γ-positive cells in the male PPARα−/− group (Fig. 3.10A). On the other hand, IFN-γ responses to PMA/ionomycin were higher in male PPARα−/− as compared to WT NKT cells (Fig. 3.10B). This was evident only in males (Fig. 3.10B).

Next, we investigated IFN-γ production by male and female WT and PPARα−/− CD8+ T cells after in vitro stimulation with anti-CD3 and anti-CD28. In some experiments, CD8+ T cultures were also supplemented with the PPARα antagonist NXT-1120 or DMSO vehicle. We observed that male, but not female PPARα−/− CD8+ T cells produced higher levels of IFN-γ as compared to WT counterparts (Fig. 3.11A). Similarly, treatment with the antagonist enhanced
Fig. 3.10. PPARα negatively regulates IFN-γ production by NKT cells in males, but not females. Splenocyte mononuclear cells isolated from male WT and PPARα−/− mice were either left on ice or were stimulated with PMA/ionomycin in the presence of GolgiStop for 5 hours. Cells were stained with cell surface antibodies followed by intracellular staining for IFN-γ. NK cells were defined as CD8−TCRβ−Tetramer−NKp46+ (A) while NKT cells were defined as CD8−TCRβ−Tetramer+ (B). Left graphs show the mean + SEM frequency values obtained in individual mice (N=5/group), while right panels show representative intracellular staining. Data are representative of two independent experiments. * Indicates a significant difference (p<0.05) from the WT group using a two-tailed T-test.
Fig. 3.11. PPARα negatively regulates IFN-γ production by male, but not female, CD8⁺ T cells. (A) Total CD8⁺ T cells isolated from spleens of male WT and PPARα⁻/⁻ mice (N=5/group) were stimulated with anti-CD3 and anti-CD28 at the indicated concentrations. IFN-γ production was measured in culture supernatants after 24 h. (B) Total CD8⁺ T cells from male WT and PPARα⁻/⁻ mice were pre-incubated with 100 nM of the PPARα antagonist NXT-1120 prior to being stimulated with anti-CD3 and anti-CD28. Cytokine levels in culture supernatants were assessed after 36 h. (C) Total CD8⁺ T cells were stimulated with anti-CD3 and anti-CD28 for 2 h in the presence of 100 nM NXT-1120 or vehicle (added to cells 2 h prior to stimulation). Cells were then fixed and ChIP for H4-Ac was performed as described in Fig. 3 legend. Values are means ± SE of triplicate cultures or PCR reactions. Data are each representative of 2-3 independent experiments. * denotes a significant difference (P<0.05) from WT group and + denotes a significant difference (P<0.05) from the vehicle control group using a T-test (two-tailed).
IFN-γ production selectively by male CD8⁺ T cells above levels observed in the vehicle group (Fig. 3.11B, left panel). This enhanced IFN-γ production in antagonist-treated male CD8⁺ T cells correlated with a higher abundance of H4-Ac at the promoter and at most CNS sites examined at the Ifng locus (Fig. 3.11C, left panel). Again, the antagonist did not modulate IFN-γ production and if anything lowered H4-Ac levels in female WT CD8⁺ T cells (Fig. 3.11B and C, right panels). These results indicate that PPARα regulates IFN-γ production by male NKT, CD4⁺ and CD8⁺ T cells.

3.4.5. Treatment with NXT-1120 Protects against L. Monocytogenes Infection Exclusively in Males

IFN-γ signaling has been shown to be crucial for mounting cellular immune responses against intracellular pathogens including the gram-positive bacterium L. monocytogenes (383). Experimental infection of mice with this pathogen has been an instrumental model for deciphering the importance of IFN-γ in host immune responses (384). We therefore investigated the utility of the novel PPARα antagonist NXT-1120 to enhance IFN-γ production by innate and adaptive immune cells during infection with L. monocytogenes.

During experimental infection, L. monocytogenes primarily replicates in the liver and spleen and early IFN-γ production by NK and NKT cells in these organs is important for the initial control of the pathogen (171, 385). On the other hand, later-acting Th and cytotoxic T cells are crucial for sustaining IFN-γ production to support clearance of L. monocytogenes and to mediate long-term protective immunity against the pathogen (187, 384). To investigate the effect of NXT-1120 on IFN-γ production by NK and NKT cells, we infected male and female mice i.p. with 100,000 CFU of L. monocytogenes and treated mice twice daily with NXT-1120 (30 mg/kg) or vehicle control
starting 1 day prior to infection. We then harvested spleens from mice at 24 h, the time of the peak IFN-\(\gamma\) response. We found that NXT-1120 had the effect of enhancing IFN-\(\gamma\) responses by NKT, but not NK cells in male mice (Fig. 3.12A & B). This elevated IFN-\(\gamma\) production by male NKT cells as a result of NXT-1120 treatment was also associated with a higher level of this cytokine in the serum (Fig. 3.12C) and a lower bacterial load in the spleen (Fig. 3.12D). Differences in these measures between NXT-1120 and vehicle groups were not apparent in female mice (Fig. 3.12A-D).

To investigate whether NXT-1120 also enhanced IFN-\(\gamma\) production by adaptive lymphocytes, we repeated infection studies, measuring the production of this cytokine by splenic CD4\(^+\) and CD8\(^+\) T cells at 7 days post-infection after recall stimulation \textit{ex vivo} with heat-killed \textit{L. monocytogenes}. For these studies, mice were infected with a sublethal dose of 20,000 CFU to avoid confounds associated with animal mortality that occurs between 4-5 days post-infection. Similar to findings for NKT cells, splenocytes isolated from vehicle-treated females tended to exhibit higher IFN-\(\gamma\) responses to stimulation with \textit{L. monocytogenes} in both the CD4\(^+\) and CD8\(^+\) T cell compartment as compared to vehicle-treated male cells (Fig. 3.12E-G). Treatment with NXT-1120 enhanced these IFN-\(\gamma\) responses exclusively in male mice (Fig. 3.12E-G).

Finally, we explored whether the effect of NXT-1120 in enhancing IFN-\(\gamma\) production would impact mouse survival during \textit{L. monocytogenes} infection. Male and female mice were infected with the LD50 dose of the pathogen, which was found in preliminary studies to be 100,000 CFU for males and 150,000 CFU for females (Fig. 3.12H). When challenged with these pathogen doses, NXT-1120 increased the percentage survival of male, but not female mice (Fig. 3.12I). In fact, treatment with the antagonist essentially abrogated the sex-difference in mouse survival upon infection with the same dose of 100,000 CFU of the pathogen (compare male
vehicle survival curve in Fig. 3.12I and female 100,000 CFU survival curve in Fig. 3.12H).

Altogether, these results suggest that the PPARα antagonist NXT-1120 is a novel adjuvant that can be used to boost innate and adaptive IFN-γ responses in males.
Fig. 3.12. NXT-1120 treatment enhances IFN-γ responses selectively in males during *L. monocytogenes* infection.

(A-D) Female and male C57BL/6 mice (N=3-5/group) were infected with 100,000 CFU *L. monocytogenes* and were treated twice daily by gavage with 30 mg/kg NXT-1120 or vehicle, starting 1 d prior to infection. Spleens were harvested after 24 h and processed for intracellular cytokine staining. Shown are the frequencies of IFN-γ+ cells in the gates of NK (CD8 TCRβ tetramer/NKp46+) (A) and NKT cell (CD8 TCRβ tetramer+) (B). (C) Shows IFN-γ levels in the serum and (D) shows bacterial load in the spleen after 48 h. (E-G) Female and male C57BL/6 (N=3-8/group) were infected with 20,000 CFU *L. monocytogenes* and were treated as described. After 7 d, spleens were harvested and cultured with heat-killed pathogen (1:1 ratio of bacterium:cell) for 24 h with GolgiStop added in the last 4 h of culture. Shown are the frequencies of gated CD4+ (E) and CD8+ (F) cells that produced IFN-γ and the levels of IFN-γ in culture supernatants (G). (H) Step-wise dose-escalation studies were performed to determine the LD50 dose of *L. monocytogenes*. Mice were infected i.p. with the pathogen and followed for survival to end-points for 14 days. Each curve represents one independent experiment that used 7-8 mice. (I) Female and male mice (N=18/group) were treated as described after infection with the LD50 dose and followed for survival to endpoints. Values are means +/- SEM of values obtained from individual mice (A-F) or triplicate cultures (G). Data are representative of 2-3 independent experiments. * Significantly different (P<0.05) from vehicle by T-test (two-tailed) (A-G) or Log-Rank test (I).
3.5. Discussion

It has been known for thirty years that females exhibit more robust IFN-γ responses to vaccination than males (2). Since this initial report, a number of hypotheses have been proposed to explain the female Th1 bias in the immune response including a higher production of IL-12 by APC and enhanced T cell responsiveness to this cytokine in females (239, 308). In addition, we previously observed that CD4+ T cells in male mice and humans have a lower intrinsic ability to produce IFN-γ compared to their female counterparts, which correlates with the higher expression of PPARα in male T cells (283). Our present studies identify Ifng as the gene target of PPARα regulation in male T cells and also provide evidence that this nuclear receptor inhibits IFN-γ mRNA expression in effector CD4+ and CD8+ T lymphocytes by decreasing histone acetylation at key regulatory cis elements in the Ifng locus. Furthermore, we show that treatment of mice with the small molecule PPARα antagonist NXT-1120 abrogates sex differences in IFN-γ production by NKT, CD4+ and CD8+ T cells during L. monocytogenes infection. These findings not only indicate that PPARα activity is the factor controlling sex differences in IFN-γ responses, but further suggest that this drug can be used as a sex-specific adjuvant to boost cellular immune responses in males.

PPARs are known to function both as transcriptional activators and repressors (386). The transactivating activity of PPARα has been demonstrated in studies in the liver where treatment with synthetic or diet-derived fatty acid ligands leads to the induction of >100 genes that are involved in the transport and oxidation of fatty acids (311). PPARα induces expression of these genes via heterodimerizing with the retinoic X receptor and binding to peroxisome proliferator response elements in gene promoter regions (386). On the other hand, the repressive activities of PPARs feature more in inflammation control and are associated with the downregulation of
inflammatory genes, a mechanism that has been termed ligand-dependent transrepression (386). Our finding that treatment with the PPARα ligand inhibited IFN-γ production by CD4+ T cells without increasing IL-4 or IL-17A production suggested that PPARα operates to repress the Th1 pathway directly. Furthermore, the finding that PPARα−/− CD4+ T cells expressed elevated mRNA levels of IFN-γ, but not T-bet or Eomes under conditions of IFN-γ neutralization implicated IFN-γ as the gene target of PPARα in this repression. This concept was further reinforced by the observation that fenofibrate induced the recruitment of PPARα to key cis elements in the Ifng locus and decreased IFN-γ mRNA levels without affecting T-bet expression.

Until recently, the molecular underpinnings of this transrepression mechanism were not understood. Insights into this molecular complexity have been provided by investigations of the effects of PPARγ ligands on immune cells (319, 320). In macrophages, treatment with rosiglitazone was shown to lead to the sumoylation of PPARγ. This post-translational modification associated with the enhanced recruitment of PPARγ and the stabilization of HDAC3/NCOR-containing corepressor complexes at the inos promoter, rendering the gene refractory to LPS stimulation (319). Similarly in T cells, repression of IL-17A production by the ligand pioglitazone was shown to associate with the enhanced presence of an NCOR-like Silencing Mediator for Retinoid or Thyroid-hormone receptors (SMRT) at the RORγT gene (320). Our finding that treatment of CD4+ T cells with fenofibrate induced the recruitment of PPARα to key regulatory elements at the Ifng gene (CNS-22, CNS-34, promoter) and increased the presence of NCOR at these sites, suggested that PPARα operates via a similar mechanism to repress Ifng expression in T cells. Further supporting the involvement of HDACs in this repression, we observed that fenofibrate treatment reduced H4-Ac at these same three cis regulatory elements.
One inconsistency between the effects of NXT-1120 and fenofibrate on histone acetylation was that NXT-1120 increased H4-Ac at most CNS sites, whereas fenofibrate influenced H4-Ac only at CNS-22, CNS-34, and the *Ifng* promoter. This difference likely relates to the distinct types of Th cells used in these ChIP studies and effect of the short-term drug treatments on T-bet expression in these cells. In the ChIP experiments using antagonist, naïve CD4+ T cells were used that exhibited low baseline T-bet expression and H4-Ac. Stimulation of naïve CD4+ T cells over 4 h in the presence of the antagonist had the effect of elevating T-bet expression, a necessary pre-requisite for inducing the broad-scale changes in H4-Ac at the *Ifng* locus (226). On the other hand, the ChIP experiments using fenofibrate were conducted using Th1-polarized cells that expressed high levels of T-bet at baseline. This expression was not impacted in the short-term by ligand treatment; hence no broadscale changes in H4-Ac were evident. Another reason why the influence of fenofibrate on H4-Ac was restricted to CNS-22, CNS-34, and the *Ifng* promoter is because these sites are considered to be pioneering elements within this gene locus, being readily accessible (i.e., showing DNAse-sensitivity) even in quiescent T cells (222). These three *cis* elements also contain binding sites for NF-κB, a transcription factor that is regulated by PPARα activity in T cells (283) and acts downstream of TCR and CD28 signals to induce *Ifng* gene expression (55). Together, these findings emphasize that at least some effects of PPARα on histone acetylation are direct and enacted through CNS-22, CNS-34 and the *Ifng* promoter, while others are indirect and occur through induced changes in T-bet expression.

The findings that PPARα regulates IFN-γ expression through the CNS-34 site may also explain why NK cell IFN-γ production was not significantly impacted by treatment with NXT-1120. While CNS-22 is involved in the establishment of H4-Ac and *Ifng* transcription in both NK cells and adaptive lymphocytes, the human homolog of CNS-34 (CNS-30) is only important in the regulation of IFN-γ in CD4+ T cells and NKT cells, but not in NK cells (222, 235). Thus,
the PPARα-dependent control of Ifng may be diluted in NK cells due to the lack of importance of CNS-34 in this regulation.

The negative regulation of histone acetylation at Ifng locus in male T cells is likely just one facet of a larger mechanism of IFN-γ expression regulation by PPARα. It was previously reported that PPARα also inhibits the DNA binding and transactivating activity of NF-κB p65 (283, 355, 380). Since these observations of NF-κB p65 were made using oligo-based DNA binding- or plasmid transactivation assays, they are distinct from the PPARα-dependent epigenetic changes described here. Our data here further suggest that these effects of PPARα on NF-κB are not due to alterations in the kinetics of IκB degradation. Future studies will investigate whether PPARα regulates the activity of kinases that phosphorylate specific serine residues on NF-κB p65 to alter the DNA binding or transactivation potential of this transcription factor (387).

Another potential aspect of Th1 control that remains to be addressed is the metabolic functioning of PPARα. If PPARα functions in T cells as it does in the liver, the absence of PPARα activity in PPARα−/− CD4+ T cells would be anticipated to lead to a decrease in the expression of genes involved in the oxidative phosphorylation of fatty acids and reduced expression of pyruvate dehydrogenase kinase, the gate keeper of pyruvate flux into the mitochondrion (311). The net effect of these gene expression changes would be shift towards an increased utilization of glucose and glycolysis for ATP production. Indeed, a similar metabolic shift from the oxidative phosphorylation of fatty acids to aerobic glycolysis has been shown to occur in naïve CD4+ T cells upon anti-CD3 and anti-CD28 stimulation and a recent study demonstrated that increased glycolytic activity is directly linked via GAPDH to an increased
stability of the IFN-γ transcript (198). Further experiments are needed to address the metabolic function of PPARα in T cells and effects of this nuclear receptor on IFN-γ mRNA stability.

Our in vivo treatment studies using NXT-1120 also provided the proof of concept that antagonizing PPARα activity may be a novel approach of boosting IFN-γ production in males to aid in the clearance of intracellular pathogens. In addition to its role in combating bacterial infection, the production of IFN-γ is crucial for anti-tumor immune responses and recombinant IFN-γ or IL-12 have been used with some success in clinical trials to treat various malignancies (388, 389). Because systemic administration of these cytokines is associated with side effects and dose-related toxicities (389, 390), alternative approaches have been pursued to enhance IFN-γ signaling including injection of tumors with adenovirus encoding IFN-γ (389) and administration of small molecule activators of STAT1 signaling (391). We propose that treatment with NXT-1120 may serve as another approach to boost IFN-γ responses and anti-tumor immune responses in the male sex. Future studies will test NXT-1120 in pre-clinical tumor models in mice and will investigate the safety of this compound in humans to establish whether this drug should be pursued as a male-specific treatment for cancer or chronic infection in immunocompromised individuals.
CHAPTER 4

Summary and Future Directions
4. Summary and Future Directions

4.1. Further Investigation of Sex-specific Th Immune Responses

It has been known for 30 years that female murine CD4+ T cells produce higher levels of the Th1 cytokine IFN-γ compared to their male counterparts upon stimulation (2). This sex difference in Th1 cytokine production was later observed in the context of EAE: the development of more severe EAE in female mice correlates with a higher IFN-γ production by myelin-reactive T cells (240, 392). More recently, peripheral blood mononuclear cells isolated from female MS patients were found to produce higher levels of IFN-γ compared to those of their male counterparts (3, 4), indicating that a sex bias in Th1 immunity may exist in humans as well. These mouse and human studies also reported that male Th cells are biased towards Th2; however, since these studies were conducted prior to the discovery of the Th17 subset, whether sex differences exist in Th17 immunity was not known.

In Chapter 2 of this thesis, we confirmed that CD4+ T cells from female SJL/J mice and women produce higher levels of IFN-γ. We also made the novel observation that male T cells have a higher propensity to make IL-17. Since both Th17 cells and IL-17 production are thought to be pro-inflammatory in EAE and MS, our findings raise a number of additional questions:

1) Is the Th1 bias responsible for the increased susceptibility to central nervous system autoimmunity in females?

2) What are the implications of the sex bias in Th1 and Th17 responses on inflammation and the health of the organism?

3) What is the evolutionary advantage of having a more robust immune response in females and why are PPARs involved in this regulation?
4.1.1. Does the Th1 Bias Underlie the Higher Susceptibility of Females to Develop Central Nervous System Autoimmunity?

MS affects women three times more often than men (251). Similarly, female mice of the SJL strain develop EAE with a higher incidence and severity compared to male mice (240, 254). It has been demonstrated through adoptive transfer studies that the enhanced EAE incidence in females is due to the higher encephalitogenicity of female Th cells (240). Furthermore, the finding of a correlation between the enhanced Th1 response and the development of more severe EAE in females lead to the hypothesis that the higher IFN-γ production is the cause of the worse disease in this sex (7, 240). However, both Th1 and Th17 cells are pathogenic in EAE (81), and some reports have indicated that Th17 cells are able to induce even more extensive neuronal injury than Th1 cells in the central nervous system during EAE (393). These latter findings raise the possibility that the more severe EAE in female SJL mice is due to some other property or properties of female Th cells.

To address this possibility, we performed preliminary adoptive transfer studies where we generated PLP p139-151-reactive IL-12-expanded Th1 and IL-23-expanded Th17 cell lines from male or female SJL/J mice and then adoptively transferred these cells into healthy female SJL/J recipients. Given that female SJL mice do not respond to HY male antigen, cross-sex transfers are possible in this mouse strain (258). Growing PLP p139-151-reactive T cell lines in the presence of IL-12 was shown to result in predominantly IFNγ-producing cells, while T cells cultured in the presence of IL-23 were shown to secrete large amounts of IL-17 as well as small amounts of IFN-γ (81). Regardless of the Th cytokine profile, we observed that female donor T cells consistently induced worse EAE symptoms compared to male T cells (Fig. 4.1A and B). We also did not observe overt sex difference in the cytokine profiles of male or female PLP
Fig. 4.1. Regardless of Th status, female PLP p139-151-reactive T cells are more highly encephalitogenic than male T cells. (A-D) Female and male SJL mice were immunized with PLP p139-151 and CFA. Ten days later, lymph node cells were harvested and cultured for 4 days with PLP p139-151 in complete media containing either IL-12 (Th1) or IL-23 (Th17) to expand PLP p139-151-reactive Th1 and Th17 cells, respectively. An equivalent number of PLP p139-151-reactive Th cells were transferred into naïve female recipients (N=8-10/group) mice. Mice were then followed for the development of classical EAE signs as previously (283). (A) shows the mean clinical scores over time in Experiment #1. (B) shows the mean clinical scores of mice over time for Experiment #2 with Th1 cell lines only. (C-D) show the levels of IFNγ and IL-17A produced by PLP p139-151 cells in culture prior to transfer in Experiment #1 as determined by ELISA (C) or by flow cytometric analysis of intracellular cytokine production for Experiment #2 (D). Values are means +/- SEM of individual mouse or + SEM of triplicate cultures. * indicates a significant difference (P < 0.05) from the male Th1 cells using an independent t-test (two-tailed).
p139-151-reactive T cell lines prior to transfer as determined either by intracellular cytokine staining or ELISA (Fig. 4.1C and D). These findings suggest that another aspect of female T cells may contribute to the initiation of more severe EAE.

Future plans include testing whether the higher encephalitogeneic potential of female T cells relates to the enhanced proliferation or survival of these cells. We have observed in Chapter 2 and Dunn et al. (283) that murine female T cells proliferate more than male T cells and secrete more IL-2 in vitro upon stimulation with anti-CD3 and anti-CD28 (283). Though we did not observe a significant sex difference in T cell proliferation in humans in Chapter 2, an enhanced T cell proliferative response in women has been observed by another group in a larger study of MS patients compared to healthy controls (394). Since we observed that sex differences in IFNγ cytokine production were not apparent when male and female PLP p139-151 T cells were cultured in the presence of IL-12 (Fig. 4.1C and D), it will be possible to use this adoptive transfer system to study sex differences in T cell proliferation. We will repeat our adoptive transfer studies using Th1-polarized cell lines except that PLP p139-151 T cell lines will be grown from congenic CD90.1 SJL male or female mice. We also intend to enumerate the myelin-specific donor cells with PLP p139-151 dextramers to ensure that the same number of antigen-specific male and female T cells are transferred into recipient mice. We will then label these cells with carboxyfluorescein succinimidyl ester (CFSE) before transferring them into male or female CD90.2 SJL/J recipients in order to track proliferation in vivo. At various times points after transfer, we will measure CFSE dilution and assess the extent of cell death by differentiating between the early (Annexin V+ propidium iodide−) and late (Annexin V+ propidium iodide+) stages of apoptosis in CD90.1 donor cells using flow cytometry. These results will indicate whether female T cells expand more robustly or experience less cell death than male T cells. Conducting transfers into mice of both
sexes will also provide insights into whether the sex of the host can influence T cell proliferation or survival.

### 4.1.2. What are the Implications of the Sex Bias in Th1 and Th17 on Immunity and Health of the Organism?

Since our adoptive transfer studies indicated that both IL-12-polarized Th1 and IL-23-polarized Th17 cells induce EAE, the implications of the sex bias of Th immunity on central nervous system inflammation are not clear. Although we did not observe a major difference in EAE clinical scores, others have shown that IL-12-driven EAE is associated with a higher infiltration of monocytes, whereas IL-23-driven EAE is associated with more neutrophilic inflammation (81). In addition, it is reported that Th17 cells cause more extensive neuronal injury than Th1 cells in this disease (393). Thus, it is possible when autoreactive T cells become activated in response to a natural infection or tissue damage in MS, that the subtle sex difference in Th1 cytokine production may differentially impact the degree of tissue damage in the brains of male and female patients. Indeed, it has been observed that men experience a more rapid progression of their disease once they develop MS (395). One magnetic resonance imaging study has shown that lesions in male MS patients are less inflammatory and more destructive in nature compared to those in female MS patients (396). However, more studies are needed to address whether myelin-reactive Th cells in male MS patients exhibit a Th17 bias and whether this is associated with more extensive neuronal pathology.

Although our adoptive transfer system highlighted that some other features of Th cells may drive the sex bias in EAE, there is evidence that the higher IFN-γ production in females may directly lead to autoimmune tendencies in mice. Mice that are deficient in the AU-rich element in
the 3’ UTR of the IFN-γ gene show an enhanced stability of IFN-γ mRNAs, higher production of IFN-γ by T lymphocytes, NK and NKT cells, and a chronic elevation of IFN-γ in the serum (159). Interestingly, this higher IFN-γ production led to enhanced levels of anti-nuclear antibodies in the circulation, which are also associated with autoimmune diseases such as SLE (159).

In addition to promoting autoimmunity, the higher Th1 immune responses in females also explain why women exhibit a lower prevalence of infection with bacteria and viruses such as *Mycobacterium tuberculosis* (397) and West Nile Virus (6), respectively. Sex differences in antiviral immunity have also been observed in mice (398). For example, male C57L/J mice are less able to mount a murine encephalomyelitis virus-specific Th1 response and clear the virus compared to their female counterparts (399). The higher Th1 production in females may also explain the sex differences in the incidence of cancer. It is documented that men are 1.3-fold more likely to develop cancer and exhibit a higher mortality due to cancer compared to women (5). Future studies should investigate whether tumor-specific T cells in females produce higher levels of IFN-γ than their male counterparts either during human malignancies or after administration of anti-tumor vaccines (400).

On the other hand, our finding that males are more prone to mount Th17 responses may explain why inflammation is more severe in males in certain diseases that exhibit a male predominance. For example, men are more likely to succumb to viral myocarditis (401) and male mice are more susceptible to virus-induced myocarditis than their female counterparts (402). Interestingly, one study indicated that the more severe disease in male mice correlated with a higher expression of IL-17 at both the mRNA and protein level (402). Another example of a disease that is more predominant in men and male rodents is hypertension (403). Recently, it has
been demonstrated that this more severe disease in males is due to T cell functioning: adoptive transfer of male T cells into Rag1−/− male mice led to the development of Angiotensin II-induced hypertension in the recipients, whereas female T cells were not prohypertensive (403). It was also shown that this elevated pathogenicity of male T cells associated with the elevated production of IL-17 (403). Allergic rhinitis is also a disease that exhibits a male predominance (404). Male patients with allergic rhinitis exhibit a higher number of IL-17-producing CD4+ T cells in peripheral blood than female patients (405). Together, these studies illustrate that the female Th1 bias and male Th17 bias may differentially impact the development of diseases beyond EAE and MS.

4.1.3. What is the Evolutionary Advantage of Having a More Robust Immune Response in Females and Why are PPARs Involved in This Regulation?

Our observations in Chapters 2 and 3 are in line with past literature that indicated that adaptive immune responses are more robust in females (2, 261). Why do sex differences exist in immune responses? Though it is thought that the evolution of immune genes is invoked by host-pathogen interactions, certain sex-specific host behaviours may further influence immune responses (406). Indeed, it has been hypothesized that intrinsic differences in reproductive strategies between males and females determine the level of investment of each sex in immunity (406). It is proposed that females invest more energy into offspring than males, and thus acquire longevity through the development of greater immune-competence (407). On the other hand, males are thought to devote more energy into acquiring larger body size to better compete for opportunities of sexual reproduction, and are therefore less immune-competent (407).
Evidence of the negative correlation between immunity and body size was demonstrated by a meta-analysis of reports of 106 mammalian species, including rodents and non-human primates (408). A larger body size in males was shown to positively correlate with susceptibility to parasitic infection and mortality and to negatively correlate with immunity (408). Another study showed that the stronger immunity in females enhances longevity, but is also associated with fitness costs in that the enhanced immune response can trigger autoimmunity. This concept of having an “optimal” immune response was demonstrated in an eco-immunological study of wild Soay sheep that have natural variations in antibody responses (409). In this sheep population, females exhibit elevated autoantibody responses and enhanced survival than male sheep during harsh winters (409). It was also observed that female sheep with the highest autoantibody levels had the most elevated antibody responses in general and higher survival rates, but were less likely to have had an offspring in the previous year, indicating a fitness cost of having a more robust immune response (409).

Although eco-immunological studies have drawn correlations between parental investment and immune-competence, whether varying parental investment leads to altered immunity was not tested until recently in a study of the insect species Kawanaphila nartee that is capable of varying parental investment (410). When environmental changes result in a decreased availability of food, it was shown that males increase parental investment as well as immune-competence (410) as measured by increased phenoloxidase activity in the haemolymph and an increased ability to encapsulate a foreign organism (410). Whether sex hormone changes accompany this decrease in food availability and affect immune-competence was not addressed. Nonetheless, these findings provide direct evidence that sex differences in immune responses may be driven by differences in reproductive investment (410).
The latter study also highlighted the influence of environmental factors such as nutrition on reproductive strategies and immune investment. Since PPARα is the major sensor of dietary fatty acids present in the environment (311), this molecule may have evolved overtime to fine-tune immune responses or liver metabolism based on energy availability in the organism. It is still unclear why PPARα expression was put under the control of androgens. We speculate that having a higher ability to metabolize fat gave males a greater ability to compete in sexual reproduction. The less robust immune response in males is likely just a by-product of putting PPARα under the control of androgens.

**4.2. Further Investigation of the Regulation of Ifng by PPARα**

We and others previously reported that PPARα<sup>−/−</sup> CD4<sup>+</sup> T cells express higher levels of IFN-γ compared to PPARα<sup>+/+</sup> CD4<sup>+</sup> T cells (283, 350). This phenotype was more prominent in males due to the higher expression of PPARα in male T cells (283). In Chapter 2 of this thesis, we expanded on these initial findings by demonstrating that PPARα is an intermediary molecule between androgens and Th1 immune responses: androgens induce PPARα expression in CD4<sup>+</sup> T cells, resulting in the suppression of IFN-γ production. Chapter 2 and 3 further presented data that advanced the understanding of how PPARα suppresses IFN-γ production. Treating male mouse CD4<sup>+</sup> T cells with either PPARα siRNA knockdown or with the small-molecule modulators (i.e. ligand fenofibrate or the antagonists NXT-592 and NXT-1120) affected IFN-γ production without influencing either IL-4 or IL-17 production, suggesting that PPARα controls Th1 production directly.

Our studies in Chapter 2 and 3 also furthered our understanding of the molecular mechanism of how PPARα limits Th1 cytokine production in male CD4<sup>+</sup> T cells. We observed
in Chapter 2 that knocking down PPARα using siRNAs in human CD4+ T cells led to an enhanced abundance of the permissive histone mark H4-Ac, and an increased recruitment of NF-κB at two CNS sites on the IFNG locus upon TCR/CD28 co-stimulation. In Chapter 3, we conducted additional studies in mouse CD4+ T cells and observed that ligand-activation of PPARα lead to a decreased abundance of H4-Ac, and elevated recruitment of PPARα and the co-repressor at CNS-34, CNS-22 and the promoter of Ifng. Conversely, we found that antagonizing PPARα using a novel small molecule was associated with global de-repression at the Ifng locus that was partly related to effects on T-bet expression. These findings indicated that PPARα represses IFN-γ gene expression by inducing histone modifications at the Ifng locus that are less conducive to transcription. Despite these important advances in understanding IFN-γ gene regulation by PPARα, several important questions remain unanswered:

1) What is the role of NF-κB in the PPARα-dependent regulation of Ifng expression?

2) Does PPARα regulate IFN-γ expression though other mechanisms?

4.2.1. What is the Role of NF-κB in the PPARα-dependent Regulation of Ifng Expression?

Following T cell activation, NF-κB activates IFN-γ production by directly binding to cis elements in the enhancer and promoter regions of the Ifng gene (133, 233, 234). NF-κB is normally sequestered by its inhibitor IκBα in the cytosol (132). Upon T cell activation, IκBα is phosphorylated by the IκB kinase and goes through ubiquitin-mediated degradation, leading to the nuclear translocation of NF-κB and activation of genes associated with T cell effector function and proliferation (132). We and others have demonstrated that PPARα inhibits the DNA binding and trans-activating activity of NF-κB p65 (283, 355, 380). Previous findings that nuclear extracts prepared from PPARα−/− mouse CD4+ T cells exhibited enhanced NF-κB p65 binding in an oligo-based DNA binding assay (283) suggested that some effects of PPARα on
NF-κB activity may be distinct from the PPARα-dependent changes on histone acetylation at the IFN-γ locus. Thus, how PPARα regulates NF-κB activity is still not fully understood and needs to be further addressed.

Our findings in Chapter 3 showed that there was no difference in the degradation of total IκB between male mouse PPARα−/− and PPARα+/− CD4+ T cell upon anti-CD3 and anti-CD28 stimulation by western blot analysis. This indicated that the control of NF-κB activity by PPARα may be downstream of this signaling event. We speculate that PPARα may regulate NF-κB activity in mice by modulating the activity of NF-κB kinases that alter the DNA binding or transactivation potential of NF-κB through phosphorylation (387). In future experiments, we will stimulate PPARα+/+ and PPARα−/− male naïve CD4+ T cells with anti-CD3 and anti-CD28 in the presence of a cross-linking antibody for various time points, isolate both cytosolic and nuclear extracts from these cells, and examine the state of NF-κB phosphorylation using western blots. Since NF-κB is phosphorylated at various serine residues by different NF-κB kinases (387), antibodies will be used to distinguish amongst the phosphorylation variants. If we find that the phosphorylation of NF-κB is regulated by PPARα, we will further investigate this interaction by conducting co-immunoprecipitation experiments with PPARα and the NF-κB kinase of interest.

4.2.2. Does PPARα Regulate IFN-γ Expression through Other Means?

We observed that male PPARα−/− CD4+ T cells expressed elevated IFN-γ at both the protein and mRNA levels as compared to PPARα+/+ CD4+ T cells. We then showed that these differences in IFN-γ mRNAs between PPARα−/− and PPARα+/+ CD4+ T cells correlated with changes in histone acetylation at the Ifng locus. However, these differences in IFN-γ mRNA expression between male PPARα+/+ and PPARα−/− CD4+ T cells may also be due to effects of PPARα on the stability of IFN-γ...
γ mRNAs. IFN-γ mRNAs turn over quickly (i.e. within the first few hours) in quiescent T cells (159); however, the mRNAs become more stable upon activation of T cells with anti-CD3 and anti-CD28 (192) or upon the addition of IL-12 (195, 411). This change in mRNA stability is regulated through ARE in the 3’ UTR of the transcript (412). Naïve mice that lack the ARE region on the 3’UTR in the Ifng transcript in all cells exhibit highly stable IFN-γ mRNAs and produce elevated levels of IFN-γ in NK, NK, CD4+ and CD8+ T cells (159). The stability of IFN-γ mRNAs is known to be regulated through the activation of p38 (196) and certain RNA-binding proteins (193, 198). Nuclear receptors can also regulate the stability of IFNs. For example, treatment of a mouse fibroblast cell line with glucocorticoids, which are ligands of the glucocorticoid receptor, results in the destabilization of the IFNβ transcript in these cells (412).

To explore whether PPARα regulates IFN-γ mRNA stability, we will stimulate PPARα+/+ and PPARα+/− CD4+ T cells with anti-CD3 and anti-CD28 in the presence of actinomycin D, an antibiotic that interferes with the elongation of RNA polymerase. We will then collect mRNA at different time points after activation, measure transcript levels with real-time PCR, and analyze the abundance of IFN-γ transcripts over time to determine the half-life of the transcript. Given the relationship between PPARα and metabolism, it would also be interesting to explore the link between PPARα, glycolytic metabolism and IFN-γ mRNA stability (see section 4.3.1 below).

4.3. Metabolic Functions of PPARα

Compared to the immune functions of PPARα, the metabolic functions of this nuclear receptor have been studied more extensively (310, 328, 413). This metabolic function of PPARα was revealed by studies in the liver that showed that treatment of PPARα+/+ mice with either Wy14643 or dietary fatty acids induced PPARα expression that associated with increased hepatic
gene expression (333, 414). On the other hand, PPARα−/− mice not only exhibit enhanced plasma fatty acids but also greater hypoglycemia as compared to PPARα+/− counterparts under conditions of fasting (334, 415, 416). It was found that these metabolic defects in PPARα−/− mice related to the function of PPARα in inducing fatty acid oxidation (311) and inhibiting glucose metabolism in the liver (335). Although PPARα is most abundant in the liver, it is also expressed in the heart, kidney, intestine and brown adipose tissue, tissues that also exhibit high levels of fatty acid catabolism (315). However, whether PPARα also regulates cell metabolism in immune cells has not been yet explored. We have conducted some preliminary experiments to measure the metabolism of male PPARα+/− and PPARα−/− CD4+ T cells both under conditions of quiescence or after stimulation for 48 hours with anti-CD3 and anti-CD28. We measured the level of extracellular acidification rate (ECAR), an indicator of aerobic glycolysis, and oxygen consumption rate (OCR), an indicator of mitochondrial oxidative phosphorylation (OXPHOS), using the XF Analyzer from Seahorse Bioscience (Fig. 4.2A). We observed that after 48 hours of TCR/CD28 co-stimulation, both ECAR and OCAR levels were enhanced in stimulated PPARα−/− compared to PPARα+/− CD4+ T cells, whereas these differences were not present in quiescent cells (Fig. 4.2B and C). The implication of these findings on the role of PPARα in regulating IFN-γ production and T cell proliferation will be discussed in the following sections.

4.3.1. Does Metabolism Factor into the Regulation of IFN-γ Production by PPARα?

Upon activation, quiescent naïve T cells become metabolically active, increase glycolytic activity, and switch from the utilization of fatty acids to carbohydrates for ATP generation (417). Interestingly, an increase in glycolytic flux has been linked to optimal IFN-γ production; IFN-γ production is significantly compromised when glycolysis is inhibited in activated T cells (198). This is due to the fact that IFN-γ production is regulated by GAPDH, which functions as both a
Fig. 4.2. PPARα−/− male naïve CD4+ T cells exhibit higher levels of both OCAR and ECAR after anti-CD3 and anti-CD28 stimulation. (A) The cartoon shows the major metabolic pathways and read-outs of glycolytic activity through ECAR and mitochondrial oxidative phosphorylation through oxygen consumption rate, OCAR. (B-C) Naive CD4+ T cells isolated from PPARα+/+ and PPARα−/− male mice were stimulated with anti-CD3 and anti-CD28 for 48 hours before being analyzed with the XF Analyzer from Seahorse Bioscience as described in Chang et al (198). The basal levels of OCAR (B) and ECAR (C) are shown for either freshly isolated (0 h) or stimulated PPARα+/+ and PPARα−/− CD4+ T cells (at 48 h of culture). Values are mean + SEM of triplicate cultures. + indicates a significant difference (P < 0.05) from the PPARα+/+ group in the same stimulation condition using an independent t-test (two-tailed). G6P, glucose-6-phosphate; TCA, tricarboxylic acid cycle or the Krebs Cycle; ETC, electron transport chain; ROS, reactive oxygen species; NADH, nicotinamide adenine dinucleotide; ATP, adenosine triphosphate.
glycolytic enzyme and an RNA-binding protein that binds to the ARE in the 3’ UTR to destabilize the IFN-γ transcript (198). When T cells ramp up glycolysis upon T cell activation, GAPDH switches from its role as an RNA-binding protein to a glycolytic enzyme, and this switch results in increased stability of IFN-γ mRNAs. Hence, our finding that male PPARα−/− CD4+ T effector cells exhibited higher ECAR than PPARα+/+ counterparts upon TCR/CD28 co-stimulation raises the possibility that PPARα may regulate IFN-γ mRNA stability through effects on T cell metabolism.

In the future, we will test this hypothesis in the following studies. We will activate naïve CD4+ T cells from PPARα+/+ and PPARα−/− male and female mice with anti-CD3 and anti-CD28 in medium that contains pyruvate in the presence of either glucose or galactose. We will then measure IFN-γ mRNA stability and the extent of association of GAPDH with ARE at the 3’UTR at Ifng by chromatin immunoprecipitation. Cells cultured in galactose alone cannot generate energy through glycolysis and are instead forced to utilize OXPHOS (418, 419). As a result, culturing T cells with galactose instead of glucose may bypass any control imposed on IFN-γ functioning by changes in glycolytic flux and GAPDH functioning. Thus, if PPARα regulates IFN-γ stability by controlling T cell metabolism, we will see a difference in IFN-γ production between PPARα+/+ and PPARα−/− T cells cultured with glucose but not with galactose.

4.3.2. Does Metabolism Factor into the Control of T Cell Proliferation Play by PPARα?

We have observed that male, but not female PPARα−/− CD4+ T cells proliferate more robustly and produce higher levels of IL-2 as compared to PPARα+/+ counterparts in response to anti-CD3 and anti-CD28 stimulation (283). These data suggest that PPARα also works in males to regulate T cell proliferation, which requires ATP from both OXPHOS and glycolysis (198, 420,
It has been further shown that mitochondria-derived reactive oxygen species (ROS) that are produced as a by-product of OXPHOS induced IL-2 production by enhancing the nuclear translocation of NFAT (421). Given the connection between the production of IL-2, mitochondrial ROS and our finding of higher OXPHOS in PPARα<sup>-/-</sup> versus PPARα<sup>+/+</sup> CD4<sup>+</sup> T cells, future experiments will investigate whether the higher proliferation of PPARα<sup>-/-</sup> CD4<sup>+</sup> T cells is due to higher production of mitochondrial ROS and IL-2. We will determine whether PPARα<sup>-/-</sup> CD4<sup>+</sup> T cells produce more ROS than PPARα<sup>+/+</sup> CD4<sup>+</sup> T cells using the MitoSOX Red Mitochondrial Superoxide Indicator. We will also assess whether the difference in proliferation between male PPARα<sup>+/+</sup> and PPARα<sup>-/-</sup> CD4<sup>+</sup> T cells disappears in the presence of exogenous IL-2, or the addition of antioxidants (e.g. Mito-Vitamin E and N-acetyl-L-cysteine) to the medium that can scavenge ROS.

4.4. Can a PPARα Antagonist Be Used to Boost Male-specific IFN-γ Production in an Anti-tumour Mouse Model?

We observed that treatment with the PPARα antagonist NXT-1120 boosted IFN-γ production by CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and NKT cells in male mice and improved male mouse survival upon infection with <i>Listeria monocytogenes</i>. These studies provided the proof of concept that antagonizing PPARα activity is a novel way of boosting IFN-γ production in males. Given the broad importance of IFN-γ in immunity, these findings have implications that extend beyond bacterial infection. The production of IFN-γ is also vital in the generation of anti-tumor responses (388). NKT cells and NK cells produce IFN-γ upon recognition of tumors, which leads to the activation of macrophages and tumor cell killing. The lysis of tumor cells by NK and NKT cells and the production of IL-12 by macrophages together lead to the generation of tumor antigens, which prime adaptive tumor-specific immune responses (388). The presence of IFN-γ
and IL-12 also promotes a Th1 environment and primes cytotoxic T cell responses (388). IFN-γ production within the tumor induces MHC class I expression on tumour cells and enhances tumor immunogenicity (388). Hence, the use of this novel antagonist NXT-1120 may be useful in increasing the cellular immune responses in the context of cancer in males.

To address this question, we plan to utilize a mouse lung melanoma model where we transfer a C57BL/6-derived melanoma cell-line B16/F10 that has been transfected with whole chicken ovalbumin (B16-OVA) (422) into female and male C57BL/6 mice. Recipient female and male C57BL/6 mice will then be treated with the antagonist or vehicle by oral gavage at various times after the adoptive transfer. We will then profile the immune cells in the lungs and draining lymph nodes, and measure tumor size as well as the number of foci (423). In addition, in some experiments, we will co-transfer CD45.1 OTII TCR transgenic-CD4+ T cells, which recognize OVA p323–339 in the context of I-A\(^b\), or CD45.1 OTI TCR transgenic-CD8+ T cells, which recognize OVA p257-264 in the context of H2-K\(^b\), in order to assess OVA-specific IFN-γ production by T lymphocytes in the tumors, spleens and draining lymph nodes using OVA-specific tetramers and intracellular cytokine staining. Given our results in the \textit{L. monocytogenes} model, we expect that the antagonist will increase tumor antigen-specific IFN-γ cytokine production selectively by male T cells, leading to smaller tumors and better survival as compare to vehicle-treated males. Regardless of treatment, female mice will produce abundant quantities of tumor antigen-specific IFNγ, have smaller tumor sizes, and exhibit elevated survival as compared to males.

4.5. Conclusions

Our findings indicate that human CD4+ T cells exhibit a sex difference in the production of IFN-γ and IL-17A that are driven by androgen-mediated expressions of the nuclear receptors
PPARα and PPARγ, respectively. We also dissected the underlying molecular mechanism of how PPARα regulates Th1 immunity in male murine CD4⁺ T cells, and extended this dissection to other IFNγ-producing cells that include CD8⁺ T, NK, and NKT cells. Finally, we demonstrated the utility of a novel small molecule antagonist of PPARα, NXT-1120, to boost cellular immunity in male mice during infection with the classic Th1-associated pathogen *Listeria monocytogenes*. 
References


