Effects of Acute Alcohol Exposure on Post Burn Intestinal Immunity: Role of IL-23

Juan L. Rendon

Loyola University Chicago

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LOYOLA UNIVERSITY CHICAGO

EFFECTS OF ACUTE ETHANOL EXPOSURE ON POST BURN INTESTINAL IMMUNITY: ROLE OF IL-23

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CELL BIOLOGY, NEUROBIOLOGY AND ANATOMY

BY
JUAN L. RENDON
CHICAGO, ILLINOIS
MAY 2013
ACKNOWLEDGEMENTS

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# TABLE OF CONTENTS

**ACKNOWLEDGEMENT** iii

**LIST OF TABLES** vi

**LIST OF FIGURES** vii

**LIST OF ABBREVIATIONS** ix

**CHAPTER ONE: INTRODUCTION** 1
  - Hypothesis 2
  - Specific aims 2

**CHAPTER TWO: REVIEW OF LITERATURE** 6
  - Alcohol Intoxication and Burn Injury 6
  - Alcohol intoxication, Burn Injury and Gut Barrier Dysfunction 6
  - Introduction to Th17 Lymphocytes 7
  - IL-23, Th17 Lymphocytes and Intestinal Immunity 10
  - Regulation of Th17 Development and IL-17/IL-22 Production 13
  - Th17 Cells and Burn Injury 19
  - Protective Role of IL-22: Underlying Mechanism and Therapeutic Potential 24
  - Significance 30

**CHAPTER THREE: ETHANOL EXPOSURE SUPPRESSES BONE MARROW-DERIVED DENDRITIC CELL INFLAMMATORY RESPONSES INDEPENDENT OF TLR4 EXPRESSION** 32
  - Abstract 32
  - Introduction 33
  - Materials and Methods 36
  - Results 38
  - Discussion 47

**CHAPTER FOUR: THE ROLE OF ARYL HYDROCARBON RECEPTOR IN IL-23 DEPENDENT RELEASE OF IL-22 FOLLOWING ETHANOL EXPOSURE AND BURN INJURY** 53
  - Abstract 53
  - Introduction 54
  - Materials and Methods 56
  - Results 60
  - Discussion 74

**CHAPTER FIVE: IL-22 MODULATES GUT EPITHELIAL AND IMMUNE BARRIER FUNCTIONS FOLLOWING ACUTE ALCOHOL EXPOSURE AND BURN INJURY** 81
Abstract 81
Introduction 82
Materials and Methods 84
Results 87
Discussion 99

CHAPTER SIX: SUMMARY AND DISCUSSION 104
Ethanol and Burn Injury 104
Summary of Results 105
Suppressed Th17 Effector Functions, Gut Bacterial Translocation and Barrier Function 108
Future Directions 110
Clinical Implications 112

APPENDIX A: SUPPLEMENTAL DATA 114

APPENDIX B: SPECIFIC METHODS 117
Mouse Model of Ethanol Intoxication and Thermal Injury 118
Growing Bone Marrow Derived Dendritic Cells In Vitro 119
Cell Preparation from PPs 120
Tissue Sonication Procedure 121

REFERENCES 122

VITA 144
LIST OF TABLES

**TABLE 1**: Summary of TLR4 and TLR2 expression on DCs  
Page 44

**TABLE 2**: Percentage of T cells, dendritic cells and macrophages in PPs after EtOH intoxication and burn injury  
Page 60
LIST OF FIGURES

FIGURE 1: IL-23/Th17 Axis 12

FIGURE 2: Representative FACS plots of bone marrow-derived dendritic cell population 39

FIGURE 3: BM-DC cytokine release following ethanol exposure and LPS stimulation 41

FIGURE 4: TLR4 expression on DCs 45

FIGURE 5: TLR2 expression on DCs 47

FIGURE 6: EtOH and burn injury suppresses PP Th17 effector cytokines IL-17 and IL-22 62

FIGURE 7: PP IFN-γ and IL-2 are decreased following EtOH and burn injury 64

FIGURE 8: PP IL-23 75

FIGURE 9: EtOH exposure and burn injury does not influence IL-23 receptor expression67

FIGURE 10: IL-23 specifically restores PP IL-22, but not IL-17, following EtOH intoxication and burn injury 69

FIGURE 11: AhR modulates IL-23 dependent restoration of IL-22 following combined injury 71

FIGURE 12: IL-17 requires intracellular calcium signaling following EtOH and burn injury 73

FIGURE 13: Intestinal tissue IL-22 is decreased following EtOH and burn injury 88

FIGURE 14: Increased gut permeability one day post EtOH exposure and burn injury 89

FIGURE 15: Decreased intestinal AMP expression one day post EtOH and burn injury 91

FIGURE 16: Increased Gram-negative bacterial growth one day after EtOH exposure and burn injury 93
FIGURE 17: IL-22 treatment prevents increased gut permeability 95

FIGURE 18: Treatment with IL-22 prevents the decrease in intestinal AMP expression one day post EtOH exposure and burn injury 96

FIGURE 19: IL-22 treatment prevents increased Gram-negative bacterial growth in the intestine 98

FIGURE 20: Schematic of Th17/gut mucosal interactions 107
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>Alcohol/Ethanol</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer’s patch</td>
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<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>ROR</td>
<td>Retinoic acid-related orphan receptor</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>Mφ</td>
<td>Macrophage</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>B. fragilis</td>
<td>Bacteroides fragilis</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>Klebsiella pneumoniae</td>
</tr>
<tr>
<td>C. albicans</td>
<td>Candida albicans</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut associated lymphoid tissue</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL-23R</td>
<td>IL-23 receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>CFB</td>
<td>Cytophaga-flavobacter-bacteroidetes</td>
</tr>
<tr>
<td>SFB</td>
<td>Segmented filamentous bacteria</td>
</tr>
<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>FICZ</td>
<td>6-formylindolo[3,2-b]carbazole</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>ILC22</td>
<td>IL-22 producing innate lymphoid cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>LTi</td>
<td>Lymphoid tissue-inducer cell</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>CLP</td>
<td>Cecal ligation and puncture</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sodium sulfate</td>
</tr>
<tr>
<td>BM-DC</td>
<td>Bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>MLN</td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>1,2-diacylglycerol</td>
</tr>
<tr>
<td>Reg</td>
<td>Regenerating</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
</tr>
<tr>
<td><em>C. rodentium</em></td>
<td><em>Citrobacter rodentium</em></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
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CHAPTER ONE
INTRODUCTION

Each year over 50,000 hospitalizations and 5,000 deaths within the United States are associated with burn injury [1]. Multiple studies demonstrate that greater than 50% of adult burn patients have a measurable blood alcohol/ethanol (EtOH) level at the time of hospital admission [2-11]. These patients are shown to have an increased risk of infection and higher mortality when compared to burn patients without EtOH exposure at the time of injury [2-6, 11-13]. Yet, the exact role of EtOH on post burn pathogenesis remains to be elucidated.

Published data from our laboratory have demonstrated that acute EtOH intoxication prior to burn injury exacerbates intestinal T cell immune suppression and enhances bacterial translocation [14-21], though the mechanism responsible for these observations remain unknown. Peyer’s patches (PPs) are important intestinal secondary lymphoid organs that play a critical role in T cell immunity, as well as gut bacterial translocation and its containment. Current evidence suggests that T helper (Th) 17 lymphocytes are crucial in regulating mucosal immunity. Within the gut, Th17 lymphocytes help maintain intestinal immune homeostasis and protect against extracellular bacteria [22-26]. Moreover, recent reports demonstrate that Th17 lymphocytes, in concert with dendritic cells (DCs), promote epithelial cells to release antimicrobial peptides (AMPs) into the intestinal lumen [27-30].
Interleukin (IL)-23, a cytokine released by mucosal antigen presenting cells, plays a central role in Th17 differentiation and intestinal AMP release. IL-23 acts via signal transducer and activator of transcription (STAT)-3 to maintain upregulation of transcription factor retinoic acid-related orphan receptor (ROR)-γt, which is indispensible in the expression of Th17 effector cytokines IL-17 and IL-22 and the release of AMPs from epithelial cells [31-34].

Gaining an understanding of the mechanisms that contribute to gut immune suppression in a murine model of EtOH intoxication and burn injury may reveal valuable therapeutic targets for patients who sustain burn injury after EtOH exposure. The studies performed herein utilize a murine model of EtOH exposure and scald burn injury to examine the effects of acute EtOH intoxication on post burn Th17 effector function within PP, explore the role of IL-23 in mediating Th17 effector function and establish a role for IL-22 in modulating AMP expression, gut permeability and bacterial growth following combined insult.

**Hypothesis**

EtOH intoxication prior to burn injury perturbs Th17 effector functions within PPs and intestinal tissue, contributing to decreased gut immune function and increased gut damage.

**Specific Aim 1**

Demonstrate the effects of acute EtOH exposure on Th17 effector cytokines IL-17 and IL-22 in PPs and intestinal tissue after burn injury.
**Rationale**

IL-17 and IL-22 are cytokines central to the effector functions of Th17 cells [23, 28, 35-37]. IL-17 is crucial in the protection against gut pathogens [22-25, 38], while IL-22 is critical in preserving epithelial barrier function as well as enhancing mucosal immunity through induction of AMP release from epithelial cells [23, 29, 39]. Moreover, perturbation of IL-23 following EtOH exposure and burn injury may negatively impact Th17 effector function, facilitating gut leakiness and subsequent bacterial translocation. Therefore, the experiments in this aim will determine if EtOH exposure prior to burn injury results in altered IL-23 and whether this impacts Th17 effector function within the gut. Changes in IL-23 and subsequent alterations in IL-17 and IL-22 may give insight to possible mechanisms by which EtOH intoxication prior to burn injury leads to increased bacterial translocation across the epithelial barrier.

**Specific Aim 2**

i) Determine if modulation of IL-23, by administration of recombinant IL-23, *in vitro*, ameliorates alterations in PP Th17 effector functions following EtOH intoxication and burn injury.

ii) Define the role ROR-γt and the aryl hydrocarbon receptor (AhR) on IL-23-dependent modulation of IL-17 and IL-22 following EtOH intoxication and burn injury.

**Rationale**

IL-23, a member of the IL-12 family, is heterodimeric cytokine composed of the p19 and p40 subunits, the p40 subunit is shared by IL-12 while the p19 subunit is unique to IL-23 [34]. Previous studies indicate that IL-23 is required for the expression of Th17
cytokines IL-17 and IL-22 [31, 40-42]. Many of these same studies illustrate that *in vitro* and *in vivo* reconstitution of IL-23 with recombinant IL-23 (rIL-23) effectively restores the IL-23/Th17 axis [31, 40, 41]. Thus, experiments in this aim are designed to test whether reconstitution of IL-23 *in vitro* ameliorates Th17 effector functions following EtOH intoxication and burn injury. Successful restoration of Th17 responses by modulation of IL-23 may give insight into a possible mechanism by which to prevent Th17 suppression and increased bacterial translocation following EtOH and burn injury.

Binding of heterodimeric IL-23 to its receptor complex, activates Jak2-mediated phosphorylation of tyrosine residues on the cytoplasmic domain of the IL-23R subunit and subsequent phosphorylation of STAT3 proteins, which homodimerize and translocate to the nucleus to upregulate expression of ROR-γt [43]. More recent implicate the aryl hydrocarbon receptor (AhR) in regulation of IL-22, but not IL-17 [44, 45]. Most interesting, it remains unclear whether IL-22 is dependent on ROR-γt. These studies highlight the crucial role of AhR in expression of IL-22, but disagree as to whether ROR-γt is necessary for the expression of IL-22. Moreover, neither study offers a mechanism by which AhR modulates IL-22. Therefore, the experiments proposed in this aim will determine the mechanism by which rIL-23 mitigates the IL-23/Th17 axis following EtOH intoxication and burn injury.

**Specific Aim 3**

Elucidate the role of IL-22 in intestinal gut barrier damage and epithelial cell antimicrobial peptide (AMP) expression following EtOH exposure and burn injury.
**Rationale**

Our lab has previously demonstrated increased gut damage following combined EtOH exposure and burn injury [20, 21, 46]. Recent findings from murine models of EtOH induced liver damages demonstrate protective effects of IL-22 [47]. Together these data support the use of rIL-22 *in vivo* to test its role in gut damage and AMP expression following EtOH and burn injury. In these experiments, we will test the effects of IL-22 on gut leakiness following combined insult by measuring gut permeability *in vivo* [21]. In regards to AMPs, Reg3γ and Reg3γ have been demonstrated to be crucial to gut immunity [48]. Our experiments will test the effects of combined insult on AMP expression as well as determine whether administration of IL-22 *in vivo* ameliorates AMP expression.
CHAPTER TWO

REVIEW OF LITERATURE

Alcohol Intoxication and Burn Injury

An estimated more than one million burn injuries are reported yearly within the United States [1]. These injuries translate into an approximate 500,000 emergency room visits and 40,000 hospitalizations annually [1]. Interestingly, greater than 50% of these injuries occur under the influence of alcohol/ethanol (EtOH) intoxication [2-11]. Furthermore, burn victims who sustain injury post EtOH exposure exhibit significantly higher rates of morbidity and mortality than patients without EtOH exposure at the time of injury [2-6, 11-13]. Despite clear correlations between post burn pathogenesis and EtOH intoxication, the mechanism by which EtOH enhances post burn morbidity and mortality remains to be elucidated. Previous data suggest that gut pathogens and their products may play a pivotal role in the subsequent development of sepsis and multiple organ failure reported in burn and trauma patients [6, 14, 49-58]. In line with this hypothesis, our laboratory has demonstrated increased intestinal tissue damage, leakiness and bacterial translocation as well as intestinal T lymphocyte suppression following EtOH intoxication and burn injury [14-21].

Alcohol Intoxication, Burn Injury and Gut Barrier Dysfunction

Under healthy conditions the intestine maintains a barrier which prevents intestinal bacteria and their products from reaching systemic organs. However, this barrier is compromised following insult, including EtOH exposure, burn and other traumatic injuries [12, 14, 59-61]. In such instances, intestine-derived bacteria and their products
present a major clinical problem to burn/trauma victims, patients with EtOH exposure as well as ICU patients and patients developing multiple organ failure. Several lines of evidence indicate that burn patients who sustain injury under the influence of EtOH have significantly longer hospital stays, require more surgical procedures and have more complications compared to patients who sustain similar extent of burn injury but were not intoxicated at the time of injury [2-4, 12, 13]. Moreover, intoxicated patients are more likely to die of smaller burns [2]. Although, both chronic and acute alcohol intoxication are likely to increase susceptibility of injured host, studies have shown that the majority of alcohol-exposed burn-injured patients have consumed alcohol on an acute basis before injury [4, 13]. Utilizing rodent models of acute EtOH intoxication and burn injury, we examined the influence of acute ethanol exposure on post burn intestinal tissue damage and leakiness. We found that as compared to either ethanol intoxication or burn injury alone, combined insult causes intestinal tissue damage, leakiness and bacterial translocation [14, 16, 18, 20, 21, 46]. Thus, delineating the mechanism by which EtOH intoxication alters gut barrier integrity and function following burn injury warrants further investigation.

**Introduction to Th17 Lymphocytes**

T helper (Th) cells have long been recognized as vital components of the adaptive immune system [62, 63]. Classically, T helper cells are generated following antigen presentation by antigen presenting cells (APCs), including dendritic cells (DCs) and macrophages (Mϕ), in secondary lymphoid tissues [64]. Until recently, CD3⁺CD4⁺ T helper cells were divided into either cell mediated Th1 (Interferon-γ producing) or
humoral Th2 (Interleukin-4 producing) responses. The effector cytokines produced by Th1 and Th2 were further predicted to regulate the development and function of the alternate subset [62]. However, the Th1-Th2 hypothesis failed to accommodate the more recently described IL-17 producing CD3⁺CD4⁺ Th17 cells [65]. Today, the major T helper cell subsets include Th1, Th2, Th17 and T regulatory (Treg) cells, each of which produce specific effector cytokines under unique transcriptional regulation [66]. Specifically, Th17 cells produce effector cytokines IL-17, IL-21 and IL-22 under the regulation of retinoic acid-related orphan receptor ROR-γt [36, 67, 68].

IL-17 cDNA was originally cloned from murine hybridomas, where it was noted to share 58% homology with open reading frame 13 from the T lymphotropic Herpesvirus saimiri [69, 70]. However, IL-17 was not identified as a T helper cytokine until 2000, when Infante-Duarte et al. demonstrated IL-17 production from T helper cells in response to microbial lipopeptide challenge [65]. IL-22 was first cloned from T cells and found to share 22% amino acid identity with IL-10 [71]. Further analysis confirmed that IL-22 expression required and induced activation of the signal transducer and activator of transcription (STAT) proteins [71]. Co-expression of IL-17 and IL-22 eventually led to the recognition of these molecules as effector cytokines of Th17 cells [28, 72].

Th17 effector cytokines IL-17 and IL-22 have been immensely studied since the early 2000s [30, 36, 73]. Dysregulation of Th17 effector cytokines IL-17 and IL-22 has been implicated in psoriasis [39, 74], rheumatoid arthritis [32, 75], and both forms of inflammatory bowel disease (IBD) [76-78]. Although, Th17 lymphocytes were first
described as orchestrators of neutrophil recruitment and activation and as key players in chronic inflammation and autoimmunity [36, 79], more recent evidence suggest that Th17 lymphocytes and their effector cytokines, IL-17 and IL-22, play a crucial role in maintaining mucosal immunity. While IL-17 has long been recognized as a proinflammatory cytokine essential for the recruitment of neutrophils and protection against extracellular pathogens [36], IL-22 does not appear to affect immune cells but rather regulates the functions of epithelial cells [48].

Th17 lymphocytes have been shown to protect against various pathogens, including *Bacteroides fragilis* (*B. fragilis*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Candida albicans* (*C. albicans*) [22-26]. Specifically, IL-17 was first recognized as protective against extracellular infections when IL-17R knockouts demonstrated a decreased ability to recruit neutrophils in response to a *K. pneumoniae* infection [26]. Further studies confirmed IL-17’s role in neutrophil accumulation and protection against extracellular bacteria, when it was found to be necessary for *in vivo* formation of intra-abdominal abscesses in response to *B. fragilis* [22]. Thus, Th17 lymphocytes are crucial to the containment of various gut pathogens, including *B. fragilis* and *K. pneumoniae*, which if not contained properly may become sources of local or systemic infection following traumatic injury, such as alcohol intoxication and burn injury [22-25]. Moreover, Th17 lymphocytes have been proposed to enhance tight junction formation through extracellular signal-related (ERK) mitogen-activated protein kinase (MAPK) signaling and subsequent upregulation of tight junction proteins [80].
Contrary to IL-17, IL-22 does not act on immune cells, but rather on epithelial cells of the skin, pancreas, intestine, liver, lung and kidney [27, 48], where it is involved in chemotaxis [81], antimicrobial expression [27-30], tissue repair [73, 82] and epithelial cell survival [83, 84], proliferation [23] and differentiation [39]. Together these findings suggest that Th17 effector cytokines must be tightly regulated in order to effectively maintain mucosal immunity and barrier function.

**IL-23, Th17 Lymphocytes and Intestinal Immunity**

Under healthy conditions the gut epithelium and its associated lymphoid tissue (GALT) provide both a physical and immunological barrier to effectively regulate the translocation of intestinal-derived bacteria and their products. In the gut, APCs, including DCs, continuously sample luminal content to effectively maintain innate and adaptive components of the intestinal immune system and regulate intestinal epithelial barrier function [85-87]. Following the uptake of antigen, DCs shuttle microbes to regional lymphoid tissue, such as PPs, and present antigen to naïve T lymphocytes to generate Th1, Th2, or Th17 responses [64]. Though Th1 and Th2 lymphocytes have long been recognized as vital components of the adaptive immune system [62, 63] and have been studied in a variety of injury and inflammatory conditions, Th17 lymphocytes remain vastly unexplored. In regards to gut immunity, Th17 lymphocytes have long been implicated in both forms of IBD, Chron’s disease and ulcerative colitis [76-78]. Given their central role in mediating gut mucosal immunity and barrier function, Th17 lymphocytes should be explored in the context of injury, such as EtOH intoxication and burn.
Recent data suggest that IL-23, a pro-inflammatory cytokine secreted predominantly by DCs, is critical for the differentiation of Th17 lymphocytes [31-34]. IL-23, a heterodimeric cytokine and member of the IL-12 family, plays a critical role in the development, expansion and survival of Th17 lymphocytes [31-33, 67, 88]. Binding of IL-23 to its receptor complex, IL-12Rβ1 and IL-23R, on differentiating T helper lymphocytes activates STAT3 to maintain upregulation of transcription factor ROR-γt, which is indispensable in the production and secretion of Th17 effector cytokines IL-17 and IL-22 (Figure 1) [89-93].
Figure 1. IL-23/Th17 axis
Regulation of Th17 Development and IL-17/IL-22 Production

The development of Th17 cells has been extensively studied and reviewed in mice and humans [35-37, 68]. Murine models demonstrate that Th17 lymphocytes begin differentiation under the control of TGF-β and IL-6 [91-93]. Alone TGF-β, induces the expression of Foxp3 and differentiation of Tregs. However, IL-6 induces the expression of IL-21 in a STAT3 dependent manner [94]. Subsequently, IL-21, along with IL-6 and TGF-β, further amplifies STAT3 activation and prompts downstream expression of ROR-γt [67, 88, 95]. In this manner, IL-21 acts in an autocrine loop on differentiating Th17 cells, similar to the actions of interferon (IFN)-γ and IL-4 on Th1 and Th2 cells, respectively [88, 94, 96]. IL-21 has further been suggested to exert its actions by inducing expression of the IL-23 receptor (IL-23R) [67, 88, 95] and suppressing Foxp3 expression [94, 96]. Lastly, IL-23 expands and stabilizes Th17 cells through further activation of STAT3 driven ROR-γt expression and subsequent production of Th17 effector cytokines IL-17 and IL-22 (Figure 1) [31-33, 89].

IL-23, a member of the IL-12 family, is composed of the p19 and p40 subunits; the p40 subunit is shared by IL-12 while the p19 subunit is unique to IL-23 [34, 97]. IL-23 is synthesized and produced by a variety of cells, including DC and Mϕ [34, 97]. Binding of IL-23 to its receptor complex, IL-12Rβ1 and IL-23R, results in Jak2-mediated phosphorylation of tyrosine residues on the cytoplasmic domain of the IL-23R subunit [43]. While 12Rβ1 is also part of the IL-12 receptor, IL-23R is unique to the IL-23 receptor complex. Phosphorylated tyrosine residues serve as docking sites for the STAT3 proteins, which in turn become phosphorylated [43]. Once phosphorylated, STAT3
proteins homodimerize and translocate to the nucleus to upregulate downstream signaling pathways, including the expression of Th17 hallmark transcription factor ROR-γt [43, 67, 88, 95, 98].

Recently, hypoxia-inducible factor (HIF)-1 was implicated in the regulation of Th17 differentiation [99]. HIF-1 regulates the metabolic switch from oxidative phosphorylation to aerobic glycolysis during hypoxic conditions [100], and is a target gene of activated STAT3 [98]. Dang et al. found increased HIF-1 expression in Th17 cells and demonstrated that naïve T cells from CD4+ specific HIF-1−/− mice failed to differentiate into IL-17 expressing cells or upregulate mRNA expression of IL-17 and IL-23R [99]. Further experimentation revealed that HIF-1 is necessary for STAT3 dependent expression of ROR-γt and that CD4+ specific HIF-1−/− mice are resistant to experimental autoimmune encephalitis, a Th17 dependent process. Together, in vitro and in vivo data supported a role for HIF-1 in modulation of Th17 cells. Chromatin immunoprecipitation analysis of the ROR-γt binding site on the IL-17 promoter region showed localized binding of ROR-γt, HIF-1 and transcription factor p300, which modulates HIF-1 mediated gene expression [99]. Collectively, these results demonstrate that Th17 differentiation is regulated by changes in cellular metabolism, including hypoxia.

Given their role in maintaining mucosal immunity, it is not surprising that recent investigation suggest that Th17 cells may be regulated in the gut through host-commensal bacteria interactions [101-103]. Ivanov et al. took advantage of ROR-γt<sup>gfp/+</sup> mice to demonstrate that after birth TCRβ<sup>+</sup>CD4<sup>+</sup>IL-17<sup>+</sup>GFP<sup>+</sup> cells appear in the small intestine lamina propria at day 25, and eventually represent the majority of IL-17 producing cells
by day 33 [102]. These data suggest that the presence of Th17 cells depends on colonization of the intestine with commensal bacteria. This notion is further supported by results indicating that germ-free mice, which are devoid of bacteria and fungi, lack Th17 cells. Colonization of germ-free mice with fecal homogenates from control mice induced Th17 cells in the gut lamina propria within two weeks after colonization [102]. Treatment of control mice with antibiotics known to affect commensal bacteria diminished the frequency of Th17 cells in the lamina propria. Through analysis of commensal bacteria from Th17 sufficient mice, the authors concluded that the presence of Th17 cells in small intestine lamina propria correlated with the quantity of Gram-negative cytophaga-flavobacter-bacteroidetes (CFB) bacteria present in the gut [102]. This group later demonstrated that colonization of germ-free mice with segmented filamentous bacteria (SFB), Gram-positive spore-forming bacteria, also induced IL-17 and IL-22 producing Th17 cells in the gut lamina propria [101]. Wu et al. demonstrated that gut colonization of mice with enterotoxigenic B. fragilis induced expression of IL-17 in a STAT3 dependent manner [104]. Increased IL-17 was not seen with nontoxigenic B. fragilis or in CD4 STAT3-KO mice [104]. Together, these data highlight the role of host-commensal bacteria interactions in regulation of immune homeostasis. Moreover, the duodenum, through upregulation of Th17 chemokine CCL20, has recently been implicated in the regulation of Th17 immune cells. In a murine model of Staphylococcus aureus (S. aureus) sepsis, Esplugues et al. reported increased Th17 cells in the duodenum of S. aureus treated mice 3 days post bacterial injection, which was not noted in the spleen [105]. Further analysis of duodenal Th17 cells revealed that these cells exhibited a
suppressive phenotype, as marked by increased expression of IL-10 [105]. While the mechanism by which these Th17 cells acquire a suppressive phenotype remains to be elucidated, these data suggest that the gut is critical in maintaining equilibrium of Th17 cells, which are necessary to clear infection, but are immunopathogenic in excess.

Most recently, the aryl hydrocarbon receptor (AhR) has been implicated in Th17 immunity, particularly in the regulation of IL-22 [44, 45, 106-108]. AhR is a ubiquitous transcription factor found in the cytoplasm of vertebrate cells. Upon ligand-binding, AhR translocates to the nucleus where it regulates gene expression. Ligands for AhR include dioxin and natural chemicals, including bacterial metabolites, derivatives of tryptophan and phytochemicals. Initially, Veldhoen et al. found selective expression of AhR in mouse and human Th17 cells, as compared to other T helper cells and further demonstrated that addition of 6-formylindolo[3,2-b]carbazole (FICZ), a high affinity ligand of AhR, greatly increased expression of IL-17 and IL-22 in vitro [106]. Moreover, AhR−/− T cells failed to respond to FICZ treatment, while retroviral transduction of AhR restored IL-22 expression [106]. Quintana et al. provided evidence suggesting that AhR controls Th17 differentiation in a ligand dependent manner. In these experiments, in vivo activation of AhR by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) reduced the frequency of IL-17+CD4+ cells, and induced the frequency of TGF-β secreting CD4+Foxp3+ Tregs, which were capable of attenuating experimental autoimmune encephalomyelitis (EAE) [108]. In line with Veldhoen et al., in vivo activation of AhR by FICZ boosted Th17 cell differentiation and exacerbated EAE [108].
As AhR drew attention in the field of Th17 immunity, more recent studies revealed that AhR plays a greater role in the modulation of IL-22, than IL-17 [44, 45, 107]. Two groups simultaneously demonstrated a subset of human T helper cells that produce IL-22 but neither IL-17 nor IFN-γ, namely, Th22 cells [44, 45]. Both demonstrated a vital role for AhR; however, these groups presented conflicting data regarding the role of ROR-γt in Th22 production of IL-22. Duhen et al. reported low expression of RORC (ROR-γt in humans) in their Th22 population and concluded that RORC is non-essential for Th22 development [44]. The authors further showed that de novo induction of Th22 cells required IL-6 and tumor necrosis factor-α. Conversely, Trifari et al. used siRNA to demonstrate that silencing of RORC or AhR diminished IL-22 production from memory CD4+ T cells, while IL-17 was only affected by silencing of RORC [45]. This group found that IL-1β and IL-23 are required for de novo induction of Th22 cells, while the presence of TGF-β induced IL-17 production [45]. The authors confirmed that FICZ treatment increases IL-22 production, yet provided protein and mRNA data suggesting that FICZ decreases IL-17 [45], which challenges data by Veldhoen et al. A third group, further confirmed increased IL-22 and decreased IL-17 in response to FICZ treatment, but also reported increased IL-22 following TCDD treatment, which Quintana et al. had demonstrated inhibits IL-17 but did not assess its effect on IL-22 [107]. Collectively, the consistent data demonstrating a role for AhR in expression of IL-22 and the discrepancies in the requirements for RORC and effect of FICZ/TCDD on IL-17 production support the notion that the requirements for IL-22 and IL-17 production are distinct. Regardless, of whether IL-22 and IL-17 are produced by
one cell type, Th17 cells, or two cell types, Th17 and Th22, further studies exploring the molecular mechanism by which AhR regulates IL-22 production are warranted.

There are multiple mechanisms by which AhR may control IL-22. One such possible mechanism is the interaction between STAT molecules and AhR, where STAT1 and STAT5 have been co-immunoprecipitated with AhR [109]. It is possible AhR and STAT3 interact to differentially regulated ROR-γt and expression of IL-17 and IL-22. Most recently, AhR induced Notch signaling has been implicated in the development of gut IL-22 producing innate lymphoid cells (ILC22), including natural killer (NK)p46 and lymphoid tissue-inducer (LTi) cells, as well as in the formation of postnatal lymphoid tissues [110]. While these data focus on innate producers of IL-22, the mechanisms involved may affect the development of adaptive IL-22 producing T helper cells as well as the secondary lymphoid tissues necessary for the differentiation of Th17 cells. In line with previous literature, Lee et al. found reduced production of IL-22 in gut related lymphoid organs and impaired response to C. rodentium infection in AhR−/− mice [110]. The authors also noted decreased ILC22 cells and the lack of cryptopatches and isolated lymphoid follicles in the small intestines of AhR−/− mice, which they attributed to the absence of AhR signaling. Administration of TCDD induced expression of Notch1 and Notch2, which was absent in AhR−/− mice [110]. Moreover, mice lacking expression of RBP-Jκ, the DNA-binding protein that associates with the intracellular regions of all Notch molecules to mediate transcriptional activity of Notch, demonstrated less ILC22 cells, as compared to wild-type mice [110]. Together these data suggest that the development of ILC22 cells is regulated by AhR mediated Notch activity, a mechanism
which may also be involved in the adaptive immune response. Further studies should test
the role of AhR and Notch in Th17 cell development as well as establish whether AhR
signaling produces differential immune responses when activated by endogenous versus
exogenous ligands.

**Th17 Cells, Ethanol and Burn Injury**

While the role of Th17 lymphocytes has been extensively studied in the context of
regulating infection, little work has focused on these cells in the context of injury.
Following traumatic injury, such as burn, there are global changes to the systemic
immune response, including immunosuppression and increased susceptibility to infection.
Traumatic injury, including burn, outcomes are further confounded by the presence of
EtOH intoxication, which is associated with increased susceptibility to infection and
mortality [12, 14-19, 49-51, 53, 111-115]. Burn trauma, regardless of EtOH exposure, is
associated with remote organ injury, affecting the lung [116, 117], kidney [118-120], gut
[61, 121-124] and bone marrow compartment [125-127] in human and animal studies.
Alteration in gut barrier function may facilitate the translocation of gut derived bacteria,
and/or their products, and contribute to the development of sepsis, systemic inflammatory
response syndrome and multiple organ dysfunction syndrome in critically-ill burn
patients [61, 123, 128]. Thus, it is imperative to investigate the interrelationship between
EtOH, burn, remote organ injury and immune suppression. Given their central role in
mediating mucosal immunity, including the gut and lungs, Th17 lymphocytes may play a
role in immune perturbations following EtOH intoxication and burn injury burn injury.
Murine models of burn injury demonstrate that Th17 responses are elicited by burn injury. At the site of burn injury, Th17 cytokines IL-17 and IL-22 have been shown to be elevated ~3-fold as compared to sham injury, within 3 hours of burn in the absence of significant changes in IL-6, IL-23 or transforming growth factor (TGF)-β [129]. While these changes are transient, an early perturbation of IL-17 and IL-22 post burn injury may disrupt the wound healing process and promote burn wound sepsis [130]. Moreover, heightened IL-17 has been observed at distant sites and in the systemic circulation. IL-17 is elevated in cardiac tissue 3 hours post burn injury [131] and in the circulation one and seven days post injury [132]. Together these data indicate that local and systemic Th17 immune responses are elicited early on post burn injury, and that sustained elevations in levels of IL-17 may contribute to the burn induced inflammatory response and subsequent immune aberrancies.

In the lung, Oppeltz et al. used cells isolated from bronchoalveolar lavage fluid 7 days post burn injury to show heightened IL-17 production following stimulation with TLR2 agonist zymosan [133]. While this study did not suggest a specific source for IL-17, nor identify a cell type responsive to zymosan, it further highlights the importance of IL-17 at mucosal barriers, such as the lung, and provides reason to investigate the role of Th17 cells, and their immunomodulatory products, in acute lung injury following burn. In regards to the effects of EtOH on Th17 immunity in the lung, Shellito et al. found increased mortality and decreased IL-17/myeloperoxidase (MPO) levels in response to pulmonary *K. pneumoniae* inoculation following 2-weeks of 20% EtOH consumption [134]. These results were further substantiated when local delivery of the IL-17 gene
improved mortality and restored pulmonary MPO levels following chronic EtOH feeding and *K. pneumoniae* inoculation [134]. A more recent study utilized a single dose of EtOH and pulmonary *K. pneumoniae* infection model to demonstrate that EtOH suppresses mRNA expression of IL-23 in the lung following pulmonary infection [135]; thus, suggesting a role for APCs in impaired Th17 immunity. Happel *et al.* confirmed their findings by demonstrating that EtOH inhibits the mRNA expression of IL-23 in alveolar macrophages stimulated directly with *K. pneumoniae* in a dose-dependent manner [135]. At the protein level, a dose-dependent decrease in splenocyte IL-17 production was elicited by supernatants from EtOH treated alveolar macrophages [135]. Perturbation of Th17 immunity may be exacerbated following combined EtOH exposure and burn injury, which may result in host immunosuppression and susceptibility to infection.

Other experimental models of burn injury demonstrate bimodal skewing of Th17 lymphocytes. Neely *et al.* reported increased Th17 cells (IL-17+CD4+) 3 and 14 days post burn injury in draining peripheral lymph nodes, but not spleens of injured mice [136]. More importantly, this group demonstrated a decreased Th17/Th1 ratio day 3 post injury, which normalized at day 7 and increased at day 14 [136]. Early post burn injury, a decreased Th17/Th1 ratio may contribute to increased susceptibility to extracellular pathogens, including *K. pneumoniae* and *C. albicans*. Conversely, late post burn injury, an increased Th17/Th1 ratio may perturb Th1 dependent immune responses.

Patient data specific to the Th17 axis following burn injury, and its possible role in remote organ damage, is quite limited. However, data from the *Inflammation and Host Response Glue Grant* indicate that serum IL-17 is elevated in adult and pediatric burn
patients within one week post injury [137, 138]. Heightened levels of IL-17 are exacerbated in pediatric as compared to adult patients [137], suggesting an age dependent difference to burn injury, which may play a role in the development of a hypermetabolic state. Recently a single center study examined Th17 cell development in 26 burn patients with third-degree thermal injuries. The results revealed that peripheral blood mononuclear cells isolated from these patients had a decreased ability to express ROR-γt and produce IL-17 in response to TCR activation and C. albicans challenge, which has been shown to increase mortality in burn patients [139, 140]. Inatsu et al. further reported measureable levels of IL-10 in sera from burn patients, and demonstrated an inhibitory role for IL-10 in the development of CD4^+ROR-γt^+ IL-17 producing T cells [139]. Though the molecular mechanism by which burn injury induced IL-10 inhibits IL-17 remains to be elucidated, this study highlights the perturbation of Th17 cells following burn injury and warrants further investigation in the context of traumatic injury, including burn. In the context of EtOH, human PBMCs demonstrate impaired expression of Th17 transcription factor ROR-γt, as well as decreased frequency of IL-17 producing CD4^+ T cells [141]. Conversely, EtOH treatment increased expression of T reg transcription factor Foxp3 [141], suggesting a skew towards a T reg phenotype [142]. Thus, suppression of antigen specific Th17 responses, following EtOH and/or burn injury, may lead to increased susceptibility to bacterial and fungal infection, particularly at mucosal interfaces, including the lungs and gut. Studies focusing on the role of the Th17 axis following EtOH exposure, burn and other traumatic injury are necessary to define the
normal temporal dependent Th17 responses to injury and to identify whether these changes contribute to remote organ damage, susceptibility to infection and mortality.

Given the limited literature on Th17 cells in response to EtOH and/or burn trauma, the role of Th17 cells in clinical sepsis was reviewed. To date, no direct perturbations of CD3+CD4+ lymphocyte derived IL-17 have been reported in clinical data or experimental models of sepsis. However, Flierl et al. did show increased IL-17 in a murine model of cecal ligation and puncture (CLP), though this was attributed to γδ T cells, not CD3+ αβ T cells [143]. Moreover, the authors demonstrated that neutralization of IL-17 correlated with decreased bacteremia, increased survival and decreased plasma levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 [143]. Together these data suggest that γδ T cell derived IL-17 negatively impacts outcomes from sepsis, through modulation of pro-inflammatory cytokines [143]. This observation was confirmed when γδ T cell knockout mice showed improved survival and decreased plasma IL-17 following CLP [143]. More recently, these findings were substantiated when the same laboratory took advantage of Rag-1 knockout mice, which lack T and B cells, to demonstrate that lack of CD3+ T cells does not affect outcome of severe sepsis following CLP [144], again eliminated the role of CD3+ Th17 cells.

IL-22, the other major immunomodulatory secretory product of Th17 cells, has also been shown to negatively affect outcomes during septic injury. While IL-22 is generally considered a protective cytokine, models of peritonitis, as well as clinical data from patients with abdominal sepsis suggest that higher levels of IL-22 may facilitate bacterial burden and septic complications. Specifically, Weber et al. demonstrated
increased levels of IL-22 in kidney and spleen following CLP peritonitis [145]. Inhibition of IL-22 following CLP enhanced bacterial clearance, promoted phagocyte recruitment, attenuated organ dysfunction and decreased expression of IL-10 [145]. Similarly, a rat model of CLP revealed measurable levels of IL-22 in serum from septic animals [146]. Clinically, a single center hospital study of 16 patients that developed abdominal sepsis following surgery, demonstrated significant elevations in serum IL-22 as compared to healthy volunteers and control surgery patients without septic complications [147]. As compared to control groups, IL-10 was also significantly increased in patients with abdominal sepsis [147]; again highlighting that IL-22 may modulate IL-10 levels resulting in heightened bacterial growth [145]. Together these findings indicate an interdependent relationship between IL-22 and IL-10, which modulates a critical balance between bacterial burden and host immune/barrier homeostasis. As such, adequate levels of IL-22 must be maintained to regulate host immune/barrier integrity while higher levels may induce increased expression of IL-10, subsequently enhancing bacterial growth and increasing the risk of sepsis. Further studies evaluating this interdependency between IL-22, IL-10, bacterial burden and host immune/barrier defense may provide a mechanism by which these interactions are regulated and lead to the identification of novel therapeutic targets for patients who develop septic complications.

**Protective Role of IL-22: Underlying Mechanism and Therapeutic Potential**

The protective and regenerative effects of IL-22 have led to the investigation of IL-22 as a therapeutic agent, which has produced promising results, particularly in the field of gastrointestinal related disease. In a model of concanavalin A (ConA)-induced T
cell-mediated hepatitis, *in vivo* treatment with IL-22 prevented liver injury, as marked by increased levels of hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [84]. Conversely, treatment of animals with anti-IL-22 antibody exacerbated ConA-induced ALT and AST levels [84]. This observation was later confirmed, when IL-22<sup>−/−</sup> mice were shown to be more susceptible to ConA-induced hepatitis than wild-type counterparts [83]. Radaeva *et al.* further demonstrated a role of STAT-3 activation in IL-22 dependent modulation of liver injury. Using the human HepG2 heptoma cell line, IL-22 was shown to inhibit apoptosis through induction of anti-apoptotic proteins Bcl-xL, Bcl-2 and Mcl-1 and promote proliferation through activation of c-myc, cyclin D1 and retinoblastoma (RB) 2 [84]. *In vivo*, Radaeva *et al.* injected IL-22 transfected or null HepG2 cells into mice, and found that mice injected with IL-22 transfected HepG2 cells developed larger tumors with heightened levels of activated STAT3, Bcl-xL and Rb2, compared to null HepG2 injected mice [84]. Together, these results demonstrate a protective role for IL-22 in T cell-mediated liver injury, which is dependent on activation of STAT3, and subsequent induction of proliferation and inhibition of apoptosis in hepatocytes, both *in vivo* and *in vitro*. To further elucidate the protective role of IL-22 in ConA-induced hepatitis, Zenewicz *et al.* injected differentiated Th17 cells from IL-22<sup>−/−</sup> or wild-type mice into IL-22<sup>−/−</sup> mice and found that recipients of IL-22<sup>−/−</sup> Th17 cells exhibited higher ALT and AST levels [83]; confirming a protective role for IL-22 against T cell mediated liver injury.

More recently, the same group demonstrated that treatment with a single dose of IL-22 attenuates chronic-binge alcohol induced liver injury [47]. Ki *et al.* used a modified
Lieber-DeCarli diet and single high dose of ethanol by oral gavage to produce fatty liver and liver injury, as marked by increased levels of hepatic enzymes ALT and AST, increased hepatic triglyceride levels and steatosis. Though the authors did not establish decreased IL-22 in the context of alcohol induced liver injury, their data did demonstrate increased expression of IL-22R1 in mice and patients with alcoholic hepatitis [47]. Thus, IL-22 may be required for normal liver homeostasis and, in the presence of alcohol, the IL-22 pool may not be sufficient for normal hepatic functions. Ki et al. found that IL-22 treatment prevented alcohol induced elevations in serum AST/ALT and hepatic triglyceride levels and steatosis. The authors attributed the protective effect of IL-22 to induction of liver antimicrobial lipocalin 2 and the antioxidant gene metallothione as well as decreased expression of a fatty acid transport protein, though modulation by STAT3 was variable [47]. These results were later confirmed using liver specific IL-22 transgenic (IL-22TG) mice, which proved resistant to ConA-induced T cell hepatitis [148]. IL-22TG mice also recapitulated upregulation of anti-oxidant and mitogenic genes noted with exogenous IL-22 treatment [148]. Collectively, these data highlight the antioxidant, antisteatotic and antimicrobial effects of IL-22 and elucidate a possible role for IL-22 in the treatment of alcoholic liver disease.

Most recently, IL-22 was proposed to modulate pancreatitis through regulation of autophagy related proteins [149]. In a mouse model of cerulein-induced pancreatitis, IL-22KO and IL-22TG mice were utilized to elucidate the vital role of IL-22 in mediating pancreatic injury and function. IL-22KO mice demonstrated a similar phenotype as wild-type mice in response to acute and chronic cerulein injections; both strains had increased
pancreatic weight to body weight ratios, amylase, lipase, MPO+ cells infiltration and apoptosis [149]. Knocking out of IL-22 did not exacerbate injury. Moreover, liver specific overexpression of IL-22, in IL-22TG mice, showed no pathologic response to cerulein treatment [149]. The potential role for IL-22 as a possible therapeutic option for the treatment of pancreatitis was further highlighted when treatment of wild-type animals with IL-22, prior to acute cerulein injection, also ameliorated changes in amylase, lipase and inflammatory cell infiltration. Similarly, IL-22 treatment via adenoviral delivery ameliorated chronic cerulein-induced pancreatitis [149]. As previously mentioned, IL-22 inhibits apoptosis through activation of anti-apoptotic genes, including Bcl-2 and Bcl-xL. To dissect the mechanism by which IL-22 modulates the development of pancreatitis, Feng et al. tested the role of Bcl-2 and Bcl-xL in the regulation of autophagy, which has been implicated in the progression of pancreatitis [150]. Moreover, Bcl-2 proteins inhibit Beclin-1 dependent autophagy [151]. In line with these finding, cerulein-treated IL-22TG mice had decreased formation of autophagosomes as compared to wild-type counterparts [149]. Feng et al. further showed that IL-22TG mice displayed increased Bcl-2/Bcl-xL and Beclin-1 interactions, as compared to wild-type mice [149], suggesting a role for IL-22 mediated induction of Bcl-2/Bcl-xL and Beclin-1 interactions, which inhibit pancreatic pathology and autophagy.

Studies of IBD consistently emphasize the role of Th17 cells in the pathogenesis of ulcerative colitis and Chron’s disease [68, 152]. Yet, most of these studies highlight the role of IL-17, and pay less regard to IL-22. Zenewicz et al. eloquently explored the role of IL-22 in IBD by taking advantage of B and T cell null Rag1−/− (lack T cells) and
IL-22−/− mice. Rag1−/− mice lack T cells, including regulatory T cells; thus, when naïve CD4+ T cells from wild-type mice are adoptively transferred to Rag1−/− mice, they rapidly expand and acquire effector functions in an uncontrolled manner causing the development of massive inflammation in the gut, similar to that found with IBD. These adoptive transfer studies revealed increased colitis and colonic levels of IFN-γ, IL-17 and IL-22 as well as weight loss, as compared Rag1−/− mice that did not receive T cells [153]. To clarify the role of IL-22 in intestinal colitis, IL-22−/−Rag1−/− mice were injected with naïve CD4+ T cells from IL-22−/− or wild type mice. Both treatments resulted in the development of colitis, yet IL-22−/−Rag1−/− mice injected with naïve T cells from IL-22−/− donor mice demonstrated more severe colitis and weight loss than mice that received wild-type T cells [153]; suggesting a protective role for IL-22 producing cells in the development of gut inflammation. Interestingly, Rag1−/− mice injected with T cells from IL-22−/− donors, demonstrated the same expression of IL-22 as IL-22−/−Rag1−/− mice that received T cells from wild type mice [153], indicating a host derived source of IL-22. Further analysis revealed that innate NK cells are a major contributor of IL-22 in the gut. This graded response, in which abolishment of IL-22 in recipient mice and donor T cells, as compared either alone, produced increasingly worse colitis and weight loss convincingly indicates that IL-22 plays a role in the development of gut inflammation. To further underscore the protective role of IL-22, Zenewicz et al. utilized a model of innate colitis, dextran sodium sulfate (DSS)-induced colitis, which causes disruption of colonic epithelial integrity and subsequent inflammation and colitis. IL-22−/− mice treated with DSS lost significantly more weight and exhibited greater mortality than DSS treated
wild-type mice [153]; confirming the protective role of IL-22. In yet another model of intestinal colitis, Sugimoto et al. used IL-22 gene delivery in a murine model of Th2-mediated colitis. Their results indicate that IL-22 treatment increased goblet cell expression and reduced colonic diameter and thickness in a STAT3 dependent manner [154]. These results were extended in a DSS model of colitis, where peak levels of colonic IL-22 correlated with increased production of membrane bound mucins (Muc1, -3, -10 and -13) during the recovery phase of DSS colitis [154]. IL-22 failed to modulate colitis in mice treated with a mucolytic agent, which removes the colonic mucus layer, blocking the actions of IL-22 on mucin production. Similarly, mice concurrently treated with IL-22 neutralizing antibody and IL-22 exhibited greater weight loss, decreased STAT3 activation and decreased goblet cell accumulation as compared to mice treated with control antibody and IL-22 [154], confirming that IL-22 restores goblet cells in a STAT3 dependent manner.

These studies demonstrating the protective role of IL-22 in liver, pancreas and intestinal tract provide promising evidence for the use of IL-22 in the treatment of gastrointestinal related disease, which may be extended to gut pathology induced by injury, including EtOH and/or burn trauma. The early animal studies provide a strong foundation and offer a unique challenge to translation research teams. At the bench, studies exploring whether IL-22, IL-22R and/or its signaling pathway is perturbed in patients with gut related pathologies, may reveal epigenetic changes or genetic polymorphisms specific to patients who develop disease or remote organ damage following EtOH and burn. These findings would help advance the treatment of disease
through the development of new therapeutic agents, as well as through modulation of
gene expression with gene therapy. Clinically, research exploring the possibility of
treating patients with recombinant IL-22 should be considered in patients with severe
gastrointestinal disease who have failed standard therapy. Results from these studies
would give a better understanding of the short and long term effects of IL-22 and help
redirect related basic research. Lastly, investigation of IL-22 as a therapeutic agent may
prove beneficial in the treatment of critically-ill patients who sustain secondary organ
damage, such as burn and trauma patients.

**Significance**

EtOH intoxication prior to injury augments post burn pathogenesis [2-6, 11-13],
yet the mechanisms by which this occurs remain largely unexplored. Gut barrier
dysfunction is frequently associated with ethanol exposure and major injury. Our
laboratory has previously demonstrated that acute EtOH exposure prior to burn injury is
associated with intestinal T cell suppression and increased bacterial translocation [14-21].
Th17 lymphocytes maintain intestinal immune homeostasis and barrier function [22-25,
39, 85, 86]. Th17 cells are now well established modulators of immune and host
epithelial homeostasis, particularly at host/environment interfaces. While ROR-γt
remains the hallmark Th17 transcription factor, new data scrutinizing the regulation of
Th17 effector cytokines IL-17 and IL-22 propose complex networks involving metabolic
changes, host/commensal bacteria interactions and environmentally or endogenously
derived chemicals in the maintenance of proper Th17 balance. Where impaired Th17
responses diminish bacterial clearance and increase epithelial vulnerability, uncontrolled
Th17 effector functions promote autoimmune disease. Thus, the multiple pathways regulating Th17 immunity are interdependent and must be tightly regulated. Injuries, such as burn, trauma and clinical sepsis have global systemic consequences, many of which affect the delicate mechanisms that govern Th17 immunity. Suppression of antigen specific Th17 responses, as noted with burn injury, may contribute to infection, remote organ damage and mortality. Given the importance of this emerging subset, the role of Th17 cells following clinically relevant host injury remains relatively unexplored and warrants deeper investigation.
CHAPTER THREE

ETHANOL EXPOSURE SUPPRESSES BONE MARROW-DERIVED DENDRITIC CELL INFLAMMATORY RESPONSES INDEPENDENT OF TLR4 EXPRESSION

Abstract

Acute alcohol (ethanol) exposure is linked with increased susceptibility to infection and increased mortality in trauma and burn patients. Dendritic cells (DCs) are central mediators in innate and adaptive immune responses, and play a role in presentation of pathogens to adaptive immune cells. We investigated the effects of acute ethanol exposure on bone marrow-derived dendritic cell (BM-DC) responses. Total bone marrow cells, obtained from 8-10wk old C57BL/6 male mice, were cultured in the presence of GM-CSF and IL-4 for 7 days. BM-DCs were harvested and treated with increasing doses of ethanol (50, 100 and 250 mM) at the time of, or 3 hours prior to, lipopolysaccharide (LPS). Following LPS, supernatants were collected for cytokine measurement and cells were harvested for flow cytometry. Concurrent acute ethanol exposure and LPS treatment resulted in a dose-dependent suppression of IL-6, IL-12p40, IL-23 and IL-10. Additionally, ethanol exposure prior to LPS, dysregulated the IL-12p40/IL-23 balance and more profoundly suppressed IL-6 and IL-10 secretion by BM-DCs, as compared with cells treated concurrently with ethanol and LPS. Ethanol treatment did not affect toll-like receptor (TLR)4 or TLR2 expression. In summary, our study demonstrates that acute ethanol exposure suppresses BM-DC LPS-induced responses irrespective of affecting TLR4 or TLR2 expression.
Introduction

Numerous bodies of literature indicate that acute and chronic ethanol consumption lead to aberrant immune responses to bacterial and viral pathogens [6, 155, 156]. Specifically, acute ethanol exposure results in increased infectious complications following trauma and burn [4, 6, 157]. *In vivo* and *in vitro* studies further demonstrate that acute ethanol exposure downregulates lipopolysaccharide (LPS)-induced immune responses, including reduced production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 from murine and human macrophages and monocytes [113, 114, 158-162]. Closely related to macrophages, dendritic cells (DCs) are a subset of antigen presenting cells (APCs) that arise from myeloid precursors [163]. Despite the overwhelming amount of evidence indicating ethanol as an immunomodulatory agent in LPS-induced macrophage responses, only a small number of studies have examined the effects of ethanol on LPS-mediated DC immune responses [164-167].

DCs comprise a migratory group of bone marrow-derived leukocytes which are able to uptake, transport, process and present antigen [168-170]. Following uptake of antigen, DCs migrate from the periphery to draining lymph nodes and undergo maturation, marked by increased expression of major histocompatibility complex (MHC) Class II and other genes that promote antigen presentation. In secondary lymphoid organs, DCs present antigen via MHC II to naïve T lymphocytes to generate T helper (Th)-1, Th-2 or Th-17 responses [64]. Thus, in their role as APCs, DCs are a critical link between the innate and adaptive arms of the immune system.
Like monocytes/macrophages, DCs express various pattern recognition receptors, including toll like receptors (TLRs). Receptor ligation of TLRs by specific ligands induces various signaling pathways to modulate immune responses. Specifically, LPS activates immune cells through its interaction with TLR4 [171], which results in activation of multiple downstream signaling pathways and transcription factors, including the MyD88-dependent and mitogen activated protein kinase (MAPK) pathways [171]. Ultimately, this leads to the release of multiple cytokines, including TNF-α, IL-1, IL-6, IL-10, IL-12 and, most recently, IL-23, which is known to be a critical mediator of Th-17 polarization [113, 114, 172-177].

Currently, the literature addressing the effects of ethanol exposure on DC immune responses is quite limited, particularly in the context of acute ethanol exposure. To date, the effects of acute ethanol exposure on DC function are limited to DCs derived from monocytes isolated from healthy human donors exposed to ethanol prior to venipuncture [164, 178]. These studies demonstrate that DCs derived from volunteers exposed to ethanol had decreased expression of co-stimulatory molecules CD80 and CD86, which are required for T cell activation [164]. Functionally, DCs from ethanol exposed volunteers induced T cell anergy, inhibited Th1 activation and resulted in decreased T cell proliferation [164, 178]. In line with published literature on monocytes and macrophages, these studies showed decreased secretion of Th1 polarizing IL-12 and increased secretion of anti-inflammatory IL-10 [164]. Similarly, Lau et al. demonstrate that acute ethanol exposure inhibited differentiation of BM-DC, subsequent co-signaling molecule expression, naïve T cell activation and impaired production of IL12p70 [165].
Murine models of chronic ethanol abuse demonstrate that chronic ethanol consumption suppressed the ability of DCs to stimulate activation and proliferation of naïve T cells, both *in vivo* and *in vitro* [165-167]. In regards to cytokine production, DCs from ethanol-fed mice produced less IL-12p40, TNF-α and IFN-α [167]. These data provide evidence to suggest that ethanol exposure results in aberrant DC immune responses, which may result in an imbalanced immune response and detrimental outcomes following infection or trauma. However, the effects of acute ethanol exposure on differentiated DCs remain to be elucidated.

To explore the impact of acute ethanol on DC cytokine production, our study utilized bone marrow-derived dendritic cells (BM-DCs) to evaluate the concurrent and pre-treatment, as well as dose-dependent, effects of ethanol on DC cytokines known to mediate T cell responses, IL-12p40, IL-6, IL-23 and IL-10. Using differentiated DCs and LPS, our study models a scenario in which the host is immunologically challenged while under the influence of ethanol intoxication. Thus, our study directly measures the effects of acute ethanol exposure on differentiated DCs, rather than exploring the effects of ethanol on DC differentiation. We also sought to determine if TLR expression contributes to ethanol-mediated cytokine dysregulation. Herein, we show that acute ethanol exposure results in a dose-dependent perturbation of IL-6, IL-12p40, IL-23 and IL-10. Moreover, our data suggest that acute ethanol exposure has more profound effects if DCs are pre-treated with ethanol than if they are exposed to ethanol at the time of LPS stimulation. Lastly, we demonstrate that acute ethanol exposure blunts LPS-induced cytokine responses independent of TLR4 or TLR2 expression.
Materials and Methods

Animals

Male C57BL/6 mice (Harlan Laboratories), 8-9 week old, weighing 22-25 g, were used in the experiments. Animals were allowed to acclimate to the animal facility for 2 weeks before being used for experiments. All experiments were conducted in accordance with the guidelines set forth in the Animal Welfare Act and were approved by the Institutional Animal Care and Use Committee at the Loyola University Chicago Health Sciences Division.

Bone Marrow Isolation and DC Differentiation

BM-DCs were generated in vitro from total bone marrow, as previously described [179-181]. Briefly, femurs and tibias were dissected from unmanipulated mice. Both ends of each bone were cut open and bone marrow flushed out with 3 ml cold RPMI-1640 medium (Thermo Fisher Scientific) using a 22 gauge needle. Subsequently, bone marrow was brought to single cell suspension by pipetting and passage through a 70 µm cell strainer. Total bone marrow cells were counted by hemocytometer, washed in RPMI medium, adjusted to a concentration of 2 x 10^6 cells/ml. Bone marrow cells, 10 x 10^6 cells, were cultured in complete RPMI-1640 supplemented with 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 10 mM HEPES, 50 µg/ml gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% heat-inactivated FCS (Thermo Fisher Scientific), 50 ng/ml granulocyte/monocyte-colony stimulating factor (GM-CSF, R&D Systems) and 100 U/ml IL-4 (R&D Systems) in 100-mm tissue culture dishes at 37°C and 5% CO₂ for 7-10 days. Every 3 days, cell cultures were washed and media replaced with complete RPMI-1640
containing GM-CSF and IL-4. On days 7-10, BM-DC were harvested using Accutase detachment medium (eBioscience), washed, counted by hemocytometer and resuspended in complete RPMI-1640 medium at a concentration of $2 \times 10^6$ cells/ml. The purity of DCs by co-expression of CD11c and MHC II was assessed via FACS analysis as described below.

**Ethanol Exposure and LPS stimulation in vitro**

Following differentiation, BM-DC ($2 \times 10^5$ cells/well) were cultured in the presence of increasing doses of ethanol (0, 50, 100 and 250 mM) for either 3 hours prior to, or in parallel with, LPS (1µg/ml) stimulation in 96-well plates at 37°C and 5% CO$_2$ for 24 h in a humidified chamber. Following ethanol exposure and LPS stimulation, cell supernatants were harvested for the measurement of IL-6, IL-10, IL-12p40 and IL-23 by ELISA. Additionally, cells were harvested for measurement of cell viability (see Appendix A1), TLR4 and TLR2 expression by flow cytometry, as described below.

**CD11c, MHC II, TLR2 and TLR4 Expression**

Flow cytometric analysis was performed to determine DC purity as well as TLR2 and TLR4 expression following ethanol exposure and LPS stimulation. Briefly, BM-DC ($4 \times 10^5$ cells) were collected and resuspended in staining buffer (PBS containing 5% FCS and 2 mM EDTA). Cell suspensions were blocked with anti-CD16/32 (0.5 µg/100 µL staining buffer) and rat IgG for 20 min at 4°C and subsequently stained with PE conjugated anti-CD11c (0.25 µg/100 µL staining buffer), FITC conjugated anti-MHC II (0.25 µg/100 µL staining buffer), PE-Cy7 conjugated anti-TLR4 (0.5 µg/100 µL staining buffer) and Alexa 647 conjugated anti-TLR2 (0.1 µg/100 µL staining buffer) for 30 min.
at 4°C. Cells were washed twice, resuspended in 500 µL staining buffer and analyzed at the Loyola University Medical Center FACS Core Facility using a FACSCanto I (BD Bioscience). All FACS data analyses were performed using FlowJo software (Treestar). All antibodies used for flow cytometry were purchased from eBioscience.

**Cytokine Measurement**

Cytokine production in BM-DC cultures was determined by ELISA. The IL-6 and IL-12p40 ELISAs were purchased from BD Bioscience and the IL-10 and IL-23 ELISAs were purchased from R&D System. IL-12 and IL-23 are both members of the IL-12 family that share the p40 subunit [31, 32]. Given that we measured IL-23 by an ELISA kit that does not react with IL-12, we used IL-12p40 as a proxy for IL-12.

**Statistical Analysis**

Results are presented as mean + standard error of the mean. ANOVA with Tukey post-hoc testing was used to compare between experimental groups. All statistical analyses were performed using InStat 3.0 (GraphPad Software). p<0.05 was considered statistically significant.

**Results**

**DC purity**

DCs were differentiated from total bone marrow cells in the presence of GM-CSF and IL-4, as previously described [180, 181]. Following differentiation, BM-DC purity was determined based on co-expression of DC markers CD11c and MHC II [87, 179, 182]. Culture of total bone marrow cells with GM-CSF and IL-4 yielded an average of >90% CD11c*MHC II* cells (Fig. 2).
Figure 2. Representative FACS plots of bone marrow-derived dendritic cell population. Following 7-10 day culture in complete RPMI media containing GM-CSF (50 ng/ml) and IL-4 (100 U/ml), cells were harvested and analyzed for expression of dendritic cell markers, CD11c and MHC II. Cell suspensions were stained with FITC-labeled anti-MHC II and PE-labeled CD11c antibodies. A, Live gate on a forward versus side scatter plot. B, Percentage of total live cells expressing MHC II and CD11c - value represents mean ± SEM, n=7 animals from two independent experiments.

Ethanol exposure leads to BM-DC cytokine dysregulation

To determine the effect of ethanol exposure on secretion of pro- and anti-inflammatory cytokines, BM-DCs were cultured with LPS (1 µg/ml) in the presence of increasing doses of ethanol (50, 100 and 250 mM) or following 3 hours of ethanol pre-treatment at the same doses (Fig. 3). We evaluated IL-12p40, IL-6 and IL-23, three pro-inflammatory cytokine generated by DCs that are important for Th1 (IL-12) and Th17 (IL-6 and IL-23) polarization. We also examined IL-10, an anti-inflammatory cytokine associated with a Th2 immune response. Cell viability by flow cytometry, as determined
by the percent of cells in the live gate, was not affected by ethanol or LPS treatment (see Appendix A1).

Concurrent treatment of BM-DC with LPS and ethanol led to a dose dependent decrease in IL-12p40, IL-23 and IL-10 with maximum suppression of IL-6 seen at 50 mM (Fig 3). Introduction of LPS and 50 mM ethanol in parallel resulted in a 25% decrease in IL-6 secretion from BM-DCs compared with LPS alone (Fig. 3A, p<0.05 for 50 mM ethanol + LPS group as compared with LPS alone). At increasing doses no additional suppression was seen, though it should be noted that there was no statistical significance between the three ethanol doses in respect to IL-6 secretion. This ethanol mediated cytokine suppression was also seen with IL-23 (Fig. 3C, p<0.001 as compared with LPS alone). Specifically, concurrent addition of LPS and ethanol (50, 100 and 250 mM) resulted in ~40%, 55% and 68% reduction of IL-23 release, respectively. In response to concurrent ethanol exposure and LPS stimulation, IL-12p40 secretion was decreased as compared with the LPS alone group, but only reached significance when treated with 250 mM ethanol (Fig. 3B, p<0.05). While 50 mM ethanol treatment administered simultaneously with LPS did not affect IL-10 secretion, 100 and 250 mM ethanol exposure plus LPS stimulation resulted in 30% and 75% reductions in IL-10 release, respectively (Fig. 3D, p<0.05 compared with LPS alone). Co-culture with 250 mM ethanol and LPS resulted in a significant decrease in IL-10 release as compared with co-cultures with 50 and 100 mM ethanol (Fig. 3D, p<0.05). Together these data suggest that ethanol treatment at the time of immune challenges leads to global BM-DC cytokine suppression.
Figure 3. **BM-DC cytokine release following ethanol exposure and LPS stimulation.** BM-DCs (2 x 10^6 cell/well) were cultured in 96-well plates in the presence of LPS (1 µg/ml) and varying concentrations of ethanol for 24 hours at 37°C. Supernatants were harvested for the measurement of IL-6, IL-12p40, IL-23 and IL-10 by ELISA. Values are means + SEM, n=4-7 animals/group from two independent experiments. *, p<0.05 as compared to LPS only group; #, p<0.001 as compared to all groups; †, p<0.05 as compared to corresponding ethanol dose + LPS group; §, p<0.05 as compared to corresponding ethanol (50 mM) group by ANOVA with Tukey post-hoc test.
To further elucidate if ethanol exerts differential effects on BM-DC cytokine secretion following immune stimulation, BM-DCs were pre-treated with ethanol for 3 hours prior to culture with LPS. Pre-treatment of BM-DCs with increasing doses of ethanol (50, 100 and 250 mM) resulted in ~25%, 40% and 60% IL-6 secretion in response to LPS, respectively (Fig. 3A, p<0.05, as compared with LPS alone group). Moreover, pre-treatment of DCs with 250 mM ethanol resulted in a 50% reduction in IL-6 release as compared with BM-DCs pre-treated with ethanol 50 mM (p<0.05) and to DCs concurrently cultured with LPS and ethanol 250 mM (p<0.05). Ethanol treatment prior to LPS stimulation lead to a marked dose-dependent suppression (~30%, 45% and 75% at 50, 100, and 250 mM ethanol, respectively) of IL-23 as compared with LPS alone (Fig. 3C, p<0.001). However, this suppression was irrespective of the timing of ethanol exposure relative to LPS treatment and was most profound in cells exposed to 250 mM ethanol as compared with lower doses (Fig. 3C, p<0.05). While these two cytokines exhibited a similar, or slightly more extensive, immune suppression with ethanol pre-treatment, IL-12p40 was paradoxically elevated in supernatants from BM-DCs treated with 50 mM ethanol prior to LPS stimulation, as compared with LPS treatment alone (Fig. 3B, p<0.05). Likewise, compared with BM-DCs treated with 50 mM and 100 mM ethanol in parallel with LPS, IL-12p40 secretion from corresponding pre-treated cells was significantly elevated (p<0.05). However, pre-treatment of cells with higher ethanol concentrations (100 mM and 250 mM) did not lead to significant changes in IL-12p40 production when compared with LPS treatment alone. These data suggest the ethanol may exert differential effects on the mechanisms that modulate these
cytokines, and may alter the ability of DCs to effectively induce one T cell subset over another. Pre-treatment with ethanol also seems to more profoundly impact the ability of BM-DC to exert immunosuppressive effects. Prior exposure of BM-DCs with 50, 100 and 250 mM ethanol resulted in 60%, 81% and 100% decline in IL-10 release, respectively (Fig. 3D, p<0.05 as compared with LPS alone). When compared with BM-DCs exposed to LPS and ethanol concurrently, ethanol exposure 3 hours prior to LPS exacerbated suppression of IL-10 secretion. Prior incubation with 50 mM and 100 mM ethanol diminished IL-10 release by 56% and 73%, as compared with BM-DCs at the respective doses in the concurrent treatment group (Fig. 3D, p<0.05). Notably, pre-treatment of DCs with 250 mM ethanol abolished IL-10 release, whereas co-culture with ethanol and LPS diminished IL-10 release by 75% (Fig. 3D, p<0.05, as compared with 250 mM EtOH plus LPS group).

**Ethanol does not alter BM-DC TLR4 or TLR2 expression**

In order to determine whether the difference in cytokine levels with ethanol exposure were a result of alterations in the receptor for LPS, we used flow cytometry to analyze expression of TLR4 on BM-DCs (Fig. 4). LPS stimulation resulted in a ~25% decrease in the percentage of DCs expressing TLR4, as compared with unstimulated cells (Fig. 4A, p<0.05). Similarly, LPS challenge resulted in a ~50% decrease (p<0.05) in the mean fluorescence intensity (MFI) of TLR4, as compared with unstimulated cells (Table 1). This reduction in percentage as well as per cell expression of TLR4 expression (summarized in Table 1) may be due to receptor ligation or internalization. DCs exposed to ethanol, prior to or at the time of LPS challenge, demonstrated a similar reduction in
### Table 1. Summary of TLR4 and TLR2 expression on DCs.

MFIs are expressed as fold difference in order to combine data obtained from independent experiments. Data represented as means ± SEM, n=4-7 animals/group from two independent experiments. *, p<0.05 as compared to unstimulated, ethanol 50 mM and 100 mM groups; #, p<0.05 as compared to ethanol 250 group; **, p< 0.05 as compared to unstimulated and ethanol only groups; ##, p<0.0001 as compared to unstimulated and ethanol only groups by ANOVA with Tukey post-hoc test.

<table>
<thead>
<tr>
<th></th>
<th>TLR4</th>
<th>TLR2</th>
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<tbody>
<tr>
<td></td>
<td>% CD11c⁺, MHC II⁺, TLR4⁺</td>
<td>TLR4 Expression (Fold Difference)</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>95.77 ± 0.57</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>LPS</td>
<td>71.77 ± 7.10 *</td>
<td>0.52 ± 0.02 **</td>
</tr>
<tr>
<td>Ethanol (50 mM) + LPS</td>
<td>77.07 ± 4.80</td>
<td>0.51 ± 0.01 **</td>
</tr>
<tr>
<td>Ethanol (100 mM) + LPS</td>
<td>78.00 ± 4.40</td>
<td>0.50 ± 0.01 **</td>
</tr>
<tr>
<td>Ethanol (250 mM) + LPS</td>
<td>65.48 ± 1.21 *#</td>
<td>0.49 ± 0.01 **</td>
</tr>
<tr>
<td>Ethanol (50 mM) 3hrs, LPS</td>
<td>77.20 ± 5.17</td>
<td>0.53 ± 0.03 **</td>
</tr>
<tr>
<td>Ethanol (100 mM) 3hrs, LPS</td>
<td>76.59 ± 5.65</td>
<td>0.56 ± 0.05 **</td>
</tr>
<tr>
<td>Ethanol (250 mM) 3hrs, LPS</td>
<td>74.40 ± 5.42 *</td>
<td>0.50 ± 0.02 **</td>
</tr>
<tr>
<td>Ethanol 50 mM</td>
<td>95.97 ± 0.29</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td>Ethanol 100 mM</td>
<td>96.01 ± 0.55</td>
<td>0.95 ± 0.05</td>
</tr>
<tr>
<td>Ethanol 250 mM</td>
<td>94.28 ± 0.52</td>
<td>0.85 ± 0.03</td>
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Figure 4. TLR4 expression on DCs. BM-DCs (2 x 10^6 cell/well) were cultured in 96-well plates in the presence of LPS (1 µg/ml) and varying concentrations of ethanol for 24 hours at 37°C. Cells were harvested and cell suspensions were stained with PE-conjugated anti-CD11c, FITC-conjugated anti-MHC II and PE-Cy7 conjugated anti-TLR4. Representative FACS plots were taken from a single animal (ethanol dose – 50 mM), and demonstrate the percentage of TLR4+ cells (A) and MFI of the TLR4 antibody conjugate (B) within the DC (MHC II^+CD11c^+) gate. Numbers indicate the percentage of cells in each quadrant. Solid black line, unstimulated cells; gray histogram, LPS only control; dashed line, respective treatment.

The percentage and per cell expression of TLR4 (Fig. 4), suggesting that ethanol does not influence levels of TLR4 following LPS stimulation. Together these data suggest that the cytokine changes found following ethanol exposure are independent of TLR4 expression and may suggest aberrant downstream signaling.
LPS has been reported to induce expression of TLR2 via activation of TLR4-dependent pathways [104, 183, 184]. To assess whether ethanol exposure affects LPS induction of TLR2 expression, we determined the effects of ethanol on BM-DC expression of TLR2 by flow cytometry (Fig. 5). Though LPS stimulation alone significantly increased the percentage of DCs expressing TLR2 by 2.3 fold as compared with unstimulated cells (Fig. 5A, p<0.05), ethanol treated BM-DCs also demonstrated similar increases in percent of TLR2+ DCs. Similarly, LPS challenge heightened the MFI of TLR2, as compared with unstimulated cells (Table 1, p<0.0001). Neither paradigm of ethanol exposure altered the MFI of TLR2, as compared with BM-DCs cultured with LPS alone. These results are summarized in Table 1 and indicate that ethanol exposure does not perturb LPS-induced TLR2 expression.
Figure 5. TLR2 expression on DCs. BM-DCs (2 x 10^6 cell/well) were cultured in 96-well plates in the presence of LPS (1 µg/ml) and varying concentrations of ethanol for 22 hours at 37°C. Cells were harvested and cell suspensions were stained with PE-conjugated anti-CD11c, FITC-conjugated anti-MHC II and Alexa-647 conjugated anti-TLR2. Representative FACS plots were taken from a single animal (ethanol dose – 50 mM), and demonstrate the percentage of TLR2^+ cells (A) and MFI of the TLR2 antibody conjugate (B) within the DC (MHC II^+CD11c^+) gate. Numbers indicate the percentage of cells in each quadrant. Solid black line, unstimulated cells; gray histogram, LPS only control; dashed line, respective treatment.

Discussion

The results presented in this article clearly demonstrate that exposure to ethanol suppresses BM-DC secretion of pro- and anti-inflammatory cytokines that contribute to T cell polarization, in a manner independent of TLR4 and TLR2 expression. These effects
are dose-dependent, and are further influenced by the timing of alcohol administration relative to the LPS challenge. Despite the differential cytokine profiles depending on the treatment paradigm used, neither ethanol pre-treatment nor concurrent culture with ethanol and LPS altered TLR4 and TLR2 expression. Our results provide strong evidence to suggest that ethanol exposure suppresses BM-DC immune responses in a dose-dependent manner. While we recognize that our studies were carried out in vitro and may have limited physiological implications, our results provide strong evidence to suggest that ethanol exposure suppresses BM-DC immune response in a dose-dependent manner.

The activation of cytokine production and secretion in response to LPS, the main component of the outer membrane of Gram-negative bacteria, predominantly depends on TLR4 signaling. When combined with LPS-binding protein, LPS is transferred to TLR4 by CD14, making CD14 a critical adaptor molecule for TLR signaling. Additionally, LPS ligation to TLR4 requires accessory protein MD-2 to activate TLR4 signaling and induce transcription of immune response genes [174, 185, 186].

As LPS predominately affects changes in cytokine production via the TLR4 pathway, and indirectly via TLR2, we hypothesized that the cytokine dysregulation observed in our study may be correlated with altered expression of these pattern recognition receptors. Previously, several groups have demonstrated that in vivo and in vitro acute ethanol exposure disrupts macrophage TLR4/CD14 clustering by interfering with lipid rafts and actin cytoskeleton reorganization following LPS stimulation [187-189]. Our study directly examined the effect of ethanol on TLR expression. Though no alteration in TLR expression was observed in our study, it is possible that these
mechanisms play a role in the cytokine changes found in DCs following ethanol exposure and LPS challenge. Additional experiments will explore TLR4/CD14 clustering and cytoskeleton reorganization in response to LPS to answer whether ethanol exerts its immunosuppressive effects on DCs by inhibiting proper receptor interactions and/or subsequent downstream signaling. Moreover, our study found that LPS-induced expression of TLR2 was not affected by the presence of ethanol; yet, we did not directly test the functionality of TLR2. Future experimentation with TLR2 specific agonists will elucidate whether ethanol abrogates TLR2 dependent immune responses.

In our study, we also sought to determine how the timing of ethanol exposure relative to LPS stimulation affects cytokine secretion. For the Th1 polarizing cytokine IL-12, ethanol exposure (50 mM) prior to LPS stimulation generated significantly higher IL-12 levels compared with LPS alone and those cells treated concurrently. This is an interesting observation, suggesting that how ethanol interacts with cellular machinery to regulate cytokine production may be time dependent. Of note, Mandrekar et al. and Lau et al. demonstrated that ethanol exposure perturbs differentiation of DCs and subsequent IL-12 production [164, 165]. In contrast to our study, these studies used ethanol during the differentiation process, potentially altering additional cell subsets that may impact the ability of DC precursors to effectively differentiate into functional DCs. Moreover, in vivo exposure to a single dose of ethanol (2.9 g/kg) results in decreased LPS-induced IL-12 production from splenic macrophages, 6 hrs after ethanol injection [158]. Taken together, these results suggest that the stage of immune cell differentiation and timing of ethanol exposure may be important factors in driving ethanol induced perturbation of IL-
12p40 secretion. Further, these data suggest that ethanol exposure may skew the ability of DCs to aid in T cell polarization following immune challenge.

In reference to IL-6 and IL-23, DC secreted cytokines that work in concert to drive naïve T cells toward the Th17 phenotype, pre-treatment with ethanol exerted a more robust cytokine suppression after LPS stimulation as compared with co-cultured cells. As mentioned previously, IL-23 is a member of the IL-12 family, and both cytokines share the p40 subunit. The role of the p40 subunit has long been described as crucial for the biological functions of IL-12, as the IL-12R recognizes the heterodimeric structure. However, until the discovery of the IL-23 p19 subunit, it was thought that excess p40 formed homodimers, which exert antagonistic effects to IL-12 [190]. It is now understood that the p40 subunit may combine with p35 to form IL-12p70, p19 to form IL-23 or form homodimers [34, 190, 191]. Today, IL-23 is recognized for its role in promoting the production of Th17 effector cytokines IL-17 and IL-22 [28, 31, 33]. Interestingly, in BM-DCs treated with ethanol prior to LPS culture, there was a dramatic decrease in IL-23 with a relative increase in IL-12. On the other hand, concomitant treatment with ethanol and LPS resulted in a reduction of both cytokines. These data may suggest that ethanol exposure in advance of an immune challenge may preferentially shift the utility of the p40 subunit towards IL-12 production. Although not examined in this study, others have shown that LPS/TLR4 mediated induction of IL-23 p19 expression depends on NF-κβ and AP-1 [172], which are modulated by MyD88 and MAPK pathways, respectively. In regards to AP-1, Liu et al. were the first to establish that AP-1 acts to promote expression of IL-23 p19 under the direction of ERK [172]. Of note, ERK is also a known regulator
of the p40 subunit of IL-23, the subunit which is shared with IL-12; however, while ERK promotes expression of p19, it inhibits expression of p40 [192], suggesting that ethanol may impact ERK activity and relative expression of these subunits.

Alterations in ERK activity, in conjugation with other members of either the MAPK or MyD88 pathway have been attributed to alterations in IL-6 following acute ethanol treatment. In vivo acute ethanol exposure reduces LPS-induced IL-6 production from splenic macrophages [113, 114, 158]. Goral et al. further demonstrated that ethanol modulates IL-6 by transiently down-regulating the activation of p38 and ERK in unstimulated and LPS challenged splenic macrophages [113, 114]. Our results indicate that when pre-treated with ethanol, LPS-induced DC IL-6 production is suppressed in a dose dependent manner, suggesting that ethanol modulates IL-6 release in fully differentiated DCs. While further experimentation to determine the effect of ethanol on p38 and ERK pathways in BM-DCs in response to LPS, these previous studies support the idea that ethanol decreases ERK activity. As mentioned above this ethanol-mediated reduction in ERK activity could explain the diminished secretion of the Th17 polarizing cytokines IL-6 and IL-23, and the elevation of Th1 skewing IL-12p40 in a time-dependent manner. The temporal role of ERK in DC cytokine production as well as other functional measures of DC activity will be the focus of future studies.

While our pro-inflammatory cytokine data supports previous findings regarding ethanol and immune cell subsets, it is clear that more research needs to be conducted to examine the impact of ethanol on DC immune function. Moreover, even less is understood about the effects of ethanol on DC anti-inflammatory profiles. Current
literature focusing on DCs IL-10 production is limited to studies of human peripheral
blood monocyte derived DCs. In their study, Mandrekar *et al.* report an increase in LPS-
induced IL-10 from DCs generated in the presence of ethanol [164]. While the range of
IL-10 levels we obtained correlate with these data, we demonstrate a dose dependent
suppression of LPS-induced IL-10, which is further exacerbated if DCs are pre-treated
with ethanol prior to LPS stimulation. Moreover, Mandrekar *et al.* demonstrated
increased IL-10 from DCs generated in the presence of ethanol, irrespective of LPS
stimulation. In our studies, we found that ethanol alone does not induce a detectable level
of IL-10. These disparities in our data may be due to differences in cell population and
differentiation methods as discussed previously. Together, these data imply that ethanol
has a differential role in anti-inflammatory cytokine production that may be due to the
impact of ethanol on DC differentiation and maturation.

In conclusion, our study demonstrates that acute ethanol exposure dysregulates
LPS-induced immune responses irrespective of affecting TLR4 or TLR2 expression.
These effects also appear to be related to the timing of ethanol exposure relative to
immune challenge, as our data suggests that ethanol exposure prior to LPS impairs IL-23
and IL-6 secretion, and shunts the immune response towards an IL-12 response. While
further studies need to be conducted to elucidate the immunomodulatory mechanisms
altered by ethanol in DCs, the ethanol induced cytokine dysregulation observed in this
study is an important consideration when investigating the role of ethanol in DC/Th17
cell mediated pathologies such as rheumatoid arthritis and inflammatory bowel disease.
CHAPTER 4
THE ROLE OF ARYL HYDROCARBON RECEPTOR IN IL-23 DEPENDENT RELEASE OF IL-22 FOLLOWING ETHANOL EXPOSURE AND BURN INJURY

Abstract

T helper (Th)-17 lymphocytes play a crucial role in the maintenance and regulation of gut immunity. Our laboratory has demonstrated that acute ethanol (EtOH) exposure prior to burn injury results in intestinal T cell suppression and enhanced bacterial translocation. We examined the effects of EtOH exposure and burn injury on Th17 responses within intestinal lymphoid Peyer’s patches (PP). We further investigated whether restitution of IL-23 modulates PP cell IL-17 and IL-22 following EtOH and burn injury. Male mice, ~25g, were gavaged with EtOH (2.9mg/kg) prior to receiving a ~12.5% total body surface area full thickness burn. One day post injury, PP mixed cells were cultured in the presence of plate bound anti-CD3/soluble anti-CD28 in presence or absence of rIL-23 for 48 h. Supernatants were harvested for IL-17 and IL-22 levels. When combined with EtOH intoxication, burn injury significantly decreased IL-17 and IL-22, as compared to sham injury. Restitution of IL-23 successfully restored IL-22, but not IL-17 following EtOH intoxication and burn injury. However, IL-23 restoration of IL-22 was prevented in PP cells treated with CH-223191, an inhibitor the aryl hydrocarbon receptor (AhR). To further delineate the mechanism of suppressed IL-17 and IL-22, PP cells were treated with phorbol 12-myristate 13-acetate (PMA) and ionomycin. We found that, PMA and ionomycin significantly prevented the decrease in
IL-17 but not IL-22. Collectively, these findings suggest that IL-23 mediated restoration of IL-22 is dependent on the AhR following EtOH and burn injury. Conversely, IL-17 requires activation of PKC and intracellular calcium signaling.

**Introduction**

More than one million burn injuries are reported yearly within the United States [1]. These injuries translate into an approximate 500,000 emergency room visits and 40,000 hospitalizations annually [1]. Interestingly, nearly one-half of these injuries occur under the influence of alcohol/ethanol (EtOH) intoxication [2-4, 6, 8, 11]. Furthermore, burn victims who sustain injury post EtOH exposure are more susceptible to infection, exhibit higher morbidity, and are more likely to die than patients without EtOH exposure at the time of injury [2-4, 6, 11-13]. Previous data suggest that gut pathogens and their products may play a pivotal role in the development of sepsis and multiple organ failure reported in burn and trauma patients [14, 59, 128, 193]. In line with these studies, our laboratory has demonstrated increased intestinal tissue damage, leakiness, and bacterial translocation as well as T lymphocyte, particularly T helper (Th) cell, suppression following EtOH intoxication and burn injury [14-21, 112]. We observed that ethanol combined with burn injury results in decreased Th1 cytokine IFN-γ production [16, 17, 112].

Recent findings suggest that Th17 lymphocytes maintain intestinal immune homeostasis and barrier function [22-25, 39, 85, 86]. Furthermore, IL-23, a heterodimeric cytokine and member of the IL-12 family, has been shown to play a critical role in the development, expansion and survival of Th17 lymphocytes [31-33]. Binding of IL-23 to
its receptor complex on differentiating T helper lymphocytes activates signal transducer and activator of transcription (STAT)-3 to maintain upregulation of transcription factor retinoic acid related orphan receptor (ROR)-γt, which is indispensable in the production and secretion of Th17 effector cytokines IL-17 and IL-22 [67, 88-91]. IL-17 has been shown to protect against gut pathogens, including *Bacteroides fragilis* and *Klebsiella pneumoniae* [22-25], as well as to enhance tight junction formation [80]. Conversely, IL-22 acts on epithelial cells to induce release of antimicrobial peptides (AMPs) [29, 30] and stimulate epithelial cell regeneration and proliferation [23, 47]. Furthermore, dysregulation of Th17 lymphocytes has been implicated in both forms of inflammatory bowel disease [76-78].

More recently, suppression of Th17 generation was reported in patients with severe burn injuries [139]. Our study examined whether Th17 effector cytokines IL-17 and IL-22 are affected by combined EtOH and burn injury in gut associated secondary lymphoid organs, Peyer’s patches (PPs). PPs are important intestinal secondary lymphoid organs that play a critical role in T cell immunity, as well as gut bacterial translocation and its containment [85, 194]. Our results demonstrate that EtOH and burn injury results in suppressed CD3-dependent Th17 cytokines IL-17 and IL-22. Moreover, restitution with IL-23 successfully restores IL-22, but not IL-17, suggesting a differential role for IL-23 following EtOH intoxication and burn injury. Lastly, we treated PP cells with an inhibitor the aryl hydrocarbon receptor (AhR) as well as phorbol 12-myristate 13acetate (PMA) and ionomycin to determine the molecular mechanism by which EtOH and burn injury modulates Th17 cytokines IL-17 and IL-22. We found that following EtOH and
burn injury, IL-23 dependent restoration of IL-22 is regulated, at least in part, by the AhR. On the other hand, IL-17 is induced by PMA and ionomycin, suggesting a role for intracellular calcium signaling in perturbation of IL-17 following EtOH and burn injury.

**Material and Methods**

**Animals and reagents**

Male C57BL/6 mice, 6-7 wk old, were obtained from Harlan Laboratories. Hamster anti-mouse CD3e and anti-mouse CD28 were obtained from BD Biosciences (San Diego, CA). Rat affinity purified anti-mouse CD16/32, hamster PE-conjugated anti-mouse CD11c, rat FITC-conjugated anti-mouse MHC II, rat APC-conjugated anti-mouse F4/80, hamster PE-Cy7-conjugated anti-mouse CD3e and recombinant IL-12 and IL-23 were obtained from eBioscience (San Diego, CA). Goat APC-conjugated anti-mouse IL-23R and IL-17 and IL-22 ELISA kits were obtained from R&D Systems (Minneapolis, MN). Collagenase D was obtained from Roche Applied Science (Indianapolis, IN). Concanavalin A, ionomycin calcium salt, phorbol 12-myristate 13-acetate (PMA), and AhR inhibitor CH-223191 were obtained from Sigma-Aldrich (St. Louis, MO).

**Mouse model of acute EtOH intoxication and burn injury**

As previously described [195], adult C57BL/6 male mice were randomly divided to receive sham or burn injury and either ethanol (EtOH) or vehicle (water) to yield four experimental groups: sham vehicle (SV), sham EtOH (SE), burn vehicle (BV) and burn EtOH (BE). Mice were gavaged with either 0.4 ml of 25% EtOH in water (~2.9 g/kg) or water. Four hours after gavage, mice were anesthetized by i.p. injection of ketamine hydrochloride/Xylazine cocktail (~80 mg/kg and 1.2 mg/kg, respectively). Dorsal
surfaces were shaved, and animals were transferred into a template fabricated to expose ~12.5% of the total body surface area (TBSA). TBSA was calculated by using Meeh’s formula as described by Walker and Mason [196]. Burn-injured mice were immersed into a water bath maintained at 85–87°C for 7 s, which results in a 3rd degree full thickness scald injury. Sham-injured mice were subjected to identical anesthesia and treatment, but immersed into isothermic water (37°C) for 7 s. Immediately after burn or sham procedure, animals were dried and resuscitated with 1.0 ml physiological saline by i.p injection. Animals were allowed food and water ad libitum. All experiments were conducted in accordance with the guidelines set forth in the Animal Welfare Act and were approved by the Institutional Animal Care and Use Committee at the Loyola University Health Sciences Division.

**Cell isolation from PPs**

One day after injury, mice were anesthetized and the abdominal cavity exposed via midline incision. PPs were collected aseptically and placed into a Collagenase D bathe for 15 min at 37°C, as described previously [197]. Collagenase D (0.5 mg/ml) was prepared in Hank’s Buffered Saline Solution (HBSS, Fisher Scientific) containing Ca^{2+} and Mg^{2+} and supplement with 10 mM HEPES, 50 µg/ml gentamicin, 100 U/ml penicillin with 100 µg/ml streptomycin and 5% FCS. Following Collagenase D treatment, PPs were crushed to prepare single-cell suspensions, filtered through a 70 µm Nylon filter, washed and resuspended at a concentration of 5x10^6 cells/ml in complete media (RPMI 1640 supplemented with 2 mM L-glutamine, 10 mM HEPES, 50 µg/ml gentamicin, 100 U/ml penicillin with 100 µg/ml streptomycin and 10% FCS).
Flow cytometry analysis

For the measurement of PP T cell, macrophage and dendritic cell populations, PP mixed cells were resuspended in FACS buffer (PBS with 5% FCS) at a concentration of 2 x 10^6 cells/ml. Cells suspensions were blocked with purified anti-mouse CD16/32 for 20 min at 4°C and stained with PE-Cy7 conjugated anti-mouse CD3e, PE conjugated anti-mouse CD11c, APC conjugated anti-F480 and FITC conjugated anti-mouse MHC II for 30 min in the dark at 4°C. For the measurement of IL-23 receptor expression, PP mixed cells (2 x 10^6 cells/mL) were cultured in 96-well plates in the presence of plate bound anti-CD3 (5µg/ml) and soluble anti-CD28 (1 µg/ml) for 4 h. Cells were collected, blocked with purified anti-mouse CD16/32 for 20 min at 4°C and stained with PE-Cy7 conjugated anti-CD3e and PE conjugated anti-IL-23R for 30 min in the dark at 4°C. The cells were washed twice and resuspended in 0.5 ml FACS buffer. All samples were analyzed at the Loyola University Health Sciences Division FACS Core Facility using a six-color flow cytometer (BD FACSCanto) and FlowJo Software (Treestar). CD3e^+ cells were identified as T cells, CD11c^+MHC II^+ cells were considered dendritic cells and F480^+ cells were considered macrophages.

Measurement of IFN-γ, IL-2, IL-17 and IL-22

Experiment 1: To determine the effects of EtOH exposure and burn injury on T helper effector cytokines IFN-γ, IL-2, IL-17 and IL-22, mixed cells (2 x 10^6 cells/ml) were cultured in the presence of ConA (5 µg/ml) or T cell specific stimuli, plate bound anti-CD3 (5 µg/ml) and soluble anti-CD28 (1 µg/ml), in 96-well plates at 37°C and 5%
CO₂ for 48 h [33]. Following culture, supernatants were harvested and tested for IFN-γ, IL-17 and IL-22 levels, using respective ELISA kits.

Experiment 2: To assess whether rIL-23 restores IL-17 and IL-22 following combined injury, mixed cells (2 x 10⁶ cells/ml) were cultured in the presence of plate bound anti-CD3 (5 µg/ml) + soluble anti-CD28 (1 µg/ml) +/- rIL-23 (10 ng/ml) +/- rIL-12 (10 ng/ml) in 96-well plates at 37°C and 5% CO₂ for 48 h [31, 33, 89]. Supernatants were harvested and IFN-γ, IL-17 and IL-22 measured by ELISA.

Experiment 3: To investigate the role of the AhR transcription factor in IL-23 dependent restoration of IL-22, mixed cells (2 x 10⁶ cells/ml) were pre-treated with AhR inhibitor CH-223191 (10 µM) for 2 h, as previously described [198], prior to being cultured in the presence plate bound anti-CD3 (5 µg/ml) + soluble anti-CD28 (1 µg/ml) +/- rIL-23 (10 ng/ml) in 96-well plates at 37°C and 5% CO₂ for 48 h. IL-17 and IL-22 were measured in culture supernatants.

Experiment 4: To explore the role of intracellular calcium signaling on Th17 effector cytokines IL-17 and IL-22 following EtOH and burn injury, mixed cells cells (2 x 10⁶ cells/ml) were cultured in the presence plate bound anti-CD3 (5 µg/ml) + soluble anti-CD28 (1 µg/ml) +/- PMA (10 ng/ml) and ionomycin (50 ng/ml) in 96-well plates at 37°C and 5% CO₂ for 48 h. IL-17 and IL-22 were measured in culture supernatants.

Statistical analysis

The data, wherever applicable, are presented as means ± SEM and were analyzed using ANOVA with Tukey’s post hoc test or Student’s test (GraphPad InStat). p<0.05 was considered statistically significant.
**Results**

**PP immune cells following ethanol and burn injury**

We determined the effect of EtOH and burn injury on PP T cells (CD3e⁺), dendritic cells (CD11c⁺MHC II⁺) and macrophages (F480⁺) by flow cytometry. As summarized in Table 2, the percentage of PP immune cells remained unaffected following EtOH and/or burn injury.

<table>
<thead>
<tr>
<th></th>
<th>T cells (%) (CD3e⁺)</th>
<th>Dendritic Cells (%) (CD11c⁺MHC II⁺)</th>
<th>Macrophages (%) (F4/80⁺MHC II⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham Vehicle</td>
<td>19.57 ± 1.74</td>
<td>3.23 ± 0.21</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>Sham EtOH</td>
<td>18.97 ± 1.84</td>
<td>3.03 ± 0.20</td>
<td>0.26 ± 0.06</td>
</tr>
<tr>
<td>Burn Vehicle</td>
<td>24.46 ± 2.25</td>
<td>3.09 ± 0.20</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>Burn EtOH</td>
<td>22.58 ± 1.82</td>
<td>3.21 ± 0.18</td>
<td>0.25 ± 0.02</td>
</tr>
</tbody>
</table>

**Table 2.** Percentage of T cells, dendritic cells and macrophages in PPs after EtOH intoxication and burn injury. PPs were harvested one day post injury, processed for single cell suspension and resuspended in FACS buffer at a concentration of 2 x 10⁶ cells/ml. Cells suspensions were blocked with purified anti-mouse CD16/32 for 20 min at 4°C and stained with PE-Cy7 conjugated anti-mouse CD3e, PE conjugated anti-mouse CD11c, APC conjugated anti-F480 and FITC conjugated anti-mouse MHC II for 30 min in the dark at 4°C. The cells were washed twice, resuspended in 0.5 ml FACS buffer and analyzed by flow cytometry. CD3e⁺ cells were identified as T cells, CD11c⁺MHC II⁺ cells were considered dendritic cells and F480⁺MHC II⁺ cells were considered macrophages. Data represent frequency of T cells, dendritic cells and macrophages per live events. Data are represented as mean ± SEM, n=8-12 animals/group from two independent experiments.
**EtOH and burn injury suppresses PP Th17 effector cytokines IL-17 and IL-22**

Our laboratory has previously demonstrated that EtOH intoxication and burn injury suppresses gut associated T cell IFN-γ and IL-2 in a rat model [17, 18, 112]. To further elucidate the effects of EtOH intoxication and burn injury on T helper responses, we examined whether combined insult affects Th17 effector responses in PPs. To test this, PP mixed cells were cultured with ConA (5 µg/ml, see Appendix 2A) or T cell specific stimuli, plate bound anti-CD3 (5 µg/ml) and soluble anti-CD28 (1 µg/ml), for 48 h. Following culture, cell supernatants were harvested, and IL-17 (Fig. 6A) and IL-22 (Fig. 6B) were measured. As demonstrated in Fig. 6, we found no significant change in PP IL-17 and IL-22 in mice gavaged with EtOH alone. Following burn injury, IL-17 and IL-22 were both decreased as compare to sham vehicle. Of note, burn induced suppression of IL-17 and IL-22 was significantly exacerbated in the presence of EtOH intoxication. In our preliminary studies we used ConA as a T cell stimulant and found similar results to T cell specific CD3/CD28. However, to explicitly study the effects of EtOH and burn injury on CD3/CD28 mediated Th17 effector responses, further experiments utilized anti-CD3 and anti-CD28 as T cell stimuli. Moreover, the greatest suppression of Th17 effector cytokines was found in animals subjected to combined EtOH exposure and burn injury; thus, the remaining studies were carried out using only the sham vehicle and burn EtOH groups.
**Figure 6.** *EtOH and burn injury suppresses PP Th17 effector cytokines IL-17 and IL-22.* PP mixed cells (2 x 10⁶ cells/ml) were cultured in 96-well plates in the presence of plate bound anti-CD3 (5µg/mL) and soluble anti-CD28 (1µg/mL) for 48 h at 37°C. Supernatants were harvested for the measurement of IL-17 (Panel A) and IL-22 (Panel B) (IL-17 and IL-22 ELISA Kits, R&D Systems). Values are means + SEM, n=5-11 animals/group, from three independent experiments. *, p<0.001 as compared with sham vehicle; †, p<0.01 and ‡, p< 0.001 as compared with sham EtOH by ANOVA with Tukey’s post hoc test. §, p<0.05 as compared with burn vehicle by student’s t-test.
To test whether the decreased Th1 effector cytokines, IFN-γ and IL-2, exist in mouse PPs, mixed cells were cultured with ConA for 48 h. Following culture, cell supernatants were harvested for the measurement of IFN-γ and IL-2. As shown in Fig. 7, combined insult suppressed Th1 effector cytokines IFN-γ (Fig. 7A) and IL-2 (Fig. 7B), as compared with sham injury.

**PP IL-23 and IL-23 receptor expression**

IL-23 is synthesized by a variety of cells, including monocytes, macrophages, dendritic cells, T cells, B cells and endothelial cells [34, 97]. Given its central role mediating Th17 effector responses, we tested whether EtOH and burn injury perturbs IL-23. Mixed cells were cultured in the presence of LPS (1 µg/ml) for 24 h. Following culture, cell supernatants were collected for protein measurement and cells lysed for intracellular IL-23 protein levels. In our initial studies we measured the p40 subunit of IL-23, which is also shared by IL-12. As demonstrated in Fig. 8A, EtOH and burn injury suppressed IL-12/23p40 in cell culture supernatants, as compared with sham injury. We next measured IL-23p19, the subunit unique to IL-23, in cell culture supernatants and found that the levels of IL-23p19 were below the level of detection (data not shown). To circumvent this, we measured IL-23p19 in cell lysates. Our results, Fig. 8B, show that IL-23p19 is detectable only in sham vehicle samples, suggesting that combined EtOH and burn injury suppresses IL-23p19.
**Figure 7.** PP IFN-γ and IL-2 are decreased following EtOH and burn injury. PP mixed cells (2 x 10^6 cells/ml) were cultured in 96-well plates in the presence of ConA (5µg/ml) for 48 h at 37°C. Supernatants were harvested for the measurement of IFN-γ (Panel A) and IL-2 (Panel B) (IFN-γ and IL-2 ELISA Kits, BD Bioscience). Values are means + SEM, n=4-6 animals/group. *, p<0.05 and †, p<0.005 as compared with sham vehicle group by student’s t-test.
Figure 8. PP IL-23. PP mixed cells (2 x 10^6 cells/ml) were cultured in 96-well plates in the presence of LPS (1 µg/ml) for 24 h. Supernatants were harvested for the measurement of IL-12/23p40 (panel A) (IL-12p40 ELISA Kit, BD Bioscience). Cell culture supernatants were collected for IL-23p19 protein measurement and cells lysed for intracellular IL-23p19 protein levels (Panel B) by ELISA (IL-12/23p40 ELISA kit, BD Sciences; IL-23p19 ELISA kit, R&D Systems). Values are means ± SEM, n=3-8 animals/group. *, p<0.05 as compared with sham vehicle by student t-test.
We further determined whether EtOH exposure and burn injury perturb expression of the IL-23 receptor (IL-23R). To accomplish this, PP cells were stimulated with plate bound anti-CD3 (5 µg/ml) and soluble anti-CD28 (1 µg/ml) for 4 h. After incubation, cells were harvested and stained with PE-Cy7 conjugated anti-mouse CD3e and PE conjugated anti-mouse IL-23R. CD3e and IL-23R co-expression was determined by flow cytometry (Fig. 9). We found that only 2-3% of PP cells co-express CD3e and IL-23R following 4 h of culture. However, EtOH intoxication and burn injury did not significantly affect the frequency (percent) of CD3e⁺IL-23R⁺ event (data not shown), nor the per cell expression of IL-23R within CD3e⁺ cells.
Figure 9. *EtOH* exposure and burn injury does not influence IL-23 receptor expression. PP mixed cells (2 x 10⁶ cells/mL) were cultured in 96-well plates in the presence of plate bound anti-CD3 (5µg/ml) and soluble anti-CD28 (1 µg/ml) for 4 h. Cells were collected and stained with PE-Cy7 conjugated anti-CD3e and PE conjugated anti-IL-23R. Expression of CD3e and IL-23R were determined by flow cytometry. Panel A, representative FACS plots demonstrate the MFI of IL-23R antibody conjugate: gray histogram, fluorescence minus one control; solid black line, respective treatment. Panel B, cumulative IL-23R MFI expression data, values are means + SEM from 4 animals/group.
**IL-23 specifically restores IL-22, but not IL-17, following combined injury**

Differentiation of Th17 lymphocytes depends on IL-1β, IL-6 and TGF-β; however, IL-23 is crucial to Th17 effector function. IL-23 shares the p40 subunit of IL-12, where p40 joins with the p19 subunit to form IL-23, p40 can also join with the p35 subunit to form IL-12 [34]. We tested whether IL-23 restitution prevents decreased IL-17 and IL-22 following EtOH and burn injury. Since IL-23 shares one subunit with IL-12 and we have previously shown decreased IL-12 following EtOH and burn injury [17], we confirmed whether IL-12 influences IL-17 and IL-22. PP mixed cells were cultured with plate bound anti-CD3 (5 µg/ml) and soluble anti-CD28 (1 µg/ml) in the presence or absence of rIL-12 (10 ng/ml) or rIL-23 (10 ng/ml) for 48 h. Following culture, cell supernatants were harvested, and IFN-γ (Fig. 10A), IL-17 (Fig. 10B) and IL-22 (Fig. 10C) were measured by ELISA. Similar to our previous findings [17], the results presented in Fig. 10A confirm that IL-12 restores IFN-γ following EtOH intoxication and burn injury in PP cells. This effect is specific to IL-12 as treatment with IL-23 does significantly affect IFN-γ. In regards to Th17 effector cytokines, our results demonstrate a differential role of IL-23 in modulation of IL-17 and IL-22. The results in Fig. 10B demonstrate that neither IL-12 nor IL-23 treatment modulates PP IL-17. Conversely, IL-23 treatment significantly increased IL-22 in both sham and burn EtOH samples, suggesting the IL-23 restores IL-22 following ethanol intoxication and burn injury. Moreover, IL-12 did not affect IL-22 in either sham or burn EtOH, confirming that restoration of IL-22 is truly IL-23 dependent. Together these results suggest that IL-23 restores IL-22, but not IL-17 following ethanol and burn injury.
Figure 10. IL-23 specifically restores PP IL-22, but not IL-17, following EtOH intoxication and burn injury. PP mixed cells (2 x 10^6 cells/mL) were cultured in 96-well plates in the presence of plate bound anti-CD3 (5µg/ml) and soluble anti-CD28 (1 µg/ml) in the presence or absence of rIL-23 (10 ng/ml) or rIL-12 (10 ng/ml) for 48 h. Supernatants were harvested for the measurement of IFN-γ (Panel A), IL-17 (Panel B) and IL-22 (Panel C) (IFN-γ ELISA Kit, BD Bioscience; IL-17 and IL-22 ELISA Kit, R&D Systems). Values are means ± SEM, n=6-14 animals/group, representative of two independent experiments. *, p<0.001 as compared with all groups; †, p<0.05 as compared with respective sham vehicle; ‡, p<0.001 as compared with respective anti-CD3/28 and anti-CD3/28 + rIL-12 groups by ANOVA with Tukey’s post hoc test. §, p<0.05 as compared with respective sham vehicle by Student’s t-test.
AhR modulates IL-23 dependent restoration of IL-22 following EtOH and burn injury

Our results indicating divergent roles of IL-17 and IL-22, in response to IL-23, led us to examine the role of the AhR, a ubiquitous transcription factor found in the cytoplasm of vertebrate cells, in IL-23 dependent restoration of IL-22. While the AhR has been recently implicated in the regulation of IL-22 [44, 45, 106, 107], and less so IL-17, the mechanisms by which IL-23 and the AhR modulate IL-22 remain poorly described. We examined whether IL-23 dependent restoration of IL-22 is modulated by AhR. Mixed PP cells were pre-treated with AhR inhibitor CH-223191 (10 µM) for 2 h, prior to being cultured with plate bound anti-CD3 (5 µg/ml) and soluble anti-CD28 (1 µg/ml) in the presence or absence of rIL-12 or rIL-23. Following 48 h of culture, cell supernatants were collected and tested for IL-17 (Fig. 11A) and IL-22 (Fig. 11B). Our data demonstrate that PP cells treated with an inhibitor to the AhR, prior to restitution of IL-23, produced significantly less IL-22. However, inhibition of the AhR did not affect IL-17. Together these results suggest that IL-23 dependent restoration of IL-22 is modulated, at least in part, by the AhR and further support divergent roles of IL-23 in Th17 effector functions following ethanol intoxication and burn injury.
**Figure 11.** AhR modulates IL-23 dependent restoration of IL-22 following combined injury. PP mixed cells (2 x 10⁶ cells/mL) were treated with AhR inhibitor CH-223191 (10 µM) for 2 h prior to being cultured in 96-well plates in the presence of plate bound anti-CD3 (5 µg/ml) and soluble anti-CD28 (1 µg/ml) in the presence or absence of rIL-23 (10 ng/ml) for 48 h. Supernatants were harvested for the measurement of IL-17 (Panel A) and IL-22 (Panel B) (IL-17 and IL-22 ELISA Kit, R&D Systems). Values are means + SEM, n=9-17 animals/group, representative of three independent experiments. *, p<0.01 as compared with respective sham vehicle; ‡, p<0.01 as compared with respective anti-CD3/28 and anti-CD3/28 + CH-223191 groups by ANOVA with Tukey’s post hoc test. †, p<0.05 as compared with respective sham vehicle by Student’s t-test.
**IL-17 requires intracellular calcium signaling following EtOH and burn injury**

To further decipher the differential effects of EtOH exposure and burn injury on IL-17 and IL-22 following, PP cells were treated with PMA and ionomycin, which directly activates Protein kinase C (PKC) and intracellular Ca^{2+} signaling. Mixed PP cells were cultured with plate bound anti-CD3 (5 µg/ml), soluble anti-CD28 (1 µg/ml), PMA (10 ng/ml) and ionomycin (50 ng/ml) for 48 h. Following cell culture, cell supernatants were collected and tested for IL-17 (Fig. 12A) and IL-22 (Fig. 12B). We found that treatment of cells with PMA and ionomycin restores PP IL-17 levels to that of sham vehicle levels. However, PMA and ionomycin treatment does not affect IL-22. Together, these data highlight the importance of PKC and intracellular Ca^{2+} in the induction of IL-17, but not IL-22, which supports recent data indicating differential regulation of IL-17 and IL-22.
Figure 12. *IL-17 requires intracellular calcium signaling following EtOH and burn injury*. PP mixed cells (2 x 10^6 cells/mL) were cultured in 96-well plates in the presence of plate bound anti-CD3 (5µg/ml), soluble anti-CD28 (1 µg/ml), PMA (10 ng/ml) and ionomycin (50 ng/ml) for 48 h. Supernatants were harvested for the measurement of IL-17 (Panel A) and IL-22 (Panel B) (IL-17 and IL-22 ELISA Kit, R&D Systems). Values are means + SEM, n=6-16 animals/group, representative of three independent experiments. *, p<0.05 as compared with respective sham vehicle (t-test for IL-22); †, p<0.01 as compared with respective anti-CD3/28 group by ANOVA with Tukey’s post hoc test.
Discussion

The data presented in this article clearly demonstrate that acute EtOH exposure prior to burn injury results in suppressed CD3/28 dependent expression of Th17 effector cytokines IL-17 and IL-22 in PP cells. While our findings suggest that IL-23 restitution effectively restores IL-22, it does not modulate IL-17. We further found that IL-23 mediated restoration of IL-22 is dependent on the AhR. IL-17, on the other hand, requires activation of PKC and intracellular calcium signaling, as demonstrated by increased levels of IL-17 following treatment with PMA and ionomycin. Altogether, our results provide novel evidence suggesting that IL-23 differentially regulates Th17 effector cytokines IL-17 and IL-22 following EtOH and burn injury, and further indicate a cross-talk between IL-23 and the AhR.

T helper cells, including Th17 cells, are of critical importance to mucosal immunity, barrier function and the containment of pathogenic gut bacteria [22, 23, 23-25, 29, 30, 80]. Gut bacterial pathogens are often implicated in pathogenesis associated with EtOH and/or burn injury. For these studies we focused on gut associated lymphoid organs PPs, since they are of central importance to gut bacteria translocation and their containment [85, 199, 200]. Specifically, PPs are secondary lymphoid organs along the small intestine, which are lined by specialized epithelial cells, “M” cells. Similar to antigen presenting cells, including dendritic cells, M cells come in direct contact with luminal content. Under normal conditions, pathogens that cross the epithelial barrier are limited from invading the host by the immune cells within PPs, including T helper cells [85, 86, 200]. Several studies exploring the role of T cells demonstrate that athymic
(nu/nu) mice, which lack mature T cells, have increased bacterial translocation to mesenteric lymph nodes (MLN), spleen and liver [201]. Together these findings define an interdependent relationship between PPs, T cells and containment of gut bacteria. In the context of EtOH exposure, chronic EtOH feeding has been linked with decreased total, T and B cells in PPs of animals subjected to chronic EtOH feeding as compared to control animals [194]. In regards to EtOH and burn, our laboratory has demonstrated decreased T cell proliferation and Th1 immune functions in T cells from PPs and MLN, as well as increased bacterial translocation to MLN [14-17, 19-21, 112]. Additionally, our laboratory found that depletion of CD3+ T cells from normal animals increases bacterial accumulation in MLN. We further showed that depletion of T cells in animals receiving EtOH and burn injury results in systemic invasion of bacteria, including spleen and systemic circulation [18]. The results presented in this article are the first to elucidate the effects of EtOH and burn injury on Th17 immune responses within PPs. Since Th17 cells have been implicated in defense against gut pathogens [22-25], a suppression of Th17 effector functions may contribute to the increased bacterial translocation and gut barrier dysfunction following combined insult of EtOH intoxication and burn injury.

Our initial results indicating suppression of Th17 effector cytokines IL-17 and IL-22 following EtOH exposure and burn injury, led us to examine the effects of injury on PP cell composition. As demonstrated in Table 2, we did not find any change in the percent (frequency) of T cells, macrophages or dendritic cells. Therefore, it is possible that the ability of T cells to respond to antigenic stimuli is diminished following EtOH exposure and burn injury. Moreover the suppression of Th17 cytokines could also result
from a decrease in IL-23, a cytokine needed for Th17 differentiation. Thus, we focused on restituting IL-23 and defining the pathways involved in modulation of IL-17 and IL-22 post EtOH exposure and burn injury.

We observed a decrease in IL-23 levels in PP following ethanol and burn injury. Since IL-23 plays a role in Th-17 differentiation [31-33], we investigated whether the restituation of IL-23 prevents Th17 cytokine suppression following ethanol and burn injury. Our laboratory recently demonstrated decreased IL-12 following EtOH and burn injury, as well as successful restoration of Th1 cytokines IFN-γ and IL-2 following restituation of IL-12 [17]. In this study, we attempted to measure IL-23, as well as IL-12/23p40, in PP cell culture supernatants stimulated with LPS. While we found decreased IL-12/23p40 in cells from EtOH and burn injured mice, we were only able to measure IL-23 in cell lysates from the shame vehicle group. Levels of IL-23 were undetectable in cell lysates from the EtOH and burn group. As shown in Table 2, there was no change in the percent of macrophages or dendritic cells following EtOH and burn injury; nonetheless, it possible that their ability to produce IL-23 is impaired following EtOH and burn. IL-12 and IL-23 share a common subunit, the p40 subunit, and have been suggested to have common roles in driving Th1 and Th17 adaptive immune responses, though this remains controversial [34, 41, 202]. Therefore, we tested the effects of IL-12 and IL-23 on IFN-γ, IL-17 and IL-22 in our murine model of EtOH exposure and burn injury. As previously published [17], our results demonstrate effective restoration of IFN-γ following restituation of IL-12, with no significant change in IFN-γ following restitution of IL-23. Though we expected restoration of IL-17 and IL-22 following restitution of IL-23, the
addition of IL-23 only induced IL-22, not IL-17, with no effects in response to IL-12. These effects were not due to changes in IL-23 receptor expression on CD3+ T cells. It is possible that IL-17 requires restitution of other Th17 inducing cytokines, including IL-6 and TGF-β. Differential requirements for effective expression of IL-17 and IL-22 might explain our divergent results. Recently, Duhen et al. reported low expression of RORC (ROR-γt in humans) in the IL-22 producing cells, and concluded that RORC is non-essential to expression of IL-22 [44]. It may be that EtOH and burn injury inhibit IL-23 dependent induction of ROR-γt, and thus prevent IL-23 induction of IL-17. Further studies will examine the effects of EtOH and burn injury on the IL-23 signaling pathway, including activation of STAT3 and expression of ROR-γt.

While our results partly support previous literature indicating a requirement for IL-23 in expression of Th17 effector cytokine IL-22 [37, 203], our IL-17 results suggest a divergent role for IL-23. New data investigating the molecular pathways that control IL-17 and IL-22 expression increasingly demonstrate differential regulation of these Th17 effector cytokines [44, 45, 101, 102, 107, 110, 110]. Among the multiple regulators of IL-17 and IL-22 are various transcription factors, including STAT3, ROR-γt and, most recently, the AhR. While it is generally accepted that IL-6 and IL-23 activate STAT3 [88], to in turn activate expression of Th17 hallmark transcription factor ROR-γt, how AhR regulates IL-22 remains unclear. The AhR is a cytosolic ligand-activated transcription factor. Upon ligand binding, AhR translocates to the nucleus, where it dimerizes with AhR nuclear translocator and activates gene transcription [204]. In our study, we utilized an inhibitor to that binds the AhR and prevents translocation to the
nucleus [198], to demonstrate that IL-23 dependent induction of IL-22 utilizes the AhR. While our data indicate cross-talk between IL-23 and the AhR, further studies will be carried out to test how these two pathways converge. Previous studies have used co-immunoprecipitation to demonstrate interaction of the AhR and various STAT proteins [109]. Thus, it is possible that IL-23 activates AhR/STAT interactions. Moreover, determining the role of ROR-γt in IL-22 immunity also warrants further investigation, since controversy regarding the importance of ROR-γt in IL-22 expression still exists [44, 45]. Duhen et al. reported low expression of RORC (ROR-γt in humans) in the IL-22 producing cells, and concluded that RORC is non-essential to IL-22 [44]. Conversely, Trifari et al. used siRNA to demonstrate that silencing of RORC or AhR diminished IL-22 production from memory CD4+ T cells [45].

To further decipher the intracellular mechanism of suppressed Th17 effector cytokines following EtOH intoxication and burn injury, we determined whether restitution of intracellular calcium and activation of PKC prevents the suppression of Th17 effector function. Several lines of evidence suggest that the activation of PKC and the sustained elevation of intracellular calcium are required for T cell activation [205, 206]. These studies suggest that signals emanating from TCR result in the activation of protein tyrosine kinases and phospholipase C-γ [207, 208]. PLC-γ hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 causes a calcium release from intracellular stores followed by calcium influx through the plasma membrane leading to a sustained elevation in intracellular calcium concentration [207, 208]. DAG activates PKC [209]. In line with
these findings, burn injury, regardless of EtOH, has been correlated with suppressed intracellular calcium signaling [210]. Our current findings suggest that PP cells cultured with plate bound anti-CD3 (5 µg/ml), soluble anti-CD28 (1 µg/ml) in the presence of PMA (10 ng/ml) and ionomycin (50 ng/ml) restored IL-17 levels to that of sham vehicle levels. On the other hand, PMA and ionomycin treatment did not affect IL-22. Together, these data suggest the importance of effective intracellular Ca\(^{2+}\) influx and PKC activation in the induction of IL-17. Furthermore, our findings support recent data indicating differential regulation of IL-17 and IL-22. However the mechanism underlying this divergent regulation of PP T cell IL-17 and IL-22 remains to be explored.

In summary, our findings suggest that ethanol combine with burn injury differentially regulate the expression of PP T cell IL-17 and IL-22. While IL-22 is successfully restored by restitution of IL-23, IL-17 restoration requires reestablishment of PKC and intracellular calcium signaling. The divergent transcriptional regulation of IL-17 and IL-22 may offer a target for immunomodulation in the treatment of patients who sustain burn injury under the influence of EtOH intoxication. Specifically, IL-22’s role in epithelial immune and barrier function maintenance, in addition to its transcription regulation by AhR, offers a unique opportunity for targeted therapy. Among the ligands for AhR are components of various vegetables, including broccoli, cabbage and brussel sprouts [204]. Following injury, including EtOH and burn, these foods may be incorporated into diet regimens to induce IL-22 and promote epithelial cell regeneration, proliferation and boost innate immunity through induced expression of AMPs, without
inducing pathogenic levels of IL-17. Thus, future studies will explore the translational potential of modulating IL-22 following EtOH and burn injury.
CHAPTER FIVE

IL-22 MODULATES GUT EPITHELIAL AND IMMUNE BARRIER FUNCTIONS FOLLOWING ACUTE ALCOHOL EXPOSURE AND BURN INJURY

Abstract

Interleukin (IL)–22 maintains gut epithelial integrity and expression of antimicrobial peptides (AMPs) Reg3β and Reg3γ. Burn patients with a measureable blood ethanol level at the time of hospital admission have an increased risk of morbidity. Our lab has shown that acute alcohol/ethanol (EtOH) exposure prior to burn injury results in increased gut permeability, intestinal T cell suppression and enhanced bacterial translocation. Furthermore, we also found a decrease in intestinal IL-22 levels and Reg3β and Reg3γ expression, which were not noted following ethanol or burn alone. We examined whether in vivo restitution of IL-22 modulates gut permeability, Reg3β and Reg3γ and bacterial load within the intestine following ethanol and burn injury. Male mice, ~25g, were gavaged with EtOH (2.9 mg/kg) prior to receiving a ~12.5% TBSA full thickness burn. Mice were immediately treated with saline control or IL-22 (1 mg/kg) by i.p. injection. One day post injury animals were sacrificed and intestinal permeability, Reg3β and Reg3γ expression and bacterial load measured. EtOH combined with burn injury resulted in decreased expression of Reg3β and Reg3γ, as compared to sham injury. This was attenuated following treatment with IL-22. Qualitatively, combined insult resulted in increased bacterial load in intestine luminal content and intestine tissue, which was prevented in half of IL-22 treated animals. IL-22 treatment also prevented increased
gut permeability following ethanol and burn injury. Our data indicate that treatment with IL-22 maintains gut epithelial and immune barrier integrity following ethanol and burn injury; thus, the IL-22/AMP pathway may provide a novel therapeutic target for the treatment of patients who sustain burn injury under the influence of EtOH.

**Introduction**

IL-22, a cytokine that uniquely does not act on immune cells, maintains gut epithelial integrity and mucosal immunity by acting on epithelial cells. Specifically, IL-22 is involved in chemotaxis [81], antimicrobial expression (AMP) [27-30], tissue repair [73, 82] and epithelial cell survival [83, 84], proliferation [23] and differentiation [39]. Recent evidence further implicates IL-22 and IL-22-induced AMPs Reg3β and Reg3γ, two C-type lectins, in the containment of Gram-negative and Gram-positive gut pathogens [23, 29, 211, 212].

In the United States, more than one million burn injuries are reported yearly [1]. Nearly one-half of these injuries occur under the influence of alcohol/ethanol (EtOH) intoxication [2-4, 6, 8, 11]. Burn victims who sustain injury post EtOH exposure exhibit increased clinical complications, resulting in significantly longer hospital stays, more surgical interventions and increased susceptibility to infection and higher morbidity [3, 4, 6, 11-13]. Moreover, patients with EtOH in their blood at the time of hospital admission are more likely to die of smaller burns than non-EtOH exposed patients [2]. Though, the mechanism(s) by which EtOH confounds clinical outcomes following burn injury remain(s) unknown, previous data suggest that gut pathogens and their products may play a pivotal role in the development of sepsis and multiple organ failure reported in burn and
trauma patients [6, 14, 49-58]. In line with these studies, our laboratory has demonstrated increased intestinal tissue damage, leakiness, and bacterial translocation as well as T lymphocyte suppression following EtOH intoxication and burn injury [14-21]. Therefore, further studies aimed at targeting the modulation of gut immune barrier function and immunity are required to improve clinical outcomes of patients who sustain burn injury under the influence of EtOH. Since IL-22 has been implicated in the maintenance of gut epithelial barrier, perturbation of IL-22 or AMP expression following EtOH and burn injury may severely impair the immune defense and barrier integrity.

Our present study evaluated the effects of EtOH and burn injury on gut IL-22 levels, leakiness, bacterial growth, and Reg3β and Reg3γ expression in our recently established murine model [195]. Additionally, we have determined whether in vivo treatment with rIL-22 prevents increased gut leakiness and bacterial growth and modulates Reg3 expression following EtOH exposure and burn injury. Our results provide a novel role for rIL-22 in the immunomodulation of gut barrier function, bacterial containment and AMP expression. We found that when combined, EtOH and burn injury resulted in decreased levels of IL-22 as well as decreased expression of Reg3β and Reg3γ in the small intestine. We further confirmed that dual insult results in increased gut leakiness and gut bacterial growth. IL-22 treatment successfully restored expression of Reg3 and prevented increased gut permeability following combined EtOH exposure and burn injury. Qualitatively, IL-22 treatment prevented increased gut bacterial load in half of IL-22 treated animals. Thus, IL-22 maintains gut epithelial and immune barrier integrity following EtOH and burn injury. The IL-22/AMP pathway may provide a novel
therapeutic target for the treatment of patients who sustain burn injury under the influence of EtOH.

Material and Methods

Animals and reagents

Male C57BL/6 mice, 6-7 wks old, were obtained from Harlan Laboratories. Recombinant IL-22 was obtained from GenScript (Piscataway, NJ). IL-22 ELISA kit was obtained from R&D Systems (Minneapolis, MN). Protein Assay was obtained from Bio-Rad (Hercules, CA). RNeasy Mini Kit was obtained from Qiagen (Valencia, CA). TaqMan Expression Assays for Reg3γ, Regβ and GAPDH were obtained from Applied Biosystems (Carlsbad, California). Fluorescein isothiocyanate (FITC)-conjugated dextran was obtained from Sigma Aldrich (St. Louis, MO).

Treatment of mice with rIL-22 following EtOH and burn injury

As described in Chapter 4 [195], adult C57BL/6 male mice were randomly divided to receive sham or burn injury and either ethanol (EtOH) or vehicle (water) to yield four experimental groups: sham vehicle, sham EtOH, burn vehicle and burn EtOH. Mice were gavaged with either 0.4 ml of 25% EtOH in water (~2.9 g/kg) or water. Four hours after gavage, mice were anesthetized by i.p. injection of ketamine hydrochloride/Xylazine cocktail (~80 mg/kg and 1.2 mg/kg, respectively). Dorsal surfaces were shaved, and animals were transferred into a template fabricated to expose ~12.5% of the TBSA. Immediately after sham or burn injury, a group of sham vehicle and burn ethanol injured mice were treated with rIL-22 (1 mg/kg body weight in sterile PBS, GenScript) by i.p. injection [47]. Animals were resuscitated with 1.0 ml
physiological saline by i.p injection. Animals were allowed food and water *ad libitum*.

All experiments were conducted in accordance with the guidelines set forth in the Animal Welfare Act and were approved by the Institutional Animal Care and Use Committee at the Loyola University Health Sciences Division.

**Measurement of intestinal tissue IL-22 levels**

One day post injury, mice were anesthetized and the abdominal cavity exposed via midline laparotomy and a 3-cm-long segment of small intestine was removed, cleaned, snap-frozen in liquid nitrogen and stored at −70°C [20, 21]. Equal weights of tissue (100 mg) from each experimental group were suspended in 1 mL of a buffer (PBS containing protease inhibitor cocktail) and sonicated at 30 cycles twice for 30 sec on ice. Homogenates were cleared by centrifuging at 12,000 rpm at 4°C. The supernatants were stored at −70°C. Protein levels in the homogenates were determined using the Bio-Rad Protein Assay. IL-22 levels in the intestinal tissue homogenates were measured by enzyme-linked immunosorbent assay (ELISA) following manufacturer’s instructions.

**Measurement of Reg3β and Reg3γ expression**

One day post injury, mice were anesthetized and the abdominal cavity exposed via midline laparotomy and a 1-cm-long segment of small intestine was removed, cleaned, snap-frozen in liquid nitrogen and stored at −70°C [20, 21]. Equal weights of tissue (25 mg) from each experimental group were used for RNA extraction using the Qiagen RNeasy Mini Kit. RNA was quantified using a Nanodrop Spectrophotometer ND-1000 and cDNA synthesized using the High Capacity Reverse Transcriptase Kit (Applied Biosystems). Quantitative RT-PCR was performed with the Applied Biosystems 7500
Fast Real-Time PCR system, using specific TaqMan Expression Assays (Applied Biosystems) for Reg3β, Reg3γ and GAPDH. Values were then normalized to GAPDH expression via the ΔΔCT method, as previously described [213].

**In vivo gut permeability assay**

A separate group of animals was used for the measurement of intestinal permeability, with minor modifications to previously described method [20, 21]. One day post sham or burn procedure and treatment with IL-22, animals were anesthetized and a midline laparotomy performed. The renal artery and vein of both kidneys were ligated and a 10 cm segment of small intestine (lower jejunum and ileum) isolated without damaging intestinal and mesenteric structures. An 18 gauge intravenous catheter was inserted into the isolated intestine. A solution of 4 kDa FITC-conjugated dextran in PBS (100 µl of 25 mg/ml) was injected into the isolated small intestine. Blood samples were collected from the heart 90 min post FITC-dextran injection and plasma FITC-dextran concentration was determined using a fluorescence spectrophotometer at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The plasma concentration of FITC-dextran in each sample was calculated using a standard curve prepared from dilutions of FITC-dextran in PBS.

**Intestinal bacterial content**

One day post injury, mice were anesthetized and the abdominal cavity exposed via midline laparotomy and a 1-cm-long segment of small intestine, ileum, was aseptically removed, cleaned and rinsed in sterile PBS one time. Tissues were weighed and homogenized in sterile PBS (10 µl/mg tissue weight), one cycle for 30 sec.
Separately, fecal luminal content was harvested from terminal small intestine, weighed and resuspended in PBS (40 μl/mg fecal luminal content weight) by vortexing suspension on high for 30 sec. 25 μl of intestinal tissue or fecal luminal content were plated and cultured on trytic soy agar (TSA) or MacConkey agar for the analysis of total and Gram negative bacteria, respectively, for 24 h at 37°C. Following incubation, TSA and MacConkey agar plates were assessed for bacterial colony forming units (CFUs) and photographed.

**Statistical analysis**

The data, wherever applicable, are presented as means + SEM and were analyzed using ANOVA Tukey post-hoc test or Student’s test (GraphPad InStat). *p*<0.05 was considered statistically significant.

**Results**

**Intestinal tissue IL-22 is decreased following EtOH and burn injury**

To determine whether EtOH and burn injury perturbs IL-22 in the intestinal tissue, we measure IL-22 in intestinal tissue homogenates one day post EtOH exposure and burn injury. As shown in Fig. 13, there was no difference in IL-22 in the intestinal tissue of sham-injured animals, regardless of EtOH. However, while burn injury alone trended towards an increase in intestinal IL-22, this was not found to be significantly different from sham animals. The intestinal tissue from the EtOH burn group demonstrated significantly decreased levels of IL-22, as compared to sham vehicle and burn vehicle groups.
Figure 13. Intestinal tissue IL-22 is decreased following EtOH and burn injury. One day post insult, terminal small intestines were collected and equal weights of tissue (100 mg) from each experimental group were suspended in 1 mL of a buffer (PBS containing protease inhibitor cocktail) and sonicated. Homogenates were cleared by centrifugation and supernatants were analyzed for IL-22 by ELISA (IL-22 ELISA Kits, R&D Systems). IL-22 levels were normalized to protein levels. Values are means ± SEM, n=6-9 animals/group from two independent experiments. *, p=0.0381 as compared with sham vehicle by Student’s t test with Welch correction; †, p<0.01 as compared with burn vehicle by ANOVA with Tukey’s post-hoc test.

Increased gut permeability one day post EtOH exposure and burn injury

Our laboratory has previously demonstrated increased gut permeability in a rat model of EtOH exposure and burn injury [20, 21]. To confirm these findings in our recently established mouse model [195], we measured intestinal permeability one day after injury. The results presented in Fig. 14 indicate that there was no difference in the
plasma FITC-dextran levels in either sham group. Similarly, plasma FITC-dextran levels in burn vehicle group were also not found to be different from shams controls. However, FITC-dextran levels were higher in burn ethanol group relative to other groups. These results suggest that the combined insult of ethanol and burn increases intestinal permeability when compared to either ethanol intoxication or burn injury alone. Moreover, our findings confirm that similar to rats, mice also exhibit a disruption in the intestinal barrier following ethanol and burn injury.

**Figure 14.** *Increased gut permeability one day post EtOH exposure and burn injury.* On Day 1 after EtOH and burn injury, intestinal permeability was determined by monitoring the transfer of FITC-dextran from the isolated intestinal lumen to the systemic circulation, blood was drawn by cardiac puncture 90 min after FITC-dextran injection. Values are means + SEM, \( n=4-5 \) animals/group. *, \( p<0.01 \) as compared to all groups by ANOVA with Tukey’s post-hoc test.
EtOH intoxication and burn injury suppresses intestinal AMP expression

Among the various AMPs regulated by IL-22, Reg3β and Reg3γ are key components of gut mucosal immunity [29, 211, 214]. To determine whether EtOH exposure and burn injury affects expression of Reg3β and Reg3γ we measured mRNA levels of Reg3β and Reg3γ in small intestinal tissue one day following EtOH exposure and burn injury. As demonstrated in Fig. 15, neither EtOH alone, nor burn injury alone affect Reg3β and Reg3γ expression, as compared to sham injury. However, when combined EtOH exposure and burn injury resulted in a significant suppression of Reg3β and Reg3γ expression, as compared with sham vehicle. Thus, these data suggest that EtOH and burn injury results in decreased intestinal AMP expression.
Figure 15. Decreased intestinal AMP expression one day post EtOH and burn injury. One day post injury, small intestine was harvested and equal weights of tissue (25 mg) from each experimental group were used for RNA extraction using the Qiagen RNeasy Mini Kit. Reg3β, Reg3γ and GAPDH were measured by qRT-PCR using specific probes. AMP expression was normalized to GAPDH expression via the ΔΔCT method. Values are means + SEM, n=3-10 animals/group from two independent experiments. *, p<0.05 as compared to sham vehicle; †, p<0.05 as compared to burn vehicle by ANOVA with Tukey’s post-hoc test.
Increased gut bacterial growth one day post EtOH exposure and burn injury

Previously, our laboratory has demonstrated increased gut bacterial translocation to regional draining mesenteric lymph nodes following EtOH and burn injury [16, 18]. To determine whether EtOH intoxication and burn injury facilitates gut bacterial growth, we measured total and Gram-negative bacterial load in small intestinal tissue as well as in small intestinal luminal content (feces). Intestinal tissue samples were weighed and homogenized in PBS and luminal content samples weighed and resuspended in PBS. Homogenates were cultured on specific agar plates for total (TSA) and Gram-negative (MacConkey). We measured Gram-negative bacteria as they are the major cause of sepsis and organ dysfunction in critically ill burn patients [215, 216]. As demonstrated in Fig. 16, our results show that EtOH and burn injury alone do not cause a significant change in gut bacterial load. However, the combined insult of EtOH and burn injury resulted in a robust increase in total (data not shown) and Gram-negative bacteria in the intestinal tissue, as well as in the luminal content.
Figure 16. *Increased Gram-negative bacterial growth one day after EtOH exposure and burn injury.* One day post injury, a 1-cm-long segment of small intestine was aseptically removed, cleaned and rinsed in sterile PBS. Tissues were weighed and homogenized in sterile PBS (10 μl/mg tissue weight). Separately, fecal luminal content was harvested from terminal small intestine, weighed and resuspended in PBS (40 μl/mg fecal luminal content weight). 25 μl of intestinal tissue or fecal luminal content were plated and cultured on MacConkey agar for the analysis of Gram-negative bacteria, for 24 h at 37°C. Following incubation, plates were assessed for bacterial colony forming units (CFUs) and photographed. Pictures are representative of n=5-9 animals/group.
Treatment with IL-22

To perform these studies, sham vehicle and burn EtOH injured animals were treated with IL-22 (1 mg/kg body weight) immediately after sham or burn injury. In these and subsequent experiments, we did not include all four experimental groups. Rather, we used only sham vehicle and burn EtOH, since only the burn EtOH group demonstrated decreased intestinal IL-22, increased gut permeability and decreased AMP expression.

Treatment with IL-22 prevents increased gut permeability

The effect of IL-22 treatment on gut leakiness was observed one day post injury. As demonstrated in Fig. 17, IL-22 treatment did not alter gut permeability in sham treated animal. However, IL-22 treatment prevented the increase in plasma FITC-Dextran levels following EtOH and burn injury. These data suggest that IL-22 prevents the increase in gut permeability following EtOH exposure and burn injury.
Figure 17. **IL-22 treatment prevents increased gut permeability.** In a group of sham vehicle and burn EtOH animals, IL-22 (1 mg/kg body weight) was injected immediately after injury. One day after EtOH and burn injury, intestinal permeability was determined by monitoring the transfer of FITC-dextran from the isolated intestinal lumen to the systemic circulation, blood was drawn by cardiac puncture 90 min after FITC-dextran injection. Values are means ± SEM, n=4-6 animals/group. *, p<0.05 as compared to all groups by ANOVA with Tukey’s post-hoc test.

**IL-22 treatment promotes intestinal AMP expression**

To test whether IL-22 treatment modulates AMP expression one day following EtOH and burn injury, small intestinal tissue was collected from saline control and IL-22 treated animals. Tissues were processed for RNA extraction and the expression of Reg3β and Reg3γ analyzed by qRT-PCR. The results presented in Fig. 18 demonstrate that IL-22 treatment does not affect expression of Reg3β or Reg3γ in sham animals. However, IL-22 treatment prevented the suppression of Reg3β and Reg3γ expression in animals that received EtOH and burn injury.
Figure 18. Treatment with IL-22 prevents the decrease in intestinal AMP expression one day post EtOH exposure and burn injury. Day one after injury and IL-22 treatment, small intestine was harvested and equal weights of tissue (25 mg) from each experimental group were used for RNA extraction using the Qiagen RNeasy Mini Kit. Reg3β, Reg3γ and GAPDH were measured by qRT-PCR using specific probes. AMP expression was normalized to GAPDH expression via the ΔΔCT method. Values are means + SEM, n=4-14 animals/group from three independent experiments. *, p<0.05 as compared to all groups; †, p<0.05 as compared to sham vehicle; ‡, p<0.001 as compared to burn vehicle by ANOVA with Tukey's post-hoc test.
Gut bacterial load and IL-22 treatment

Lastly, we examined whether IL-22 treatment has any relationship with gut bacterial load. Small intestine tissue and luminal content were harvested from saline and IL-22 treated animals, one day post sham or EtOH and burn injury. Intestinal tissue samples were weighed and homogenized in PBS and luminal content samples weighed and resuspended in PBS. Homogenates were cultured on specific agar plates for total (TSA) and Gram-negative (MacConkey) bacteria. As depicted in Fig. 19, IL-22 treatment did not substantially affect Gram-negative bacterial growth in sham animals. However, 4 out of 8 mice treated with IL-22 exhibited a substantial decrease in Gram-negative bacterial load, both in the intestinal tissue samples and luminal content. IL-22 did not appreciably affect total bacteria (data not shown). These findings suggest that the decrease in IL-22 may be responsible in part for the increase in gut bacterial load following EtOH and burn injury.
Figure 19. *IL-22 treatment prevents increased Gram-negative bacterial growth in the intestine.* One day post injury and IL-22 treatment, a 1-cm-long segment of small intestine was aseptically removed, cleaned weighed and homogenized in sterile PBS (10 μl/mg tissue weight). Separately, fecal luminal content was harvested from terminal small intestine, weighed and resuspended in PBS (40 μl/mg fecal luminal content weight). 25 μl of intestinal tissue or fecal luminal content were plated and cultured on MacConkey agar for the analysis of Gram-negative bacteria, for 24 h at 37°C. Following incubation, plates were assessed for bacterial colony forming units (CFUs) and photographed. Pictures are representative of n=5-8 animals/group.
**Discussion**

EtOH intoxication is a confounding factor in postburn pathogenesis. Yet, few studies have evaluated the role of EtOH in post burn complications. In the present study, we examined the role of EtOH intoxication on postburn gut permeability, intestine expression of IL-22 and AMP expression as well as total and Gram-negative gut bacterial load. Additionally, we treated animals with IL-22 to test whether it plays any role in the modulation of gut barrier, immune function and bacterial containment following EtOH and burn injury. We found that EtOH combined with burn injury resulted in a decrease in intestinal tissue levels of IL-22 as well as suppression of Reg3β and Reg3γ expression, as compared to sham injury. This was attenuated following treatment with IL-22. IL-22 treatment also prevented increased gut permeability following ethanol and burn injury. The restoration of AMP and gut barrier was accompanied with a demonstrable decrease in Gram-negative gut bacterial load in half of the animals treated with IL-22 after ethanol and burn injury. Specifically, with IL-22 treatment samples continued to exhibit a substantial increase in total bacteria (TSA) post EtOH and burn injury (data not shown); yet, there was decreased growth of Gram-negative bacterial colonies in intestine tissue homogenates and luminal content. This finding suggests Gram-negative bacteria are likely controlled by IL-22 and/or AMPs. Thus, our data indicate that IL-22 treatment maintains gut epithelial immune and barrier integrity following ethanol and burn injury.

Unlike most cytokines, which exert their actions on immune cells, the IL-22 receptor is only expressed on epithelial cells of the skin, intestine, liver, pancreas, lung and kidney [39, 73, 217]. IL-22 is of particular interest to gut immunity and barrier
integrity [39, 73]. The IL-22 receptor is composed of the IL-22R and IL-10R2 subunits [73]. Binding of IL-22 to IL-22R1 induces its complex formation with IL-10R2 and propagation of the JAK-STAT pathway, phosphorylation/homodimerization of STAT3 and subsequent activation of gene transcription [218]. Among the various genes activated by IL-22 are AMPs, including Reg3β and Reg3γ, which are recognized for their role in gut immunity and barrier integrity [23, 29, 211]. Moreover, IL-22 has been implicated in tissue repair and regeneration. Therapeutically, IL-22 shows improvement in models of EtOH induced liver injury, pancreatitis, hepatitis and inflammatory bowel disease [47, 83, 84, 149, 153, 154].

Our current data, suggests that IL-22 treatment modulates AMP expression, bacterial load and gut permeability one day post EtOH exposure and burn. Together these data highlight the critical role that IL-22 places in maintaining gut immune function and barrier integrity. While further experiments will need to be performed to delineate a mechanism by which IL-22 treatment improves intestinal AMP expression, bacterial load and permeability, our data support the use of IL-22 as a therapeutic agent. Mechanistically, it is likely that IL-22 maintains gut epithelial integrity through induction of genes related to regeneration and proliferation in a STAT3 dependent manner. Recently, our laboratory demonstrated IL-18 dependent increased apoptosis and altered tight junction formation follow EtOH and burn injury [219]. It is likely that uncontrolled IL-18 driven damage is due, in part, to decreased protective effects of IL-22. To determine the mechanism by which IL-22 prevents increased gut permeability following
EtOH and burn injury, future experiments will explore the role of IL-22 dependent proliferation and regeneration in counterbalancing the damaging effects of IL-18.

In regards to immune function, it is possible that IL-22 exerts its action as a direct immune modulator and through induction of AMP expression. Recently, IL-22 was demonstrated to protect against *K. pneumoniae* infection, a Gram-negative gut pathogen [23]. *In vivo* inoculation with *K. pneumoniae* induced IL-22 in the lungs of infected animals, where blockage of IL-22 increased mortality as well as local and systemic bacterial dissemination. In line with our current findings, Aujla *et al.* further demonstrated a requirement for IL-23 in expression of IL-22 [23]. Following EtOH and burn injury, it is possible that decreased intestinal IL-22, as presented in this study, may facilitate survival of Gram-negative bacteria, including *K. pneumoniae*. Conversely, the immuno-protective effects of IL-22 result from induction of Reg3β and Reg3γ. Initially, Reg3β and Reg3γ were shown to bind peptidoglycan and kill Gram-positive bacteria [211]. More recent evidence, suggest that IL-22 plays a role in protective against Gram-negative bacteria [29, 212], though the mechanism by which this occurs remains unknown. Using an *in vivo* model of *C. rodentium* infection, a Gram-negative pathogen, in IL-22 knockout mice, Zheng *et al.* demonstrated a requirement for IL-22 in survival of *C. rodentium* [29]. IL-22 knockout mice demonstrated decreased survival, while treatment with recombinant mouse Reg3γ-Ig fusion protein improved survival [29]. Previously, Reg3β was shown to induce aggregation of *E. coli* [212]. Together these data suggest support the notion that Reg3β and Reg3γ play a role in the protection against Gram-positive as well as Gram-negative bacteria. In our model, IL-22 dependent
induction of Reg3β and Reg3γ correlated with decreased Gram-negative bacterial burden in small intestinal tissue and luminal content. Further studies dissecting the role of Reg3β and Reg3γ in the containment of gut Gram-negative bacteria are need. It is possible that Reg3β and Reg3γ do not directly kill Gram-negative bacteria, but rather prevent their invasion into intestinal crypts.

Our data indicate that IL-22 treatment prevents increased gut permeability and increased bacterial load following EtOH exposure and burn injury. However, we recognize that gut permeability did not return completely back to that of sham vehicle animals following IL-22 treatment. Similarly, decreased bacterial load was only noted in half of animals treated with IL-22. Together this suggests that suppression of IL-22 is only one mechanism by which gut permeability is increased following EtOH and burn injury. Other mechanisms that contribute to increased gut permeability and increased bacterial load may include IL-18 mediated gut damage, such as neutrophil infiltration and increased apoptosis, and suppression of other T helper cells, including Th1 interferon-γ [15, 17, 20, 112, 219-221]. To test this possibility, future experiments will explore the effects of combined anti-IL-18 antibody and IL-22 on gut barrier and immune function. These studies will begin to identify a pathway by which to maintain proper gut homeostasis combined EtOH exposure and burn injury.

In conclusion, our findings suggest that administration of IL-22 following EtOH and burn injury prevents increased gut leakiness, gut bacterial load and decreased AMP levels in the gut. While more studies are needed to elucidate the mechanism by which IL-
22 influences these parameters, the IL-22/AMP pathway may provide a novel therapeutic target for the treatment of patients who sustain burn injury under the influence of EtOH.
CHAPTER SIX

SUMMARY AND DISCUSSION

Ethanol and Burn Injury

More than one million burn injuries are reported yearly within the United States [1]. These injuries result in approximately 500,000 emergency room visits and 40,000 hospitalizations annually [1]. Greater than 50% of these injuries occur under the influence of alcohol/ethanol (EtOH) intoxication [2-11]. Burn victims who sustain injury under the influence of EtOH exhibit significantly higher rates of morbidity and mortality than patients without EtOH exposure at the time of injury [2-6, 11-13]. To date, the mechanism by which EtOH confounds post burn clinical outcomes remains unclear. Several lines of evidence suggest that gut pathogens and/or their products may play a pivotal role in the subsequent development of sepsis and multiple organ failure reported in burn and trauma patients [6, 14, 49-58]. In line with this hypothesis, our laboratory has demonstrated increased intestinal tissue damage, leakiness and bacterial translocation as well as intestinal T lymphocyte suppression following EtOH intoxication and burn injury [14-21].

Th17 cells are implicated in gut homeostasis and in the containment of gut pathogens. We investigated the effects of EtOH exposure and burn injury on intestine associated lymphoid PP Th17 effector functions. We further examined whether in vivo
modulation of IL-22 influences gut barrier function and bacterial load following EtOH and burn injury.

**Summary of Results**

Our murine model of EtOH intoxication and burn injury demonstrates decreased CD3/CD28 dependent expression of Th17 effector cytokines IL-17 and IL-22 in PP cells one day post injury, Chapter 4. We further demonstrate restoration of IL-22 following restitution of IL-23, as well as restoration of IL-17 in response to PMA and ionomycin. Mechanistically, we found that IL-23 dependent induction of IL-22 is regulated, at least in part, by the AhR, Fig. 20. While Th1 responses have been extensively studied in the context of EtOH [141], trauma [111], burn [17, 18, 112, 116, 222, 223], and sepsis [144], our current results are the first to demonstrate perturbation of Th17 responses following EtOH exposure and burn injury. Our *in vitro* data further offer novel information regarding the role of the AhR in regulation of IL-22.

While the exact mechanism by which EtOH and burn injury results in suppression of IL-17 and IL-22 remain unclear, it is possible that EtOH modulates expression of IL-23, a key molecule involved in Th17 differentiation. As presented in Chapter 3, we found that EtOH exposure suppresses LPS-induced DC IL-23, in a dose dependent manner. This observation agrees with previously published literature indicating suppression of IL-23 in an *in vivo* model of EtOH exposure and pulmonary infection [135]. Additionally, this data is recapitulated in our own *in vivo* model, where EtOH and burn injury resulted in decreased IL-12/23p40 in supernatants harvested from PP mixed cells stimulated with LPS. Though, IL-23p19 was measurable only at very low concentrations in cell lysates
from LPS stimulated PP mixed cells obtained from sham animals, IL-23p19 was undetectable in cell lysates from EtOH and burn injured mice. The direct measurement of IL-23 has proved challenging to us and other investigators [135]. Nonetheless, these data support our hypothesis that EtOH and burn injury perturbs IL-23 leading to suppression of Th17 effector functions and contributing to decreased gut immune function and increased gut damage, Fig. 20.

Recently, IL-22 has gained interest as a possible therapeutic agent. Specifically, IL-22’s role in modulating epithelial cell proliferation, regeneration, immune function and expression of AMPs [73], makes IL-22 a promising therapeutic target. Several gastrointestinal disease models show improved outcomes following treatment with IL-22 [47, 83, 84, 149, 154]. In applying our observations to the modulation of Th17 effector function in vivo, we further took advantage of our murine model to test whether in vivo treatment with IL-22 improved gut barrier and immune functions following EtOH and burn injury. As described in Chapter 5, we started by testing whether IL-22 dependent expression of AMPs Reg3β and Reg3γ were affected by EtOH and burn injury. We found decreased levels of Reg3β and Reg3γ, as well as increased gut permeability and bacterial growth, one day post EtOH exposure and burn injury. In line with recent literature highlighting the protective role of IL-22, we found increased expression of Reg3β and Reg3γ, as well as decreased gut permeability and bacterial growth in animals treated with IL-22. While these are correlative observations, they support the possible use of IL-22 as therapeutic agent.
Figure 20. Schematic of Th17/gut mucosal interactions. Under healthy conditions, IL-23 drives expression of Th17 cytokines, IL-17 and IL-22. These Th17 effector cytokines maintain gut barrier integrity and immune function, as well as promote expression of antimicrobial peptides, together these T cell effector functions prevent evasion of gut microbes. Following ethanol and burn injury, we find an increase in gut bacterial and increased gut permeability in the context of decreased IL-23, IL-17, IL-22 and antimicrobial peptides Reg3β and Reg3γ.
Suppressed Th17 Effector Function, Gut Bacterial Translocation and Barrier Function

Several lines of evidence suggest that gut pathogens and their products play a pivotal role in the development of sepsis and multiple organ failure reported in burn and trauma patients [14, 59, 128, 193]. Therefore, the focus of our laboratory has been to delineate the effects of EtOH exposure and burn injury on gut immune and barrier function. To date, our laboratory has demonstrated increased intestinal tissue damage, leakiness, and bacterial translocation to regional lymph nodes as well as T lymphocyte, particularly T helper (Th) cell, suppression within 24 hours following EtOH intoxication and burn injury [14-21, 112]. Specifically, ethanol combined with burn injury results in decreased Th1 cytokine IFN-γ production [16, 17, 112].

Mechanistically, our laboratory has found a decrease in IL-12 to be the major determinant in suppression of T cell effector functions, namely Th1 IFN-γ production [17]. Increased IL-18, on the other hand, was found to cause neutrophil-dependent intestinal barrier disruption as well as remote tissue damage, such as the lung, following ethanol combined with burn injury [21, 46, 195, 219-221, 224]. Th17 cells are a third subset of T helper cells and are implicated in cellular immunity, neutrophil recruitment and clearance of bacteria as well as in epithelial cell integrity and tissue repair [36, 37, 48, 73]. Thus, in further dissecting T cell effector function and intestinal barrier integrity following combined insult, our laboratory has recently focused on Th17 cells.

Our current findings indicating suppression of Th17 effector cytokines IL-17 and IL-22 following combined EtOH exposure and burn injury complement our laboratory’s earlier findings. In regards to increased bacterial translocation to MLN and gut leakiness,
our current studies suggest that this may be due, at least in part, to perturbation of Th17 effector functions. Under healthy conditions, a few indigenous bacteria continuously translocate to the MLN, but because of the intact immune defense these bacteria do not survive. Decreased IL-17 and IL-22 following EtOH and burn injury, may allow for to the passage of viable gut pathogenic bacteria across the epithelial barrier to MLN and distant organs. Alternatively, IL-22 increases expression of AMPs, including Reg3β and Reg3γ, which directly bind and kill bacteria [29, 30, 211]. In our data, we demonstrate increased bacterial growth in the context of suppressed IL-22 and decreased expression of Reg3β and Reg3γ. Following treatment with IL-22, we find decreased bacterial growth and restoration of Reg3β and Reg3γ expression. It is possible that Reg3β and Reg3γ help prevent evasion of gut microbes by limiting their invasion into intestinal crypts and attachment to the gut epithelium.

In regards to intrinsic barrier function, it is possible that suppressed IL-17 and IL-22 promotes gut leakiness. Decreased levels of IL-22 may facilitate epithelial barrier dysfunction by altering the balance between epithelial cell renewal and loss. Under normal conditions, the rapid rate of epithelial cell proliferation is counterbalanced by apoptosis, thus maintaining the mucosal architecture and a constant number of cells. Inappropriately high rates of intestinal epithelial cell apoptosis, such as those found after EtOH and burn injury [219], or decreased proliferation may lead to an imbalance, thereby causing intestinal tissue injury and facilitating gut leakiness and the evasion of gut pathogens. Similarly, IL-17 has been implicated in the enhancement of tight junction proteins [80]. Our laboratory has recently demonstrated that EtOH and burn injury alter
intestinal tight junction protein phosphorylation in an IL-18 dependent manner [219]. Thus, it is plausible that IL-17 and IL-22 work to counteract the actions of IL-18 \textit{in vivo}. Following EtOH and burn injury, decreased IL-17 and IL-22 in the context of increased IL-18 may promote epithelial cell apoptosis, decrease proliferation and deregulate tight junction formation.

**Future Directions**

Our current data analyzing the effects of combined EtOH exposure and burn injury on Th17 effector functions and their relationship with gut immune and barrier function offer an opportunity for further exploration. Further studies can focus on determining whether EtOH plus burn injury directly or indirectly affects DC expression of IL-23 or whether our observation are intrinsic deficiencies in T cells and their ability to respond to the local cytokine milieu. In regards to IL-23/AhR dependent modulation of IL-22, future experiments should elucidate the molecular mechanism by which AhR regulates IL-23 dependent restoration of IL-22. \textit{In vivo} potential studies include determining whether or not IL-22 directly improves gut barrier function following EtOH and burn injury.

First it is necessary to delineate whether EtOH exposure and burn injury suppresses IL-23. In the gut, DCs are likely the largest contributors of IL-23 [34]. Moreover, as demonstrated in Chapter 3, EtOH suppresses BM-DC expression of IL-23 in a dose dependent manner. Thus, it seems logical to test the effects of combined injury on PP DC derived IL-23. However, given the small number of cell obtainable from PPs ($\sim 2 \times 10^6$ total cells/animal) and the low percent of CD11c$^+$ DCs ($\sim 3\%$ of total live events, Table1) found in PP, it is not feasible to isolate a sufficient number of DCs from one animal.
Therefore, to circumvent this challenge, future experiments would isolate CD11c^+ DCs from PPs and small intestine lamina propria. Isolating cells from PPs and lamina propria would provide the opportunity to effectively isolate sufficient cells from all four experimental groups to test whether combined injury suppresses DC ability to produce IL-23 in response to antigenic challenge, such as LPS. If IL-23 were found to be affected by combined injury, studies could be expanded to determine how EtOH and burn affect DC derived IL-23. Utilizing current literature, these studies could explore the effects of EtOH on TLR expression and downstream signaling, lipid raft formation, gene expression, antigen presentation and/or expression of co-stimulatory receptor necessary for efficient adaptive immune activation [158, 165-167, 188, 189, 225, 226].

In regards to IL-17 and IL-22, it is necessary to understand the relationship among IL-23, STAT proteins, RORγt and the AhR. Though the AhR is essential for expression of IL-22, it is still unclear how it modulates IL-22 and whether it plays a role in IL-17 expression [106, 107, 109]. Additionally, whether RORγt is necessary for IL-22 expression also remains controversial [44, 45]. Therefore, future experiments should determine whether AhR utilizes ROR-γt and/or STAT3 in modulating IL-23-mediated induction of IL-22. Though our current studies used PP mixed cells, future studies would need to utilize purified PP T cells to accurately establish the regulation of IL-17 and IL-22 in T cells. Our current data, demonstrating lack of IL-17 induction following IL-23 restitution, indicate that suppressed CD3 dependent expression of IL-17 and IL-22 may be due to impaired intracellular signaling. Thus, initial experiments would explore the effects of EtOH and burn injury on STAT3 phosphorylation, as well as RORγt and AhR.
expression. Later experiments will utilize an inhibitor to the AhR to determine whether AhR regulates IL-23 dependent activation of STAT3 and/or expression of ROR-γt. Finally, how AhR modulates IL-23 dependent induction of IL-22 will be determined by testing whether AhR physically interacts with STAT3 and/or ROR-γt. Collectively, these studies will elucidate the mechanism by which EtOH and burn injury impair Th17 immune responses, describe a pathway by which AhR modulates expression of IL-17 and/or IL-22, and determine whether or not ROR-γt is necessary for expression of IL-22.

**Clinical Implications**

The data presented herein, are applicable to variety of clinical settings and offer novel information regarding IL-22 as a potential therapeutic. First, it is possible that Th17 effector functions are perturbed following injuries other than burn, such as trauma and sepsis. In these situations, studying the *in vivo* effects of protective proteins, such as IL-22, may provide new targets by which to decrease clinical complications related to suppressed gut immune and barrier functions; thus, decreasing the development of sepsis and multiple organ dysfunction and improving clinical outcomes and survival. Conversely, if EtOH directly suppresses IL-17 and IL-22, investigating how this occurs may prove applicable to the treatment of clinical diseases involving hyperactive Th17 dependent responses, such as inflammatory bowel disease, psoriasis, atopic dermatitis and rheumatoid arthritis. In this arena, where immunomodulation remains at the forefront of treatment, identifying a soluble molecule responsible for EtOH dependent immune suppression may lead to the development of new therapeutic treatments. Our data, demonstrating suppressed IL-17 and IL-22 following EtOH and burn injury and recusing
of gut immune and barrier function with IL-22 treatment implicate IL-22 as a possible therapeutic in the treatment of gastrointestinal related pathology.
APPENDIX A:

SUPPLEMENTAL DATA
Appendix 1A. Ethanol exposure did not influence the percentage of live cells. BM-DCs (2 x 10^6 cell/well) were cultured in 96-well plates in the presence or absence of varying concentrations of ethanol for 24 hours at 37°C. Cells were harvested and cell suspensions were analyzed for cell viability by flow cytometry. Representative FACS plots were taken from a single animal and demonstrate the percentage of viable cells within live gate of total events collected. Numbers indicate the mean percentage of cells in live gate ± SEM, n=4. p>0.05.
Appendix 2A. IL-17 secretion is decreased after EtOH intoxication and burn injury. PP mixed cells (5x10^6 cells/mL) were cultured in the presence of T cell mitogen ConA (5µg/mL) for 48hr at 37°C. Supernatants were harvested for measurements of IL-17. Values are means ± SEM from two independent experiments, n=3-9 animals/group. *, p<0.001 as compared with sham vehicle by ANOVA.
APPENDIX B:

SPECIFIC METHODS
Mouse Model of Ethanol Intoxication and Thermal Injury

Adult C57BL/6 male mice (7-8 weeks old, 22-25 g body weight, Harlan Laboratories, IN) were chosen randomly for all experiments. Animals received sham or burn injury and either ethanol (EtOH) or vehicle (water) to yield four experimental groups: sham vehicle (SV), sham EtOH (SE), burn vehicle (BV) and burn EtOH (BE). Mice were gavaged with either 25% EtOH in water (~3 g/kg) or water. Four hours later, when blood EtOH levels were in the range of 90-100 mg/dL, mice were anesthetized by intraperitoneal (i.p.) injection of ketamine hydrochloride/Xylazine cocktail (~80 mg/kg and 1.2 mg/kg, respectively), dorsum shaved, and transferred into a template fabricated to expose ~15% of the total body surface area (TBSA). Burn-injured mice were immersed into a water bath (85-87°C) for ~7 seconds, which resulted in a 3rd degree full thickness scald injury. Sham-injured mice were subjected to identical anesthesia and treatment, but immersed into an isothermic water bath (37°C). Immediately after burn or sham procedure, mice were resuscitated with i.p. injection of 1 mL physiologic saline. Animals were allowed food and water ad libitum. Mice were sacrificed and organs collected one day post injury.

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**Time: 0**
- Gavaged with either 0.4 mL 25% EtOH (2.9 g/kg) or H₂O

**4 hrs**
- Sham Injury: 37°C, 7 sec
- Burn Injury: 85-90°C, 7 sec (results in ~15% TBSA full thickness burn)
- IP resuscitation with saline

**One day**
- Animals sacrificed
- Peyer’s patches/intestinal tissue collected
- In vivo gut permeability
Growing Bone Marrow Dendritic Cells *In Vitro*

1. Sacrifice mice per protocol.

2. Sterilize the abdomen and hind legs with 70% ethanol.

3. Make an incision in the midline of the abdomen. Clip outward to expose the hind legs.

4. Dissect femurs from surrounding muscle tissue.

5. Place femurs (and/or tibia) from each mouse in a 1.5ml microcentrifuge tube containing cold, sterile PBS.

From this point onwards, work in the tissue culture hood.

6. Cut off both ends of the each femur/tibia with a blade and flush the bone with 3 ml media (without GM-CSF or IL-4) using a 22g needle and a 3mL syringe. (if use two tibias and two femurs will have 12mL of media)

7. Pipette the bone marrow cells up and down to bring the cells into single-cell suspension.

8. Pass the cells through a 70μm cell strainer. Wash the strainer with another 3 ml of media (total 15mL of media)

9. Count cells using a hemocytometer (15mL). Wash cells once in complete media. Adjust cell concentration to 2x10^6 cells/ml.

10. Plate 10x10^6 cells at a concentration of 2x10^6 cells/ml in a 100mm tissue culture petri dish.

11. Differentiate cells in a humidified incubator with 5% CO₂ at 37°C.

12. Feed cells at Day 2 with 5ml complete media. Wash cells and replace media every 2-3 days.

13. Harvest bone marrow DC’s between Day 7 and 10 using accutase and then cell scraper.

14. Check for purity of DC by staining for the cell surface markers CD11c and MHC II and analyze via flow cytometry.

Complete media: RMPI supplemented with 10% FBS, 1% Pen/Strep and L-glutamine and 50ng/mL GM-CSF and 100U/mL IL-4 (~3ng/mL)

Notes: Expect about 60 million BM cells if use both femurs and both tibias.
Cell Preparation from PPs

Supplies
- HBSS Media
- Collagenase 1mg/ml
  - Prepare enough to have 10mL per sample of PP (10mL per animal)
- Small petri dishes
- 70μm nylon filter
- Syringe tops
- Conical tubes
- 1.5mL microcentrifuge tubes
- Transfer pipets
- Trypan blue

Procedures
1. Collect PP into conical tube containing 5mL plain HBSS
2. Add 5mL HBSS containing 1mg/ml Collagenase
3. H₂O bath @ 37°C for 15 min
4. Crush PPs on nylon filter using syringe top
5. Collect cell suspension into new conical tube
6. Centrifuge suspension @ 4° 1200RPM for 15min
7. Discard supernatant
8. Re-suspend pellet in RPMI media (~10mL) - to wash
9. Centrifuge suspension @ 4° 1200RPM for 15min
10. Discard supernatant
11. Reconstitute cell pellet in 800μL RPMI media
12. Separate 10μL into microcentrifuge tube
13. Add 90μL trypan blue to 10μL cell suspension
14. Count cells
Tissue Sonication Procedure

Media (PIC protease inhibitor cocktail in PBS – 1 pill per 10mL of PBS)

1. Before you start do the following
   a. Label one of the 2mL tubes for each sample
   b. Label three 1.5mL eppendorf tubes for each sample
   c. Make the PIC cocktail in PBS (do NOT use 10x PBS by mistake)
   d. Turn on mini-centrifuge set to 4°C and press fast cool.
2. Take samples out of freezer and place box on a piece of dry ice so that the samples do not thaw
3. Put each piece of tissue in 2mL tubes (make sure tissue weighs ~100mg)
4. Add 500µL PIC to each sample
5. Keep on ice
6. Sonicate for 30sec each, twice
   a. KEEP dial between 3 and 4
   b. Sonicate on ice (put ice into the small beaker and place 2mL tubes into this beaker)
   c. Make sure all the tissue is broken up – if not repeat cycle
   d. Between samples dip sonicator in 70% alcohol and then in PBS
7. Add 500µL PIC to each sample – to total 1mL
8. Centrifuge 10,000 RPM for 15min @ 4°C
9. Transfer supernatant to new eppendorf tubes, aliquot 250µL per sample – collect only the clear supernatant part – do not let the pipet touch the bottom (make 3 aliquot per sample)
REFERENCES


IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe*. 4, 337-349.


VITA

Juan L. Rendon was born on March 5, 1984 to Jose and Virginia Rendon. He received his secondary education at the Granada Hills Math, Science and Technology Magnet High School in Granada Hills, California. In August 2002, Juan entered Georgetown University, where he majored in Nursing and Health Studies. Upon graduation, Juan spent a year working as a registered nurse on the Cardiothoracic Intensive Care Unit at the UCLA Medical Center in Los Angeles, California.

In July 2007, Juan began his medical education at the Loyola University Chicago Stritch School of Medicine. As a medical student, Juan served as a student representative for the Technology Information’s Committee and as secretary for the Anesthesiology Interest Group. He also participated in the MD with Honors in Research Program. In May 2009, he was accepted into the combined MD/PhD program and entered the laboratory of Dr. Mashkoor A. Choudhry studying the role of ethanol exposure on post burn gut immunity and barrier function.

From 2010 to 2011, Juan held a predoctoral position on the Alcohol Research Program training grant, under the directorship of Dr. Elizabeth J. Kovacs. From 2011 to 2013, he received research and tuition support through a predoctoral Ruth L. Kirchstein National Research Service Award from the National Institutes of Health. Juan served as MD/PhD representative for the Dean’s Medical Council from 2009 to 2011 and as co-president of the Graduate Student Council from 2011 to 2012. Upon completion of
medical and graduate school, Juan will pursue a residency in Plastic and Reconstructive Surgery to continue his training as a Physician-Scientist.