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NOVEL MECHANISMS IN INFLAMMATORY BOWEL DISEASE

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Abstract of Dissertation

Razvan I. Arsenescu, MD

The Graduate School
University of Kentucky
2011
NOVEL MECHANISMS
IN
INFLAMMATORY BOWEL DISEASE

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine, Microbiology, Immunology & Genetics at the University of Kentucky

By

Razvan I. Arsenescu, MD
Lexington, Kentucky

Director: Dr. Alan Kaplan, Professor of Microbiology, Immunology & Genetics
Lexington, Kentucky
2011

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Inflammatory Bowel Diseases, Crohn's Disease and Ulcerative colitis, are idiopathic chronic conditions with multifactorial determinants. In general, terms, intestinal inflammation results from abnormal host-microbe interactions. Alterations in homeostasis involve host genetic factors, environmental cues and unique luminal microbial niches. We have examined the coordinated expressions of several molecular targets relevant to the mucosal immune system and identified signature biomarkers of IBD. Qualitative and quantitative changes in the composition of microbiota can be related to unique immuno-phenotypes. This in turn can have more systemic effects that involve energy metabolism. Adiponectin, an adipose tissue derived adipokine, can restore cellular ATP levels and fulfills innate immune functions. We have concluded that IBD might represent a state of adiponectin resistance relating to chronic inflammation and obesity status. Lastly we hypothesized that activation of xenobiotic pathway (AHR-aryl hydrocarbon receptor) can further modulate host immune and metabolic responses, and thus contribute to IBD phenotypes. We found that IBD is associated with robust mucosal, aryl hydrocarbon receptor pathway and related to proinflammatory cytokine secretion. We conclude that IBD heterogeneity is reflected through distinct immunophenotypes. Furthermore, environmental cues that involve the AhR receptor and adipose tissue derived adiponectin are important regulators of the inflammatory process in IBD.
KEYWORDS: Inflammatory Bowel Disease, Mucosal Immunity, Macrophages, Adiponectin, Aryl Hydrocarbon Receptor

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June 28th, 2011
NOVEL MECHANISMS IN
INFLAMMATORY BOWEL DISEASE

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June 28th, 2011
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DEDICATION

Violeta, Victor, Silvia and Paul
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Table of Content

Acknowledgments...........................................................................................................iii

List of Tables..................................................................................................................vi

List of Figures..................................................................................................................vii

Chapter One......................................................................................................................1
  Introduction....................................................................................................................1

Chapter Two: SIGNATURE BIOMARKERS IN CROHN 'S DISEASE: TOWARD A MOLECULAR CLASSIFICATION.................................................................25
  Synopsis .........................................................................................................................25
  Introduction ...................................................................................................................26
  Materials and Methods .................................................................................................28
  Results ............................................................................................................................32
  Discussions ...................................................................................................................40
  Table Legends ...............................................................................................................46
  Figure Legends .............................................................................................................47

Chapter Three: ADIPOnectin AND PLANT DERIVED-MAMMALIAN ADIPOnectin - HOMOLOG EXERT A PROTECTIVE EFFECT IN MURINE COLITIS.................................................................63
  Synopsis .........................................................................................................................63
  Introduction ...................................................................................................................64
  Materials and Methods .................................................................................................67
  Results ............................................................................................................................73
  Discussions ...................................................................................................................78
  Figure Legends .............................................................................................................84
Chapter Four: ROLE OF THE XENOBIOTIC RECEPTOR IN INFLAMMATORY BOWEL DISEASE

Synopsis

Introduction

Materials and Methods

Results

Discussions

Figure Legends

Chapter Five

Conclusion

Future Directions

References

Vita
List of Tables

Table 2.1: Comparison of clinical characteristics and molecular phenotypes of CD patients

Table 2.2: Effects of medications at the time of biopsy on molecular phenotypes of CD patients
List of Figures

Figure 1.1: IBD Paradigm.............................................................................................................21
Figure 1.2: Epithelial Innate Immune Molecular Targets.......................................................22
Figure 1.3: Adiponectin in IBD...............................................................................................23
Figure 1.4: AhR and T cell polarization..................................................................................24
Figure 2.1: Biomarker expression in CD patients and normal controls...............................55
Figure 2.2: Comparison of gene expression in colon and ileum..............................................56
Figure 2.3: Effects of local inflammation on gene expression..............................................57
Figure 2.4: Multifactorial analysis of gene expression patterns............................................58
Figure 2.5: Comparison of individual biomarker expression in CD patients grouped by molecular phenotype ..................................................................................................................59
Figure 2.6: Serum IgA levels and localization of plgR and IgA in colonic mucosa of normal controls and CD patients.........................................................................................60
Figure 2.7: Clinical characteristics of CD patients classified in sets 1 – 3 based on colon gene expression..........................................................................................................................61
Figure 2.8: Model for inflammation due to dysregulated mucosal gene expression in Crohn’s disease...............................................................................................................................62
Figure 3.1: The severity of DSS-induced colitis is decreased in mice receiving Adiponectin adenovirus......................................................................................................................88
Figure 3.2: Efficacy of adenoviral delivery..............................................................................89
Figure 3.3: Expression of pro-inflammatory cytokines during DSS-induced colitis was decreased in mice overexpressing adiponectin...............................................................90
Figure 3.4: Adiponectin treatment promoted an anti-inflammatory milieu during DSS administration......................................................................................................................91
Figure 3.5: Adiponectin treatment reduces the pro-inflammatory adipokines during DSS induced colitis......................................................................................................................92
Figure 3.6: Adiponectin reduced cellular stress and apoptosis during DSS induced colitis.................................................................................................................................93
Figure 3.7: Osmotin - a plant derived adiponectin homolog - is beneficial in DSS colitis model.................................................................................................................................94
Figure 3.8: PPARγ agonist and retinoic acid reversed the LPS induced adiponectin resistance in dendritic cells..........................................................................................................................95

Figure 4.1: The severity of DSS-induced colitis is attenuated in AhR −/+ mice …118

Figure 4.2: Decreased histological severity in AhR −/+ mice during DSS-induced colitis.........................................................................................................................................................119

Figure 4.3: The expression of pro-inflammatory cytokines and macrophage marker are reduced in AhR −/+ mice during colitis...................................................................................................................120

Figure 4.4: Differential expression of master regulators for Treg and Th17 cells in the colon of WT and AhR −/+ mice.......................................................................................................................................................121

Figure 4.5: Proinflammatory adipokines are decreased in AhR+/− mice……….122

Figure 4.6: Adiponectin is negatively regulated during colitis.................................123

Figure 4.7: Reduced macrophage recruitment during DSS colitis in the AhR −/+ mice........................................................................................................................................................................124

Figure 4.8: AhR activation in patients with IBD.........................................................125

Figure 4.9: Epithelial cellular stress is decreased in AhR −/+ mice exposed to DSS........................................................................................................................................................................126

Figure 4.10: Decreased expression of Secretory leukoprotease inhibitor [SLPi] in WT compared to AhR −/+ mice exposed to DSS, consistent with reduced inflammation in the AhR heterozygote mice during colitis................................................127

Figure 4.11: Decreased renin angiotensin system [RAS] components in AhR −/+ mice correlates with a better outcome of DSS-induced colitis in AhR heterozygote mice.........................................................................................................................128
Chapter 1

Introduction:

Crohn's Disease (CD) and Ulcerative Colitis (UC) are chronic intestinal disorders known as idiopathic inflammatory bowel diseases (IBD)\(^1\). IBD affects approximately 1.4 million Americans with a peak onset occurring at 15-30 years of age. The development of CD and UC requires a genetic predisposition, a dysregulated immune response and an environmental trigger. Candidate genes include those that regulate innate immunity and epithelial barrier function\(^2\text{-}^5\) (Figure 1.1). Additional genetic studies have revealed that gene products involved in the elimination of endogenous small organic cations, drugs and environmental toxins are linked to CD and UC etiology\(^6\text{-}^8\) (Figure 1.1).

Lifestyle choices such as diet are thought to contribute to CD and UC by altering the commensal flora (prebiotics) or promoting obesity related inflammatory responses\(^9\) (Figure 1.1). Thus, the interactions between genetic and environmental factors will shape the gut epithelial-innate immune interface and lead to unique phenotypes in patients with Inflammatory Bowel Diseases. Current CD therapies are rather broad in action and not particularly focused on patient characteristics. Understanding the contribution of host and environmental factors will promote individualized management of this heterogeneous patient population.

The intestinal barrier is a dynamic multilayered structure that consists of bacterial biofilm, mucus, epithelium cells and innate immune cells. Dysfunction of this complex barrier precedes the development of inflammation in patients with
Crohn's Disease\textsuperscript{10}, as well as established animal models of Inflammatory Bowel Diseases\textsuperscript{11}. The intestinal barrier must balance the need for selective permeability of nutrients with protection from microbial invasion. This daunting task is accomplished by physical means such as tight junction proteins and mucus production on one hand and innate immune exclusion mechanisms on the other hand.

Epithelial cells interact with luminal microbial antigens through specialized extracellular TLR receptors (toll-like receptors) and intracellular NLR (NOD-like receptors)\textsuperscript{12}. Downstream signaling through these receptors promotes the production of antimicrobial peptides (defensins and cryptidins) and microbial antigen presentation through upregulation of MHC molecules (major histocompatibility complex). NOD2/Card15 receptor (Nucleotide binding Oligomerization Domain2/Caspase activating recruiting Domain 15 receptor) mutations and defective HBD (human β-defensin) production have been found in IBD-Crohn's Disease patients\textsuperscript{13}. Polymorphisms of NOD2/Card15 gene are believed to alter Paneth cell function and thus explain the impairment in antimicrobial peptides. Mechanistically, NOD2 senses the intracellular bacterial antigen MDP (muramyl dipeptide) and recruits adaptor proteins with subsequent activation of the NF-κB pathway. This pathway commonly results in transcriptional upregulation of proinflammatory genes. Therefore, defective NOD2 signaling in Inflammatory Bowel Diseases patients seemed, at first counterintuitive.

Recent studies addressing the role of epithelial NF-κB pathway, have
furthered our understanding of the pathophysiology of Inflammatory Bowel Diseases and patient response to therapy\textsuperscript{14}. For example, inhibition of NF-κB signaling, in epithelial cells, was found to result in impaired antimicrobial defense, enhanced bacterial penetration and a heightened inflammatory response\textsuperscript{15}. The NF-κB family of transcription factors consists of five members: RelA (p65), c-Rel, RelB, p50/p105 and p52/p100\textsuperscript{16}. Dimers of these proteins are kept inactive by interacting with inhibitory proteins of the IκB family. The IκB kinase (IKK) complex can target the IκB proteins for proteosome-mediated degradation. The canonical NF-κB signaling pathway requires the regulatory protein IKKγ/NEMO and the catalytic subunit IKK2 (IKKβ) and leads to RelA nuclear translocation. Proinflammatory cytokines such as TNF-α and bacterial antigen can trigger this process.

The alternative pathway involves IKK1 and promotes nuclear accumulation of p52/RelB dimers. This pathway is important for lymphoid organogenesis and lymphocyte development. Mice lacking the regulatory protein NEMO, within the epithelial cells, develop spontaneous colitis at an early age\textsuperscript{17}. Some of the early events are innate immune system activation and increased epithelial cell apoptosis. Interestingly intestinal epithelial deletion of IKK2, another component of the canonical pathway does not lead to this phenotype since the alternative pathway subunit IKK1 has a compensatory role. The proinflammatory, NF-kB dependent effects in macrophages and T cells during later stages of colitis, may overshadow the initial protective role of epithelial NF-κB. In line with these observations, loss of IKKβ in macrophages and neutrophils was able to attenuate
chronic colitis in IL10 KO mice\textsuperscript{18}.

Inflammatory Bowel Diseases (Crohn's Disease and Ulcerative Colitis) have a relapsing/remitting pattern. Nevertheless, some patients enjoy prolonged periods of remission while others have rather continuous activity and poor response to anti-inflammatory treatment. In light of the opposing role of NF-\kappa B in acute vs. chronic colitis and epithelial vs. immune cells, it will be important to determine whether cell specific impairment in this signaling pathway correlates with clinical course and response to therapy.

An important target of NF-\kappa B/RelA in intestinal epithelial cells is the polymeric immunoglobulin receptor (plgR)\textsuperscript{19}. This receptor is responsible for the active transport of IgA into the gut lumen. The plgR is synthesized in the endoplasmic reticulum and delivered to the basolateral membrane of the intestinal epithelial cell via the Golgi apparatus. At this level, plgR binds an IgA dimer and the receptor/ligand complex reaches the apical membrane through the endosomal compartment. Cleavage of the plgR extracellular domain releases the SC (secretory component) with or without IgA dimer. The SC fragment prevents plgA (polymeric IgA) degradation and thus enhances humoral immunity. Moreover, the SC fragment can bind bacterial components such a \textit{C. Difficile} toxin A and fimbriae of enterotoxigenic \textit{E. coli}, and thus limit their pathogenicity\textsuperscript{20-21}.

In addition to a direct effect on epithelial cells, bacteria can induce pro-inflammatory cytokines and chemokines with subsequent immune cell influx and increased tissue damage. Free SC can bind IL-8 and scavenge this
proinflammatory chemokine thus limiting neutrophil infiltration\textsuperscript{22}. Mice lacking plgR expression are more susceptible to DSS (dextran sodium sulphate) induced colitis\textsuperscript{23}. Interestingly these mice have increased IgA production possibly reflecting a role of plgR on B cells, or more likely a response to increase bacterial antigen exposure due to a barrier defect.

The incidence and relevance of plgR abnormalities in patients with Inflammatory Bowel Disease has not been established. Moreover, plgR polymorphisms have not been associated with increased risk of Crohn's Disease or Ulcerative Colitis. It is conceivable that variations of plgR expression reflect the activity of epithelial NF-kB. Recent in-vitro studies, using the HT-29 human colon carcinoma line, highlighted the importance of the canonical NF-kB/RelA component. Upon stimulation with TNF\(\alpha\), these cells respond by quickly upregulating IL-8 secretion. In contrast, plgR expression peaked at 24 hrs at the time when the proinflammatory chemokine IL-8 was downregulated. Given prior evidence that the SC fragment can bind and neutralize IL-8, we may consider the late plgR expression as a defense mechanism needed to terminate the acute inflammatory response.

The other important observation is that chronic exposure of intestinal epithelial cells to TNF\(\alpha\) maintains high plgR expression\textsuperscript{24}. Mucosal TNF\(\alpha\) is elevated in patients with Inflammatory Bowel Diseases and thus the abnormal expression of plgR could reduce the defense mechanisms of the epithelial barrier. Furthermore, although TNF\(\alpha\) blockade is highly successful in early versus late disease, the net effect of plgR expression may lead to epithelial cell immune
dysfunction and partly explain the less optimal long-term success. It also implies that knowledge of pIgR expression in mucosal biopsies may help select the best candidates for anti-TNF therapy in patients with Inflammatory Bowel Diseases.

As outlined above, one of the main functions of polymeric immunoglobulin receptor is IgA transport across the epithelial cells. IgA plays an important role in mucosal immunity. IgA has two subclasses, IgA1 and IgA2. IgA2 predominates in the mucous secretions whereas IgA1 is more abundant in the serum. The dimeric form of IgA but not the monomer interacts with the pIgR at the basolateral surface of the epithelial cells. This complex is released at the apical site together with the secretory component (SC) which retards its digestion in the gut lumen. It is important to realize that compared to IgG antibodies, IgA does not trigger complement activation. Given the enormous amount of IgA produced in response to commensal flora this property prevents an inappropriate innate immune response to commensals, as seen in patients with Inflammatory Bowel Diseases.

Evaluation of patients with inflammatory bowel diseases has demonstrated an increase in IgG1 antibody subclass, which is a rather potent activator of the complement system. Aside from the subsequent induction of pro-inflammatory cytokines and chemokines, this also reflects a break in tolerance to gut bacteria. B cell numbers are increased in Inflammatory Bowel Diseases. This may theoretically compensate for the IgG shift. Nevertheless, J chain expression in the lamina propria B cells is decreased which means less IgA dimers are formed. Since only polymeric IgA can be transported by pIgR this may translate
Induction of NF-kB downstream of TNFα and toll receptor signals can also trigger regulatory molecules. The zinc finger protein A20 is part of this negative feedback regulatory loop. A genome wide association study has identified A20 as a susceptibility gene for Crohn's Disease. In support of its role in inflammation control, A20 KO mice develop severe multi-organ inflammation. Specific deletions of A20 in enterocytes do not result in spontaneous colitis. Nevertheless, these mice are more susceptible to DSS induced colitis. Although NF-kB expression protects enterocytes from apoptotic signals, overactivation in the absence of A20 may have the opposite effect. The TLR-MyD88-NF-kB signaling axis may also confer protection form apoptosis. Activation of A20 secondary to the pro-inflammatory cytokine TNFα may act cooperatively but independent of the former pathway since the A20/MyD88 double KO mice are more susceptible to colitis then either single KO strain. Since enterocyte, specific A20 KO mice do not develop spontaneous colitis, it is possible that A20 plays a more important role under pathologic conditions characterized by unchecked proinflammatory cytokine secretion such as TNFα. On the other hand, transfer of A20 KO bone marrow into irradiated wild type mice leads to spontaneous colitis that can be prevented by antibiotic treatment. This suggests that myeloid but not enterocyte A20 expression prevents inflammatory response against gut flora under normal conditions.

In chapter 2, I propose that the coordinated expression of NF-kB/RelA, pIgR, IgA, A20, TNF and IL-8 defines unique phenotypes in patients with
Inflammatory Bowel Diseases. Figure 1.2 depicts my proposed model whereby bacterial and pro-inflammatory signals converge on the NF-kB transcriptional hub to induce epithelial defense mechanisms: anti-apoptotic (RelA), bacterial immune exclusion (plgR, IgA), anti-inflammatory (A20), pathogen elimination (TNFα, IL-8). Deregulation of these mechanisms in IBD patients can generate a state of mucosal innate immune deficiency with secondary activation of the acquired immune arm. Homeostatic functions of the gut epithelial barrier such as IgA, mucus and antimicrobial peptide secretion require a high level of synthetic capacity and metabolic activity.

Dysfunction of the protein folding process or insufficient generation of ATP is likely to promote ER (endoplasmic reticulum) and mitochondrial stress responses followed by apoptosis and inflammatory mediators. Under these conditions, restitution of homeostasis requires adequate protein and organelle turnover. Autophagy is an evolutionary conserved pathway triggered by ER stress and starvation. In the process of autophagy, cytoplasmic material (protein, organelles) is engulfed in membrane-coated autophagosomes, which subsequently fuse with endosomes and lysosomes in order to form autolysosomes that degrade the ingested material.

The autophagy process is also utilized to remove intracellular bacteria. A genome wide analysis linked Crohn's Disease to the autophagy gene ATG16L1 (ATG16L1T300A). Importantly this gene was associated with impaired clearance of *Salmonella typhimurium*. Moreover, studies in mice hypomorphic for ATG16L1 protein expression, as well as ileal biopsies form IBD patients revealed defective
Paneth cell function\textsuperscript{30}. These cells are found at the bottom of the intestinal crypts and regulate the bacterial microenvironment by secreting antimicrobial peptides.

Morphological analysis of these Paneth cells also revealed degenerating mitochondria. Surprisingly these cells expressed higher levels of PPAR\textsubscript{\gamma} (peroxisome proliferator-activated receptor), a master regulator of adipocyte differentiation as well as adiponectin an adipose tissue anti-inflammatory adipokine. Intestinal microbiota in obese patients is characterized by an increased ratio of \textit{Firmicutes} relative to the \textit{Bacteroides} species\textsuperscript{31-32}. Interestingly the abundance of the \textit{Bacteroides} species in the murine gut is positively correlated with the expression of \(\alpha\)-defensins\textsuperscript{33}. Furthermore, metabolic disorders associated with low-grade systemic inflammation (type II diabetes, obesity) are associated with increased gut permeability. Thus, both inflammatory bowel disease and metabolic disorders appear to share Paneth cell deregulated function and dysbiosis. Upregulation of adipose tissue signature factors, PPAR\textsubscript{\gamma} and adiponectin may represent a defense mechanism since both exert protective effects in gut inflammatory states\textsuperscript{34-35}. Therefore, adipose tissue specific factors can regulate innate immune response in the gut mucosa.

The epidemic of obesity has affected several autoimmune conditions. In spite of the malabsorptive nature of Inflammatory Bowel Diseases the percent of overweight/obese patients is rising\textsuperscript{36}. Moreover, increased incidence of metabolic syndrome can create a background of low-grade systemic inflammation. A signature feature of Crohn’s Disease is the development of mesenteric fat inflammation\textsuperscript{37}. Macroscopically, the fat tissue wraps around the diseased bowel
segment, and give rise to what is called “creeping fat”. Given the transmural nature of the inflammation in Inflammatory Bowel Disease, the adipose tissue inflammation has generally been dismissed as a secondary event. Moreover, until recently, adipose tissue was primarily viewed as an energy depot.

Emerging data from multiple medical fields clearly demonstrate that adipocytes and resident adipose tissue macrophages function as bacterial sensors and thus promote chronic inflammation. More importantly, adipose tissue is a source of pro-inflammatory cytokines such as angiotensin, TNF-α, IL-6- as well as the anti-inflammatory adipokine, adiponectin and IL-10. The “creeping fat” that surrounds the bowel can thus become a source of inflammatory mediators at the expense of the anti-inflammatory adiponectin and contribute to chronic bowel inflammation. Both macrophages and adipocytes share regulatory pathways relevant to metabolism and innate immunity. Signaling through adiponectin receptors regulates overlapping pathways responsible for energy balance, insulin sensitivity and macrophage polarization. If we acknowledge the plasticity of adipose cells that acquire a macrophage like phenotype during inflammation, the LPS buffering capacity of adiponectin and the availability of endogenous TLR4 ligands in the fat depot, then metabolism and inflammation can no longer be considered as separate processes.

Previous studies addressing the role of adiponectin in experimental colitis yielded controversial results. In chapter 3 I address the effects of adiponectin overexpression in a model of DSS and the therapeutic benefit of a plant homologue. Adiponectin (AdipoQ) is unique among adipokines, because
its circulating levels are inversely related to the adipose mass\textsuperscript{44-45}. The human adiponectin gene contains a signal sequence, a collagen like domain, and a globular domain similar to the complement factor C1q. Biological effects of adiponectin depend upon the formation of multimeric complexes. The basic unit is a trimer, which can associate through disulfide bonds to generate hexamers and dodecamers referred to as low, medium and high molecular weight adiponectin (LMW, MMW, HMW) respectively\textsuperscript{46}.

Cleavage of the adiponectin molecule by leukocyte esterase can release the globular part, which retains biological activity. It is important to distinguish between these isoforms since they may have opposing effects on inflammation. Both pro- and anti-inflammatory effects have been described for all forms of adiponectin\textsuperscript{47-50}. This is in part explained by the experimental conditions and cell type, although LPS contamination is another important factor\textsuperscript{51}. Recent studies suggest that HMW weight adiponectin is the main anti-inflammatory moiety\textsuperscript{52}. In vitro experiments have shown that globular adiponectin induces NF-kB and pro-inflammatory cytokines, but prolonged exposure blocks further activation. In contrast, HMW adiponectin can quickly prevent NF-kB activation\textsuperscript{53}.

Studies in Crohn’s disease patients and experimental colitis have reported total adiponectin level rather than HMW. Adiponectin production is regulated at transcriptional and post-translational levels. During adipogenesis, several transcription factors, including PPAR\textgamma, bind its promoter to upregulate mRNA expression\textsuperscript{54}. Following translation, adiponectin undergoes hydroxylation of proline and lysine residues as well as glycosylation of hydroxylysines. These
processes along with formation of disulfide bonds are required for HMW complex assembly\textsuperscript{55-56}. Furthermore, these bonds are essential for adiponectin secretion.

An endoplasmic reticulum (ER) chaperone protein, ERp44, retains adiponectin within the cell. Ero1-Lα, another chaperone, competes with adiponectin for binding to ERp44\textsuperscript{57}. Ultimately, the balance between the two ER proteins may regulate the amount of secreted adiponectin. The volume of adipose tissue influences plasma levels of adiponectin. Obese patients have lower adiponectin levels. Weight loss through caloric restriction, exercise or bariatric surgery increases adiponectin or at least the ratio of HMW to total adiponectin\textsuperscript{58-61}. Similar to findings in Crohn's Disease, obesity is associated with adipocyte ER stress, which leads to decreased adiponectin exocytosis and hypoadiponectemia\textsuperscript{62}. \textit{In-vivo} and \textit{in-vitro} studies suggest that the visceral rather than the subcutaneous fat is the main source of adiponectin. Importantly, the size of adipocytes correlates with the amount of secreted protein. Large, insulin insensitive adipocytes have lower secretion\textsuperscript{61,63}.

Two main adiponectin receptors with homology to G protein-coupled receptors have been identified. These receptors have distinct tissue specificities within the body and have different affinities to the various forms of adiponectin (mono or multimers)\textsuperscript{64}. Adiponectin binds to the extracellular COOH terminus of adiponectin receptors (AdipoR1 / AdipoR2) and recruits the adapter APPL1 (adaptor protein containing PH domain) which in turn activates AMPK (AMP-activated protein kinase)\textsuperscript{65}. APPL1 is rather promiscuous and interacts with at least 14 proteins that modulate apoptosis\textsuperscript{66}, endosomal localization of proteins\textsuperscript{67},
and chromatin remodeling. Recruitment of APPL1 by adiponectin receptors induces AMPK (5'-AMP activated protein kinase) phosphorylation. This process requires the upstream LKB1 kinase and to a lesser extent Ca^{2+}/calmodulin dependent protein kinase kinase (CaMKK)^{68}. Activation of AMPK by CaMKK can provide a direct link between ER stress and autophagy. Decreased cellular ATP during starvation or states of mitochondrial dysfunction can also activate AMPK. Under these circumstances, the upregulation of adiponectin in Paneth cells carrying the \textit{ATG16L1}^{T300A} mutation may be interpreted as a trigger for autophagy.

APPL1 and AMPK also modulate PI3K/AKT (phosphoinositide-3-kinase and protein kinase B) and mTOR (mammalian target of rapamycin), which function as regulatory hubs in both metabolic and immune processes. Signaling cascades that polarize T cell and macrophage responses incorporate these molecules^{69}. Therefore, adiponectin can regulate both the acquired and innate arms of the immune responses. AICAR (5 aminoimidazole-4-carboxamide ribose) a non-specific AMPK activator ameliorates experimental autoimmune encephalitis^{70-71}, a model dependent on Th1/Th17 effector cells.

Analysis of both murine and human macrophage cell lines found high basal expression AMPK, predominantly the \(\alpha1\) isoform^{72}. The cytokine milieu can regulate its expression in these cells. Anti-inflammatory cytokines IL-10 and TGF\(\beta\) upregulate while TLR4 agonist, LPS downregulate its expression. Inhibition of macrophage AMPK amplifies the TNF\(\alpha\) and IL-6 response to LPS. Mechanistically the AMPK enhances Akt activity, which in turn phosphorylates
and inactivates its substrate GSK3-β. This Akt target promotes Th1 cytokine expression. In addition, GSK3-β can negatively regulate CREB (cyclic AMP responsive element binding protein) expression, a known activator of IL10 production in macrophages. Moreover, AMPK prevents IκB degradation and indirectly the NF-κB translocation to the nucleus. Overall, the activity of AMPK in macrophages appears to promote an M2 (alternatively activated) anti-inflammatory phenotype.

Previous unpublished data from my laboratory indicate decreased mucosal adiponectin receptor (AdipoR1/R2) expression in subsets of patients with moderate to severe Crohn’s disease. It is currently unknown whether this is a global event, or is restricted to certain cell types. Peroxisome proliferator-activated receptor gamma (PPARγ) can increase adiponectin sensitivity by upregulating its receptors, while the opposite may be true for the pro-inflammatory adipokine, angiotensin. Thus, PPARγ can enhance macrophage response to adiponectin.

Intestinal epithelial cells produce angiotensin converting enzyme (ACE) that mediates the conversion of angiotensinogen (produced by adipocytes and macrophages) into the biologically active angiotensin I (ANG-I). Angiotensin has pro-inflammatory and profibrotic activity and can negatively regulate adiponectin and adiponectin receptors. A significant number of Crohn’s Disease patients develop fibrotic strictures and require surgical intervention. Adiponectin can prevent fibrosis by reducing the TGFβ signaling in an AMPK and PPARα dependent manner. Angiotensin can downregulate AMPK /PPARα and enhance
TGFβ expression\textsuperscript{76}. Therefore, the relative balance of adiponectin and angiotensin could modulate TGFβ dependent epithelial-to-mesenchimal transition and determine the Inflammatory Bowel Disease behavior.

Despite normal/reduced ACE activity in the serum, ACE levels are increased in the mucosa of Crohn’s patients and correlate with disease activity\textsuperscript{77}. Since macrophages and adipocytes produce angiotensinogen (AGT)\textsuperscript{78-80}, epithelial ACE may promote inflammation within the gut and mesenteric fat. Medications that block angiotensin increase adiponectin, adiponectin receptor expression, and ameliorate experimental colitis, thus supporting these observations\textsuperscript{81}. The relative antagonism of adiponectin and angiotensin as well as competition for PI3/AKT and p38MAPK pathways could theoretically alter epithelial cell cycle, and sensing of bacteria\textsuperscript{82}. This in turn would influence NF-kB dependent targets such as pIgR (polymeric IgA receptor), tight junction proteins, and inflammatory cytokines.

Adiponectin induces mucosal IL-10 production (unpublished data) and activates p38MAPK (p38 mitogen-activated protein kinase). The p38MAPK/IL-10/STAT3 axis inhibits inflammation-induced ER stress response mechanism\textsuperscript{83}. STAT3 expression positively correlates with AdipoR2 expression in Crohn’s Disease patients, and adiponectin reduces ER stress response in DSS colitis. Thus, adiponectin may activate p38MAPK/IL-10/STAT3 axis during states of inflammation. AMPK activation downstream of adiponectin receptor could dampen the Th1/Th17 immune response during acute and chronic phases of colitis while promoting the M2 type macrophages (Figure 1.3)
Environmental factors can affect adipose tissue volume and distribution. Prior studies in our laboratory identified a link between Aryl hydrocarbon pathway (AhR), abnormal adipocyte metabolism and inflammation\textsuperscript{84}. Specifically, AhR agonist PCB77 promoted adipose tissue Th1 cytokine expression, activation of the renin angiotensin system and decrease adiponectin. Cigarette smoke contains a mixture of AhR agonists and is associated with complicated forms of Crohn's Disease. It is well known that the gene/environment interplay contributes to the pathogenesis of chronic disease states such as IBD.

Perhaps one of the best examples of this type of gene/environment interplay is that of the pregnane X receptor (PXR) signaling pathway. The association between IBD and the PXR pathway was first identified following gene expression profiling of inflamed tissues obtained from patients with Ulcerative Colitis and Crohn Disease. Increasing evidence supports the idea that activation of the AhR by environmental factors may similarly impact the severity of IBD. Like the PXR, the AhR regulates both xenobiotic metabolism and the inflammatory response. For example, exposure to environmental agents such as PCB 77 and cigarette smoke activates the AhR leading to the upregulation of phase I (CYP1A1 and CYP1B1), and phase II (UGT1A1 and GSTA1) of the xenobiotic metabolism.

The AhR is a basic helix-loop helix transcription factor that in its latent form, resides in the cytosol as a complex comprised of a dimer of heat shock protein of 90 kDa (HSP90), an immunophilin (ARA9) and p23\textsuperscript{85}. The presence of an AhR agonist triggers translocation of the AhR into the nucleus where it
dimerizes with its DNA binding partner, aryl hydrocarbon nuclear translocator (ARNT) and binds specific DNA recognition sites, the xenobiotic response elements (XRE) and thus either upregulates or downregulates a battery of genes. Prototypical AhR target genes most commonly studied are those involved in xenobiotic metabolism, in particular, CYP1A1 and CYP1B. These genes encode enzymes that are highly induced following exposure to AHR agonists. AhR translocation to the nucleus can also trigger an auto-regulatory negative feedback thorough aryl hydrocarbon receptor repressor (AHRR).

Proinflammatory metabolites of arahidonic acid (i.e. lipoxin A4) and tryptophan were recently identified as relatively potent AhR agonists. This indicates that the AhR signaling pathway plays a role in the regulation of the inflammatory responses. For example, 6-formylindolo [3, 2-b] carbazole (FICZ) a tryptophan derivative, generated by UV light exposure, is a potent AhR inducer and can be detected in human urine. Interestingly, several gram positive and negative bacteria present in the human gut can also process tryptophan, and thus generate AhR ligands in the form of indoles. On the other hand, indole-negative bacteria produce oxygenases that interfere with indole signaling. Taken together these observations suggest that the relative distribution of indole positive/negative bacteria can modulate intestinal epithelial and immune cells function.

Dietary habits can influence the amount of tryptophan intake and alter the composition of bacterial biofilm. AhR agonists that are typically used in research to induce the AhR signaling pathway are environmental contaminants, such as
Dioxin (TCDD or 2, 3, 7, 8-Tetrachlorodibenzo-p-Dioxin) and other polychlorinated hydrocarbons such as PCBs. In addition, studies have shown that polycyclic aromatic hydrocarbons present in cigarette smoke act as potent AhR agonists. In fact, the AhR has been recently identified as a major regulator of responses elicited by exposure to cigarette smoke. The aryl hydrocarbon receptor is strongly expressed in the gut, particularly in small bowel intestinal epithelial cells. Furthermore, either oral or intraperitoneal administration of AhR agonists resulted in rapid induction of CYP1A1 expression in the intestinal crypts. Thus, a number of ubiquitous xenobiotics, such as PCBs and cigarette smoke, are capable of activating the AhR signaling pathways in cells of the gastrointestinal mucosa.

The canonical AhR pathway activates nuclear enzymes important for endo- and xenobiotic metabolism (CYP450). AhR can also interact with NF-kB subunits (non-canonical pathway) and modulate inflammation. Several mechanisms have been proposed regarding the AhR /NF-kB interaction: 1) direct binding NF-kB to CYP (cytochrome) genes; 2) transcriptional repression involving factors such as PPAR, RXR and LXR; 3) post-transcriptional regulation of CYP activity through heme-oxygenase.

The differentiation of naïve CD4 T cells into Th1 is important for controlling intracellular bacterial infections while Th2 cells initiate antibody responses against extracellular pathogens. Aryl hydrocarbon receptor plays an important role in the development of both Th memory and Th effector cells (Figure 1.4) and by extension in the development of chronic inflammatory conditions like IBD.
The CD4⁺ Th17 cells are defined by a high AhR expression and a combination of IL17A, IL17F and IL22 cytokines⁹⁵. Activation of Th17 cells by FICZ (short acting AhR agonist) augment these cytokines production and induce the expression of AhR battery genes. Supporting the relationship between AhR and Th17 cells is the fact that Th17 cells from AhR KO mice cannot produce IL22, implying that AhR activation is required for IL-22 production in this T cell subset. In addition, since macrophages and dendritic cells induce IL22 through IL-23 release indicates that IL23 functions downstream of the aryl hydrocarbon receptor (AhR).

Recent studies have also demonstrated that AhR can bind the IL17 promoter and this interaction is negatively regulated by the nuclear receptor liver X receptor (LXR)⁹⁶. Th17 development is impaired in AhR KO mice. On the other hand, Th17 polarizing cytokines, TGFβ and IL6 induce AhR expression in these cells⁹⁷. Increased AhR expression was correlated with lower signal transducer and activator of transcription 1 (STAT1) levels. This may suggest that AhR can antagonize the negative effect of IFN-γ (STAT1 activator) on Th17 cells development⁹⁷.

Intestinal epithelial cells (IEC) can also express IL22 that promotes release of antimicrobial peptides. There is no evidence that AhR ligands are driving IEC IL22 secretion but given the role of NF-kB in Paneth cells and NF-kB – AhR interaction, the latter may modulate the innate immune function of the gastrointestinal mucosa. In favor of this interaction is the fact that IL22 expression is upregulated in the gut mucosa of Crohn's Disease patients and plays an important role in experimental colitis as well.
Short acting AhR ligands, such as FICZ promote inflammation. In contrast, the long acting agonist TCDD has been associated with immunosuppression. Several mechanisms may be involved in this process: 1) direct effect on T cells; 2) modulation of antigen presenting cell (APC) function; 3) toxic effect on bone marrow stem cells. In a mouse model of IBD (TNBS induced colitis), TCDD induced forkhead box P3 (FoxP3) expression and decreased the severity of colitis\(^9\). A similar mechanism has been proposed in a model of experimental autoimmune encephalitis (EAE) \(^9\). Dendritic cells (DC) can polarize naïve T cells toward the Treg phenotype by expressing IL10 and decreasing the availability of tryptophan. AhR is required to induce indoleamine 2, 3-dioxygenase (IDO) expression in DC. This enzyme catabolizes tryptophan into metabolites like kynurenine (Kyn) which promotes inducible Treg (iTreg) development. Taken together, the AhR pathway can regulate the Th17/Treg balance.

Although animal studies using TCDD indicate that AhR ameliorates inflammation this may not apply to human conditions such as IBD (excluding accidental exposure). The naturally occurring ligands are rapidly metabolized, whereas synthetic, long acting compounds such as TCDD persist and will accumulate in the body. Therefore, experiments in AhR KO and heterozygote mice will allow us to determine the overall effects of endogenous ligands on the development of Inflammatory Bowel Diseases.
Figure 1.1 Inflammatory Bowel Diseases paradigm
Figure 1.2 Epithelial Innate Immune Molecular Targets
Figure 1.3 Role of Adiponectin in Inflammatory Bowel Disease
Figure 1.4 Aryl hydrocarbon receptor and T cell polarization
Chapter Two

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SIGNATURE BIOMARKERS IN CROHN ’S DISEASE: TOWARD A MOLECULAR CLASSIFICATION

Synopsis

In an effort to develop a molecular classification scheme for Crohn ’ s disease (CD), mucosal biopsies from 69 CD patients and 28 normal controls were analyzed for expression of the RelA subunit of nuclear factor (NF)- kB, A20 (a negative regulator of NF- kB), polymeric immunoglobulin receptor (pIgR), tumor necrosis factor (TNF), and interleukin (IL)-8. Principal component analysis was used to classify individuals into three subsets based on patterns of biomarker expression. Set 1 included normal subjects and CD patients with mild disease and good responses to therapy, thus defining “normal” biomarker expression. CD patients in set 2, characterized by low expression of all five biomarkers, had moderate to severe disease and poor responses to immunosuppressive and anti-TNF therapy. Patients in set 3, characterized by low expression of RelA, A20, and pIgR, normal TNF and elevated IL-8, had acute inflammation that responded well to therapy. Classification of CD patients by these biomarkers may predict disease behavior and responses to therapy.
Introduction

Crohn’s disease (CD) is a chronic disorder characterized by patchy transmural inflammation of the small and/or large bowel, thought to result from an inappropriate inflammatory response to normal components of the intestinal microbiota in genetically predisposed individuals.\(^1\)\(^-\)\(^3\) Progress has been made in characterizing the effector cells and molecules that comprise the inflammatory response, and current biological therapies are targeted toward neutralizing these effectors.\(^4\)\(^-\)\(^7\) However, recent evidence suggests that the primary defect in CD may actually be a defective innate immune response within the intestinal mucosa.\(^8\),\(^9\) There is general agreement that molecular biomarkers for CD are needed to improve diagnosis and guide therapies.\(^10\) The aim of this study was to identify a set of biomarkers representative of the innate immune response in the gut mucosa, which could be used to classify CD patients into molecular phenotypic subsets predictive of disease behavior and responses to therapy.

The association of nuclear factor (NF-\(\kappa\)B) signaling with induction of pro-inflammatory cytokines has led to the hypothesis that excessive activation of NF-\(\kappa\)B is central to the pathogenesis of CD.\(^11\),\(^12\) Recent findings, however, have suggested that activation of NF-\(\kappa\)B may be crucial for regulation of intestinal inflammation and maintenance of epithelial barrier function.\(^13\)-\(^15\) Thus "physiological" inflammation induced by RelA signaling may be important for maintaining epithelial homeostasis in the gut, and reduced expression of RelA could contribute to the "innate immunodeficiency" seen in CD.
NF-κB induces expression of A20, an ubiquitin-modifying enzyme that negatively regulates the NF-κB activation pathway.\textsuperscript{16,17} Mice genetically deficient in A20 are hypersensitive to tumor necrosis factor (TNF)- and Toll-like receptor (TLR) induced NF-κB activation, and develop severe intestinal inflammation.\textsuperscript{18} We hypothesized that reduced expression of A20 in the intestinal mucosa could therefore be a risk factor for CD.

Secretory antibodies of the IgA class (SIgA) form the first line of antigen-specific immune protection at mucosal surfaces and promote homeostasis between the intestinal epithelium and the commensal microbiota.\textsuperscript{19,20} Transport of IgA across intestinal epithelial cells into the gut lumen is mediated by the polymeric immunoglobulin receptor (pIgR).\textsuperscript{21,22} Following transport, the extracellular domain of pIgR is cleaved to form secretory component (SC), which remains associated with SIgA and confers additional innate immune functions.\textsuperscript{23,24} Targeted deletion of the \textit{Pigr} gene in mice causes elevated serum IgA, increased mucosal permeability, and increased susceptibility to experimental colitis.\textsuperscript{25–27} Expression of pIgR in intestinal epithelial cell is upregulated by TNF and TLR signaling via activation of NF-κB.\textsuperscript{21,22} We hypothesized that diminished expression of pIgR in the intestinal mucosa could lead to increased inflammatory responses to the commensal microbiota.

The pro-inflammatory cytokine TNF is associated with mucosal inflammation in CD, and therapies designed to neutralize TNF activity have shown promise in a subset of CD patients who are refractory to conventional therapies.\textsuperscript{6,28} TNF also has protective roles in innate immunity, including
induction of NOD2 and plgR expression, epithelial restitution, and control of potentially pathogenic luminal bacteria. We hypothesized that although optimal expression of TNF in the intestinal mucosa may promote physiological inflammation, reduced TNF could lead to defective innate immunity.

Interleukin (IL)-8 is a potent chemoattractant for neutrophils, the major component of the cellular infiltrate in acute inflammation. Although neutrophils contribute to mucosal inflammation through the release of soluble mediators, they also play an important role in removal of bacteria and foreign debris that could otherwise promote a granulomatous response by mucosal macrophages. Accordingly, we investigated IL-8 as a potential biomarker for acute inflammation in CD patients.

Given the heterogeneous clinical presentation in CD, we anticipated that no single biomarker would accurately predict disease behavior. We utilized a multifactorial approach to develop a molecular classification scheme, based on expression levels of RelA, A20, plgR, TNF, and IL-8 in the intestinal mucosa of CD patients and normal controls.

**Materials and Methods**

*Study subjects.* Peripheral blood and mucosal biopsies were obtained from individuals undergoing colonoscopy at the University of Kentucky Medical Center, after institutional review board approval and written informed consent. For CD patients, the indication for colonoscopy was either to evaluate disease exacerbation or to screen for dysplasia and colorectal cancer. Diagnosis of CD
was based on clinical, radiological, and endoscopic criteria according to the
Montreal classification, supported by histopathological findings. Active disease
was defined by a Harvey–Bradshaw index >4 or endoscopic evidence of active
inflammation. Control subjects aged 50 years or older underwent screening
colonoscopies for colon cancer in accordance with current guidelines. Control
subjects under 50 years of age underwent colonoscopy for evaluation of
constipation or chronic abdominal pain. Individuals were classified as “normal”
when endoscopic, radiologic, and pathologic evaluation of randomly obtained
biopsies revealed no disease of the small or large bowel.

**Analysis of mRNA levels in colonic and ileal mucosa.** Biopsied tissue was
immediately immersed in an RNA-stabilizing solution (RNAlater; Qiagen,
Valencia, CA) and stored at −80 °C. Total RNA was purified using the Qiagen
RNeasy Protect mini kit (Qiagen) according to the manufacturer’s directions.
RNA was reverse-transcribed to generate cDNA templates using the TaqMan
Gold RT-PCR kit (Applied Biosystems, Foster City, CA). Specific mRNA levels
were quantified by real-time reverse transcription PCR, using the ABI Prism 7700
Sequence Detection System (Applied Biosystems) as previously reported. The
level of 2-microglobulin mRNA, which did not vary between normal subjects and
CD patients, was used to normalize mRNA levels for test genes according to the
following formula: \((2^{-\Delta \Delta C_{\text{test} - \Delta C_{2\text{-microglobulin}}}}) \times 100\%\).

Preliminary studies indicated that gene expression did not vary significantly in
different regions of the large bowel (cecum, transverse colon, and rectum) within
each individual. Accordingly, biopsies of non-inflamed mucosa from different regions of the colon were pooled into a single sample for mRNA analyses.

*Measurement of serum IgA and immunolocalization of IgA and plgR.* Serum IgA levels were measured using a commercial ELISA kit for human IgA (Bethyl Laboratories, Montgomery, TX). Mucosal biopsies were immediately fixed in formalin and then embedded in paraffin. The sections (5 – 7 μm) were mounted on glass slides, deparaffinized, rehydrated, quenched with 3 % H₂O₂, and treated for 10 min with a citric acid-based antigen-unmasking solution (Vector Laboratories, Burlingame, CA). Sections were blocked with normal goat serum (1 % in phosphate-buffered saline) and incubated overnight at 4°C with primary antibodies: monoclonal mouse anti-human IgA, (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc66185) (diluted 1:50) and polyclonal rabbit anti-human SC (the extracellular domain of plgR) (diluted 1:200). Sections were washed and incubated for 1 h with a mixture of Cy2-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; catalog nos. 115225-146 and 111-165-144). Sections were counterstained with 4’, 6-diamidino-2-phenylindole dihydrochloride (Molecular Probes Invitrogen, Eugene, OR) to visualize nuclei and mounted with VECTASHIELD medium (Vector Laboratories). Stained sections were imaged with an Olympus BX51 microscope, using a ×20 objective. ImagePro software was used to generate the composite images.
Analysis of NOD2 mutations. Genomic DNA was extracted from whole blood using the AquaPure Genomic DNA Isolation kit (BioRad, Hercules, CA). The R702W, G908R, and 1007fsinsC polymorphisms in the NOD2 gene were detected by restriction fragment length polymorphism analysis of PCR-amplified DNA, as described. 42

Statistical analyses. All statistical analyses were performed using StatView software (SAS Institute, Cary, NC). Because biomarker expression did not follow normal Poisson distributions, non-parametric statistical analyses were used for all comparisons. Mann – Whitney analysis was used to test for significant differences among groups of individuals. Paired sign analysis was used to compare differences in gene expression in paired biopsies within individuals. Spearman correlation analysis was used to test for correlations in mRNA levels among different biomarkers. PCA 36 was used to reduce the five variables (biomarkers) into two PCs. Expression levels for biomarkers from non-inflamed colon or ileum mucosa from all subjects in the cohort (normal controls and CD patients) were included in the PCA. The goodness-of-fit of the two PCs for the colon and ileum data sets was tested by χ2 analysis. To assign individual scores for PC1 and PC2, the expression level for each of the five biomarkers for each individual was first normalized to the mean for that biomarker for all subjects in the cohort. Individual scores for PC1 and PC2 were then calculated as the sum of the normalized value for each biomarker times the weight for that biomarker. Individuals were classified into subsets according to their scores for PC1 and
PC2. The $\chi^2$ analysis was used to test the effects of clinical parameters and medications at time of biopsy on distribution of CD patients into phenotypic subsets.

**Results**

*Biomarker expression in CD patients and normal controls*

Mucosal biopsies from the colon and/or terminal ileum were obtained at colonoscopy from 69 CD patients and 28 normal controls (Figure 2.1 a). Based on visual inspection and histology, tissues were classified as inflamed or non-inflamed. To test the hypothesis that the innate immune response is inherently dysregulated in CD patients, we compared mRNA levels in the colon and ileum for five potential biomarkers in non-inflamed tissue from CD patients and healthy controls (Figure 2.1 b and c). Levels of RelA, A20, and TNF mRNA were significantly lower in non-inflamed colon mucosa of CD patients than in normal controls. Expression of pIgR in colon mucosa was highly heterogeneous in normal controls and was not significantly different from CD patients. However, the observation that many CD patients had low pIgR expression suggested that this biomarker might be used to identify a subset of CD patients with deficient pIgR-mediated IgA transport. IL-8 expression was extremely low in the majority of normal controls and CD patients. However, elevated IL-8 expression in some CD patients suggested that this biomarker could be useful in a multifactorial analysis of gene expression. There were no significant differences in expression in the ileum mucosa between normal controls and CD patients for any of the five
biomarkers, suggesting that dysregulated expression of RelA, A20, and TNF may be a unique feature of the large bowel in CD. To determine whether expression of these biomarkers varies between the colon and ileum, paired biopsies (within the same individual) were analyzed from 19 normal controls (Figure 2.2 a) and 22 CD patients (Figure 2.2 b). Expression of plgR was higher in the colon than in the ileum of both normal controls and CD patients, likely due to enhanced TLR signaling from greater numbers of commensal bacteria. We have previously reported that plgR expression is significantly higher in the colon than in the ileum in mice and is reduced in mice with deficient TLR signaling. Expression of RelA was slightly but significantly higher in the colon than in the ileum of CD patients, but not of normal controls. No differences in expression of A20, TNF, or IL-8 were observed between the colon and ileum of either normal controls or CD patients. We conclude that regional differences in gene expression between the colon and ileum do not account for the observation that dysregulated expression of RelA, A20, and TNF in CD patients was restricted to the large bowel.

To test the hypothesis that local inflammation influenced biomarker expression, we analyzed paired biopsies of non-inflamed and inflamed colon mucosa from 17 CD patients (Figure 2.3 a). Remarkably, there were no significant differences in mRNA levels for any of the five biomarkers, suggesting that the observed dysregulation of gene expression in CD patients was an inherent condition of the colon mucosa, and not secondary to local inflammation. Because access to the ileum during colonoscopy was limited to the terminal 10 – 20 cm, we were only able to obtain a single biopsy of the ileum mucosa per individual. Accordingly,
each CD patient was classified as having either non-inflamed or inflamed ileum, even though regional differences in inflammation could have existed in other parts of the small bowel. Interestingly, no differences in the expression of RelA, A20, plgR, or TNF were observed among different CD patients with or without ileal inflammation (Figure 2.3 b). However, expression of IL-8 was significantly elevated in mucosa of CD patients with visible inflammation in the terminal ileum, suggesting that this gene may be a biomarker for acute ileal disease.

*Multifactorial analysis of five biomarkers defines molecular phenotypic subsets in CD*

The heterogeneous patterns of gene expression in both normal controls and CD patients suggested that a multifactorial scheme might be more robust than individual biomarkers for classifying molecular phenotypes. Principal component analysis (PCA) is a statistical approach that is used to reduce the number of variables in a complex data set and to classify relationships between variables. A recent study utilized PCA to identify a subset of IBD patients with significant abnormalities in the composition of the colonic microbiota. We used PCA to classify our study cohort (including both normal controls and CD patients) into molecular phenotypic subsets based on expression of RelA, A20, plgR, TNF, and IL-8 mRNA in non-inflamed intestinal mucosa. The first step in this analysis was to examine patterns of correlated expression among the five potential biomarkers (Figure 2.4 a). In the colon, highly significant correlations were observed among the expression levels of RelA, A20, plgR, and TNF, consistent with the function
of NF-κB in inducing transcription of the A20, plgR, and TNF genes. Expression of TNF but not RelA was positively correlated with IL-8, suggesting that TNF may promote IL-8 gene transcription through a RelA-independent mechanism. In the ileum, A20 expression was positively correlated with RelA and TNF, but RelA and TNF were not correlated with each other. In contrast to the colon, IL-8 expression was positively correlated with plgR but not TNF.

Principal component analysis was used to reduce the five variables to two factors or principal components (PCs) (Figure 2.4 b). The χ² analysis demonstrated that most of the variability in the data set could be described by PC1 and PC2 (P < 0.0001 for the colon and P = 0.0255 for the ileum). On the basis of colon gene expression, PC1 was strongly weighted toward RelA, A20, and plgR, with an intermediate weight for TNF and a negative weight for IL-8 (see Factor tables in Figure 2.4 b). PC2 was strongly weighted toward TNF and IL-8, with a low weight for RelA and negative weights for A20 and plgR. For ileum gene expression, PC1 was strongly weighted toward RelA, A20, plgR, and TNF and weakly weighted toward IL-8. PC2 was strongly weighted toward IL-8, weakly weighted toward plgR and TNF, and negatively weighted toward RelA and A20. Each normal control and CD patient was assigned a score for PC1 and PC2 based on the sum of weighted expression levels for all five biomarkers (see Methods). Scatter plots of the scores for PC1 vs. PC2 demonstrated that individuals could be classified into three subsets based on colon or ileum gene expression (Figure 2.4 c). Set 1 is defined as individuals with a high score for PC1 and a low score for PC2, set 2 is defined as individuals with low scores for both PC1 and PC2, and set 3 is
defined as individuals with a high score for PC2. On the basis of colon gene expression, 81 % of normal controls were classified into set 1, with 8% in set 2 and 11 % in set 3 (Figure 2.4 d). On the basis of ileum gene expression, the percentages of normal controls in sets 1, 2, and 3 were 57, 38, and 5 %, respectively. These findings suggested that high scores for PC1 and low scores for PC2, particularly for the colon, define a “normal” molecular phenotype. In contrast, 42 % of CD patients were classified in set 1 based on colon gene expression, with 45 % in set 2 and 13 % in set 3. On the basis of ileum gene expression, the percentages of CD patients in sets 1, 2, and 3 were 33, 45, and 22 % , respectively.

Patterns of colon gene expression differed significantly for CD patients classified into the three molecular phenotypic subsets (Figure 2.5). Individuals in set 2 had significantly reduced expression of RelA, A20, plgR, and TNF compared to individuals in set 1 (the “normal” phenotype). Individuals in set 3 had significantly reduced expression of RelA, A20, and plgR, normal expression of TNF, and elevated expression of IL-8. On the basis of these findings, we propose that CD patients in set 2 have an inherent deficiency in innate immunity in the colon mucosa. CD patients in set 3 had selected features of innate immunodeficiency (reduced RelA, A20, and plgR), but elevated IL-8 expression in this group was suggestive of acute inflammation. Classification of individuals based on ileum gene expression was less informative than for the colon. CD patients in ileum set 2 had some evidence of innate immunodeficiency (reduced levels of RelA and A20), but expression of plgR and TNF was normal. CD patients in ileum set 3
had elevated IL-8 expression, but no evidence of innate immunodeficiency. We conclude that RelA, A20, plgR, TNF, and IL-8 are potentially useful biomarkers for classification of CD patients into molecular phenotypic subsets based on gene expression in non-inflamed colon mucosa. Accordingly, we compared clinical findings in CD patients distributed into the three colon subsets.

**Aberrant localization of plgR and SlgA in a subset of CD patients**

Deficient plgR expression in mice has been associated with reduced transport of SlgA into the colon lumen and elevation of serum IgA.\textsuperscript{25,26} We observed that serum IgA levels tended to be lower in CD patients than in normal controls (Figure 2.6 a), but given the overall heterogeneity, this difference did not achieve statistical significance. However, serum IgA levels were significantly elevated in CD patients in set 2 (Figure 2.6 b), who had decreased expression of plgR in the colonic mucosa (Figure 2.5 a). Serum IgA levels also appeared to be elevated in CD patients in set 3, although due to the relatively small number of individuals in set 3 the difference from set 1 was not statistically significant. To examine whether reduced plgR-mediated transport of IgA contributed to the elevation in serum IgA, we compared the localization of plgR and IgA in colonic biopsies from a normal individual in set 1 and from a CD patient in set 2 (Figure 2.6 c). In the normal colon mucosa, plgR and IgA were colocalized at the basolateral surface, within the cytoplasm, and at the apical surface of epithelial cells, consistent with normal transport of IgA. In the lamina propria, IgA was observed in association with plasma cells, but no plgR was detected. In contrast, staining for plgR was
markedly reduced in epithelial cells of non-inflamed mucosa from the set 2 CD patient, and there was reduced evidence of transepithelial transport of IgA. In addition, more IgA was observed in the lamina propria, compared with the normal control. A punctuate border of colocalized plgR / SC and IgA at the apical surface of the epithelium most likely represented SIgA bound to the mucus layer. In the inflamed mucosa from the same CD patient, dense colocalization of IgA and plgR / SC in the lamina propria was suggestive of deposition of SIgA along the basement membrane. Leakage of excess IgA (either free or bound to SC) from the lamina propria into the systemic circulation could account in part for the elevated levels of serum IgA in set 2 CD patients.

Correlations between molecular phenotypes and clinical outcomes of CD patients

A review of the clinical histories of CD patients in this cohort indicated that, in general, patients in set 1 tended to have mild disease and good responses to therapy; patients in set 2 had moderate to severe disease and poor responses to immunosuppressive and anti-TNF therapy; and patients in set 3 had acute disease that responded promptly and durably to therapy.

Although young age at diagnosis of CD has been reported to be associated with more severe disease, we observed no differences among the three subsets in age at diagnosis (Mann – Whitney test, \( P>0.05 \)). We did find that set 3 patients tended to be more recently diagnosed, independent of the age at diagnosis (Figure 2.7). Set 3 patients also had elevated serum C-reactive protein, a marker of acute inflammation in CD (Figure 2.7). These data suggest that some CD
patients may present initially with acute inflammation (set 3), but the inherent innate immunodeficiency characterized by reduced expression of RelA, A20, and plgR (and likely other genes) results in a failure to resolve the underlying trigger of inflammation. Over time, these patients may progress from acute to chronic inflammation, characteristic of set 2.

Recent evidence suggests that inflammatory mediators produced by visceral adipose tissue may promote intestinal inflammation in CD. However, we observed no differences in body mass index among molecular phenotypic subsets, indicating that excess body fat did not influence expression of the biomarkers we analyzed. There were also no significant differences among subsets in Harvey–Bradshaw index of disease activity at the time of colonoscopy, suggesting that subjective evaluation of disease activity at one point in time may not be informative with respect to the underlying molecular pathogenesis of the disease. Other clinical characteristics that did not affect distribution of CD patients into molecular phenotypic subsets included gender, smoking behavior, and disease location (colon vs. ileum, or both) (Table 2.1).

Crohn’s disease patients with an inflammatory or stricturing phenotype (Montreal classification) were evenly distributed among the three molecular phenotypic subsets (Table 2.1). In contrast, patients with penetrating disease were significantly biased toward set 3 and away from set 2 (P = 0.019). Given that only three patients in our cohort had penetrating disease, these data must be interpreted with caution. However, the possibility that the combination of innate
immunodeficiency and acute inflammation that characterizes set 3 may predispose patients to penetrating disease deserves further investigation.

Only one patient in our cohort (classified in set 1) was homozygous for the L1007fsinsC polymorphism in the NOD2 gene, which confers increased risk for CD 42 (Table 2.1). Several additional patients were heterozygous for other NOD2 polymorphism - but these did not affect their distribution among the molecular phenotypic subsets. These findings suggest that the abnormalities in innate immunity identified by our classification scheme are unrelated to NOD2 polymorphisms.

We next investigated whether the medications that CD patients were receiving at the time of biopsy affected their patients among the three subsets (Table 2.2). We conclude that the observed abnormalities in mucosal gene expression in sets 2 and 3 reflected an underlying pathology characteristic of each individual, and did not result from acute effects of medications they were receiving at the time of biopsy. This finding does not rule out the possibility that medications received earlier in the course of the disease may have had long-term effects on mucosal gene expression.

DISCUSSION

Signature biomarkers for CD

Here we describe a molecular classification scheme, based on expression of RelA, A20, pIgR, TNF, and IL-8 in the colon mucosa, which identifies unique phenotypic subsets of CD patients. Several findings support the concept that
these are molecular phenotypes. The $\chi^2$ analysis revealed no significant effect of any medication on distribution of CD “signature biomarkers” for CD. First, biomarker expression appeared to be an inherent characteristic of each CD patient, and was not affected by local variations in tissue inflammation or current medications. Second, these biomarkers identified a potential state of innate immunodeficiency in the majority of CD patients in our cohort, accompanied by chronic (set 2) or acute (set 3) mucosal inflammation. Third, classification of CD patients into these molecular phenotypic subsets was predictive of disease behavior and responses to therapy. Our findings provide strong support for prospective studies of this molecular classification scheme in a larger population of CD patients.

**A model for dysregulated innate immunity in CD**

The pathology of CD arises from an imbalanced immune response to exogenous and endogenous molecular cues. Traditionally, CD has been viewed as an exaggerated inflammatory response to microbial antigens caused by a dysregulated adaptive immune response. However, recent evidence suggests that the primary defect in CD may actually be a deficient innate immune response in the intestinal mucosa, resulting in inadequate clearance of bacteria and other foreign material from the lamina propria and generation of a chronic inflammatory state. The intestinal epithelium is a crucial player in maintaining this balance, going beyond the simple role of a physical barrier to orchestrate the
physiological inflammatory response and the innate immune response to dietary and microbial antigens.

We propose that expression of RelA, A20, plgR, TNF, and IL-8 may be useful for assessing innate immune function in the colon mucosa. A model describing the activities of these molecules in normal and diseased mucosa is presented in Figure 2.8. In healthy mucosa, cross talk between epithelial cells and constituents of the commensal microbiota is essential for maintaining normal expression of the RelA subunit of NF-κB. Constitutive low-level activation and nuclear translocation of NF-κB result in physiological inflammation, characterized by high expression of plgR and other proteins involved in epithelial polarity and function, as well as moderate expression of TNF, IL-8, and other effectors of innate immunity. Coordinate expression of negative regulatory molecules such as A20 protects epithelial cells from excessive activation and nuclear translocation of NF-κB. IgA produced by plasma cells in the lamina propria is transported across the epithelial layer by plgR, thus promoting intracellular and extracellular neutralization of microbes and their products and regulating immune effector molecules such as IL-8. 23, 24, 43 Reduced expression of RelA in the colon mucosa of some CD patients may compromise the ability of epithelial cells to mount a physiological inflammatory response to the commensal microbiota. Reduced expression of plgR could inhibit normal transport of IgA, resulting in diminished regulation of microbial products and IL-8, compromised barrier function, and deposition of SIgA complexes in the lamina propria. Low mucosal expression of RelA may also predispose CD patients to periodic flares.
of pathological inflammation, due to reduced expression of negative regulators such as A20, diminished anti-inflammatory activity of SIgA, increased access of microbial antigens to the lamina propria, activation of macrophages and generation of an adaptive immune response.

*Protective roles for NF-κB / RelA in the intestinal epithelium*

Association of NF-κB signaling with induction of pro-inflammatory cytokines in T cells and inflammatory cells such as macrophages and neutrophils has supported the use of therapies designed to inhibit activation of NF-κB. When considering these therapeutic approaches, it should be kept in mind that physiological NF-κB signaling is important for maintenance of intestinal homeostasis. It has recently been reported that selective inhibition of NF-κB activation in intestinal epithelial cells, by targeted deletion of genes encoding subunits of the IκB-kinase complex, actually increases the susceptibility of mice to experimental colitis. \(^{13,14}\) A critical role of the RelA subunit of NF-kB in intestinal homeostasis was highlighted by the recent report that mice with a targeted deletion of *Rela* in intestinal epithelial cells exhibited reduced expression of pro-restitution genes, elevated epithelial apoptosis and proliferation, and increased susceptibility to chemically induced colitis.\(^ {15}\) Our data suggest that reduced expression of the RelA subunit of NF-κB in the gut mucosa, with concomitant downregulation of key target genes, may be a contributing factor for the development of CD in humans.
NF-kB signaling in the intestine is kept under tight control by a complex network of negative regulatory molecules, many of which are target genes of NF-kB. To represent this group of negative regulators in our panel of biomarkers, we analyzed expression of A20, a ubiquitin-editing enzyme that downregulates NF-kB signaling initiated by pro-inflammatory cytokines and TLRs. A20 null mice have been shown to develop severe intestinal inflammation, suggesting that A20 and other negative regulators may fulfill the role of a “brake” and set the threshold for NF-kB activation. We consistently observed down-regulation of mucosal A20 expression in CD patients, which may hamper their ability to regulate pathological NF-kB activation induced by acute inflammatory responses. Significantly, we found that A20 levels did not increase in inflamed regions of the colon in CD patients (Figure 2.3). Paradoxically, CD may be characterized both by a defect in physiological NF-kB signaling and by a reduced capacity to regulate pathological NF-kB signaling.

Compartmentalization of IgA in the intestinal epithelium is crucial for barrier function

Compartmentalization of IgA antibodies in the gut enhances barrier function by allowing a local antigen-specific response to commensal microorganisms without stimulating potentially inflammatory systemic immune responses. We observed that reduction of pIgR expression in some CD patients (set 2) led to reduced transport of IgA and accumulation of dense membranous deposits of SIgA (Figure 2.6). Elevated serum IgA levels in this group of CD patients could have
resulted from leakage of IgA from the lamina propria into the systemic circulation, which has been reported in plgR-deficient mice. Elevated serum IgA could also result from deficient epithelial barrier function and access of the commensal microbiota to the lamina propria, resulting in a loss of tolerance and a systemic immune response to microbial antigens.

Potential applications of a molecular classification scheme for diagnosis and management of CD

The results of this study support the concept of developing a molecular classification scheme for CD based on patterns of gene expression in the colon mucosa. Using this scheme, we classified a cohort of CD patients into three molecular phenotypic subsets that were generally predictive of clinical findings and responses to therapy. It will be important to validate this model in a larger cohort and to evaluate the utility of additional biomarkers. Molecular classification of CD patients at the time of diagnosis may be helpful in targeting specific therapies. For example, patients classified in set 3, with evidence of acute inflammation, may respond better to TNF blockers and other biological therapies than would patients classified in set 2, who exhibited subnormal expression of TNF in the colon mucosa. This classification system may also be useful for monitoring the course of the disease and modifying therapy over time. We found that recently diagnosed patients were more likely to be classified in set 3, whereas patients with longstanding disease were more likely to be classified in set 1 or set 2 (Figure 2.7), suggesting that patients may undergo transition over
time into different subsets. Our finding of reduced mucosal expression of RelA in both set 2 and set 3, as well as the need for physiological NF-κB activation to maintain epithelial barrier function, suggests that the application of therapies designed to inhibit NF-kB activation should be used with caution.

Acknowledgments: This research was supported by grants from the Investigator-sponsored study program of AstraZeneca, the National Institutes of Health, the Kentucky Science & Engineering Foundation, the Crohn’s & Colitis Foundation of America, the Eli and Edythe Broad Foundation, and UCB SA

Table Legends
Table 2.1 Comparison of clinical characteristics and molecular phenotypes of CD patients. Abbreviation: CD, Crohn’s disease. The χ² analysis tested the hypothesis that CD patients displaying each of the listed clinical characteristics would be distributed among the molecular phenotypic subsets in the same proportions in which the entire cohort of CD patients was distributed, i.e., set 1: set 2: set 3; 22:24:7 (see Figure 2.4 d).

Table 2.2. Effects of medications at the time of biopsy on molecular phenotypes of CD patients Abbreviation: CD, Crohn’s disease. The χ² analysis tested the hypothesis that CD patients receiving each of the listed medications would be evenly distributed among the molecular phenotypic subsets in the same proportions in which the entire cohort of CD patients was distributed, i.e., set 1: set 2: set 3; 22:24:7 (see Figure 2.4 d). Medications: 5-ASA: mesalamine;
immunosuppressants: azathioprine, 6-mercaptopurine, methotrexate; steroids: budesonide, prednisone; anti-TNF: Remicade (infliximab), Humira (adalimumab). Some patients were receiving more than one medication at the time of biopsy.

**Figure legends**

Figure 2.1 *Biomarker expressions in CD patients and normal controls*. Patient characteristics (a) and gene expression in non-inflamed colon (b) and ileum (c) mucosa were compared between CD patients and normal controls. mRNA levels were measured by RT-qPCR and normalized to β2-microglobulin mRNA. Data are displayed as histograms to illustrate the heterogeneity in expression of individual biomarkers. Dashed lines indicate median expression levels for each group. Significant differences among groups were tested by Mann–Whitney non-parametric analysis; *P*-values are listed for each comparison of normal controls vs. CD patients. CD, Crohn’s disease; RT-qPCR, real-time quantitative PCR.

Figure 2.2 *Comparison of gene expression in colon and ileum*. Paired biopsies were obtained from non-inflamed mucosa of the colon and ileum for 19 normal controls (a) and 22 CD patients (b). mRNA levels were measured by RT-qPCR and normalized to 2-microglobulin mRNA. Dashed lines indicate the difference in median expression levels between colon and ileum for each gene. Significant differences among groups were tested by paired sign non-parametric analysis; *P*-
values are listed for each comparison of colon vs. ileum. CD, Crohn’s disease; RT-qPCR, real-time quantitative PCR

Figure 2.3 Effects of local inflammation on gene expression. mRNA levels were measured by RT-qPCR and normalized to 2-microglobulin mRNA. 
(a) Paired biopsies were obtained from non-inflamed and inflamed colon mucosa from 17 CD patients. Dashed lines indicate the difference in median expression levels between non-inflamed and inflamed mucosa for each gene. Significant differences among groups were tested by paired sign non-parametric analysis; $P$-values are listed for paired comparisons between inflamed vs. non-inflamed colon mucosae. (b) Histograms of gene expression in inflamed or non-inflamed ileum mucosae from different individuals. Because of limited access to the ileum during colonoscopy, a single biopsy from the terminal ileum for each CD patient was classified as either inflamed or non-inflamed, and paired biopsies could not be collected. Significant differences among groups were tested by Mann–Whitney non-parametric analysis; $P$-values are listed for each comparison of individuals with inflamed vs. non-inflamed ileum mucosa. CD, Crohn’s disease; RT-qPCR, real-time quantitative PCR.

Figure 2.4 Multifactorial analysis of gene expression patterns. (a) Non-parametric Spearman correlation analysis of gene expression data from Figure 2.1 Correlation coefficients ($r$) and $P$-values are listed for each comparison. Statistically significant correlations ($P<0.05$) are shaded. (b) Factor analysis of
NF-kB, RelA, A20, plgR, TNF, and IL-8 mRNA levels in non-inflamed colon and ileum mucosa, including both normal controls and CD patients. Weighted factors are listed for principal components (PCs) 1 and 2 for each gene. (c) Classification of individuals into molecular phenotypic subsets. Scores for PC1 and PC2 were calculated for each individual based on the sum of weighted expression levels for all five biomarkers (see Methods). Set 1 comprises individuals with a high PC1 score and low PC2 score. Set 2 comprises individuals with low scores for both PC1 and PC2. Set 3 comprises individuals with high scores for PC2. The table lists the distribution of normal controls and CD patients among colon and ileum subsets. The scatter plots represent the scores for PC1 and PC2 for each individual; open circle = normal control; filled circle = CD patient. (d) Distribution of individuals into molecular phenotypic subsets based on scores for PC1 and PC2. CD, Crohn’s disease; IL, interleukin; NF-kB, nuclear factor-kB; PC, principal component; plgR, polymeric immunoglobulin receptor; TNF tumor necrosis factor.

Figure 2.5 Comparison of individual biomarker expression in CD patients grouped by molecular phenotype. CD patients were classified in sets 1 – 3 by principal component analysis, as described in Figure 2.4. mRNA levels in colon (a) and ileum (b) mucosa were measured by RT-qPCR and normalized to 2-microglobulin mRNA. Gene expression levels for sets 1, 2, and 3 were compared by non-parametric Mann – Whitney analysis and are expressed as median + median absolute deviation. Asterisks indicate that gene expression in set 2 or 3
is different from that in set 1 (P< 0.05). CD, Crohn’s disease; RT-qPCR, real-time quantitative PCR.

Figure 2.6 Serum IgA levels and localization of plgR and IgA in colonic mucosa of normal controls and CD patients. (a) Histograms of serum IgA levels in normal controls and CD patients. Dashed lines indicate median levels for each group. Differences in serum IgA levels for normal controls vs. CD patients were tested by Mann – Whitney non-parametric analysis. (b) Comparison of serum IgA in CD patients classified in sets 1 – 3 by principal component analysis, as described in Figure 2.4. Serum IgA levels in sets 1, 2, and 3 were compared by non-parametric Mann – Whitney analysis and are expressed as median + median absolute deviation. An asterisk indicates that gene expression in set 2 or 3 is different from that in set 1 (p < 0.05). (c) Localization of plgR / SC and IgA in colonic mucosa by immunofluorescence. Representative images are shown from a normal subject (representing set 1) or matched biopsies from visibly inflamed and non-inflamed regions of the colon from a CD patient with low plgR mRNA expression (representing set 2). Red staining indicates binding of antibody to human SC and green staining indicates binding of antibody to human IgA. All samples were counterstained with DAPI to visualize nuclei. Stained tissue sections were imaged with a × 20 objective; the bottom panels are enlargements of the designated regions from merged images. CD, Crohn’s disease; DAPI, 4’, 6-diamidino-2-phenylinde dihydrochloride; E, epithelium; LP, lamina propria; plgR, polymeric immunoglobulin receptor; SC, secretory component.
Figure 2.7 Clinical characteristics of CD patients classified in sets 1 – 3 based on colon gene expression. Disease duration is defined as the time from initial CD diagnosis to when the mucosal biopsy was collected / each individual. Values for sets 1, 2, and 3 were compared by non-parametric Mann – Whitney analysis and are expressed as median + median absolute deviation. P-values indicate significant differences between CD patients in set 2 and set 3. No significant differences were observed between set 1 and sets 2 or 3. CD= Crohn’s disease.

Figure 2.8 Model for inflammation due to dysregulated mucosal gene expression in Crohn’s disease. (a) (1) In normal mucosa, bacterial – epithelial cross talk upregulates expression and activation of NF- kB (RelA) and expression of downstream targets (A20, plgR, and TNF). Modulation of these responses by A20 and other negative regulators limits the “physiologic inflammation.” (2) IgA secreted by lamina propria plasma cells binds to plgR on the basolateral surface of epithelial cells and is transported to the apical surface. (3) During transport, SlgA facilitates clearance of bacterial antigens and inflammatory chemokines. (4) SlgA promotes immune barrier function at the luminal surface. (b) (1) Defective expression of NF- kB and downstream genes by epithelial cells in CD. (2) Abnormal deposits of SlgA in the lamina propria. (3) Failure of IgA-mediated clearance of antigens and IL-8 due to defective epithelial transport. (4) Reduced SlgA at the luminal surface results in diminished barrier function. (5) Excess SlgA in the lamina propria enters the systemic circulation. (6) Chronic inflammation
with increased IL-8 and influx of neutrophils and macrophages. CD, Crohn’s disease; IL, interleukin; NF-κB, nuclear factor-κB; pIgR, polymeric immunoglobulin receptor; SIgA, secretory antibodies of the IgA class; TNF, tumor necrosis factor.
Table 2.1 Comparison of clinical characteristics and molecular phenotypes of CD patients

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b  Gene expression in non-inflamed colon

Gene expression in non-inflamed ileum

Figure 2.1 Biomarker expressions in CD patients and normal controls.
Figure 2.2 Comparison of gene expression in colon and ileum.
Figure 2.3 Effects of local inflammation on gene expression
**Figure 2.4 Multifactorial analysis of gene expression patterns**

---

### Table A

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### Table B

#### Non-inflamed colon

**Factor analysis summary**

- Number of variables: 5
- Number of factors: 2
- Number of cases: 79
- Degrees of freedom: 14
- Bartlett's Chi Square: 76.459
- P-value: <0.0001

**Factor extraction method:**

- Principal components

**Factors**

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<tr>
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#### Non-inflamed ileum

**Factor analysis summary**

- Number of variables: 5
- Number of factors: 2
- Number of cases: 48
- Degrees of freedom: 14
- Bartlett's Chi Square: 26.045
- P-value: 0.0255

**Factor Extraction Method:**

- Principal Components

**Factors**

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### Table C

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**Figure 2.4 Multifactorial analysis of gene expression patterns**

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Figure 2.5 Comparison of individual biomarker expression in CD patients grouped by molecular phenotype
Figure 2.6 Serum IgA levels and localization of plgR and IgA in colonic mucosa of normal controls and CD patients
Figure 2.7 Clinical characteristics of CD patients classified in sets 1 – 3 based on colon gene expression
Figure 2.8 Model for inflammation due to dysregulated mucosal gene expression in Crohn’s disease
Chapter Three

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ADIPONECTIN AND PLANT DERIVED-MAMMALIAN ADIPONECTIN - HOMOLOG EXERT A PROTECTIVE EFFECT IN MURINE COLITIS

Synopsis

Background: Hypoadiponectinemia has been associated with states of chronic inflammation in humans. Mesenteric fat hypertrophy and low adiponectin have been described in patients with Crohn’s disease. We investigated whether adiponectin and the plant-derived homolog - Osmotin - are beneficial in a murine model of colitis.

Methods: C57BL/6 mice were injected (i.v.) with an adenoviral construct encoding the full-length murine adiponectin gene (AN+DSS) or a reporter - LacZ (Ctr and V+DSS groups) prior to DSS colitis protocol. In another experiment, mice with DSS colitis received either Osmotin (Osm+DSS) or saline (DSS) via osmotic pumps. Disease progression and severity were evaluated using body weight, stool consistency, rectal bleeding, colon lengths, and histology. In vitro experiments were carried out in bone marrow derived dendritic cells.

Results: Mice overexpressing adiponectin had lower expression of proinflammatory cytokines (TNF, IL-1β), adipokines (angiotensin, osteopontin),
and cellular stress and apoptosis markers. These mice had higher levels of IL-10, alternative macrophage marker – arginase 1 and leukoprotease inhibitor. The plant adiponectin homolog Osmotin similarly improved colitis outcome and induced robust IL-10 secretion. LPS induced a state of adiponectin resistance in dendritic cells that was reversed by treatment with PPARγ agonist and retinoic acid.

Conclusion: Adiponectin exerted protective effects during murine DSS colitis. It had a broad activity that encompassed cytokines, chemotactic factors as well as processes that assure cell viability during stressful conditions. Reducing adiponectin resistance or using plant derived adiponectin homologs may become therapeutic options in IBD.

Introduction

Inflammatory Bowel Diseases, Crohn’s Disease and Ulcerative colitis are chronic relapsing conditions that result from an inappropriate response to gut microbiota⁹⁹. Crohn’s disease patients develop transmural inflammation of the gastrointestinal tract, which may lead to complications such as strictures and perforations. Recent advances in metabolic and cardiovascular diseases have led to a paradigm shift in which adipose tissue has been upgraded from an energy depot to a source of immunomodulatory cytokines (adipokines). In his original description of the disease, Burril Crohn directed the attention toward the characteristic mesenteric fat change¹⁰⁰. Accumulation of mesenteric fat appears to be specific for Crohn’s disease, and occurs from the onset of disease³⁷. Fat
wrapping has been defined as fat hypertrophy extending from the mesenteric attachment with > 50% coverage of the intestinal surface\textsuperscript{101}. It occurs in both the small and large bowel, and correlates with transmural inflammation, ulceration, stricture formation, increased mesenteric wall thickness, and decreased internal bowel diameter\textsuperscript{101}. Fat wrapping and mural thickening is associated with mucosal ulceration in 86% \textsuperscript{102} and with strictures in 46% of patients \textsuperscript{102}. Importantly, it was noted in 100% of patients undergoing resection, and correlated with the degree of acute and chronic inflammation\textsuperscript{103}.

Functional pattern recognition receptors (PPRs) have been recently identified in nonimmune cells like adipocytes, as well. Therefore, by expressing functional cell membrane PPRs like Nucleotide-binding oligomerization domain containing 2 (NOD2), cluster of differentiation 14 (CD14), toll-like receptors (TLR2, TLR4, and TLR5) the adipose tissue can also, respond to bacterial wall products, developing a proinflammatory phenotype\textsuperscript{104}. When we consider the fact that preadipocytes may differentiate towards the macrophage line and that NOD2 mutation is strongly associated with Crohn’s disease, the adipose tissue becomes a dynamic player for the disease phenotype. In addition, TLR signaling leads to decreased expression of the adipose tissue anti-inflammatory adipokine – adiponectin and lowers its receptors\textsuperscript{104}. It also promotes free fatty acid accumulation that act as an endogenous TLR4 ligand. Therefore, a vicious cycle is established that potentially induces a state of hypoadiponectemia and/or adiponectin resistance. Considering that adiponectin can bind and neutralize
lipopolysaccharide (LPS) the hypoadiponectemia can lead to secondary activation of the immune system\textsuperscript{105}.

Angiotensinogen is the precursor of the inflammatory adipokine angiotensin and is secreted by the adipocytes. Angiotensinogen negatively regulates adiponectin expression\textsuperscript{106}. In addition, epithelial cells and macrophages produce angiotensinogen-converting enzyme (ACE) that catalyze the conversion of the pro-inflammatory peptide - angiotensin\textsuperscript{107-108}. In turn, angiotensin promotes Th1 type cytokine production and development of colitis\textsuperscript{81}. Hence, another vicious inflammatory circle may occur between adipocytes, epithelial cells and macrophages. In contrast, agonists of peroxisome proliferator-activated receptor gamma (PPAR\gamma), a common signature marker in the early developmental phase of both adipocytes and macrophages induce both adiponectin and adiponectin receptors\textsuperscript{109}. Rosiglitazone, a PPAR\gamma agonist has been shown to be beneficial in human Inflammatory Bowel Disease and experimental colitis\textsuperscript{110}.

Obesity, a state of hypoadiponectemia, is associated with more severe Crohn’s disease and atherosclerosis. Interestingly, defective macrophage function and autophagy has been described in both conditions\textsuperscript{111-112}. Autophagy-related protein 16 (ATG16L1) upregulate adiponectin expression in Paneth cells and mutations in this gene is associated with Crohn’s disease\textsuperscript{29}. Mice ATG16L1 hypomorphic have defective autophagy processes, possibly because of impaired anti-microbial peptides production\textsuperscript{29}. Due to the fact that gut microbiota suppresses the epithelial expression of fasting-induced adipose factor (FIAF),
that promotes triglyceride accumulation, the bacterial-epithelial crosstalk could alter adipose mass, and by extension the balance of adipokines and cytokines. Current studies regarding the adiponectin role in colitis are controversial. We hereby, show that adiponectin treatment ameliorates colitis in a murine DSS model. Furthermore, we demonstrate that adiponectin alters the balance of key regulatory cytokines and adipokines relevant for both experimental and human disease. In addition, we propose that a plant derived adiponectin homolog may be considered as a viable and accessible IBD treatment.

Materials and Methods

Materials

99.9% pure Dextran Sulfate Sodium (DSS) was purchased from MP Biomedicals, Santa Ana, CA. Adiponectin adenovirus was a generous gift from Dr Jerry Olefsky, Loyola University, CA. Osmotin was purified from salt adapted cultured tobacco cells as described [36]. No contaminants were detected in the osmotin preparation by a combination of 2DGE and mass spectrometric methods. The endotoxin content was <0.03 EU/mg protein.

DSS - induced colitis and sample collection

Male, 3 months old C57BL/6 (Jackson Laboratory, n = 6 mice/group) were randomly divided into 3 groups, as described in treatment protocols below. All groups of mice were housed in a pathogen-free environment with free access to
food and water. Colitis was induced by administration of 3.5% (wt/vol) DSS in water for a week, followed by 3 days of water only. Body weight, stool consistency and rectal bleeding were monitored daily. On day ten, mice were euthanized with ketamine/xylazine (100/10 mg/kg i.p.) for blood and tissue harvest. The colons were removed and perfused with phosphate-buffered saline (PBS - pH 7.4), and measured. Half of each colon was fixed in RNAlater (Qiagen), and stored at -20°C. The other half was made in a “swiss roll”, cut and fixed in 10% buffered formalin (Sigma Chemical). The Animal Care and Use Committee at the University of Kentucky approved all procedures.

*Adiponectin overexpression*

The control (Ctr) and V+DSS mice were intravenously (i.v.) injected with adenovirus suspension (2x 10⁸ pfu, 0.1ml per mouse) carrying the reporter gene LacZ (β-Galactosidase). Mice in the AN+DSS were intravenously injected with an adenovirus suspension (2X10⁸ plaque forming units (pfu), 0.1ml per mouse) carrying the murine adiponectin gene. The Ctr group had only water while the V+DSS and AN+DSS groups were fed DSS in the drinking water. All groups received the above i.v treatments 3 days prior to DSS colitis protocol. Blood samples were taken at the end of the study to measure the adiponectin plasma level using an enzyme-linked immunosorbent assay (ELISA- mouse cytokine panel – LINCOplex, Millipore, Billerica, MA)

*Treatment with the mammalian adiponectin homolog - osmotin*
To assess the role of an adiponectin homolog in the treatment of IBD, male, 3 months old C57BL/6 (Jackson Laboratory, $n = 8$ mice/group) were divided into control (Ctr) group that received phosphate buffered saline only, a DSS group that received saline and DSS (DSS), and a group that received osmotin (73 ug/Kg/h) and DSS (OSM+DSS). All treatments (saline & Osmotin) were delivered for 2 weeks using Alzet mini-osmotic pumps (Durect Corp., Cupertino, CA). The pumps were implanted subcutaneously in the suprascapular area under sterile conditions. They ensured constant delivery of drug during the experiment. During the last 10 days, all mice except the Ctr group, received DSS in the drinking water for 7 days, followed by water for 3 days. Mice were euthanized with ketamine/xylazine and examined for macro and histopathological abnormalities. The osmotic pumps were removed and the residual volume was determined. Blood was collected for Adiponectin and IL10 measurements by ELISA (mouse cytokine panel – LINCOplex, Millipore, Billerica, MA).

**Histologic analyses and Proliferating Cell Nuclear Antigen (PCNA) assay**

Formalin fixed colons were embedded in paraffin. From these, serial sections (5 - 7 μm) were stained with hematoxylin and eosin. A pathologist blinded to the group allocation assessed the severity of colitis as described elsewhere. The sections were deparaffinized and treated with Antigen Retrieval Solution (DAKO, Carpinteria, CA) followed by incubation in 0.3% $\text{H}_2\text{O}_2$-methanol for 10 min, and wash. Sections were incubated in normal blocking serum for 90 min followed by overnight incubation in primary antibody, NCL-PCNA (1:200) (Novocastra, Leica...
Microsystems) at 4°C. The biotinilated secondary antibody (Elite ABC kit, Vector) was then applied for 2 hours. The slides were counterstained with Methyl Green (DAKO). Images were taken with an Olympus BX51 microscope (Olympus America, Inc.) at 20x magnification.

**Analysis of mRNA gene expression levels in mouse colonic tissue**

Total RNA was purified using MagnaPure Compact RNA Isolation Kit (Roche) from whole colon tissue and cDNA was obtained using Transcription High Fidelity cDNA Synthesis Kit (Roche). Specific mRNA levels were quantified by real time reverse transcription-polymerase chain reaction (RT-PCR), using the IQ iCycler (Bio-Rad), and SYBR Green qPCR Supermix (Bio-Rad). Primers were designed using the Primer 3 software (SourceForge) and the sequences are shown in Table 1. The target genes mRNA level were normalized to reference gene according to the comparative CT (cycle threshold) method, also referred to as the \(2^{-\Delta\Delta CT}\) method. The formula used: \(2^{-([\text{Mean } C_{\text{t}}\text{target}} - \text{Mean } C_{\text{t}}\text{reference}])}\) for each sample. For each treatment group the mean ± SE value has been plotted.

**Measurement of cytokines in the plasma and the colonic tissue**

At day 10, we measured cytokines in the plasma and the colonic tissue homogenate using a bead based immunoassay (Lincoplex) multianalyte detection platform (Luminex - Mouse Cytokine Panel). Values are expressed in picograms per milliliter and as mean ± SE, n=6 - 8/ group.
Western Blot Analyses

Proteins were extracted from each colon (n=6) per treatment group. Equal amounts of protein lysate were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). We blocked with 5% non-fat milk and the blots were washed in TBS-T and incubated with the primary antibody at 4°C, overnight (mouse anti-β-actin, mouse anti-COX2). Next day, blots were washed with TBS-T and incubated for 2h with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse β-actin or COX2 - Santa-Cruz Biotechnology). After careful washing the membrane was visualized by enhanced chemiluminescence (ECL)

Bone marrow-derived dendritic cells generation

A single-cell suspension of bone marrow from femur and tibia bones of C57Bl/6 mice was obtained by flushing the bones with cell culture media, RPMI 1640 medium (HyClone, Thermoscientific) supplemented with 10% fetal bovine serum (Atlanta Biologicals), L-glutamine, HEPES and Penicillin/Streptomycin (Invitrogen). After spinning, the cell pellet was resuspended in dendritic cell media (cell culture media supplemented with 20% F10.9 cell supernatant containing GMCSF) and plated at a concentration of 4x10^6 cells. On day 9, both adherent and non-adherent fraction of cells were harvested and washed free of dendritic cell media. Cells were counted before treating with the appropriate
ligands. On day 9, the cultures contained 85% CD11c+ bone marrow-derived (BM) dendritic cells (DCs).

**Ligand activation of nuclear receptors**

On day 9, BM - DCs were plated at 1X10^6 cells/ml in TC-treated 6-well plates (Falcon), in 3 replicates per treatment. Cells were incubated for 24 hours with 10µM Rosiglitazone (R) (Cayman Chemical), 1µM of 9-cis-retinoic acid (RA) (Sigma) and their combination. Cells were treated with DMSO or were incubated with 1 µg/ml LPS at the time of ligand treatment. At 24 hours, cells were harvested and washed extensively to remove residual ligands. RNA was extracted using Trizol (Invitrogen) reagent method. cDNA was obtained using Transcription High Fidelity cDNA Synthesis Kit (Roche). Specific mRNA levels were quantified by real time reverse transcription-polymerase chain reaction (RT-PCR), using the IQ iCycler (Bio-Rad), and SYBR Green qPCR Supermix (Bio-Rad). Primers were designed using the Primer 3 software (SourceForge) and the sequences are shown in Table 3.1.

**Statistical analysis**

Data are expressed as Mean ± SE. Data were analyzed using one-way analysis of variance (ANOVA) (GraphPad Prism 5) followed by Tukey’s test with significance accepted at p < 0.05. Body weight data were analyzed by one-way ANOVA with repeated measures on time. Significance was accepted at p < 0.05.
Results

The severity of DSS-induced colitis is decreased in mice receiving Adiponectin adenovirus

Viral vector administration did not alter the weight or other clinical parameters prior to DSS protocol. At the end of the DSS administration, mice receiving the reporter gene and DSS - the V+DSS group - had a significant drop in weight (Figure 3.1A, p=0.001), and hematocrit (Figure 3.1B, p=0.02) consistent with increased severity. The postmortem exam of colons from V+DSS mice group revealed swollen and shortened colons compared to control (Ctr – water+LacZ) and mice receiving Adiponectin adenovirus and DSS (AN+DSS) (Figure 3.1C, p=0.01). Microscopically, colitis was mild in the AN+DSS group as compared with significant mucosal ulceration, crypt abscesses and altered architecture present in V+DSS mice. Proliferating cell nuclear antigen (PCNA) staining revealed a significant increase in crypt proliferative activity in AN+DSS group as compared to the V+DSS group (Figure 3.1) consistent with a normal epithelial turnover. Mice in the AN+DSS group had an increased mRNA expression, colonic and plasma level of adiponectin at the end of the study compared with V+DSS and Ctr group (Figure 3.2).

Mice that overexpressed adiponectin (AN+DSS) showed decreased pro-inflammatory cytokines expression during DSS-induced colitis

During colitis, inflamed colonic tissue can become a source of inflammatory cytokines like tumor necrosis factor (TNFα) interleukin 1 (IL1β), and the key
factor in the recruitment of macrophages, the monocyte chemoattractant protein (MCP-1). In our study, adiponectin overexpression had a protective effect and led to the downregulation of aforementioned pro-inflammatory cytokines, as well as of chemokine (C-C motif), receptor2 (CCR2), the receptor for the MCP1, as compared with control (Ctr) or V+DSS mice groups (Figure 3.3). Consequently, a lower expression of MCP1 and CCR2 cytokines could decrease the ability to recruit macrophages into the colonic tissue\textsuperscript{115}.

Anti-inflammatory cytokines were increased in mice overexpressing adiponectin

IL10 is known to limit the extent of intestinal inflammation during colitis by inhibiting the macrophage and Th1 cells production of pro-inflammatory cytokines\textsuperscript{116}. Administration of adiponectin adenovirus to AN+DSS mice group upregulated the expression of the anti-inflammatory cytokine IL10 and its plasma level (Figure 3.4A, B) after DSS-induced colonic injury. DSS model of colitis leads to significant epithelial damage and recruitment of inflammatory cells\textsuperscript{117-118}. Secretory leukocyte protease inhibitor (SLPi) released by epithelial cells and macrophage can limit tissue destruction by neutralizing proteases and downregulating pro-inflammatory cytokines\textsuperscript{119-120}. We observed a robust induction of SLPi in mice receiving the adiponectin adenovirus (Figure 3.4D, p=0.001). Independent of its anti-protease activity, the SLPi can limit macrophage nitric oxide (NO) and TNF\textgreek{a} production\textsuperscript{120}. We hypothesized that higher IL10 and SLPi expression in adiponectin treated mice will promote M2 (alternatively activated) macrophages, associated with the repair processes. The
mRNA expression of arginase 1 (Arg 1), a marker of this macrophage phenotype, was increased 3 fold in AN+DSS group as compared to Ctr and V+DSS mice (Figure 3.4C, p=0.002).

Adiponectin administration reduces the pro-inflammatory adipokines during DSS induced colitis

Adipose tissue derived hormones like angiotensin II and osteopontin can fulfill pro-inflammatory cytokine-like functions. Both adipokines have been shown to promote inflammation in the DSS model of colitis. Furthermore, they are capable of downregulating adiponectin and/or its receptors. We hypothesized that adiponectin treatment will prevent activation of the renin-angiotensin system (RAS) and osteopontin and complete a regulatory loop that protects mice from colitis. Our study showed a 3-fold increase in both angiotensinogen (angiotensin precursor) and osteopontin expression in mice receiving DSS (Figure 3.5A, 3.5D). Angiotensin II is generated through the activity of angiotensin converting enzyme (ACE) while its inflammatory signal requires the angiotensin receptor 1 (AT1). There was no significant change in ACE and AT1 expression during DSS colitis in the V+DSS group receiving the reporter gene and DSS (Figure 3.5B, 3.5C) compared to the control group. In contrast, higher adiponectin levels in AN+DSS group effectively downregulated (3 fold) both ACE and AT1 (Figure 3.5B, 3.5C) and correlated with a protective effect from DSS-induced colitis.
Adiponectin upregulation in mice with colitis decreased the endoplasmic reticulum (ER) stress response and the mediators of apoptosis

Recent evidence suggests that endoplasmic reticulum (ER) stress is important in the initiation and regulation of gut inflammation\textsuperscript{126}. Human and experimental models of inflammatory bowel diseases are associated with cellular stress due to accumulation of misfolded proteins within the colonic epithelium\textsuperscript{127}. Endoplasmic reticulum - binding protein (BIP/ Grp78) acts as an intracellular sensor and is considered a marker of ER stress. Adiponectin treatment decreased BIP mRNA expression in AN+DSS mice (Figure 3.6A) implying a lower level of ER stress. In addition, since ER stress response is coupled to the cell death program, we investigated the mRNA expression of two pro-apoptotic molecules: C/EBP Homologous Protein (CHOP) and Caspase 12 (Casp12). There was a 2.5 fold downregulation of the expression of both pro-apoptotic genes in the AN+DSS mice compared with V+DSS that expressed the reporter gene (Figure 3.6B, C). Cooperation between cyclooxygenase 2 (COX-2) and TLR4 signaling pathway insures optimal proliferation and protection against apoptosis in the injured intestine\textsuperscript{128}. In our experiment, adiponectin overexpression correlated with decreased disease severity and a three-fold increase in colonic COX2 expression (Figure 3.6D, E).

**Treatment with the mammalian adiponectin homolog- osmotin- offered similar protection against DSS-induced colitis**
Osmotin, and many of its homologs, functions as defense proteins of the plant innate immune system \textsuperscript{129}. Osmotin is an adiponectin homolog and can signal through adiponectin receptors 1 and 2. Specifically, we showed that osmotin could activate the adiponectin target, AMP kinase in mammalian myocytes via adiponectin receptors \textsuperscript{130}. In our current experiment, osmotin was delivered via mini-osmotic pumps for 2 weeks and colitis was induced by administering 3.5\% DSS in the drinking water for 7 days. The osmotin treatment group OSM+DSS maintained their body weight during DSS injury while the group receiving saline (DSS group) had considerable weight loss (Figure 3.7 A). Importantly, none of the mice receiving osmotin developed hematochezia, a marker of severe colitis, as evidenced by Osm+DSS group hematocrit values in the range of control mice (Figure 3.7B). Similar to the adiponectin overexpression experiments, osmotin treatment induced a 5 fold increase in plasma level of the anti-inflammatory cytokine IL10 (Figure 3.7D). There were no statistically significant differences in colonic and plasma levels of adiponectin between the experimental groups (data not shown).

\textbf{PPAR\gamma agonists and retinoic acid reversed the state of adiponectin resistance in dendritic cells.}

Bone marrow (BM) derived dendritic cells (DCs) fulfill a major role in the initiation, maintenance and propagation of immune responses. In the gut, bacterial stimuli such as \textit{E. coli} lipopolysaccharide (LPS) activate the dendritic cells through toll-like receptor (TLR) 4 to induce the expression of IL-12 and initiate a Th1
PPARγ agonists have been shown to inhibit TLR induced activation of DCs. Furthermore, intestinal epithelial cells release retinoic acid and drive the development of tolerogenic subtype of DCs that express the anti-inflammatory cytokine IL10. Moreover, the anti-inflammatory adipokine – adiponectin - has been shown to induce IL10 release from DCs. We hypothesized that bacterial derived LPS induces a state of adiponectin resistance in DCs by downregulating its main receptors – adiponectin receptor 1 & 2. Bone-marrow derived DCs from C57Bl/6 mice were cultured in the presence or absence of LPS (Figure 3.8). There was a 3-fold decrease in both adiponectin receptors mRNA expression following LPS exposure (Figure 3.8A, B, C). Administration of Rosiglitazone (R) – a PPARγ agonist – and retinoic acid (RA – 9-cis-retinoic acid) restored the expression of both adiponectin receptors in the presence of LPS treatment (Figure 3.8A, B, C). Importantly, the combination of PPARγ agonist and retinoic acid [R/RA = Rosiglitazone + 9-cis-retinoic acid] had a synergistic effect and more than doubled the secretion of the anti-inflammatory cytokine IL10 in DCs (Figure 3.8D).

**Discussion**

Adiponectin is unique among adipokines because its circulating levels are inversely related to the adipose mass. Adiponectin gene contains a signal sequence, a collagen like domain, and a globular domain similar to the complement factor C1q. Our study indicates that adiponectin treatment can
significantly ameliorate colonic inflammation in the murine DSS model. Overexpression of adiponectin restored normal mucosal architecture and reduced the production of potent inflammatory cytokines and adipokines. The first evidence for a protective role came from the Nishihara group\textsuperscript{42}. They demonstrated that adiponectin treatment protects adiponectin KO mice from DSS induced colitis. Meanwhile, no benefit was noted in TNBS model of colitis, suggesting the fact that this model might be more dependent on T cells, while the adiponectin has a greater influence on the innate immunity. This is also supported by the fact that T cells from either wild type or adiponectin KO produce similar amounts of pro-inflammatory cytokines IL-6, IL-17, TNF\(\alpha\), and IFN\(\gamma\) upon activation with CD3/CD28 antibody\textsuperscript{136}.

In our experiment the mice overexpressing adiponectin showed a reduction in the colonic TNF\(\alpha\), IL1\(\beta\), as well as colonic and plasma IL-6 (data not shown). On the other hand, adiponectin treatment significantly elevated the IL10 level within the colon and plasma. This observation is in agreement with in-vitro studies whereby adiponectin induced macrophage IL10 expression\textsuperscript{137}. In contrast to our findings and Nishihara’s group, a recent study showed that adiponectin KO mice are protected from DSS and TNBS colitis, and adiponectin treatment induces inflammation\textsuperscript{41}. The authors have further shown that colonic explants from treated mice released more IL-6. Differences in adiponectin KO mice have been invoked since only this group showed high basal TNF-\(\alpha\) production and increased susceptibility to LPS. Several recent studies confirmed that
adiponectin effectively blocks LPS induced release of pro-inflammatory cytokines and thus, support an anti-inflammatory role\textsuperscript{50,138}.

Moreover, a study that compared the outcome of colitis in IL10 KO and adiponectin/IL10 double KO found no differences\textsuperscript{136}. It was concluded that adiponectin does not play a role in that colitis model. In accordance with our experiments, and published data\textsuperscript{50,139} we believe that IL10 production is paramount for the anti-inflammatory role of adiponectin in colitis. Therefore, the results seen in IL10 KO mice are expected. Furthermore, if adiponectin had promoted inflammation, the double KO (adiponectin/IL10) mice would have had decreased morbidity and mortality compared to IL10 single KO.

Adiponectin induced IL10 may promote alternative activation of macrophages (M2) and resolution of chronic inflammation. We now show that adiponectin treatment also resulted in higher colonic expression of arginase-1 a marker of such macrophages\textsuperscript{140}. Adipose tissue macrophages from lean mice express many genes characteristic of M2 (alternatively activated phenotype) macrophages\textsuperscript{141}, including heparin–binding lectin (Ym1), arginase-1, and IL10. Diet-induced obesity (low adiponectin state) decreases expression of these genes while increasing expression of those encoding TNF-α and nitric oxide synthase (NOS) 2 that are characteristic of classically activated (M1) macrophages\textsuperscript{141}. Furthermore, AMP-activated protein kinase (AMPK), the kinase that translates adiponectin signaling, suppresses proinflammatory responses and promotes macrophage polarization to an anti-inflammatory - M2 functional phenotype\textsuperscript{72}. Consistent with these observations, macrophages from adiponectin
KO mice have lower M2 markers\textsuperscript{142}. Influx on leukocytes during colitis requires coordinated expression of chemokines and their receptors\textsuperscript{143}. In our study, we found that adiponectin treatment effectively downregulated both MCP-1 and CCR2. It is known that CCR2\textsuperscript{−/−} mice are protected from DSS colitis and have increased IL10/TNFα ratio similar to our observations\textsuperscript{144}.

Leukocytes infiltrate the inflamed gut and breach the epithelial barrier by releasing serine proteases. Epithelial cell, in turn, produce secretory leukoprotease inhibitor (SLPi) to block their activity as well as exert broad antimicrobial properties\textsuperscript{145}. Mice receiving adiponectin, in our experiments had a significant upregulation of SLPi expression. This is also important because leukocyte proteases can cleave HMW adiponectin – the main anti-inflammatory moiety - and increase the amount of globular and LMW forms\textsuperscript{52}. In vitro experiments have shown that globular adiponectin induces NF-κB and pro-inflammatory cytokines, but prolonged exposure blocks further activation. In contrast, HMW adiponectin can quickly prevent NF-κB activation\textsuperscript{53}. Thus, indirectly, SLPi may modulate its activity at sites of inflammation. Mucosal SLPi expression is decreased in Crohn's Disease patients\textsuperscript{146}, and this observation adds to the clinical relevance of our study.

In addition to adiponectin, other adipokines can modulate colitis. We now show that adiponectin treatment downregulates the proinflammatory adipokines angiotensinogen and osteopontin in experimental colitis. Pharmacological blockade of the renin – angiotensin (RAS) system as well as absence of osteopontin protects mice from DSS colitis\textsuperscript{81,123}. In our study, adiponectin had
broad effects on the renin-angiotensin system reducing the expression of angiotensinogen, angiotensin converting enzyme (ACE) and angiotensin receptor 1 (AT1). Blockade of angiotensin can increase adiponectin levels and thus a regulatory loop may be established. A similar mechanism may operate in the case of osteopontin since KO mice have lower adiponectin levels both, under basal conditions and during DSS colitis (unpublished observation).

Adiponectin has a structure similar to complement 1q and TNFα. Interestingly, the immune system in plants utilizes thaumatin domain proteins such as osmotin, as defense effectors [35]. To date, Osmotin is the only thaumatin domain protein that has been reported to act as an adiponectin mimic in mammalian cells \(^{130,147}\). Similar to adiponectin treatment we noticed a beneficial effect of Osmotin in DSS colitis. Mice that received a two-week infusion of Osmotin showed an improved clinical outcome that correlated with a marked elevation of plasma IL10 level. Although Osmotin homologs are ubiquitous in plants and occur in significant amounts in edible plant parts, none has been examined for adiponectin-like activities. Our results suggest that Osmotin and related plant proteins are potentially promising and accessible treatments for IBD.

Decreased adiponectin levels during colitis may be secondary to TLR triggered inflammatory response. In the small intestine, the DCs precursors differentiate from circulating inflammatory monocytes and acquire mucosal functions via micro-environment cues\(^{148}\). During IBD, dysfunctional DCs may act by initiating abnormal T cell responses to the enteric environment and release of
proinflammatory cytokines. Activators of PPARγ are anti-inflammatory and have been proposed as therapeutic agents for the treatment of Th1-type inflammatory diseases. For example, rosiglitazone has been shown to enhance the production of the anti-inflammatory IL10 from activated human mature monocyte-derived dendritic cell\(^{149}\). This activity is conditioned by the retinoic acid (RA - a metabolite of vitamin A). Our current experiments in bone marrow derived DCs indicate that bacterial LPS downregulated adiponectin and its receptors. The conjugated effects of PPARγ agonists and retinoic acid reversed this state of adiponectin resistance. Moreover, this recovery was accompanied by a marked induction of IL10 expression. Our data is in agreement with the observation that PPARγ agonists induce adiponectin receptor expression in macrophages\(^{109}\). Additionally, it may further explain the protective effect of PPARγ activation in IBD colitis\(^{35,110}\).

Endoplasmic reticulum (ER) stress response may contribute to epithelial damage during DSS-induced colitis as well as in patients with IBD\(^{150}\). IL10 mediated p38 mitogen-activated protein kinase (p38MAPK) signaling can block activation of the ER stress sensor – endoplasmic reticulum-binding protein (BIP) - after exposing intestinal cells to TNFα\(^{83}\). Adiponectin can induce IL10 and activate p38MAPK\(^{151-152}\). We now show that adiponectin treatment decreased ER stress response. Furthermore, it downregulated the expression of caspase-12 (CASP12) which plays a central role in ER stress induced apoptosis\(^{153}\). Epithelial cyclooxygenase 2 (COX-2) is induced during episodes of inflammation and mediates toll-like receptor 4 (TLR4) dependent mucosal repair\(^{128,154}\). COX-2\(^{-/-}\) mice are more susceptible to DSS-induced colitis due to their inability to
produce prostaglandin E2 (PGE2)\textsuperscript{155}. The finding of enhanced COX-2 production in mice overexpressing adiponectin and reduced ER stress markers points toward a significant protective effect of adiponectin in gut mucosa during inflammatory situations.

In summary, we provide evidence that adiponectin and its plant homolog – osmotin - have an anti-inflammatory role in an established model of experimental colitis. In addition to altering the cytokine milieu, adiponectin may operate in complex regulatory networks that involve downregulation of pro-inflammatory adipokines angiotensin and osteopontin. Furthermore, adiponectin modulates the expression of epithelial protective factors, cellular stress response and immune cell traffic. In situations where adiponectin secretion is deficient, Osmotin, a plant derived adiponectin homologue or PPARγ agonists may restore its protective effects. Importantly, novel therapeutic strategies could be developed in order to mimic or enhance the anti-inflammatory action of adiponectin.

**Figure Legends**

*Figure 3.1. The severity of DSS-induced colitis is decreased in mice receiving Adiponectin adenovirus.* Mice were randomly assigned to three i.v. treatment groups: Control (Ctr) and the V+DSS groups received adenovirus reporter gene while the AN+DSS group received adenovirus adiponectin gene. The Ctr group was placed on water for 10 days. The V+DSS and AN+DSS groups were administered 3.5% DSS in drinking water for 7 days followed by 3 days of water.
(A) Body weight change between the last and first day of the experiment. (B) Hematocrit and (C) colon length at the end of the experiment. Data are expressed as mean ± SE, n=6/group. Left panels: Immunohistochemistry for epithelial cell proliferative index (PCNA) of the colonic tissue of Ctr (upper panel), V+DSS (middle panel), and AN+DSS (lower panel).

Figure 3.2. Efficacy of adenoviral delivery. Colonic adiponectin was increased at mRNA (A) and protein level (C) and correlated with plasma level (B) in mice that received adiponectin adenovirus (AN+DSS).

Figure 3.3. Expression of pro-inflammatory cytokines during DSS-induced colitis was decreased in mice overexpressing adiponectin (AN+DSS). Colonic (A) TNFα, (B) IL1β, (C) MCP1, (D) CCR2 gene expression. Data are expressed as mean ± SE, n=6/group.

Figure 3.4. Adiponectin treatment promoted an anti-inflammatory milieu during DSS administration. Increased IL10 colonic mRNA (A) and corresponding (B) plasma level in mice overexpressing adiponectin (AN+DSS). (C) Increased alternative macrophage (M2) marker - arginase 1- and (D) epithelial secretory leukoprotease inhibitor, consistent with limited tissue destruction in mice overexpressing adiponectin (AN+DSS). Data are expressed as mean ± SE, n=6/group.

Figure 3.5. Adiponectin treatment reduces the pro-inflammatory adipokines during DSS induced colitis. Angiotensinogen, the only known precursor of the pro-inflammatory angiotensin II was decreased (A) in AN+DSS group. (B)
Adiponectin overexpression downregulated the angiotensin II receptor 1 and the enzyme responsible for its generation (C) ACE. Adiponectin treatment reduced the colonic expression of the pro-inflammatory adipokine - osteopontin (D). Data are expressed as mean ± SE, n=6/group

Figure 3.6. *Adiponectin reduced cellular stress and apoptosis during DSS induced colitis. (A) Decreased mRNA gene expression of endoplasmic reticulum stress response marker – BIP – and related pro-apoptotic factors – CHOP and Casp12 (B, C) during DSS colitis in mice overexpressing adiponectin (AN+DSS). Colonic COX-2 exerts proliferative and anti-apoptotic effects. High levels of adiponectin in AN+ DSS mice correlated with increased colonic COX-2 mRNA gene expression (D) and protein level (E). Data are expressed as mean ± SE, n=6/group

Figure 3. 7. *Osmotin - a plant derived adiponectin homolog - is beneficial in DSS colitis model.* Saline (for the Ctr and DSS groups) or Osmotin (for the Osm+DSS group) were delivered as a 2 weeks infusion that overlapped the 10 days of 3.5% DSS colitis protocol. (A) Change in body weight between last and first day of DSS protocol. (B) Hematocrit at the end of the experiment. Osmotin treatment (Osm+DSS group) prevented weight loss and hematochezia during colitis. (C, D) Increased colonic and plasma level of the anti-inflammatory cytokine IL10 during DSS colitis in mice receiving Osmotin (Osm+DSS). Data are expressed as mean ± SE, n=8/group

Figure 3.8 *PPARγ agonist and retinoic acid reversed the LPS induced adiponectin resistance in dendritic cells.* Murine, bone marrow derived dendritic
cells were activated with LPS in the presence or absence of PPARγ agonist (R = rosiglitazone) and retinoic acid (RA -9-cis-retinoic acid). LPS did not affect dendritic cells’ adiponectin expression while rosiglitazone induced it (A). In the presence of LPS, both adiponectin receptors were downregulated (B, C). Rosiglitazone and retinoic acid acted synergistically to restore adiponectin receptors expression (B, C) and concurrently increased IL10 gene expression (D). Data are expressed as mean ± SE, n=3 replicates/treatment group.
Figure 3.1 The severity of DSS-induced colitis is decreased in mice receiving Adiponectin adenovirus
Figure 3.2 Efficacy of adenoviral delivery
Figure 3.3 Expression of pro-inflammatory cytokines during DSS-induced colitis was decreased in mice overexpressing adiponectin (AN+DSS)
Figure 3.4 Adiponectin treatment promoted an anti-inflammatory milieu during DSS administration
Figure 3.5 Adiponectin treatment reduces the pro-inflammatory adipokines during DSS induced colitis
Figure 3.6 Adiponectin reduced cellular stress and apoptosis during DSS induced colitis
Figure 3.7 Osmotin - a plant derived adiponectin homolog - is beneficial in DSS colitis model
Figure 3.8 PPARγ agonist and retinoic acid reversed the LPS induced adiponectin resistance in dendritic cells
Chapter Four

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ROLE OF THE XENOBIOTIC RECEPTOR IN INFLAMMATORY BOWEL DISEASE

Synopsis

Background: Gene-environment interplay modulates Inflammatory Bowel Diseases (IBD). Dioxin-like compounds can activate the Aryl Hydrocarbon Receptor (AhR) and alter macrophage function as well as T cell polarization. We hypothesized that attenuation of the AhR signaling pathway will ameliorate colitis in a murine model of IBD.

Design: DSS colitis was induced in C57BL/6 AhR null mice (AhR\(^{-/-}\)), heterozygous mice (AhR\(^{-/+}\)), and their wild type (WT) littermates. Clinical and morphopathological parameters were used to compare the groups. Patients: AhR pathway activation was analyzed in biopsy specimens from 25 IBD patients and 15 healthy controls.

Results: AhR\(^{-/-}\) mice died before the end of the treatment. However, AhR\(^{-/+}\) mice exhibited decreased disease activity compared to WT mice. The AhR\(^{-/+}\) mice expressed less proinflammatory cytokines such as TNF\(\alpha\) (6.1 versus 15.7 fold increase) and IL17 (23.7 versus 67.9 fold increase) and increased anti-inflammatory IL-10 (2.3 fold increase) compared with the AhR\(^{+/+}\) mice in the
colon. Colonic macrophage infiltration was attenuated in the AhR −/+ group. AhR and its downstream targets were significantly upregulated in IBD patients versus control (CYP1A1 – 19.9, and IL8- 10 fold increase).

**Conclusion:** Attenuation of the AhR receptor expression resulted in a protective effect during DSS-induced colitis, while the absence of AhR exacerbated the disease. Abnormal AhR pathway activation in the intestinal mucosa of IBD patients may promote chronic inflammation. Modulation of AhR signaling pathway via the diet, cessation of smoking or administration of AhR antagonists could be viable strategies for the treatment of IBD.

**Introduction**

Inflammatory Bowel Disease (IBD) is characterized by an inappropriate immune response to commensal flora.⁵⁶–⁵⁷ In Western countries, 1 in 200 patients are affected by Ulcerative Colitis (UC) or Crohn’s Disease (CD), the major forms of IBD and their incidence is steadily increasing.⁵⁸ The causes of IBD are unknown and the diagnosis is based on clinical, endoscopic, radiological and histological criteria.⁵⁹ Differences between familial and geographic clustering point toward environmental factors. In Caucasians, smoking has the strongest association with the severity of the gut inflammation.⁶⁰–⁶¹

Aryl Hydrocarbon receptor (AhR) is the only known receptor for dioxin, a potent immunomodulating environmental contaminant. Studies showed that cigarette smoke contains dioxins and dioxin-like chemicals.⁶²–⁶⁴ The unbound AhR is present in the cytoplasm of all immune system cells. Furthermore, many
genes involved in immune regulation possess multiple dioxin response elements (DREs) in their promoter region. Studies performed in AhR \(-/-\) mice have shown an enhanced inflammatory response to cigarette smoke or endotoxin, with elevated levels of tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) and interleukin-6 (IL-6). \(^{166-167}\)

AhR is essential in the regulation of cell cycle, lipid metabolism \(^{168}\) circadian rhythm \(^{169}\) and immune response. \(^{170}\) Although AhR seems to be a crucial co-factor in regulation of both homeostasis and inflammation, its role in the gut autoimmune pathology is poorly described. Surprisingly, sustained activation of AhR by its high affinity, prototypical agonist, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), resulted in altered bone marrow and thymic development, as well as susceptibility to infectious diseases and cancer in mice. \(^{171-172}\) Collectively, these observations suggest that a low to normal activation of AhR is beneficial for the protection against environmental insults, and represents an important link between environment and chronic diseases.

Our preliminary experiments indicated that AhR \(-/-\) mice are highly sensitive to DSS-induced colitis. In the current study, we examined the outcome of DSS-induced colitis in wild type and AhR heterozygous mice. Interestingly, moderation of the AhR signaling pathway in the AhR heterozygous mice was sufficient to ameliorate colitis. We also provide evidence that the level of AhR expression correlates with the profile of cytokines, adipokines and cellular stress response. These data indicate that attenuation of the AhR signaling pathway corresponds to an attenuated inflammatory response in the colon in disease-free conditions and during experimentally induced colitis. Furthermore, we extended
our observations in IBD patients were AhR pathway was significantly upregulated when compared to healthy controls.

**Materials and Methods**

*Subjects and sample collection*

Colonic biopsies were obtained from 15 healthy controls and 25 IBD (Crohn’s Disease) patients. Control subjects were healthy individuals who underwent screening colonoscopy and had no inflammatory bowel conditions. IBD patients had an established diagnosis, based on standard endoscopic, radiologic, and histologic criteria. Informed consent was obtained before participation and the study protocol was approved by the Institutional Review Board of the University of Kentucky.

To examine the AhR activation in the lower digestive tract, biopsies were taken from the colon during standard colonoscopy. Two biopsies from each subject were fixed in formalin or RNA Later until analysis. The formalin fixed subset of biopsies was subject to standard histological staining and fluorescent immunohistochemistry. The RNA later subset of biopsies was used to assess the total mRNA using MagnaPure Compact RNA Isolation Kit (Roche). cDNA was obtained using Transcription High Fidelity cDNA Synthesis Kit (Roche). Specific mRNA levels were quantified by real time reverse transcription-polymerase chain reaction (RT-PCR), using the IQ iCycler (Bio-Rad), and SYBR Green qPCR Supermix (Bio-Rad). Primers were obtained from SABiosciences, Frederick, MD. The mRNA levels for test genes were normalized to reference gene according to
the comparative $C_T$ method also referred to as the $2^{-\Delta \Delta CT}$ method. The formula used: $(2^{-\Delta C_T}) \times 100\%$.

**Animal treatments and sample collection**

Male, 3 months old C57BL/6 and AhR $^{-/-}$ mice (Jackson Laboratory), or AhR $^{-/+}$ mice bred in-house to C57BL/6 mice ($n = 10$ mice/group) were housed in a pathogen-free environment with free access to food and water. Mice were administered 3.5% (wt/vol) DSS (ICN Biochemical) in water for a week, followed by 3 days of water only. Body weight, stool consistency and rectal bleeding were monitored daily. On day ten, mice were euthanized with ketamine/xylazine (100/10 mg/kg ip) for blood and tissue harvest. The colons were removed and perfused with phosphate-buffered saline (PBS - pH 7.4), and measured. Half of each colon was fixed in RNAlater (Qiagen), and stored at -20°C. The other half was made in a “swiss roll”, cut and fixed in 10% buffered formalin (Sigma Chemical). The Animal Care and Use Committee at the University of Kentucky approved all procedures.

**Analysis of mRNA gene expression levels in mouse colonic tissue**

On day 10, total RNA was purified using MagnaPure Compact RNA Isolation Kit (Roche) from whole colon tissue and cDNA was obtained using Transcription High Fidelity cDNA Synthesis Kit (Roche). Specific mRNA levels were quantified by real time reverse transcription-polymerase chain reaction (RT-PCR), using the IQ iCycler (Bio-Rad), and SYBR Green qPCR Supermix (Bio-Rad). Primers were
designed using the Primer 3 software (SourceForge) and the sequences are shown in Table 1 (supplemental material).

The mRNA levels for test genes were normalized to reference gene according to the comparative C\textsubscript{T} method also referred to as the $2^{-\Delta\Delta C_T}$ method. The formula used: $(2^{-(C_{T_{test}} - C_{T_{reference}})}) \times 100\%$.

**Measurement of cytokines in the colonic tissue**

At day 10, we measured cytokines in the colonic tissue homogenate using a bead based immunoassay (Lincoplex) multianalyte detection platform (Luminex - Mouse Cytokine Panel). Values are expressed in picograms per milliliter and as mean ± SE, n=10 / group.

**Histology**

Serial sections (5 - 7 μm) of paraffin embedded colons (swiss roles) were stained with hematoxylin and eosin. A pathologist blinded to the group allocation assessed the severity of colitis. The scoring system evaluated the following characteristics: (1) percentage of area involved, (2) number of follicle aggregates, (3) edema, (4) erosion/ulceration, (5) crypt loss, and (6) infiltration of mononuclear and polymorphonuclear cells. The total score ranges from 0 to 26.

**Proliferating Cell Nuclear Antigen (PCNA) assay**
The sections were deparaffinized and treated with Antigen Retrieval Solution (DAKO, Carpinteria, CA) followed by incubation in 0.3% H₂O₂-methanol for 10 min, and wash. Sections were incubated in normal blocking serum for 90 min followed by overnight incubation in primary antibody, NCL-PCNA (1:200) (Novocastra, Leica Microsystems) at 4°C. The biotinilated secondary antibody (Elite ABC kit, Vector) was then applied for 2 hours. The slides were counterstained with Methyl Green (DAKO). Images were taken with an Olympus BX51 microscope (Olympus America Inc.).

**Fluorescent Immunohistochemistry**

Formalin-fixed, paraffin embedded colon sections (5-7 μm) placed on coated slides were sequentially deparaffinized and rehydrated using xylene and ethanol. Slides were then treated for 10 min with a citric acid-based antigen-unmasking solution (Vector Laboratories, Burlingame, CA). Next, slides were incubated for 1 hour at room temperature in normal blocking serum (1% in PBS) and incubated overnight at 4°C with the respective primary antibody (dilution 1:50): Angiotensin II (BGN/0856/21) - a mouse anti-human monoclonal antibody, AT1 (306: sc-579) - a rabbit anti-human polyclonal antibody or ACE (H-170: sc-20791) - a rabbit anti-human polyclonal antibody, (Santa Cruz Biotechnology, INC, CA). On the second day, after washing, sections were incubated for 1 h with a mixture of Cy2-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, catalog numbers 115-225-146 and 111-165-144). Sections were counterstained with 4′, 6-diamidino-2-
phenylindole dihydrochloride (DAPI) (Molecular Probes Invitrogen, Eugene, OR) to visualize nuclei, and mounted with VECTASHIELD® medium (Vector Laboratories, Burlingame, CA). Images were taken with an Olympus BX51 microscope, using a 20x objective. We generated the composite images using Image-ProPlus 5.0 (Media Cybernetics, Inc.) software.

**Statistical analysis**

Data are expressed as Mean ± SE. Data were analyzed using unpaired t-test and one-way analysis of variance (ANOVA) (GraphPad Prism 5) followed by Tukey’s test with significance accepted at p < 0.05. Body weight data were analyzed by one-way ANOVA with repeated measures on time. Survival percentage has been calculated using Kaplan-Meier method. Significance was accepted at p < 0.05.

**Results**

The severity of DSS-induced colitis is decreased in AhR -/+ mice

Preliminary experiments showed that AhR KO mice do not survive beyond day 7 to DSS induced colitis (Figure 4.1A). Therefore, in the current study, AhR -/+ and WT mice were analyzed. There was circa 50% reduction in AhR mRNA expression in colonic tissue of AhR -/+ mice that was maintained during colitis (Figure 4.1B). Before DSS administration, there were no differences in body weight among groups. On day ten, WT mice lost significantly more weight (Figure 4.1C. p=0.001), had a significant drop in the hematocrit (Figure 4.1D, p=0.002), and the postmortem
exam revealed swollen and shortened colons compared to AhR \(^{-/-}\) mice exposed to DSS (Figure 4.1E, \(p=0.01\)). Microscopically, colitis was mild in the AhR \(^{-/-}\) group as compared with significant mucosal ulceration, crypt abscesses and altered architecture present in WT mice and reflected in the histology score (Figure 4.1F, \(p=0.001\)), and H&E stained colon sections (Figure 4.2A).

Furthermore, Proliferating cell nuclear antigen (PCNA) staining revealed a significant decrease in crypt proliferative activity in WT group as compared to the AhR \(^{-/-}\) group (Figure 4.2B), consistent with defective epithelial turnover. Importantly, there were no significant differences between AhR \(^{-/-}\) with and without colitis and the control water fed WT mice.

**AhR \(-/-\) genotype favors Th2 over Th1 type cytokine expression**

The cytokine milieu within the gut defines the outcome of both innate and acquired immune cells activation. We investigated the expression of classical Th1/Th2 cytokines previously documented in mice with DSS induced colitis and IBD patients (27). Wild type mice exposed to DSS developed colitis that was characterized by increased expression of TNF\(\alpha\) gene and protein level (Figure 4.3A, 3B, \(p=0.001\)). Importantly, AhR \(^{-/-}\) mice had significantly lower baseline TNF\(\alpha\) gene (Figure 4.3A) and protein level (Figure 4.3B) expression as compared to that in wild type mice and did not change during colitis. Intestinal macrophages play a central role in the immune response to commensal flora and become a source of Th1 cytokines in IBD. (28) The mRNA gene expression of the macrophage marker F4/80 (Figure 4.3C, \(p=0.018\)) was downregulated in AhR
mice treated with water or DSS. The immunostaining of gut sections from AhR
mice with colitis showed a significant decrease in macrophage infiltration
(Macrophage-restricted protein-F4/80 - green, Figure 4.7), consistent with a
dampened innate immune response as compared with WT mice with colitis.
Although the monocyte chemoattractant protein (MCP1) expression increased
during colitis in AhR −/+ mice, this was significantly lower compared to wild type
mice (Figure 4.3D, p=0.005). Baseline expression of IL10 within the colon was
similar in control AhR −/+ and WT mice. Nevertheless, during DSS colitis there was
significant upregulation of this anti-inflammatory cytokine only in AhR −/+ mice
(Figure 4.3E, 4.3F, p=0.005). In addition, we observed a more prominent
induction of anti-inflammatory Secretory leukocyte protease inhibitor (SLPI) in
AhR −/+ mice (suppl. Fig 10, p=0.0001) as compared to that in the WT mice
during DSS induced colitis. SLPI is mainly expressed in the colon by the
epithelial cells and is a potent inhibitor of Th1 like cytokines. 174

Th17 to Treg shift occurs during DSS-induced colitis in AhR −/+ mice

Aside from the classical Th1/Th2 paradigm, we further investigated the
expression of master regulators for Th17 and Treg cells during colitis. Under
basal conditions there were no differences between the AhR −/+ mice and WT
(Figure 4.4A-D). During DSS colitis there was a significant up regulation of Treg
specific transcription factor FOXp3 gene expression in AhR −/+ mice as
compared to that in the WT mice (Figure 4.4A, p=0.004). In contrast, the mRNA
expression of Th17 specific transcription factor, RORγ was significantly
downregulated in AhR −/+ mice relative to the WT mice (Figure 4.4B, p=0.021).
Consistent with the latter finding we observed a significant attenuation of IL17 gene expression and protein level in AhR $^{-/-}$ mice (Figure 4.4C-D).

Pro-inflammatory adipokines are decreased in AhR $^{-/-}$ mice during DSS colitis
Mesenteric adipose tissue can become a source of inflammatory adipokines like angiotensin and osteopontin. Thus, during postmortem collection of the colons, we carefully removed the adjacent fat tissue. Nevertheless, these mediators can also be expressed in gut epithelia and lamina propria immune cells. (29-31) We confirmed that both AhR $^{-/-}$ and WT C57BL/6 mice express components of the angiotensin system (Figure 4.5 A-E) as well as osteopontin (Figure 4.5 D) within the colon. Pharmacological blockade of renin-angiotensin system has been shown to decrease the severity of colitis in several mouse models. (30, 32) We show that AhR $^{-/-}$ mice treated with DSS presented a significant downregulation of the only known precursor of angiotensin I/II - angiotensinogen (Figure 4.5A, p=0.0001), as well as the angiotensin converting enzyme ACE (Figure 4.5B, p=0.0048), and angiotensin receptor AT1 (Figure 4.5C, p=0.003) gene expression compared to the WT group. Importantly, the AhR $^{-/-}$ mice presented significant less AT1 mRNA gene expression under basal conditions (Figure 4.5C, p=0.023). Compared to WT mice with colitis, the immunohistochemistry of colon sections of AhR $^{-/-}$ mice with colitis revealed decreased angiotensin II, AT1 receptor (Figure 4.5E) and ACE expression (suppl. Figure 4.11).

Adiponectin, the anti-inflammatory adipokine is increased in AhR $^{-/-}$ mice during
DSS induced colitis

Adiponectin, the only known anti-inflammatory adipokine, is considered an exclusive product of adipose tissue. Here, we show that adiponectin gene is also expressed in the colon (Figure 4.6A). Next, we asked whether induction of colitis impairs adiponectin expression, and/or promotes a state of adiponectin resistance by down regulating its receptors. All the AhR \(^{-/+}\) mice presented a higher basal adiponectin mRNA gene expression that did not change during treatment. Importantly, adiponectin mRNA gene expression dropped significantly in the wild type mice during colitis (Figure 4.6A, p=0.0006). Consequently, adiponectin receptors (AdipoQ R1 and R2) were downregulated during DSS colitis in all groups (Figure 4.6 C, D). Interestingly, T-Cadherin mRNA gene expression, a novel adiponectin receptor that serves to anchor adiponectin to cell surface \(^{175}\), was upregulated only in the AhR \(^{-/+}\) mice and not in the WT mice with colitis (Figure 4.5B, p=0.037). Moreover, AhR \(^{-/+}\) mice exposed to DSS had a lower expression of Protein of 44 kDa (Erp44) that inhibits the secretion of adiponectin oligomers from the endoplasmic reticulum (ER). In addition, the ER oxidoreductase 1-Lα (Ero1-Lα), an ER chaperone that releases adiponectin trapped by Erp44 had similar expression in all groups. (Figure 4.6D-E, p=0.002). Therefore, by modulating the ratio of these ER chaperones the attenuated AhR signaling pathway favors the secretion of the anti-inflammatory adiponectin during experimentally induced colitis.

*The AhR+/- mice with DSS induced colitis have less endoplasmic reticulum (ER)*
stress response.

The development of colitis during DSS administration is associated with high cellular stress due to accumulation of misfolded proteins within the colonic epithelium. ER binding protein (BIP/Grp78) acts as an intracellular sensor and is considered a marker of ER stress. X-Box Binding Protein 1 (XBP1) is a transcription factor induced by ER stress that exerts a protective role and thus allows cells to recover. In our study we observed a significant downregulation of ER stress markers BIP (p=0.018) and XBP1 (p=0.002) in the AhR⁻/⁺ mice exposed to DSS as compared to the WT mice with colitis (Suppl. Figure 9). Since ER stress response is coupled to the cell death program, we investigated the mRNA expression of two pro-apoptotic molecules: C/EBP Homologous Protein (CHOP) and Caspase 12 (Casp12). There was a significant downregulation of the expression of both pro-apoptotic genes only in the AhR⁻/⁺ mice exposed to DSS (Suppl. Figure 4.9).

AhR pathway is activated in patients with Inflammatory Bowe Diseases.

In this study we have shown that AhR pathway can modulate the inflammatory response during experimental colitis. Then, using immunohistochemistry we investigated the pattern of AhR expression in healthy controls and patients with Crohn’s Disease. In human subjects without IBD, AhR expression was confined to the epithelial layer (Figure 4.8C), whereas in Crohn’s Disease there was a significant influx of AhR⁺ cells in the lamina propria compartment (Figure 4.8D) corresponding to pro-inflammatory hematopoietic cells. AhR pathway activation
was assessed by measuring the expression of its downstream target, CYP1A1. There was negligible expression in healthy controls while a 19.9 fold upregulation was noted in IBD patients (Figure 4.8A). IL-8 is a chemokine, which promotes neutrophil recruitment in patients with IBD. Human IL-8 promoter contains xenobiotic responsive elements (XRE) and thus can be upregulated by AhR signaling. Similar to the prototypical AhR target CYP1A1, IL-8 expression was upregulated 10 fold in patients with Crohn’s Disease (Figure 4.8B).

Discussion

Although the precise pathogenesis of IBD still needs to be unraveled, recent studies have reinforced the strong association between smoking, disease severity, complications and resistance to treatment. Mahid et al. found a greater prevalence of IBD patients in Kentucky, a state that ranks second in the nation in tobacco production and first in its use. Mainstream cigarette smoke contains high level of dioxins and dioxin-like chemicals that are known to be strong inducers of AhR. In addition to their prolonged half-life, these ubiquitous contaminants are highly lipophilic and accumulate in the adipose tissue, possibly leading to a prolonged activation of the AhR signaling pathway.

Persistent activation of the AhR has been extensively researched using TCDD, a high affinity AhR agonist. Administration of TCDD to laboratory animals induces inflammation by promoting tissue migration of immune cells.
185, and an increase in pro-inflammatory cytokine expression. 186-187 Surprisingly, macrophages isolated from mice lacking the AhR produce higher amounts of the inflammatory cytokines IL-1, IL-6 and TNFα in response to LPS. 188-189 Earlier studies indicated that AhR null mice develop colitis and rectal prolapse and have a propensity to develop colon cancer. 190 The inferred conclusion from the data obtained from either the ligand-activated AhR cell lineages or AhR null mice clearly states the physiological importance of this receptor in cell growth 191-192, cell apoptosis 193, elaborate cross-talk with NF-kB 194-195, and ER stress response. 196

To explore the association between the AhR pathway and IBD pathogenesis, we induced colitis in AhR −/−, AhR −/+ , and AhR +/+ mice. We found that mice lacking the AhR succumbed early to inflammation while the WT developed severe colitis. In comparison, the AhR heterozygous mice had a good clinical outcome during DSS administration and presented little structural changes in intestinal mucosa architecture.

Macrophages play an important role in the innate immune response to bacteria. In patients with Crohn’s disease, there is an influx of CD14+ macrophages within the gut, which become a rich source of TNFα. 197 Blockade of this proinflammatory cytokine induces disease remission in patients with IBD and in experimental models of colitis. 198-199 We found that AhR −/+ mice express significantly lower levels of TNFα as compared to that observed in the WT groups under both, disease-free conditions and during experimentally induced colitis. Moreover, the expression of the Macrophage-restricted protein F4/80 was
decreased in the colon of AhR −/+ mice during colitis. It is increasingly acknowledged that the AhR pathway modulates a number of immune responses. While the specific AhR-induced mechanisms that underlie its effects on the immune system are poorly understood, numerous genes activated during the immune response have been found to contain DNA recognition sites for the AhR/ARNT heterodimers. Furthermore, the AhR pathway has been shown to play a significant role in the development of both Th17, and Treg cells. The pathogenic role of Th17 cells as well as defective function of T-regulatory cells has been found in patients with Crohn’s disease and animal models of IBD. In the current study, we show that there is significant downregulation of the Th17 lineage master regulator, RORγ, with a corresponding upregulation of Treg transcription factor Foxp3 in the colon of AhR −/+ mice during colitis. Consistent with these changes, we have also noted a significant decrease in the IL-17 expression and a corresponding increase in IL-10 expression. Thus, the attenuation of the AhR signaling pathway correlates with the outcome of experimental colitis and the Th17 / Treg balance in the colon.

The adipokines and cytokines secreted by adipose tissue have been increasingly recognized as bona-fide immune regulators. Angiotensin, a proinflammatory adipokine is generated from angiotensinogen through the proteolytic activity of ACE and tissue chymases. AT1a is the main receptor that mediates the pro-inflammatory actions of angiotensin. Intestinal mucosa is a rich source of ACE, while macrophages express the full renin-angiotensin system. Increased ACE was reported in Crohn’s disease patients, while Ace/AT1
blockade protects mice from experimental colitis. In our study, AhR wild type mice that developed colitis had high expression of the angiotensin system components. Furthermore, there was a significant increase in lamina propria cells expressing both angiotensin and AT1a. We have previously shown that AhR agonists induce expression of angiotensinogen and proinflammatory cytokines in cultured adipocytes. Our current finding that AhR \(^{+/+}\) mice fail to upregulate angiotensin system during DSS colitis further implicate this system in AhR-mediated inflammation.

Adiponectin, the primary adipokine with anti-inflammatory activity was downregulated in the wild type AhR mice that developed severe colitis, while the opposite was found in AhR \(^{+/+}\) group. We have previously demonstrated that AhR activation downregulates the expression of this adipokine in fat cells. In the current study, we report that attenuated AhR expression/activity in the AhR \(^{+/+}\) mice, correlates with increased colonic adiponectin and its T-Cadherin receptor expression during DSS treatment. Moreover, our study indicates that AhR modulates the expression of ER chaperone proteins that regulate adiponectin secretion. These correlations might be very important since adiponectin has a protective role in experimental colitis as recently shown by the Nishihara group. Furthermore, it was demonstrated that adiponectin treatment protects adiponectin KO mice from developing DSS-induced colitis. In addition, adiponectin has been shown to induce IL-10, and thus, promote alternative activation of macrophages and resolution of chronic inflammation. In our study,
the increased adiponectin in AhR$^{-/-}$ mice may have had an important role in maintaining tissue homeostasis during DSS induced colitis.

Recent studies have shown that an increased endoplasmic reticulum stress response in epithelial cells promotes colitis. Importantly, mutations in XBP1, a key component of this response, have been associated with Crohn’s disease. 126 In the current study, DSS-induced colitis resulted in an increase expression of ER stress markers in wild-type mice, while they were downregulated in the AhR$^{-/-}$ mice. Furthermore, increased IL-10 expression in the latter group may have also reduced the stress response associated with colitis. 206 It is also possible that the ER stress response during colitis facilitates AhR signaling and their reciprocal induction leads to the inflammatory response associated with DSS induced colitis. 207

The effect of AhR activation on T cells is ligand dependent. TCDD induces persistent activation of AhR in T$_{reg}$ cells. 94 On the other hand dietary derived, short acting ligands, such as FICZ (6-formylindolo, 3, 2-b-carbazole) induce Th17 differentiation. 208 The relative abundance of the different ligands, along with the AhR system polymorphisms may further modulate the response. 93,209 In our DSS model, AhR activation most likely occurred through dietary (i.e. FICZ) and endogenous ligands with similar kinetics, thus explaining the upregulation of IL-17 and RORγ. The increased expression of colonic macrophages in wild type mice compared to AhR$^{-/-}$ further supports the role of this receptor in colitis. Recent in-vitro studies in macrophages described an ARNT independent, non-genomic pathway downstream of AhR that induces an inflammatory response. 210
We hypothesize that low AhR expression may be coupled to the classical signal that downregulates macrophage function whereas increased receptor expression preferentially activate the non-genomic pathway and hence promotes inflammation.

Our studies in patients without IBD indicate that AhR is mainly expressed in the epithelial layer. Nevertheless, this translated in minimal activation of this pathway and likely fulfills a physiologic role such as cell cycle regulation and metabolism of diet derived xenobiotics. AhR is important for the development of Th17 cells and can upregulate human macrophage expression of IL-8. These activities are highly relevant for the ongoing intestinal inflammation of IBD patients. The robust AhR activation shown in our Crohn’s Disease patients was secondary to the influx of lamina propria AhR+ mononuclear cells, like macrophages and T cells. Consistent with this observation, we found high IL-8 expression that mirrored the AhR classical target, CYP1A1.

In summary, we provide novel evidence that dysregulated expression of the AhR alters the outcome of colitis. The extreme phenotypes of AhR null and wild types groups indicate that AhR pathway fulfills both tissue homeostatic and inflammatory roles. Furthermore, we show that AhR pathway activation distinguishes Crohn’s Disease patients from healthy controls. Modulation of this pathway through diet, cigarette smoking cessation, as well as pharmacological antagonism of the AhR could be viable strategies for the treatment of IBD.
Figure Legends

Figure 4.1. *The severity of DSS-induced colitis is attenuated in AhR \(^{-/-}\) mice.*
Mice were administered 3.5% DSS dissolved in water for 7 days followed by 3
days of water. Control mice received water alone for 10 days. (A) Kaplan-Meier
plot showing premature lethality of male AhR\(^{-/-}\) when compared with WT and
AhR \(^{-/+}\) littermates (B) AhR mRNA gene expression in the colonic tissue (C)
Body weight (D) Hematocrit (E) Colon length (F) Histopathological grading of
colonic inflammation at the end of the experiment. Data are expressed as mean
\(\pm\) SE, n=10/group.

Figure 4.2. *Decreased histological severity in AhR \(^{-/+}\) mice during DSS-induced
colitis.* (A) H&E-staining of colons from WT (upper panel) and AhR \(^{-/+}\) mice (lower
panel). (B) Immunohistochemistry for epithelial cell proliferative index (PCNA) in
sections of the colonic tissue of WT (upper panel) and AhR \(^{-/+}\) mice (lower panel).

Figure 4.3. *The expression of pro-inflammatory cytokines and macrophage
marker are reduced in AhR \(^{-/+}\) mice during colitis.* (A) TNF\(\alpha\) gene expression, (B)
TNF\(\alpha\) protein level, (C) F4/80 gene expression (D) MCP1 gene expression, (E)
IL10 gene expression and (F) IL10 protein level in colonic tissue. Data are
expressed as mean \(\pm\) SE, n=10 / group

Figure 4.4. *Differential expression of master regulators for Treg and Th17 cells
in the colon of WT and AhR \(^{-/+}\) mice.* Increased expression of (A) FoxP3 mRNA
gene expression and attenuated gene expression of (B) ROR\(\gamma\) and (C) IL17 with
a low level of (D) IL17 protein expression in AhR \(^{-/+}\) mice treated with DSS. Data
are expressed as mean \(\pm\) SE, n=10/group.
Figure 4.5. *Proinflammatory adipokines are decreased in AhR^{-/+} mice treated with DSS.* The pro-inflammatory adipokines (A) Angiotensinogen (B) ACE, (C) AT1a receptor, (D) Osteopontin gene expression were significantly lower in AhR heterozygous mice during colitis. Data are expressed as mean ± SE, n=10/group. (E) Fluorescence immunohistochemistry for angiotensin II (green) and AT1aR (red) in AhR^{-/+} and WT mice treated with DSS. (a), (b) show dual staining.

Figure 4.6. *Adiponectin is negatively regulated during colitis only in WT mice and not in the AhR^{-/+} mice.* (A) Adiponectin gene expression in WT mice treated with DSS was significantly lower compared to AhR^{-/+} mice. (B) Decreased T-Cadherin receptor expression in WT mice with colitis. (C) and (D) Decreased adiponectin receptors in the context of low adiponectin expression may further impair this adipokine signaling pathway. (E) Increased gene expression of chaperone protein ERP_{44} relative to its partner, endoplasmic reticulum oxidoreductin (ERO) can block the secretion of adiponectin in WT mice with colitis. Data are expressed as mean ± SE, n=10/group.

Figure 4.7. *Reduced macrophage recruitment during DSS colitis in the AhR^{-/+} mice.* Fluorescent immunohistochemistry showing macrophage-restricted protein-F4/80 expression in colonic tissue of AhR^{-/+} mice with colitis. (A) Macrophage-restricted protein-F4/80 (green) in control groups, (B) Macrophage-restricted protein-F4/80 (green) in DSS-treated mice groups.

Figure 4.8. *AhR activation in patients with IBD.* (A) CYP1A1 mRNA gene expression level is increased in IBD patients compared with control. (B) IL8 mRNA gene expression increases during the inflammatory process in IBD.
patients. Control n=15, IBD n=25. Data are expressed as median. (C, D)

Fluorescent Immunohistochemistry staining of the AhR in a control patient (C) versus a patient with Crohn’s disease (D). Representative images are shown of biopsies obtained from a control subject (C) and from a patient with Crohn’s disease (D). Red staining indicates binding of the antibody to human AhR. All samples were counterstained with DAPI (blue) to visualize nuclei. Magnification is 20X. E - Enterocytes and LP- Lamina Propria.

Supplemental Figure 4. 9. **Epithelial cellular stress is decreased in AhR −/+ mice exposed to DSS.** Decreased mRNA gene expression of endoplasmic reticulum stress response proteins (BIP, XBP1) and related pro-apoptotic factors (CHOP, Casp12). Data are expressed as mean ± SE, n=10/ group

Supplemental Figure 4.10. **Decreased expression of Secretory leukoprotease inhibitor (SLPi) in WT compared to AhR −/+ mice exposed to DSS, consistent with reduced inflammation in the AhR heterozygote mice during colitis.** mRNA gene expression in colonic tissue. Data are expressed as mean ± SE, n=10/group.

Supplemental Figure 11. **Decreased renin angiotensin system (RAS) components in AhR −/+ mice correlates with a better outcome of DSS-induced colitis in AhR heterozygote mice.** Fluorescent immunohistochemistry shows expression of ACE (red – upper panels), Angiotensin (green – lower panels) and their co-localization (middle panels) in the colonic tissues of mice treated with water (left panels) compared with DSS treatment (right panels).
Figure 4.1 The severity of DSS-induced colitis is attenuated in AhR⁻/⁺ mice
Figure 4.2 Decreased histological severity in AhR\textsuperscript{−/+} mice during DSS-induced colitis
Figure 4.3 The expression of pro-inflammatory cytokines and macrophage marker are reduced in AhR−/+ mice during colitis
Figure 4.4 Differential expression of master regulators for Treg and Th17 cells in the colon of WT and AhR – /+ mice
Figure 4.5 Proinflammatory adipokines are decreased in AhR^{-/+} mice treated with DSS
Figure 4.6 Adiponectin is negatively regulated during colitis only in WT mice and not in the AhR $^{-/}$ mice
Figure 4.7 Reduced macrophage recruitment during DSS colitis in the AhR $^{-/+}$ mice
Figure 4.8 AhR activation in patients with IBD
Supplemental Figure 4.9: Epithelial cellular stress is decreased in AhR−/+ mice exposed to DSS.
Supplemental Figure 4.10 Decreased expression of Secretory leukoprotease inhibitor (SLPI) in WT compared to AhR – /+ mice exposed to DSS, consistent with reduced inflammation in the AhR heterozygote mice during colitis
Supplemental Figure 4.11 Decreased renin angiotensin system (RAS) components in AhR – /+ mice correlates with a better outcome of DSS-induced colitis in AhR heterozygote mice
Chapter 5.

Conclusions:

Inflammatory Bowel Diseases are heterogeneous chronic relapsing conditions divided into two main phenotypes: Crohn's Disease and Ulcerative colitis. Each category is further sub-typed according to established clinical criteria that take in consideration disease location, age at diagnosis and specific complications. Nevertheless, this approach does not offer insight into the immune mechanisms that contribute to a unique phenotype. Moreover, it assumes that Inflammatory Bowel Diseases are a continuum development of a common pathologic process and expected to yield similar results to treatment.

Evidence from experimental models of Inflammatory Bowel Diseases is somewhat deceptive since knockouts of specific proinflammatory cytokines appear to cure the disease in spite of the clinical results in patients. A classic example is tumor necrosis factor alpha (TNFα). TNFα-KO mice are resistant to TNBS-hapten induced colitis and overexpression of a TNFα transgene results in severe colitis. Clinical trials in IBD patients indicate that only 30% of patients on anti-TNF therapy achieve long-term remission. We have investigated the mRNA expression of TNFα in mucosal biopsies from healthy volunteers and patients with Inflammatory Bowel Diseases. In both groups, we noted a significant variation in expression and concluded that alone, this cytokine will not be informative. A similar trend was noted for other molecular targets relevant to the mucosal immune system. Genetic variations, unique microflora as well as redundant inflammatory pathways may explain the heterogeneity. On the other
hand, the concept of "physiologic gut inflammation" allows for cytokines such as TNFα to play both protective and pathologic roles, and its relative expression less significant. Given the focused approach of current biologic therapies in IBD, it is important to determine the immune phenotype of these patients. Instead of pursuing isolated molecular targets, we investigated patterns of expression, for multiple factors, relevant to the innate immune function of the gut. Principal component analysis of the relative expression of RelA, A20, plgR, TNF, and IL-8 in IBD (Crohn's type) allowed us to stratify patients into three immune phenotypes. Furthermore, they were correlated with treatment outcome and provided a "molecular signature" for Crohn's Disease. RelA, A20, plgR, TNF mainly defined the first component of this statistical analysis (PC1), while the second one, (PC2) was weighted toward TNF, and IL-8. A high PC1 score and low PC2 characterized healthy volunteers and Crohn's Disease patients with very mild disease. This suggests that RelA, A20, and plgR fulfill a protective role in the gut mucosa while TNFα and IL-8 are mainly pathogenic. Although NF-kB regulates the production of inflammatory cytokines, recent studies confirmed its importance for the integrity of epithelial barrier. Absence of ubiquitin-modifying enzyme A20 leads to excessive NF-kB stimulation downstream of TNFα and TLR signaling. Thus, it provides a negative feedback, and likely contributes to the "physiologic inflammation". Another important intestinal epithelial NF-kB target is plgR. Its baso-apical traffic ensures the continuous IgA transport in the gut lumen and thus contributes to the mucosal immune homeostasis. The subgroup of patients with the lowest plgR expression (lowest PC1) had almost absent IgA
transport. Interestingly the IgA was deposited in the lamina propria similar to patients with IgA nephropathy. They had higher than normal serum IgA as well. One explanation for the latter finding is that increased lamina propria IgA is taken up into the circulation. On the other hand, low pIgR expression may lower the epithelial defensive capacity and allow bacterial translocation, which in turn stimulates the IgA response to luminal bacteria. This conclusion is supported by evidence of barrier defects in pIgR knockout mice\textsuperscript{23} as well as increased circulating antibodies to luminal bacteria in IBD patients\textsuperscript{215-216}. Interestingly these patients had low NF-κB, TNFα and IL8 expression consistent with an immunodeficient status in the absence of immunomodulatory drugs. Clinically these patients were refractory to medical therapy. Since their previous treatment, regimens were largely immunosuppressive we can argue that this group would be candidate for immunostimulatory regimens such as GM-CSF. Our findings are in agreement with prior evidence of innate immune deficiency in Crohn's Disease patients\textsuperscript{217-218}. Similar to our patients they had a blunted IL8 response. The most common genetic mutation in CD patients involves NOD2 gene. Current studies link this intracellular bacterial sensor to protective NF-κB signaling in gut epithelium as well as mucosal defensin production by Paneth cells\textsuperscript{219}. Nevertheless, we did not find an association with our patient subsets. Crohn's Disease patients with high C reactive protein levels tend to be more responsive to anti-TNF therapy\textsuperscript{220}. Our subset of patients with high PC2 scores (higher TNF, IL8) matched these characteristics. They tended to be earlier in the disease process as well. It should be noted that TNFα and IL8 expression was elevated in
areas unaffected by disease and were not simply markers of acute inflammation. The identification of signature biomarkers of Crohn's Disease has several implications: 1) subset 1 (↑PC1↓PC2) may not require top-down therapy and/or do well with drug holidays; 2) subset 2 (↓PC1↑PC2) should be the object of immunostimulatory therapies and definitely avoid combinations of immunosuppressives; 3) subset 3 (↑PC2) can benefit early top-down, combination therapy. Future prospective studies will investigate whether early top-down therapy could switch subset 3 to subset 1 since the latter group is more likely to maintain remission in the absence of medical treatment. Furthermore, principal analysis of these signature biomarkers should be evaluated in various existing models of IBD and provide immune-phenotype specific insight for various potential treatments. In light of association between oral and intestinal inflammation, we are investigating the usefulness of oral sampling in lieu of the more invasive gut biopsies.

In light of our findings we propose that activation of downstream of extra and intracellular microbial sensors induces/activates NF-kB and its target genes plgR, IL8 and TNFα. Activation of NF-kB is limited by A20 mediated inhibition and thus prevents ongoing inflammation. Increased plgR expression assures adequate IgA transport with an important role in tolerance toward commensals and possibly neutralization of the proinflammatory chemokine IL8. The severe IBD phenotype observed in the subset of patient with low plgR expression reflects an immune deficiency of the gut epithelium. These patients have low NF-kB relA levels, which may theoretically be linked to ER stress response and
defective autophagy mechanisms. Ongoing anti-TNF treatment of these patients may aggravate the immune barrier dysfunction and favor a Th17 response to invading bacteria.

Crohn’s disease patients develop transmural inflammation of the gastrointestinal tract, which may lead to complications such as strictures and perforations. Often times, there is significant hypertrophy and inflammation within the surrounding mesenteric fat. Recent advances in metabolic and cardiovascular diseases have led to a paradigm shift in which adipose tissue has been upgraded from an energy depot to a source of immunomodulatory cytokines (adipokines). In his original description of the disease, Burril Crohn described the characteristic mesenteric fat change. Although widely recognized, the relevance of this fat hypertrophy, referred to as “creeping fat”, is unknown, and has received little research attention. Accumulation of mesenteric fat, appears to be specific for Crohn’s disease, and occurs from the onset of disease. Fat wrapping has been defined as fat hypertrophy extending from the mesenteric attachment with > 50% coverage of the intestinal surface. It occurs in both the small and large bowel, and correlates with transmural inflammation, ulceration, stricture formation, increased mesenteric wall thickness, and decreased internal bowel diameter. Fat wrapping and mural thickening is associated with mucosal ulceration in 86% of patients and with strictures in 46%. Importantly it was noted in 100% of patients undergoing resection, and correlated with the degree of acute and chronic inflammation. Adiponectin, a fat derived hormone/cytokine is involved in cell energy regulation, glucose
metabolism and modulation of immune responses. We evaluated the role of adiponectin in a DSS model of colitis. Adiponectin or LacZ (control gene) was overexpressed using an adenoviral construct. The adiponectin treatment group has a significantly better outcome as assessed by clinical and histologic criteria. We chose an innate immunity mouse model of IBD since adiponectin has structural and functional similarities to complement. The first evidence for a protective role came from the Nishihara group. They demonstrated that adiponectin treatment protects adiponectin KO mice from DSS colitis. Interestingly no benefit was noted in TNBS colitis. Since the latter model is more dependent on T cells, one may speculate that adiponectin has greater influence on innate immunity. This is also supported by the fact that T cells from either wild type of adiponectin KO mice produce similar amounts of IL-6, IL-17, TNF-α and IFNγ upon stimulation with anti CD3/CD28. In vitro studies revealed that adiponectin suppressed expression of IL-8 in the human HT-29 epithelial cell line. Surprisingly, others found that the adiponectin promotes and potentiates IL-8 in the same cell line. Nevertheless this activity was elicited by globular adiponectin at doses between 1 and 5μg/ml. Adiponectin circulates mostly as HMW at similar concentrations and it is unlikely that globular form will be generated in vivo at the same levels. We prospectively evaluated adiponectin levels in two cohorts of Crohn’s Disease patients and healthy volunteers respectively. There was no difference between total adiponectin but we detected significantly lower values in the HMW fraction. The differences between controls and IBD patients were not related to common adiponectin SNP
mutations. Nevertheless, when Crohn’s Disease patients were analyzed, a third of those with A>G 11426 in the promoter region had fistulizing or stricturing disease while less than 15% had this phenotype in the absence of mutation. This mutation lies within a PPARγ response element and may be responsible for the induced rather than the constitutive adiponectin level. In addition, inflammatory cytokines and cellular stress can modulate HMW secretion\textsuperscript{225}. Based on our studies and prior evidence we believe that TNFα, angiotensin and osteopontin play an important role. Altered receptor expression can further modulate the effects of adiponectin. Overall, no significant changes in adiponectin receptors were seen in our mouse model. This may be because we analyzed the entire colonic specimen rather specific cell fraction. Using in-vitro bone marrow derived dendritic cells, we determined that LPS downregulated AdipoR1 and AdipoR2. This may represent a state of adiponectin resistance that can be reversed by PPARγ and retinoic acid agonists. We also correlated serum HMW adiponectin and colonic adiponectin receptors expression with specific IBD subgroups. Crohn’s Disease patients in subsets 2 (↓PC1↓PC2) and 3 (↑PC2) had lower circulating HMW adiponectin and intestinal receptor mRNA expression.

In sharp contrast to our findings, Fantuzzi et al, showed that adiponectin KO mice were protected from DSS and TNBS colitis, and adiponectin treatment induces inflammation\textsuperscript{41}. In addition, colonic explants from treated mice released more IL-6. Differences in adiponectin KO mice have been invoked since only the former group showed high basal TNF-α production and increased susceptibility to LPS. Several recent studies confirm that adiponectin effectively blocks LPS
induced release of pro-inflammatory cytokines and thus support an anti-inflammatory role\textsuperscript{138,226-228}. Moreover, when the latter group investigated the outcome of colitis in IL-10 KO and adiponectin/IL-10 double KO no differences were seen\textsuperscript{222}. It was concluded that adiponectin does not play a role in this colitis model. In accordance with our preliminary experiments, and published data\textsuperscript{134,229} we believe that IL-10 production is paramount for the anti-inflammatory role of adiponectin in colitis. Therefore, the results seen in IL-10 KO mice are expected. Furthermore, if adiponectin promotes inflammation the double KO (adiponectin/IL-10) mice should have had increased morbidity and mortality. Adiponectin induced IL-10 may thus promote alternative activation of macrophages and promote resolution of chronic inflammation. Adipose tissue macrophages from lean mice express many genes characteristic of M2 (alternatively activated phenotype) macrophages\textsuperscript{230}, including Ym1, arginase 1, and IL-10. Diet-induced obesity decreases expression of these genes while increasing expression of those encoding TNF-\(\alpha\) and Nos2 (nitric oxide synthase) that are characteristic of M1 (classically activated) macrophages\textsuperscript{231}. Furthermore, AMPK, the kinase that translates adiponectin signaling, suppresses proinflammatory responses and promotes macrophage polarization to an anti-inflammatory, M2 functional phenotype \textsuperscript{232}. Our findings in the DSS model of colitis were consistent with reduced gut macrophage infiltration and increased markers of M2 polarization. The in vivo events leading to IL10 secretion are not established. Upregulation of adiponectin receptors on antigen presenting cells, as seen in our in vitro study, or increased phagocytosis of apoptotic bodies
through specific interactions between adiponectin and thrombospondin are attractive hypotheses. Osmotin, a plant-derived adiponectin receptor agonist, ameliorated DSS colitis and promoted IL10 secretion. Since Osmotin is structurally different, a direct effect rather than interaction with other inflammatory mediators is suggested. Angiotensin acts as a pro-inflammatory cytokine and promotes development of colitis. Both gut epithelium and surrounding mesenteric fat can convert angiotensinogen to active angiotensin. We have consistently seen a negative correlation between angiotensin/angiotensin receptor 1 and adiponectin levels. Thus, modulation of fat-derived adipokines by adiponectin contributes to the anti-inflammatory milieu during colitis.

Environmental factors play an important role in Inflammatory Bowel Diseases. The strongest association has been seen with cigarette smoke exposure. Cigarette smoke contains a mixture of dioxin-like compounds, which activate the aryl hydrocarbon receptor (AhR) pathway. In its inactive state, the AHR exists as a multiprotein complex. Following agonist binding, the AHR translocates to the nucleus, dimerizes with its DNA binding partner ARNT and binds specific DNA sequences (DREs). AHR activation by endogenous agonist(s) is likely transient and physiological. Activation by synthetic long-lived exogenous agonists, as TCDD is inappropriately sustained and hence, pathophysiological. Candidate endogenous AHR agonists include indole and tryptophan metabolites. Indole is produced from tryptophan by the commensal bacteria and inhibits NF-κB signaling, tight-junction resistance, and expression of inflammatory cytokines in intestinal cells. We have found that
AHR expression appears to be largely restricted to enterocytes in human patients. Thus, we speculate that the normal, physiological role of the AHR within the intestine is to sense the presence of the bacterially generated AHR agonists and regulate physiological inflammatory responses. Our experiments in AhR \(^{-/-}\), heterozygote and wild type mice yielded different phenotypes\(^{240}\). The AhR heterozygote was protected from colonic inflammation. Both AhR \(^{-/-}\) and wild type mice had severe colitis but the former exhibited increased mortality. We proposed that complete absence of AhR may alter gut permeability and promote inflammation. The results in AhR \(^{-/-}\) were consistent with observations that these mice have a heightened response to LPS stimulation. In comparison to wild type mice, the heterozygote mice had a shift toward the Treg phenotype. Specifically, we observed an increased intestinal FoxP3 expression at the expense of ROR\(\gamma\). Thus, AhR ligands (endogenous and dietary) can alter the Treg/Th17 balance\(^{241}\). Consistent with this polarization we noted increased IL10 production and decreased IL17 and TNF\(\alpha\) in the colon of AhR heterozygote mice. Furthermore these mice had lower levels of MCP-1 and colonic macrophages. The observed effects were related to the AhR level of expression. Similar conclusions can be draw from our analysis of Crohn’s Disease and healthy volunteers. We have assessed AhR activation by determining the expression of its nuclear target CypA1. In normal subjects, there was minimal AhR activation restricted to the epithelial layer. This likely reflects the AhR role in gut permeability. Treatment of the human epithelial colonic line CaCO2 with an AhR antagonist significantly decreased the transepithelial electrical resistance while the long acting agonist,
TCDD restored it. Analysis of intestinal biopsies from IBD-Crohn’s Disease patients have showed decreased epithelial expression and significant upregulation in lamina propria mononuclear cells. The upregulation of AhR expression correlated with an AhR responsive inflammatory cytokine, IL8. Although CypA1 was used as a read-out of AhR activation, interactions with NF-KB (RelA and RelB) through a non-canonical pathway are likely important. It is generally accepted that AhR and NF-kB negatively regulate each other. Therefore it is conceivable that increased epithelial AhR expression reduces NF-kB and thus alters gut permeability. Since AhR \(-/-\), mice have developmental defects we explored the effects of pharmacological blockade on DSS colitis in wild type mice. A similar outcome was noted. On the other hand, TCDD, a long acting agonist protects mice from DSS colitis. Preliminary studies in our laboratory confirmed these findings in mild (1.5% DSS) but not severe forms of colitis (3.5% DSS).

We have previously shown that AhR agonists (TCDD, PCB77) promote obesity and adipose tissue inflammation. These lipophilic compounds are widely distributed in the environment, and preferentially accumulate into the visceral fat. Furthermore, we showed that PCB77 treatment promotes perivascular inflammation and intraabdominal ectopic fat deposition in a mouse model of aortic aneurysm (manuscript submitted). Therefore, we investigated the profile of anti-inflammatory (adiponectin) and pro-inflammatory (angiotensin) during DSS colitis in mice with low (AhR\(^{-/-}\)) and normal AhR expression. AhR heterozygote mice (AhR\(^{+/-}\)) exhibited a significant downregulation of angiotensin
precursor, angiotensin receptor AT1a and angiotensin converting enzyme (ACE) correlated with a positive clinical outcome. Furthermore, serum and tissue levels of adiponectin were elevated. The link between AhR, adipokines, inflammation and obesity is of high relevance since an important number of IBD patients are either obese or at risk of metabolic syndrome. Recent associations between IBD (Crohn's Disease and Ulcerative Colitis) and BMI (body mass index) could reflect the effect of AhR on disease phenotype 36,242.

In summary, we provide evidence of unique immune phenotypes in IBD with relevance for clinical practice. Furthermore, the signature biomarkers for Crohn's Disease may serve as a guide to patient selection in clinical trials and development of relevant animal models. Our current studies link adipose tissue derived cytokines with the xenobiotic pathway, and offers a framework to further evaluate the role of environmental cues in IBD development. We propose a disease model whereby genetic and environmental factors (AhR ligands) alter gut barrier by acting on targets such as NF-kB and pIgR. This leads to bacterial translocation with specific patterns of inflammation related to the patient's immune phenotype (biomarker subset). Increased visceral adiposity (diet, AhR mediated) and accumulation of lipophilic AhR ligands induces a second wave of inflammatory mediators. Low adiponectin and activation of the renin angiotensin system within the gut and surrounding adipose tissue are expected to promote an inflammatory milieu. Ultimately, these events can promote M1 macrophage and Th1/Th17 polarization responsible for the chronic relapsing course of IBD.
Future Directions

Investigation of NF-kB related targets relevant for innate mucosal immunity, allowed us to identify the plgR/IgA system as a novel pathway in patients with Inflammatory Bowel Diseases. More importantly, it points to the fact that a pathway approach is likely to yield results in the quest for disease biomarkers. The current five-biomarker set will be expanded to include adipokines, adipokine receptors and components of the aryl hydrocarbon pathway. Selection of specific targets will be guided by in vitro studies. I am particularly interested in regulation of TLR signaling by adiponectin and downstream interactions with canonical and alternative NF-kB pathways. Ultimately, the utility of these biomarkers have to be tested in prospective clinical trials. Inflammatory Bowel Diseases naïve to immunomodulatory therapies will be randomized to treatment based on the biomarker subsets. The clinical response and mucosal healing will be assessed and correlated with subset allocation. Another important question that may be answered is whether these subsets are stable or they can be altered by successful immune therapies. These finding may have significant importance on early choice of treatments. Analysis of biomarker expression in the gut mucosa requires an invasive endoscopic procedure. On the other hand sampling of oral mucosa would be easily performed. Therefore, we would like to investigate the correlation of proposed gene targets in the gut and oral mucosa and thus offer a better modality of immune-phenotyping the IBD patients.

A significant number of IBD patients are overweight. Our results are in line
with studies that link adiponectin to innate immunity response and macrophage function. We propose that signaling downstream of adiponectin receptors alter the balance between M1 (classical) and M2 (alternative) macrophages. Targeted deletion of macrophage adiponectin receptors, and/or chimeric mouse models using macrophage transfer from receptor KO mice will be investigated in acute and chronic models of colitis. Adiponectin replacement is not considered a feasible treatment modality. Alternative options include: 1) synthetic or natural agonists; 2) increased receptor expression. We have discovered that the plant agonist Osmotin can have protective effects during colitis. Further studies will test its value in the context of impaired adiponectin activity: 1) selective adiponectin receptor deficiency; 2) adiponectin deficiency (KO mouse); 3) acquired adiponectin deficiency through diet induced obesity.

Although the role of environment in Inflammatory Bowel Diseases is widely accepted, until recently, no specific pathway was identified. Our study identified for the first time that the aryl hydrocarbon might play a role in the development of IBD. The main target of the AhR signaling in both human and experimental IBD has not been elucidated. We will investigate the consequences of AhR deletion in gut epithelial cells as immune cells. Moreover, T cell transfer from mouse donor exposed to individual AhR ligands (agonists and antagonists) as well as complex mixtures (cigarette smoke) will allow dissection of more "real-life" scenarios. We are hopeful that our research endeavors will create a better understanding of the link between environment, metabolism and immunity.

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