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The Role of Ceruloplasmin in Colitis

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THE ROLE OF CERULOPLASMIN IN COLITIS

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DEDICATION

To my parents and my brother for their steady love and support during my education. To my wife, Esen, and to my son, Mesud.
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Many thanks to my advisor Dr. Paul L. Fox for his guidance, patience, and understanding during my Ph.D. education. I thank my advisory committee Dr. Stanley L. Hazen, Dr. Xiaoxia Li, Dr. Vincent K. Tuohy, and Dr. Bibo Li for their valuable suggestions.

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THE ROLE OF CERULOPLASMIN IN COLITIS

BAKYZHAN BAKHAUTDIN

ABSTRACT

Ceruloplasmin (Cp) is an acute phase, plasma protein with multiple enzymatic activities consistent with both pro- and anti-inflammatory functions. Our laboratory has recently reported the presence of Cp in intestinal epithelial cells. To determine the potential role of Cp in intestinal inflammation, we induced colitis in Cp-deficient mice by continuous administration of dextran sodium sulfate (DSS) *ad libitum* in the drinking water. The Cp-null mice rapidly lost weight and all were moribund by day 14, while about 90% of the wild-type (WT) mice survived at least 20 days. Higher amounts of TNF-α, and neutrophil (KC) and macrophage (MCP-1) chemokines, were detected in colon culture supernatants in Cp-null mice compared to WT controls. Cp-null mice also exhibited excessive colonic bleeding after 5 days, which correlated with elevated white blood cells, neutrophils, and lymphocytes in the blood, and higher histopathology. Depletion of commensal microflora by antibiotic treatment demonstrated that excessive inflammation in Cp-null animals is microbiota-independent. Interestingly, microbiota-depleted Cp-null animals appear to have higher epithelial damage caused by DSS since they become moribund about 10 days earlier than WT controls. We have investigated the protective mechanism of Cp by injection of Cp into knockout mice. Unexpectedly, mimicking Cp secretion by liver and restoring blood Cp did not lessen the severity of inflammation. This finding was supported by the transplantation of Cp-null bone marrow (BM) into WT controls and vice versa; 90% of WT animals with Cp-null BM became moribund by day 14, whereas most of the KO mice with WT BM survived until day 20 of DSS administration. Since macrophages are the only source known to express Cp, we transferred WT macrophages to Cp-null animals all of which survived continuous DSS challenge. Our results demonstrate that Cp derived from macrophages contributes to the protection against DSS-induced damage and colonic inflammation.
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ABBREVIATIONS USED

BM, Bone Marrow

Cp, Ceruloplasmin

DSS, Dextran Sulfate Sodium

GPI, Glycosylphosphatidylinositol

HCT, Hematocrit

HGB, Hemoglobin

IBD, Inflammatory Bowel Disease

IHC, Immunohistochemistry

MPO, Myeloperoxidase

RBC, Red Blood Cell

TNF, Tumor Necrosis Factor

UC, Ulcerative Colitis

WBC, White Blood Cell
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PART A. CERULOPLASMIN

Ceruloplasmin (Cp) was first characterized by Holmberg and Laurell in 1947 as a copper-containing protein of the human plasma. Five years later Scheinberg & Gitlin discovered that Wilson's disease patients have low levels of plasma Cp and designed a biochemical test that is still in clinical use for this disease. The ferroxidase activity of Cp, implied to play an important role of iron homeostasis, was demonstrated in 1966 by a group led by Frieden. Since the determination of complete amino acid sequence and the single-chain nature of human Cp published by Takahashi et al. in 1984, the discoveries of its structure and function accelerated, and today we have the crystal structure and know that it is expressed by several tissues but mainly by the liver, that its ferroxidase activity makes it a potent antioxidant, and that it plays an important role in iron metabolism and erythropoiesis, and the genetic disorders associated with it.
Expression of Ceruloplasmin

With total length of about 65 kb, the human Cp gene has been mapped on chromosome 3q23-q24\textsuperscript{9,10}. It is composed of 20 exons that eventually yield a single polypeptide of 1046 amino acids\textsuperscript{11}. The expression of Cp is induced by inflammatory cytokines in response to inflammation, trauma, or infection\textsuperscript{12}. The main source of Cp is liver where hepatocytes synthesize Cp and subsequently secrete it into the circulation\textsuperscript{8}. Other sources of Cp include brain, lung, spleen, and testis\textsuperscript{13-15}. Interestingly, Cp is also expressed by macrophage and brain astrocytes\textsuperscript{16,17}. There are two known alternatively spliced forms of Cp, secreted and membrane-anchored. The membrane-anchored, glycosylphosphatidylinositol (GPI)-linked version is formed by alternative splicing of exons 19 and 20 in Cp gene and is found mainly in astrocytes, leptomeningeal (membrane surrounding the brain) cells and Sertoli cells (testicular cells that provide structural support)\textsuperscript{18-21}. The GPI-linked Cp is believed to have the same functions as does the secreted form since only the last 5 amino acids of secreted Cp are replaced by 30 residues (which form the trans-membrane anchor) in membrane-bound form while the rest of the protein remains the same\textsuperscript{8}.
Structure of Ceruloplasmin

Cp is a 132 KDa single chain polypeptide that folds into six structural domains where each pair forms a larger domain of about 340 residues. These three larger domains are structurally homologous to each other. Cp owes its name, light blue color, and oxidase activity to the presence of copper atoms in its structure. Thus Cp is a member of a multi-copper oxidase family including plant ascorbate oxidase. The multi-copper oxidases contain three types of copper sites classified into type I, II, and III, depending on their spectroscopic properties. There are three type I coppers present in the structure of Cp responsible for the strong absorption at 600 nm, resulting in blue color. Four imidazole nitrogens coordinate a single type II copper located close to two antiferromagnetically coupled type III coppers. The type II and type III coppers collectively are organized into a trinuclear copper cluster that serves as a site of oxygen binding necessary for catalytic activity of the protein. It has been suggested that the loosely bound copper, termed seventh copper, of human ceruloplasmin is essential for the expression of ceruloplasmin pro-oxidant activity in vitro, which opposes the previous reports of antioxidant activity.

Since Cp binds about 95% of plasma copper, Cp was thought to be a copper carrier that delivers the metal from the liver to various tissues until discovery of other copper carriers that share such function with Cp. However, the focus of Cp-based research shifted from copper to iron upon discovery of its ability to oxidize Fe$^{2+}$ to Fe$^{3+}$. Decreased ferroxidase activity in people with Cp deficiency is believed to affect the
development of several of neurodegenerative disorders such Alzheimer's and Parkinson's diseases $^{17,29,30}$. 

**Figure 1.1.** The crystal structure of human ceruloplasmin viewed by Swiss PDB Viewer.
The role of ceruloplasmin in iron metabolism

Dietary iron is absorbed by the epithelial cells of the small intestine (duodenum) and then released into blood circulation. Cp facilitates basolateral iron transport by oxidizing \( \text{Fe}^{2+} \) to \( \text{Fe}^{3+} \) to promotes incorporation of the latter into apo-transferrin. Evidence suggests that Cp also sustains iron uptake from blood circulation by cells of various organs and its GPI-anchored isoform, found in brain astrocytes, loads apo-transferrin with ferric iron in a process preceded by oxidation of highly toxic form of iron bringing the toxic \( \text{Fe}^{2+} \) in the cell to a minimum. The process briefly outlined above indicates the importance of Cp in iron metabolism and this concept has been strengthened by findings in patients with a genetic Cp deficiency termed aceruloplasminemia. In this rare genetic disorder the defect is caused by a mutation entailing premature termination of Cp-mRNA translation which ultimately leads to pathologic iron accumulation in brain and liver due to the role of Cp in iron transport.

The role of Cp is implicated in an effective control of the level of ferrous iron oxidation since the advantage of the Cp-catalyzed iron oxidation is the absence of hydrogen peroxide as an end product. Oxidation of \( \text{Fe}^{2+} \) by Cp with production of water prevents accumulation of reactive oxygen species and further cytotoxicity. In other words, Cp catalyzes the oxidation of ferrous iron to ferric in a reaction (shown below) that does not yield radicals.

\[
4 \text{Fe}^{2+} + \text{O}_2 + 4 \text{H}^+ \rightarrow 4 \text{Fe}^{3+} + 2 \text{H}_2\text{O}
\]
The importance of membrane-anchored GPI-linked Cp expressed by astrocytes has also been shown in iron metabolism \(^{38}\), where it regulates cellular iron efflux and prevents its pathologic overload and toxicity. It is believed that membrane-anchored Cp is involved in catecholamine oxidation, the disruption of which may result in accumulation of reactive oxygen species and free radical-mediated reactions, contributing to neurodegenerative disorders \(^{8}\).

Although iron is an essential cofactor of multiple proteins and enzymes, its excess is highly toxic. The iron levels in plasma is regulated by intestinal iron absorption via the duodenal enterocyte and by release from spleen and liver \(^{39}\). It is thought that ferrireductases at the apical surface of the epithelial cell reduces dietary ferric ion to ferrous \(^{40,41}\), which is further transported across the gut epithelium by divalent metal transporter-1 \(^{42}\). A ferrous ion transporter called ferroportin, facilitates the basolateral release of epithelial iron \(^{43-45}\) with the aid of another enzyme called hephaestin, a membrane-bound, Cp paralog with ferroxidase activity \(^{46}\). On the other hand, plasma Cp ferroxidase activity was thought to mobilize iron primarily from tissue stores \(^{47}\). The important role of Cp in iron metabolism is most convincingly shown by iron accumulation in liver, brain, and other tissues in patients with aceruloplasminemia \(^{48}\).

Because since its transcript is not detected in the gastrointestinal tract \(^{13-15}\) and iron absorption is undiminished in Cp\(^{-/-}\) mice \(^{37,49}\), the role of in iron absorption was omitted. Our laboratory have previously reported that the primary function of Cp under iron stress (deficiency) is to increase iron absorption from the small intestines and not to release iron.
from stores. Interestingly, the mechanism of stress-mediated iron absorption is accompanied by a remarkable relocation of Cp to the duodenal lamina propria, where Cp is thought to facilitate iron loading from ferroportin in the enterocyte basolateral membrane to transferrin for subsequent transport by blood.

**Aceruloplasminemia**

Aceruloplasminemia is a rare hereditary disease identified by Miyajima et al in 1987. This autosomal recessive disease is caused by the complete systemic absence of ceruloplasmin (Cp). Due to the role of Cp in iron homeostasis described earlier, its deficiency results in iron deposition in the liver, pancreas, basal ganglia, and brain.

Hellman and Gitlin summarize the well-studied cases of aceruloplasminemia into at least six missense mutations in Cp gene, six frame-shifts, three splice site and two nonsense mutations as causes of Cp deletion on protein level.

As has been mentioned earlier, aceruloplasminemic patients do not develop serious disorders of copper metabolism. However, many of them are diagnosed with marked hemosiderosis (an iron overload disorder), mostly low levels of serum iron, and mild anemia.

Neurological symptoms are usually detected in patients younger than the age of 40, which is a result of extensive iron accumulation in the basal ganglia and retina. Ferrous form of the iron is likely to contribute to the damaging effect of iron overload in tissues. Because astrocytes express Cp and are believed to regulate tissue iron levels, iron deposition within the nervous system is higher in astrocytes than in neurons which leads
to neurodegenerative symptoms including retinal degeneration. It is believed that free-radical stress is the cause of neuronal cell death observed in aceruloplasminemia. Due to the evidence that shows high levels of lipid peroxidation in the brain of these patients, it is likely ferrous iron contribute the increased cell death via a mechanism involving lipid peroxidation provoked. Interestingly, due to iron accumulation in the β-cells of the Langerhans islets, the disease is usually manifested by insulin-dependent diabetes mellitus.

**Cp-null mice**

Harris et al, created an animal model of aceruloplasminemia by disrupting the murine Cp gene. It has been reported that Cp−/− mice are normal at birth, but demonstrate progressive accumulation of iron in the liver and spleen over the age of the animals. The mild (compared to the aceruloplasminemic patients iron overload) phenotype in Cp−/− mice suggests at least partial compensation of ferroxidase activity by hephaestin. Hephaestin is a copper-containing transmembrane protein that transports dietary iron from intestinal enterocytes into the blood. It is widely accepted that Cp and hephaestin compensate each other in intestinal iron absorption since Cp was also demonstrated to play important role intestinal iron absorption upon acute iron deficit induced by phlebotomy.

**Wilson's Disease**

Wilson’s disease is caused by a mutation in the gene coding for P-type ATP-ase, also known as a Wilson's disease protein. The mutation greatly impairs the delivery of
copper to apo-Cp and, instead, the metal ions accumulate in mainly hepatocytes, where they cycle between Cu$^{1+}$ and Cu$^{2+}$ forms leading to production of hydroxyl radicals. Copper ions also reside in parts of brain and cornea, where (including liver) they intoxicate the cells. As a result of such pathologic accumulation of copper in above tissues causes cirrhosis, neurological degeneration, and characteristic Keiser-Fleischer rings. Kaiser-Fleischer rings observed in the eyes of all Wilson's disease patients are due to copper deposition in Descemet's membrane of the cornea. In addition, low levels of plasma Cp is characteristic in most of Wilson's disease patients.

**Bactericidal activity of Cp**

Cp, when combined with Fe$^{2+}$ at pH 5.0, is bactericidal to Escherichia coli. Interestingly, the observed effect is biphasic, i.e., killing increased with the ceruloplasmin to a maximum with Cp concentration of 1 µg/ml and then decreased as its concentration was further increased up to 100 µg/ml. It is thought that the bactericidal effect of Cp, unaffected by catalase or superoxide dismutase, is lost at higher concentrations of Cp due to more extensive oxidation and quick loss of Fe$^{2+}$.  

**Cp and myeloperoxidase (MPO) interaction**

MPO, a major protein in neutrophils, catalyzes reaction between H$_2$O$_2$ and halides to produce a highly cytotoxic product, hypochlorous acid (HClO). Cp was reported to bind and inhibit MPO's destructive enzymatic activity. Interestingly, during Cp-MPO interaction, Cp's ability to catalyze oxidation of ferrous ions and to remove peroxides remains intact.
Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal (GI) tract that affects millions of people worldwide. In addition to most common clinical symptoms such as abdominal pain, bloody diarrhea, and weight loss, progressive disease is often accompanied by granulomas (focal collection of immune cells), submucosal edema, lymphoid hyperplasia, ulcerative lesions, and fibrosis. IBD can also be complicated by extraintestinal manifestations such as pleuritis, tendinitis, and arthritis as illustrated in Figure 1.1. Two major manifestations of this disease are known as Crohn's disease and ulcerative colitis. Described by and named after Burril B Crohn, Crohn's disease may affect any part of the GI tract from mouth to anus, whereas, ulcerative colitis is limited to the large intestine. Ulcerative colitis histologically differs from Crohn's disease by localized inflammation observed in upper layers of the colon mucosa. However, both Crohn's disease and ulcerative colitis are characterized by clinical relapses and disease remissions. The diagnostic difference of Crohn's disease and ulcerative colitis is described in table 1.1.

**Epidemiology**

The incidence rates of IBD in developed countries such as USA and UK has been reported to be the highest but stabilizing, whereas the rates are on the rise in most developing countries of Europe and Asia. The variation is also observed within the countries and regions as well where incidence changes from one ethnicity or race to another. For example, the prevalence of Crohn's disease in North America among white
Figure 1.2. Common extraintestinal manifestations of IBD. The figure and legends adapted from Baumgart and Carding, 2007.
Table 1.1. Differential diagnosis of ulcerative colitis and Crohn’s disease. The figure and legends adapted from Baumgart and Carding, 2007.
and African-American individuals is 43 and 29 per 100,000, respectively, while it is only 5 per 100,000 in Asian people, which suggests that environment and lifestyle affect the incidence and prevalence of IBD.

**Crohn’s Disease**

As a relapsing inflammatory disease of the gastrointestinal mucosa Crohn’s disease is not restricted to a part of the GI tract. The disease discontinuously affects various portions of the GI tract and develops complications including abscesses, fistulas, or strictures. According to the Vienna classification, at the time of diagnosis Crohn’s disease is stricturing in 17%, penetrating (fistulas or abscesses or both) in 13%, and non-stricturing and non-penetrating in 70%, of all patients. It is also generalized that the disease behavior may fluctuate and change anatomical location. In addition, the clinical symptoms which include bowel obstruction, fever, abdominal pain, and diarrhea, also depend on the specific part affected by the disease. Statistical data shows that at the first diagnosis Crohn’s disease was observed in the terminal ileum in 47%, the colon in 28%, the ileocolon in 21%, and the upper gastrointestinal tract in 3% of patients.

**Ulcerative Colitis**

Ulcerative colitis is a relapsing inflammatory disease that is restricted to the colon. Unlike Crohn's disease, it is not transmural, i.e., affecting the entire thickness of the wall of an organ. Anatomic extent of the disease defines the subtype as pancolitis (involving the entire large intestine), left-sided colitis (involving the sigmoid colon and/or the descending colon), or proctitis (inflammation of the rectum). Occasionally patients also
develop backwash ileitis (ileal inflammation) that make it similar to Crohn's disease and complicates the differentiation of ulcerative colitis from Crohn's ileocolitis. Typical symptoms of ulcerative colitis include abdominal cramping during bowel movements, passage of pus, mucus, and bloody diarrhea 68.

A follow up study on patients with ulcerative colitis revealed overall normal life expectancy 71. Interestingly, in the first decade of their life following diagnosis, a quarter of patients are in remission and about a fifth have a relapsing disease every year. In addition, clinical remission is seen in half of the patients at a given time. Disease activity in the prior year very often serves as a significant predictor of relapse. Following the first 10 years, about a quarter of patients have colectomy and more than half the patients proximally progress during the next 25 years. In this period regression is observed in 75% of case with extensive disease 72 73. Remarkably, such a distribution of disease activity in ulcerative colitis patients is known to be constant from year to year 72.

Endoscopy and histological analysis are the primary tools in diagnosis of ulcerative colitis 74. Figure 1.2 shows a picture of the descending colon taken at endoscopy and hematoxylin & eosin staining of the mucosal biopsy of diseased colon. Three levels of disease activity are most commonly used to describe the stage of the disease. First level is called "mild", and it is characterized by up to four stools a day and lack of toxicity 75 76. Four to six stools daily with minimal toxicity comprise the "moderate" level of disease activity. And, third level, "severe", is characterized by more than six stools a day and include symptoms as anemia, fever, and tachycardia (accelerated heart beat) as general
Figure 1.3. Colonoscopy and biopsy images of UC. A) Descending colon: pancolitis with irregular surface due to extensive ulcerations, spontaneous hemorrhage, and pseudopolyps. B) Mucosal biopsy: active ulcerative colitis with marked architectural distortion, cryptitis, and crypt abscesses. Original magnification 100x, H&E. The figure and legends adapted from Baumgart and Carding, 2007.
signs of systemic toxicity. Some patients develop fulminant (rapidly progressive) ulcerative colitis with more than ten bloody stools daily and continuous bleeding. In such patients, who also develop high fevers and high levels inflammatory markers, anemia requires blood transfusion.

Oral administration of 5-aminosalicylic acid compounds, a common anti-inflammatory drug, is a major therapeutic approach to treat mild to moderately active ulcerative colitis in outpatients with left-sided disease. In addition to 5-aminosalicylic acid, enemas or foams are also used to fight more extended disease that can affect larger portions of left colon. Corticosteroid treatment (prednisolone or azathioprine) is required in cases with severe, nonfulminant disease or irresponsiveness to 5-aminosalicylic acid compounds.

Because of the risk of systemic toxicity, inpatients with severe, rapidly progressive (fulminant) colitis are often considered for emergency colectomy. However, when the severe disease is not accompanied by toxicity and is limited to focal abdominal tenderness, medical therapy is preferred to colectomy. Such therapy involves intravenous injections of corticosteroids and fluids to prevent severe dehydration.

Immune System

One of the fundamentals of human survival is uncompromised immunity. Our immune system is extremely sophisticated with its ability to distinguish self from nonself and capacity for memory. Recent advances in understanding of physiologic immune
responses explain the pathogenesis of immunologic disorders and provides new insights on therapeutic approaches. As a "body" of molecules and cells with specialized functions in defending against infection, human immune system facing a threat such as infection, deploys two fundamentally different types of responses. Innate immune responses act to the same extent regardless of the number of times it encounters the same infectious agent, whereas adaptive immunity wages a more powerful response upon each repeated exposure to a given antigen.

**Innate Immunity and Its Cellular Components**

Two major characteristics of innate immunity are lack of memory and immediate, unchanged response to the encountered antigen. Such immune responses are sometimes referred to as "primitive" as they are present in simple organisms as well. However, the human innate immune response is complex enough to cause fatalities upon defects in one or more of its mechanisms. A common example of such disorders is chronic granulomatous disease with defective killing of phagocyted microorganisms.

The cellular components of the innate immune system include natural killer cells, mast cells, eosinophils, basophils, macrophages, neutrophils and dendritic cells, all of which identify and eliminate a wide variety infectious agents. However, these cells also have distinct mechanisms of detection and elimination of a threat. For example, macrophages derived from circulating monocytes discriminate between "self" and "non-self" molecules by expressing receptors for certain microbe-specific carbohydrates.
Recruitment and activation of neutrophils and macrophages at the site of infection in an attempt to destroy pathogens is a key feature of innate immunity. This feature is an area of intense research as it may lead to the development of new anti-inflammatory therapies. Inappropriate response at this level of inflammation is known to be a key mechanism in several diseases including vasculitis and systemic inflammatory response syndrome. Activated macrophages infiltrating the infected tissue release cytokines that stimulate proliferation of myeloid precursors in the bone marrow, which ultimately leads to the release of millions of neutrophils into the blood. These cells are also recruited to a site of infection via a complex process involving expression of adhesion molecules and chemokines. In addition to phagocytosis of infectious microorganisms, there are two known mechanisms of neutrophil-mediated killing. First, by means of a respiratory burst that involves production of toxic oxygen metabolites (singlet oxygen, hydrogen peroxide, and hydroxyl radicals). Second, the so-called oxygen-independent response that utilizes the highly destructive enzymes such as myeloperoxidase and lysozyme that are stored in the cytoplasmic granules.

Another important function of macrophages and neutrophils is phagocytosis of the body's dying cells. This function eliminates the threat of necrosis as it triggers inflammation. Dying cells express phosphatidylserine on their cell surface which identifies them for phagocytosis.

Another group of blood nonphagocytic granulocytes called eosinophils are responsible for the protection of the host from nematode (round worm) infections.
Eosinophils attach to the surface of the parasite already precoated with antigen-specific IgE and release large granules containing highly cytotoxic group of proteins including major basic protein, eosinophilic cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin.

*Natural killer cells* are the major cytotoxic component of innate immunity and morphologically they resemble lymphocytes. However, natural killer cells do not act in an antigen-specific manner. They can recognize infected cells by immunoglobulin receptors (FcR) which bind antibody-coated pathogens. This is known as antibody-dependent cellular cytotoxicity. Or, they attempt to bind MHC class I on target cells by expressing specific MHC I-specific receptor. If the cell is infected and displays a foreign antigen on its MHC I, the interaction with the natural killer cell is blocked. The activated natural killer cell then lyses the target by secreting proteins called perforins onto the surface of the infected cell. Perforins form pores in the cell membrane which ultimately lead to the transfer of granzymes into the cytosol and induction of apoptosis in the target. Granzymes are serine proteases that induce apoptosis in cells by cleaving and activating caspases, one of the major mechanisms of programmed cell death.

All nucleated cells of the human body express MHC I on their surface. However, as a consequence of interference of infectious agent with the expression machinery, sometimes this ability is lost. Therefore, the lack of MHC I on the cell surface leads to the disappearance of the inhibitory signal during natural killer cell-target cell interaction,
which results in natural killer cell killing the abnormal target cell by the mechanism involving perforins and cytotoxic granzymes$^{83}$.

*Basophils and mast cells* bear high-affinity receptors for IgE and it is believed that basophils develop into tissue mast cells$^{87}$. Evidence suggest the importance of these cells in allergies, in which allergen binding to the IgE cross-links the receptor expressed on their surface. IgE cross-linking triggers the cells to secrete inflammatory mediators (histamine and prostaglandins)$^{88}$. Despite the low frequency of basophils and mast cells, their involvement in some of the most severe immunological reactions (angioedema and anaphylaxis) emphasizes the overall importance of their role in innate immune response$^{83}$.

Interdigitating dendritic cells, believed to play a key role in innate immunity, constantly endocytose and sample extracellular fluids in search of a foreign antigen. Once the pattern-recognition receptors expressed on their surface recognize pathogen-associated molecular patterns of microorganisms, dendritic cells become activated and function as antigen-presenting cells. The signals that activate these cells can be either endogenous (interferon-α and heat-shock proteins) or pathogen-associated (yeast-cell-wall mannans and lipopolysaccharides)$^{89}$. Upon activation dendritic cells increase expression of CD80 and CD86 surface markers known as costimulatory molecules. These molecules provide additional (to antigen receptor interaction) signals necessary for lymphocyte activation when activated dendritic cells migrate to draining lymph node.
Pattern Recognition Receptors

Some molecules produced by pathogen and/or microbes are not found in higher organism (vertebrate). Such microbe-specific molecules (very often essential pathogen components), also known as pathogen-associated molecular patterns (PAMPs), are recognized by the innate immune cells via pattern-recognition receptors. Two major groups of these receptors are Toll-like receptors (TLRs) and cytosolic nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs).

TLRs is a family of transmembrane proteins that consist an extracellular domain containing leucine-rich repeats (LRRs) and an intracellular domain which is a conserved signaling module called a Toll/IL-1 receptor (TIR) domain. TLRs are mainly expressed by macrophages, neutrophils, and dendritic cells, and are key to the activation of these cells by PAMPs. Twelve mammalian TLRs and a number of their naturally occurring ligands have been identified to date. The most widely known examples are TLR 3, TLR4, TLR5, and TLR9 that recognize double-stranded RNA, lipopolysaccharide (LPS), flagellin, and CpG DNA (nonmethylated bacterial DNA), respectively. Upon ligand-receptor interaction, a conserved signaling cascade is triggered and results in activation of several transcription factors including IRFs (IFN-regulatory factor transcription factors), AP-1, and NF-κB. These transcription factors initiate or increase the expression of proinflammatory genes necessary to activate innate and adaptive immunity.

The NLR family is composed of 23 cytosolic proteins characterized by the presence of a conserved Nod. The general domain structure of cytosolic receptors include an
amino-terminal effector binding region, pyrin, or baculovirus inhibitor repeat domains, and carboxyl-terminal LRRs. The effector binding region contains of protein-protein interaction domains such as caspase recruitment domains (CARD), and the LRRs are required to detect specific PAMPs. In response to the ligands (PAMPs), NRLs activate intracellular signaling cascade via RICK kinase oligomerization which leads to the activation of MAPK and NF-κB, and induction of proinflammatory genes.

**Soluble Factors in Innate Immunity**

In addition to cellular components, innate immune responses frequently involve acute-phase proteins, complement proteins, and cytokines. Besides their phagocytic activity, neutrophils and macrophages also express receptors for antibodies and complement system components, which substantially facilitates phagocytosis of microorganisms coated with antibodies and/or complement proteins. The complement system consists of about 20 serum glycoproteins and as a whole plays an important role in innate immunity. The activation of complement system proteins happens in a cascade sequence at the end of which the initial single molecular stimulus leads to the generation of thousand-fold more molecules. Three pathways of complement activation activated by various foreign substances are outlined in figure 1.3. Briefly, these pathways are the "classical", driven by antigen-antibody reactions, the "alternative" activated by
Figure 1.4. The three pathways of complement activation. Regulatory proteins are shown in orange. Components of activation pathways are shown in green. The figure and legends adapted from Parkin and Cohen, 2001.
polysaccharides from yeasts and gram negative bacteria, and the "mannon binding lectin" pathway, stimulated by mannose containing proteins of viruses and yeasts.\textsuperscript{96}

The acute-phase proteins contribute to the repair of damaged tissue and increase resistance to infection.\textsuperscript{97} The well-known example are C-reactive protein, proteinase inhibitors, coagulation proteins, serum amyloid A protein, and ceruloplasmin. As the name implies, the serum levels of acute-phase proteins changes in response to infection, inflammation, and tissue injury.\textsuperscript{79}

Cytokines, another group of soluble mediators highly involved in the regulation of immune responses, function as signaling molecules messengers necessary for communication within the immune system and between the immune system and any other tissue.\textsuperscript{98} Cells "sense" the cytokines by expressing cytokine specific receptors, which can be either in soluble or membrane-anchored forms.\textsuperscript{99,100} These signaling molecules play an important role in defense by inducing a variety of responses in a target cell. For example, the interferons, secreted by virally infected cells, induce viral resistance in the neighboring cells.\textsuperscript{101}

**Adaptive Immunity**

Adaptive immunity is characterized by the use of antigen-specific receptors on T and B lymphocytes and their activation by innate immune elements. The cellular components of the innate immunity harvest the antigen and present it to naive T or B cell. This process, which normally occurs within the lymphoid tissue, is called antigen
presentation and it leads to the activation and differentiation of the lymphocytes into an antigen specific effector cell. Depending on the cell type, the effector response can be either by the activated T lymphocytes directed to the disease site, or by mean of antibody secretion from activated B lymphocytes (plasma cells) into the blood and tissue fluids.

**Gut immunity**

Intestinal mucosa form a physical zone of interface between the outside world and immune system (Figure 1.4). Human intestinal tract bears a substantial part of the mucosa-associated lymphoid tissues and is home to very diverse microbiota that consists of over 500 species of bacteria alone.

Several important intestinal functions are regulated by the gut microflora. These functions include but limited to angiogenesis, xenobiotic metabolism, nutrient absorption, and postnatal intestinal maturation. Following birth, initial colonization of the intestines depends on such factors as delivery (vaginal or caesarean) and feeding (breast or formula), and begins with domination of aerobic species which later yield anaerobic commensals. Genetic and environmental (hygiene) factors also influence the colonization rate and type. The mechanism of establishment of oral tolerance to microbiota remains largely obscure. However, it is thought that formation of immune tolerance to commensal microflora as well as maturation of mucosal immune system take place through a complex interaction of anatomical, cellular, and humoral factors which suppresses immunologic reaction to antigens entering the GI tract via oral route.
Figure 1.5. Intestinal immunity in healthy state. M-cell = microfold cell. Th = T helper cells. Th0 = naive T cell. Th, Th1, Th2, Th17 = effector T cells. Tr, Th3 = regulatory T cells. B = B cell. B(PC) = plasma cell. NK = natural killer cell. NKT = natural killer T cells. The figure and legends adapted from Baumgart and Carding, 2007.
Epithelial barrier, a polarized single layer covered by mucus that hosts commensal microflora, is the first line of defense of the mucosal immune system. The importance of mucus in intestinal immunity has been emphasized by studies that report Crohn’s disease and ulcerative colitis patients with defective mucus production. In addition to mucus, the apical surface of single cell gut epithelium is also lined with secretory IgA and a glycocalyx (a meshwork of acidic mucopolysaccharides and glycoproteins). There are two routes that substances use to cross this layer, transcellular (through channel and membrane pumps) and paracellular (controlled by tight junctions). Paneth cells, specialized epithelial cells of small intestines, are a part of first line of defense in intestinal immunity as they regulate of microbial density and protect neighboring stem cells by secreting various antimicrobial peptides such as defensins.

Intestinal epithelium is also the site for primary level of antigen recognition. Pattern recognition receptors are constitutively expressed by epithelial cells and structurally related receptors that recognize specific microbial components, also called pathogen- (or microbe-) associated molecular patterns.

There are two important families of pattern-recognition receptors that play important role in gut immunity. The first family, TLRs, recognizes specific targets associated with a particular microbe or group of microbes. TLR recognize a wide variety of microorganisms activates both innate and adaptive antimicrobial responses via a intracellular signaling pathways. Signaling via TLR pathways activates several transcription factors such factor nuclear factor kappa B (NFκB), which ultimately results in production of proinflammatory cytokines and chemokines. However, under normal
homeostasis and absence of pathogens, TLR-ligand (microbe-associated molecular patterns) interactions are also necessary to the and maintenance of an intact epithelial barrier\textsuperscript{111}.

The second important family of pattern-recognition receptors are cytosolic NOD proteins. These proteins, namely, NOD1 and NOD2, are expressed by antigen-presenting cells and contribute to intestinal mucosa by forming an additional defense mechanism\textsuperscript{112}. In the absence of proinflammatory signals, epithelial cells also express NOD1 and NOD2 at very low level. However, the expression increases upon activation by proinflammatory cytokines\textsuperscript{113}. NOD proteins are thought to contribute to the innate immune response to pathogens because muramyl dipeptide, peptidoglycan constituent of bacterial cell wall and ligand for NOD2, triggers the activation of NF\kappa B in NOD2 overexpression models\textsuperscript{114,115}. In general, the function of NOD2 in intestinal epithelia (as the primary point of interaction with enteric microbes) requires more thorough investigation\textsuperscript{116}.

\textit{Lymphoid tissue} of mucosa consists of loose connective tissue beneath the epithelium (called lamina propria) that hosts mast cells, granulocytes, natural killer (NK) cells, T cells, and B cells. Antigens and microbes are channeled through the epithelial layer to the underlying lymphoid clusters. In these clusters, known as Peyer's patches and lymphoid follicles found in small intestines and colon, respectively, antigens are harvested by dendritic cells and macrophages\textsuperscript{117}. Dendritic cells, crucial in tolerance towards commensals and detecting pathogens, can also directly sample bacteria by sending cytoplasmic extensions (dendrites) through the tight junctions between epithelial cells\textsuperscript{118}. The distinguishing characteristic of dendritic cells is their expression of all the
TLRs and NODs, which empowers them to either activate or suppress T cell immunity in response to pathogens or commensals\textsuperscript{110}. These cells create tolerance to an antigen by stimulating naive T-cell differentiation into regulatory T cells that express transforming growth factor beta (TGF-\(\beta\)) and/or IL-10 (suppression of T cell immunity). They can also drive T-cell differentiation into effector Th1 (interferon \(\gamma^+\), interleukin 2+) or Th2 (interleukin 4+, interleukin 10+) cells, thereby, activating proinflammatory T cell immunity (activation of T cell immunity)\textsuperscript{119}.

**Intestinal immune system in disease**

Experimental evidence suggests that inappropriate inflammatory response at various levels of intestinal immunity to the commensal microflora or luminal antigens is the main trigger of IBD\textsuperscript{116}. These probably overlapping pathways shown in figure 1.5 and discussed below.

It is widely accepted that the *primary inflammatory events* in IBD begin with the "leakiness" of the epithelial barrier. Higher permeability of the epithelium and mucosa in inflamed and non-inflamed mucosa during Crohn’s disease and ulcerative colitis supports the idea that clinical onset of the disease in certain patients is led by an already present defect\textsuperscript{120,121}. Several studies have shown the genetic risk factors of epithelial permeability associated with IBD. These risk factors include, but not limited, to mutations in *CARD15* gene, dysfunction of enteric neurons, and T cell mediated disruption of tight junctions\textsuperscript{120,122,123}. 
Figure 1.6. Intestinal immune system in IBD. MLN = mesenteric lymph node. Other abbreviations spelled out in footnote of figure XXI. The figure and legends adapted from Baumgart and Carding, 2007.
Besides leakiness of the epithelium, IBD patients also have defective innate immune system at the mucosal level. For example, in healthy individuals there is a basolateral expression of TLR3 and TLR5 with slightly detectable level TLR2 and TLR4. Whereas, TLR 4 expression is higher in Crohn's disease and ulcerative colitis patients. Interestingly, epithelium affected with Crohn's disease is also characterized with downregulated TLR3. Chronic intestinal inflammation may also display upregulation of NOD2 in diseased epithelium with activated NFκB signaling pathway.

Defective pattern-recognition receptor responses also has a proinflammatory impact on gut immunity as abnormalities of antigen recognition and processing by professional antigen-presenting cells (dendritic cells and macrophages) have also been reported in people with IBD. In such cases, improper recognition of microbiota induces Th1 and Th17 mediated immunity resembling an attack against pathogens. Intestinal inflammation increases the number of these antigen presenting cells in inflamed mucosa and the extent of inflammation in IBD patients correlates with frequency and ratio of activated (mature) and naive dendritic cells. Experimental evidence also suggests that dendritic cells with defective immunoregulatory function might be responsible for the repeated activation of memory T cell populations or the absence of peripheral tolerance (deletion of over-reactive T cells), thereby, aggravating inflammation in IBD. Insufficiency of reagents specific to intestinal dendritic cells and their low frequency are the major obstacles for the investigation of their role in IBD.
In the presence of inflammatory cytokines non-professional antigen-presenting cells such as epithelial cells, gain activated phenotype and become potent effector-T-cell activators in patients with IBD \(^{128}\). In addition to classical major histocompatibility complex molecules, such epithelial cells basolaterally express CD1d known as non-classical major histocompatibility complex molecule that plays an important role in T cell activation. In addition, expression of alternative costimulatory molecules by epithelium in IBD patients might also contribute to the antigen-presenting function of these cells \(^{129}\).

Under certain circumstances activated T cells may fail to undergo apoptosis resulting in failure of either central or peripheral tolerance, presenting yet another level of abnormal gut immunity. Such a defective clearance of autoreactive T-cell populations has also been observed in people with Crohn’s disease \(^{130,131}\). Moreover, in active IBD naive T cells differentiation is driven towards the effector T cells phenotype, which results in dominance of these over the regulatory/suppressor T cells \(^{132}\). Such an imbalance of regulatory and effector T cells frequencies has a snowball effect because activated T cells in Crohn’s disease patients were found to be a primary source of such inflammatory cytokines as IL-12, IL-18, and interferon \(\gamma\), which activate macrophages. Activated macrophages, in turn, secrete TNF-\(\alpha\), IL-1, and IL-6 \(^{133,134}\).

Recent studies demonstrate that IL-17+ T cell (Th17) populations regulates proinflammatory responses in various tissues. The balance between Th17 and anti-inflammatory T cells, determined by TGF- \(\beta\), is also pivotal for the maintenance of gut
immune homeostasis. Cytokines such as TGF-β that either sustain or delete Th17 activity may play a key role in the development of chronic intestinal inflammation.

Recent studies identified that TNF-α release by macrophages can be inhibited by acetylcholine (a neurotransmitter of both central and peripheral nervous system), which emphasizes the impact of neuroimmunological interaction on inflammatory cascade. Thus psychosocial stress can attenuate the inhibitory effect of nervous system on immunity and augment the proinflammatory pathways. Patients with ulcerative colitis present overactivation of the sympathetic nerve as a result of stress, which, in turn, causes altered expression of tight junction proteins in gut epithelium, mast-cell degranulation, and increased levels of interferon γ.

*Secondary inflammatory events* commence with migration of inflammatory cells from the systemic circulation into the intestinal mucosa as result of the release of chemoattractants by professional and nonprofessional antigen-presenting cells upon antigen recognition. Activated mucosal macrophages provide simultaneous up-regulation of ligands on the vascular endothelium for the adhesion molecules on migrating cells by secreting cytokines (interleukin 1 and TNF-α). Ultimately, these events result in the tissue damage and stricture formation by the multitude of aggressive metabolites (nitric oxide, oxygen radicals, prostaglandins, leukotrienes, histamine, proteases, and matrix metalloproteinases) and that accumulate in the mucosa.
Dextran sodium sulfate induced colitis

Dextran Sodium Sulfate (DSS) induced colitis mouse model is one of most widely used animal models of ulcerative colitis which involves supplementing the drinking water of mice with DSS. The induced colitis can be either acute or chronic depending on the duration of DSS administration, continuous or alternating with water, respectively, which results in epithelial damage and a robust inflammatory response in the. After about five days of treatment with 3% (weight/volume) DSS in drinking water, mice lose weight and develop bloody diarrhea. DSS induced damage and inflammation changes overall morphology of the large intestine as it becomes thicker and shorter. In addition, extensive colonic bleeding makes animals anemic. Mice are euthanized at various days of the treatment course and dissected colons are processed for microscopic analysis.
CHAPTER II

MATERIALS AND METHODS

Animals.

Mice with targeted Cp gene deletion (Harris et al., 1999) were backcrossed for 17 to 19 generations into the C57BL/6J background. MPO−/− mice in C57BL/6 background were kindly provided by Dr. Stanley L. Hazen. 10- to 12-week-old females from Cp−/−, MPO−/−, and wild-type C57BL/6J controls (WT, The Jackson Laboratory) were used in all experiments. Animals were housed in specific pathogen-free conditions with free access to food and water. All studies followed a protocol approved by the Animal Review Committee of the Cleveland Clinic Foundation.

DSS-induced colitis.

To induce experimental colitis, 8 to 12 weeks old mice were continuously treated with 3% (w/v) DSS (M.W. 40,000 kDa; MP Biomedicals Inc.) ad libitum in drinking water. For histological, gene expression, and cytokine production studies, mice were sacrificed after DSS treatment for indicated days. In near survival studies mice were given DSS containing water for up to 30 days.
Scoring of histopathology and colonic bleeding.

Proximal and distal ends of the colons were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with H&E for histopathological confirmation of smooth muscle cell hyperplasia, epithelial damage, inflammatory infiltrate, and submucosal swelling. Scoring of histopathology and colonic bleeding was performed by blinded method.

Immunohistochemistry.

Formalin-fixed and paraffin-embedded colon sections were deparaffinized in xylene and rehydrated in several changes of alcohol. Samples were then boiled for 10 minutes in antigen retrieval solution (DAKO) and left at room temperature for 30 minutes. Prior to incubation for 1 hour at room temperature with primary antibody to CD3 (AbD Serotec), F4/80 (AbD Serotec), anti mouse neutrophil (AbD Serotec), or Cp (Santa Cruz), slides were blocked with peroxidase inhibitor (DAKO), protein block (DAKO), and biotin/avidin blocking solutions (Vector Laboratories). After incubation with biotin-conjugated secondary antibody (Abcam) and streptavidin-HRP (Vector Laboratories), positive signals were visualized by DAB kit (BioGenex) and counterstained with Mayer's hematoxylin (Sigma).

Whole-colon culture ELISA.

100–200 mg of colon tissue from rectum to cecum was washed in cold PBS supplemented with penicillin and streptomycin. Each sample was cut into small pieces
and cultured in 12-well flat bottom culture plates (Falcon) in serum-free RPMI medium containing penicillin and streptomycin. After 24 hours of incubation at 37\(^\circ\)C, each culture well was mixed with 1 ml pipette. The culture supernatant was then collected and stored at -80\(^\circ\)C until use. These whole-colon culture supernatants from DSS-treated mice at various time points were examined for KC and MCP-1 chemokines and TNF-a content with commercially available ELISA kits (R&D Systems), according to the manufacturer’s protocol. Estimated cytokine and chemokine level was normalized to total colon tissue weight. Each time point is an average of 3–4 of mice per group.

**Hematologic analysis.**

Mouse blood was obtained on various time points of DSS administration by cardiac puncture of euthanized mice into EDTA tubes (Sarstedt), After vigorous mixing 100 ul of blood was transferred into a new EDTA tube and diluted with 200 ul of 3% BSA in 1xPBS. The final mixture was analyzed by Advia 120 analyzer. Due to failure of Advia 120 to differentiate between mouse monocytes and lymphocytes, the percentage of monocytes within WBC population was estimated per sample by blood smear and the exact number calculated from Advia 120 total WBC readout.

**DAB-enhanced Prussian Blue.**

Formalin-fixed and paraffin-embedded colon and liver sections were deparaffinized in xylene and rehydrated in several changes of alcohol. Following 10 minute preincubation with peroxidase block (DAKO), tissue sections washed in DI water and incubated for one hour in fresh mixture of 10% w/v solution of potassium ferrocyanide (Sigma) in DI water
and 20% hydrochloric acid (v/v). Prussian blue signals were enhanced with DAB substrate kit (BioGenex) and counterstained with Myer's hematoxylin (Sigma).

**ABX treatment.**

6- to 8-week-old mice were continuously treated with ampicillin (1 g/l; Sigma), vancomycin (500 mg/l; Sigma), neomycin sulfate (1 g/l; Gibco), and metronidazole (1g/l; Sigma) *ad libitum* in drinking water for 4 weeks. After 4 weeks of commensal depletion, mice were then additionally treated with 3% DSS in drinking water for indicated days and then sacrificed for histological study and hematologic analysis. For the estimate of commensal depletion about 50 mg of stool collected from antibiotic-treated and untreated mice was diluted and ground in 1.5 ml PBS, further diluted 10 times with PBS, fixed with formalin, and used for SYBR green staining as described in Xiao *et al.* Briefly, fixed bacteria was filtered through 0.2 mm Whatman Anodisc 25 filter (VWR) and attached bacteria were incubated in SYBR green solution (SYBR Green 1 nucleic acid gel stain, Invitrogen). The stained filters were dried, transferred onto a slide, and covered with mounting medium. Stained microflora was visualized and counted under fluorescence microscope.

**Peritoneal macrophage purification and transfer.**

Peritoneal macrophages from wild-type and Cp<sup>−/−</sup> mice were collected by lavage 4 days after intraperitoneal injection of 1 ml 4% thioglycollate (Sigma). Cells were washed once in 1xPBS and positively purified by magnetic bead separation using a biotinylated
antibody to mouse F4/80 and anti-biotin microbeads (Multenyi Biotec). 10^7 cells were injected intraperitoneally into each recipient on days 0, 5, and 10 of DSS treatment.

**Bone marrow transplantation.**

Bone marrow was isolated from 5 to 6 weeks old C57BL/6J and Cp−/− mice (donors) and transferred through retroorbital sinus injection into 6 weeks old C57BL/6 or Cp−/− lethally irradiated recipients. 6 weeks after transfer mice were confirmed for the presence of transferred genotype in blood and given DSS.

**Carbonyl protein determination.**

Whole colon sample was isolated and homogenized in 50 mM phosphate buffer containing 5 mM EDTA and protease inhibitors. Following centrifugation at 10,000 g the supernatant was used for protein carbonyl content by using protein cabonyl assay kit (Cayman) according to the manufacturer instructions. Briefly, each sample was split into two tubes. One tube was incubated with DNPH while the other with HCl solution alone and served as a control (for corrected absorbance). Following a series of washes with trichloroacetic acid and a mix of ethanol/ethyl acetate, the final pellet was resuspended in guanidine hydrochloride. The final solution was used to measure the absorbance at 360 nm and the protein carbonyl was calculated by using the formula:

$$\text{Protein Carbonyl (nmol/ml)} = [(\text{CA})/(0.011 \ \mu\text{M}^{-1})][(500 \ \mu\text{l}/200 \ \mu\text{l})]$$
CHAPTER III
RESULTS

Experimentally induced colitis is lethal in Cp-null mice.

Induction of colitis in mice by dextran sulfate sodium (DSS) is concentration- and time-dependent. Therefore, we first confirmed that 3% DSS is sublethal in wild-type (WT) C57BL/6J female mice. Continuous administration of 3% DSS ad libitum in drinking water induced acute colitis in WT C57BL/6J mice characterized by appearance of blood in stool after day 3 and significant weight loss by day 7 (Figure 1A). After the acute peak of the disease between days 7 and 9, the WT animals showed signs of partial recovery, accompanied with stabilized weight loss and mild, non-diarrheal colonic bleeding. Despite the continuous administration of DSS and a chronic, sublethal colitis, more than 95% of these mice survived for at least 30 days (Figure 1B). Mild colonic bleeding turned into bloody diarrhea on days 7 and 9 (Figure 1C).

During the same course of DSS administration, C57BL/6 mice deficient in Cp (Cp−/−) acutely lost more weight than WT mice after day 7 (Figure 1A) and became moribund by day 14 (Figure 1B). Severe bloody diarrhea appeared in most of the Cp−/−
Figure 2.1. Experimentally induced colitis is lethal in Cp-null mice. Cp−/− and wild type (WT) C57BL/6J mice were continuously administered 3% DSS ad libitum in drinking water. (A) Percent weight change of mice normalized to their weight on day 0. (B) Percent survival (n = 20). p < 0.001 using the ANOVA test. (C) Colonic bleeding score. (D) Colon length of Cp−/− and WT mice on day 9 (n = 5). p < 0.01 using the Student T test. (E) Representative photographs of Cp−/− and WT colon on day 9. Error bars represent ± SEM.
Figure 2.2. Hematologic parameters determined by Advia 120 hematology analyzer of blood of \textit{Cp}^{-/-} and WT animals on various days of continuous DSS administration. Hematocrit, serum hemoglobin (HGB), and red blood cell (RBC) counts are normalized to day 0 (\textit{n} = 5 per time point). Error bars represent \pm SEM.
mice on day 7 and colonic bleeding (Figure 1C) increased parallel to the weight loss. Bloody stool in Cp\textsuperscript{−/−} mice worsened on day 9 and by the time animals became moribund the entire colon and caecum lacked any stool and were filled with blood (Figure 1C and 1E). In addition, by day 9 of DSS administration the colon of Cp\textsuperscript{−/−} animals was significantly shorter than the WT controls (Figure 1D and 1E), which serves as one of the first indications of more severe colitis.

As has been previously reported\textsuperscript{49,7}, due to impaired iron homeostasis, unmanipulated Cp\textsuperscript{−/−} mice are slightly anemic when compared to age and sex matched WT animals and have about 10% lower mean red blood cell volume and hematocrit. However, blood samples normalized to day 0 showed higher loss of hematocrit, red blood cells, and hemoglobin in Cp\textsuperscript{−/−} mice compared to WT controls during the course of acute colitis, which also confirms that these animals have higher colonic bleeding after day 5 (Figure 2C, 2D, and 2G).

**Cp-null mice develop excessive inflammation.**

Hematoxylin-and-eosin stained cross-sections of distal colon samples collected from Cp\textsuperscript{−/−} and WT animals on various days (0 through 9) of DSS administration were analyzed by blinded method for histopathological changes. The typical mouse colon architecture on day 0 is indistinguishable between WT and Cp\textsuperscript{−/−} animals. On day 5 of DSS consumption the mucosa from both groups of animals loses its compact epithelial cell-lined crypts. However, following day 5 the colon of Cp\textsuperscript{−/−} animals showed increasingly higher epithelial damage, smooth muscle cell hyperplasia, submucosal
Figure 2.3. Colon histology. The colon of Cp-null mice develops extensive epithelial damage and higher colonic inflammation as shown in representative photomicrographs (magnification, X50; hematoxylin and eosin staining) of colons from WT and Cp⁻/⁻ mice at days 0, 5, 7, and 9 of continuous DSS administration.
swelling, and inflammatory infiltrate compared to WT controls as shown in figure 3 and figure 4. By day 9 most of the Cp⁻/- colon exhibits a highly eroded epithelial barrier and hardly retains any crypt, while these structures are relatively intact in WT controls. Observed tissue pathology clearly coincided with colonic bleeding in both Cp-/- and WT animals.

We also determined the nature of inflammatory infiltrate on various days in both groups by immunohistochemical staining for neutrophils, macrophages, and CD3+ T cells. Both genotypes had few yet equal numbers of the above infiltrates in lamina propria of middle colon before DSS administration (day 0). The neutrophil and macrophage influx, as an indication of colonic inflammation, substantially increases by day 9 in Cp⁻/- mice compared to WT controls (Figure 5A and 5B). However, anti-CD3 immunostaining revealed that the increase in CD3+ T cell infiltration of colon lamina propria of Cp⁻/- mice was not as dramatic as for neutrophils and macrophages. T cell infiltration increased in both genotypes over the period of DSS administration but the sites of extensive epithelial damage in Cp⁻/- middle colon had as many T cells as the mild sites of inflammation in WT controls (Figure 5C). This implies that the observed severity of experimental colitis in Cp⁻/- mice is not T cell-dependent. Elevated blood levels of these major infiltrates as well as total WBC measured by Advia 120 were also higher in Cp-null mice following day 5 of DSS administration (Figure 2A, 2B, 2E, and 2F).
Figure 2.4. Histopathological analysis of colon tissue sections of WT and Cp⁻/⁻ animals on various days of DSS treatment reveals higher scoring of smooth muscle cell (SMC) hyperplasia, submucosal swelling, inflammatory infiltrate, and epithelial damage of colons from Cp⁻/⁻ mice after day 5. See Methods section for a description of the scoring algorithm. Error bars represent ± SEM. (n = 3 per time point per group).
Figure 2.5. Immunohistochemical analysis of the inflammatory infiltrate in colon shown in representative photomicrographs of for mouse (A) neutrophils (anti-neutrophil allotypic marker), (B) macrophages (anti-F4/80), and (C) T cell (anti-CD3) in WT and Cp−/− colon rolls on day 9 of DSS administration. X50 magnification.
Figure 2.6. The level of KC, MPC-1 and TNF-α in colon of WT and Cp⁻/⁻ mice determined by ELISA on culture supernatants of mouse colon incubated in RPMI with penicillin/streptomycin overnight at 37°C. Error bars represent ± SEM (n = 3 per time point per group).
Extensive inflammatory infiltration in Cp-/- mice was also confirmed by TNF-α and chemokine ELISA on colon culture supernatants. Higher amounts of TNF-α, and neutrophil (KC) and macrophage (MCP-1) chemokines were detected in Cp-/- colon after day 5 (Figure 6A, 6B, and 6C), which coincide with immunohistochemistry for inflammatory infiltrates, WBC counts, hematocrit loss and colonic bleeding.

As a result, it is clear that upon induction of acute colitis Cp-/- mice develop severe and lethal colonic inflammation accompanied by increased bloody diarrhea, complete epithelial erosion, and extensive inflammatory infiltrate primarily consisting of neutrophils and macrophages.

**Depletion of commensal bacteria by broad spectrum antibiotics implies microbiota-independent function of Cp in colonic inflammation.**

Intestinal mucosa functions as a physical and immune barrier against pathogens and commensal microflora. Inappropriate response to gut microflora is thought to be crucial to the pathogenesis of human IBD 148. Commensal bacteria also play a major role in initiation of inflammation in the DSS-induced colitis animal model 149. Depletion of commensal microflora by antibiotic treatment is commonly used to demonstrate that a given disease phenotype is dependent on the canonical involvement of microbiota. However, depletion of gut microbiota also reduces regeneration of intestinal epithelia and leads to mortality of even WT mice upon continuous administration of DSS 111.
Figure 2.7. Depletion of intestinal microflora did not decrease the extent of epithelial damage and inflammation in Cp⁻/⁻ mice caused by DSS administration. (A) Percent weight change of mice normalized to their weight on day 0. (B) Percent survival (n = 10). p < 0.05 using the one way ANOVA test. Hematologic analysis of blood of WT and Cp⁻/⁻ mice showing (C) hematocrit (HCT) loss by day 9 (normalized to day 0) and (D) white blood cell (WBC) count. (n = 5). p < 0.01 using the Student T test. Error bars represent ± SEM.
The question then was whether the severity of colitis in Cp<sup>−/−</sup> mice is microflora-dependent. This was of a particular relevance given experiments that showed ferroxidase-dependent bactericidal activity of Cp<sup>56</sup>. Therefore, we treated Cp<sup>−/−</sup> and WT mice with a mix of four different antibiotics <i>ad libitum</i> in drinking water continuously for four weeks. Following antibiotic treatment, mice were also given 3% DSS. Upon continuous challenge with DSS, microbiota-depleted WT mice gradually lost weight and became moribund between days 16 and 18 of DSS treatment. Surprisingly, Cp-null animals lost weight more rapidly and became moribund about 7 days earlier than WT controls (Figure 7A and 7B). Depletion of gut microflora was confirmed by SYBR green nucleic acid staining of homogenized stool, and was estimated to be greater than 99.5% in both groups compared to untreated controls (Figure 8B and 8C).

Hematologic analysis on day 9 of DSS challenge revealed that Cp<sup>−/−</sup> mice had higher hematocrit loss and higher WBC counts compared to WT controls indicating higher colonic bleeding and extensive inflammation, respectively (Figure 7C and 7D). Histological analysis of colon sections clearly shows that WT mice had very mild level of inflammation by day 9 of DSS administration while the epithelial damage and inflammatory infiltrate in colons from Cp<sup>−/−</sup> animals was similar to the mice of same genotype with undepleted microflora (Figure 8A). Unexpectedly, these results indicate that unlike WT controls, Cp<sup>−/−</sup> mice develop severe colitis even in the absence of intestinal microflora. Thus the DSS-induced damage and inflammation in Cp<sup>−/−</sup> mice is microbiota-independent. This finding also implies that bactericidal activity of Cp does not play a major role in colitis.
Figure 2.8. The colon of Cp-null mice develops extensive epithelial damage and higher colonic inflammation even with depleted intestinal microflora. (A) Hematoxylin and eosin staining of colon section of WT and Cp−/− mice on day 9 of DSS administration following antibiotics treatment. (B) The depletion of microflora was determined by counting SYBR green positive cells per random field. (C) Representative fields of SYBR green staining, X500 magnification. Error bars represent ± SEM.
Severe colitis in Cp$^{-/-}$ mice is not due to pathologic iron accumulation.

Neurodegenerative diseases such as aceruloplasminemia, Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease share a common significant feature: increased brain iron accumulation$^{150}$. Because Cp plays an important role in iron homeostasis by facilitating its loading into the apotransferrin, we wanted to test the possibility of iron accumulation in the colon, normal or inflamed, that could exacerbate experimental colitis. We stained colon sections for iron accumulation using DAB-enhanced Prussian blue stain. Liver sections from WT and Cp$^{-/-}$ mice were used as controls for low and high levels of tissue iron, respectively. DAB-enhanced Prussian blue staining of normal and inflamed colon sections from Cp$^{-/-}$ and WT animals did not reveal a detectable level of iron accumulation (Figure 9A). Although this method detects only ferric iron, it suggested that there are not any abnormal iron deposits in the colon before and during the inflammation that could potentially increase the severity of colitis.

Cp-null mice have higher protein oxidation during colitis.

Cp has been widely accepted as an antioxidant because of its ferroxidase activity that oxidizes toxic ferrous iron to the nontoxic ferric form, and it has been reported that Cp prevents free radical injury in the central nervous system$^{151}$. Likewise, Cp may also play a critical antioxidant role in the pathogenesis of experimental colitis by neutralizing highly reactive ferrous iron and limiting the extent of free radical-induced oxidation at the sites of inflammation. We tested this hypothesis by determining the level of protein carbonyl in the colon of Cp$^{-/-}$ and control animals upon induction of colitis.
Figure 2.9. (A) Prussian blue staining of colon sections on day 9 of DSS administration reveals no detectable accumulation of iron in mice with both genotypes. WT and Cp<sup>-/-</sup> liver was used as controls for normal iron storage levels and overload, respectively. 100X magnification. (B) Protein carbonyl content is substantially higher in the colon Cp<sup>-/-</sup> mice upon induction of experimental colitis (n = 4 per group per time point). Error bars represent ± SEM.
Protein carbonyl content (PCC) in supernatants of homogenized colons was estimated by spectrophotometric detection of hydrazone produced as a result of reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyl. Inflamed colon of Cp<sup>−/−</sup> mice on day 9 had three-fold higher PCC per colon than WT controls (Figure 9B), which indicates that colitis in Cp-null mice is characterized by higher level of protein oxidation. The above experiments indicate that the severity of experimental colitis in Cp<sup>−/−</sup> mice might result from the higher ROS activity unleashed in the absence of antioxidant function of Cp.

**Protective role of Cp in colitis may not be due to the Cp-MPO interaction.**

One of the proposed anti-inflammatory roles of Cp is its interaction with a highly destructive myeloperoxidase (MPO), an enzyme secreted by activated phagocytes. Widely used as a disease activity and neutrophil infiltration marker in DSS-induced colitis, MPO is known for its cytotoxic enzymatic activity which produces oxidants such as hypochlorous acid. In vitro studies have demonstrated that Cp binds and inhibits MPO and it has been speculated that this interaction might be a critical inflammatory control mechanism whereby Cp eliminates excessive activity of MPO. However, the in vivo impact of this interaction has not been demonstrated.

Taking advantage of the availability of MPO-null mice, we hypothesized that if the severity of disease phenotype observed in Cp<sup>−/−</sup> animals is due to Cp-MPO interaction, then the systemic absence of MPO should make mice more resistant to DSS regardless of the presence of Cp. Therefore, we induced colitis in MPO<sup>−/−</sup> mice and WT controls
Figure 2.10. Cp-MPO interaction and blood Cp do play a major role protective role in experimental colitis. (A) Percent weight change of WT and MPO$^{-/-}$ mice normalized by weight on day 0, and (B) the percent survival ($n = 10$) of MPO$^{-/-}$ and WT mice (3.5% DSS). (C) Percent weight change normalized to day 0 and (D) the percent survival of WT and Cp$^{-/-}$ animals injected with human Cp and continuous administration of 3% DSS ($n = 12$).
with 3.5% DSS lethal to the WT animals. Surprisingly, observed colitis in MPO<sup>−/−</sup> mice was similar in severity to the WT controls, and deficiency in MPO did not have a significant impact on resistance to DSS in terms of weight loss and survival during acute DSS-induced colitis (Figure 10A and 10B). Thus, contrary to the speculations that Cp-MPO interaction may play an important anti-inflammatory role, it is unlikely to account for the severe DSS-induced colitis phenotype in Cp<sup>−/−</sup> mice.

**Hepatic Cp does not protect against colitis.**

Mouse Cp bears 83% amino acid sequence identity to human Cp. Our laboratory had previously shown that purified human Cp injection into Cp<sup>−/−</sup> mice substantially increases serum iron and transferrin saturation and restores the activity of endogenous Cp in iron homeostasis<sup>7-32</sup>. The same study also demonstrated the stability and activity of intraperitoneally (I.P.) injected human Cp in Cp-null mice, and estimated the half-life of injected Cp to be about 30 hours. In addition, the injected human Cp was localized to the epithelia of small intestine<sup>7</sup>. Similarly, we attempted to rescue the C<sup>−/−</sup> mice from severe experimental colitis by injecting the animals with purified human Cp. Because the 400 µg of injected Cp stays at detectable activity levels in the blood for at least 48 hours<sup>7</sup>, we repeated Cp injections every other day for the course of acute colitis (two weeks) to mimic its synthesis in the liver and secretion to the bloodstream. It is very unlikely that I.P. injections of Cp would immunize the animals to human Cp and create neutralizing antibodies because the acute phase of the disease during which Cp<sup>−/−</sup> mice become moribund finalizes in 14 days. Such a short period of time and lack of proper adjuvants
should not lead to stimulation of a potent adaptive immune response and neutralization of over a milligram of injected human Cp.

We hypothesized that restoring circulating Cp with purified human Cp would reduce the severity of experimental colitis in Cp$^{-/-}$ mice. Cp$^{-/-}$ were given the first 500 µg Cp injection (I.P.) one day before DSS administration and injection was repeated every other day for up to two weeks during which animals were monitored for weight change and survival. Unexpectedly, Cp$^{-/-}$ mice repeatedly injected with Cp rapidly lost weight after day 5 and all became moribund within two weeks of continuous DSS administration (Figure 10C and 10D). This finding indicates that restoring circulatory Cp in Cp$^{-/-}$ mice and mimicking its secretion by liver does not have any anti-inflammatory impact and fails to reduce the severity of colitis in these animals. Since our previous studies demonstrated localization of injected Cp to the intestinal tract 7, we concluded that liver-derived Cp does not have anti-inflammatory function.

**Bone marrow-derived macrophages are the source of anti-inflammatory Cp.**

Failure to reduce inflammation in Cp$^{-/-}$ mice with Cp injections led us to conclude that anti-inflammatory effect of Cp is independent from its presence in plasma and may be derived from an extrahepatic source, e.g., spleen, lung, testes, or monocyte/macrophage lineage 13 14 154. The only source of Cp among the above candidates that would deliver the protein differently than others, e.g., by recruiting the secreting cells directly to the sites of inflammation, was monocyte/macrophage lineage. Therefore, we performed bone marrow transplantation on lethally irradiated mice and created four groups: chimeric animals, WT recipients with Cp$^{-/-}$ bone marrow and Cp$^{-/-}$ recipients with
WT bone marrow, as well as WT and Cp\textsuperscript{-/-} controls receiving WT and Cp\textsuperscript{-/-} bone marrow, respectively. All animals were rested for at least 6 weeks to allow the transplanted bone marrow to replace most of the hematopoietic cells of the host, and then given DSS. At the end of the 6-week period, all chimeric mice were screened for the presence of the transferred genotype, e.g., the blood of WT animals clearly had Cp\textsuperscript{+/-} genotype and the blood of Cp\textsuperscript{-/-} animals had WT genotype (data not shown), which shows that the transferred bone marrow successfully engrafted and completely replaced the host nucleated blood cells.

Interestingly, upon continuous treatment of these animals with DSS most of the chimeric mice developed the disease phenotype depending on the bone marrow genotype and independent from the presence of Cp in the rest of the body. WT animals with Cp\textsuperscript{-/-} bone marrow rapidly lost weight and almost all were moribund by day 14, whereas Cp\textsuperscript{-/-} mice with WT bone marrow had similar weight loss and survival parameter similar to those of WT control with WT bone marrow (Figure 11A and 11B). This result also supports Cp injection experiment since the WT animals with Cp\textsuperscript{-/-} bone marrow are expected to have WT levels of Cp in blood secreted by liver. In addition, we performed complete blood analysis of the recipients on days 0 and 9 of DSS administration and found that Cp\textsuperscript{-/-} recipients with WT bone marrow displayed lower hematocrit loss (normalized to day 0), corresponding to lower colonic bleeding, and lower WBC counts on day 9 compared to the WT recipients with Cp\textsuperscript{-/-} bone marrow (Figure 11C and 11D). In other words, colonic bleeding and WBC counts of Cp\textsuperscript{-/-} mice with WT bone marrow were similar to WT controls, whereas, the same parameters of WT mice
Figure 2.11. Bone marrow transplantation rescues Cp-/- and reverses the disease phenotype in chimeric mice. (A) Percent weight change of mice normalized to their weight on day 0. (B) Percent survival (n = 10). p < 0.05 using the ANOVA test. Hematologic analysis of blood of WT and Cp-/- mice showing (C) hematocrit (HCT) loss by day 9 (normalized to day 0) and (D) white blood cell (WBC) count (n = 5). p < 0.01 using the Student T test. Error bars represent ± SEM.
with Cp−/− bone marrow were similar to Cp−/− controls. Thus the source of anti-inflammatory Cp is bone marrow.

Because monocyte/macrophage lineage is the only known source of Cp, we attempted to rescue the Cp−/− mice from developing severe colitis by injecting them with WT thioglycollate-elicited peritoneal macrophages. Similar to the bone marrow transplantation experiment, there were four groups of macrophage recipients, Cp−/− mice injected with Cp−/− macrophages (Cp−/− controls), Cp−/− mice injected with WT macrophages, WT mice injected with WT macrophages (WT controls), and WT mice injected with Cp−/− macrophages. Each mouse received 2X10⁷ cells on days 0, 5 and 10 of DSS treatment. As a result, all Cp−/− controls rapidly lost weight after day 7 and became moribund by day 14, while all WT controls survived the DSS challenge for 30 days with about 12% weight loss during the first two weeks (Figure 12A and 12B). In contrast, all Cp−/− mice that were injected with WT macrophages were rescued regardless of the presence of Cp-null macrophages, e.g., these animals became as resistant to the DSS as WT controls (Figure 12A and 12B). However, all of the WT animals injected with Cp−/− macrophages survived the 30-day period of DSS administration, in a way similar to WT controls. Based on the above series of experiments we concluded that the source of anti-inflammatory Cp that makes Cp−/− mice less susceptible to DSS is bone marrow-derived macrophages. In addition, WT mice injected with Cp-null macrophages did not become susceptible to DSS presumably because these animals had endogenous macrophages with normal Cp production levels. This also implies that Cp-null macrophages are not
"hyperactive" and do not exert their function by excessively activating other inflammatory cells such as T cells.

Because estimated purity of macrophages isolated from the mouse peritoneum 4 days after thioglycollate elicitation is about 80% (Current Protocols in Immunology, 14.1.8), other contaminating cells, mainly, neutrophils, T cells, and dendritic cells, could potentially play a role in reversing the disease phenotype. Therefore, thioglycollate-elicited macrophages from WT and Cp⁻/⁻ mice were positively purified for F4/80 surface antigen by using magnetic bead separation technique (Mulenyi Biotec). The purity was estimated by flow cytometry for F4/80 and CD11b surface antigens to be more than 97% from both WT and Cp⁻/⁻ animals (Figure 13A). Similar to the previous experiment, each mouse was injected I.P. with F4/80⁺ cells on days 0, 5, and 10 of DSS treatment. However, the average number of cells per injection was 10⁷, half the number of cells injected in the experiment with "total peritoneal" isolate. Injection of F4/80⁺/CD11b⁺ WT cells into Cp-null animals also rescued them from lethally severe experimental colitis upon continuous administration of DSS.

In summary, the above series of experiments clearly indicate that regardless of the presence of circulatory Cp secreted by hepatocytes, animals with Cp-null macrophages develop severe and acute form of experimental colitis. Thus only macrophages recruited to the inflammatory sites express Cp that plays an anti-inflammatory role in experimentally induced colitis.
Figure 2.12. Injection of WT macrophages rescues Cp⁻/⁻ mice from lethal colitis. (A) Percent weight change of mice injected with total peritoneal isolate normalized to their weight on day 0 and (B) their percent survival (n = 10); p < 0.05 using the ANOVA test. (C) Percent weight change of mice injected with macrophage positively purified for F4/80 surface antigen normalized to their weight on day 0 and (D) their percent survival (n = 10); p < 0.05 using the ANOVA test.
**Resident and recruited colon macrophages express Cp.**

Together with the macrophage injection data, our findings in hCp injection and BM transplantation experiments indicate that in order for the Cp to exert its anti-inflammatory role, it has to be locally produced by macrophages in the diseased colon. To support this conclusion we examined at the level of Cp mRNA expression in epithelial layer of colon before and after the induction of experimental colitis. By performing qRT-PCR using primers specific to secreted form of Cp message and total RNA obtained from the above samples we found that the Cp expression in colon epithelia of WT mice treated with DSS for 9 days increases about 15 fold when compared to the level determined in WT mice on day 0 (Figure 13B). The message was not detectable in epithelium of Cp\(^{-/-}\) mice regardless of the DSS administration. However, the Cp expression was restored in epithelial isolates of Cp\(^{-/-}\) mice injected with WT macrophages to a level approximately 10 times higher than the WT controls on day 0.

It is noteworthy that the amount of Cp produced by all macrophages in a mouse is very small compared the Cp secreted into the bloodstream by hepatocytes. Then how can the macrophage Cp have an anti-inflammatory effect while the liver Cp does not? It is possible that the abundant blood Cp may not be able to reach the optimum concentration to provide antioxidant protection at the inflammatory sites in this colitis model. Another possibility for the anti-inflammatory Cp synthesized by macrophage may lie in its structural distinction from the liver Cp. There are only two known variants of Cp mRNA that translate into a protein: secreted form mainly made by hepatocytes and an alternately-spliced, GPI-linked form of expressed by brain astrocytes\(^{20, 21}\). Despite that
macrophages are known to produce only the secretory form of Cp, we hypothesized that Cp made by activated macrophages might also be GPI-linked and, thereby, differ from the canonical Cp secreted by liver. We designed primers specific for secreted and GPI-linked forms of Cp and, similar to the previous experiment, measured by qRT-PCR mRNA encoding GPI-Cp in colon epithelia of Cp^−/− and WT mice. Interestingly, GPI-linked Cp mRNA was detectable at expression levels similar to the secreted form (compare figure 13C to 13B). We detected the mRNA for membrane-bound and secreted Cp at 1:1 ratio in WT colon even before the administration of DSS. However, upon induction of colitis the GPI-Cp mRNA level did not change, and, with increasing mRNA encoding secreted Cp, the ratio decreased to 1:10. Total RNA obtained from liver and brain astrocytes were used as controls for secreted and GPI-linked encoding template, respectively. Astrocytes isolated from mouse brain expressed GPI-linked and secreted Cp at 1:10 ratio, whereas the same ratio was measured to be 1:500 in liver (data not shown).

Thus the significant amount of GPI-linked Cp mRNA in resident macrophages of the colon, and increase in secreted Cp mRNA upon induction of experimental colitis, demonstrates that Cp is effectively delivered to the sites of inflammation by resident and recruited macrophages of the colon. As a conclusion, this study reveals that anti-inflammatory function of Cp in DSS-induced colitis is macrophage-specific.
Figure 2.13. WT macrophages rescue Cp-/ mice by restoring Cp expression in the gut during experimental colitis. (A) Purity of elicited peritoneal macrophages used in macrophage injection experiments determined by flow cytometry. qRT-PCR for (B) secreted and (C) GPI-linked Cp mRNA normalized to WT day 0 expression. Error bars represent ± SEM. (D) The difference between the amino acid sequence of secreted and GPI-linked Cp at C-terminal and approximate location of RT-qPCR primers. Common /forward (red), reverse/secreted (green), and reverse/GPI-linked (blue). Adapted from Patel et. al. 21
CHAPTER IV
DISCUSSION

In this study we report that Cp plays a protective role in experimentally induced colitis and demonstrate that macrophages recruited to the sites of inflammation, and not the liver that produces its circulatory form, are the source of anti-inflammatory Cp. Our findings also suggest that the absence of antioxidant activity of Cp produced by macrophages is responsible for the severe disease phenotype observed in Cp-null mice treated with sublethal dose of DSS.

Increase in circulating levels of Cp during the acute phase of inflammation has been a hint for its anti-inflammatory function. Several studies have shown that Cp antioxidant activity may play an important role in neurodegenerative disorders such as Parkinson's and Alzheimer's diseases\textsuperscript{8,151,155}.

Our data coincide with a case report describing a Wilson's disease patient with very low level of Cp who developed a severe form of ulcerative colitis (UC) intractable to prednisolone and salazosulfapyridine treatment\textsuperscript{156}. Other complicated cases of UC in
Wilson's disease patients have also been reported as an unexpected incidence during a drug study unrelated to inflammation\textsuperscript{157}. Therefore, based on our findings and previously published research by other groups we speculate that, though they may not develop spontaneous and frequent inflammatory diseases, aceruloplasminemia and Wilson's disease patients with very low level of ceruloplasmin are likely to be prone to severe types of such diseases due to the lack of or deficiency in an important antioxidant enzyme expressed by macrophages, the ceruloplasmin.

Protein carbonylation is a widely used marker for oxidative modification of proteins by reactive oxygen species (ROS)\textsuperscript{158}. Higher protein carbonylation levels in the colon of Cp-null mice upon induction of ulcerative colitis reported in this study correlates with several clinical findings. First, evidence suggests that antioxidants may reduce the onset and severity of inflammatory bowel disease\textsuperscript{159}. Second, antioxidant activity has previously been presented as a key anti-inflammatory function of Cp and this role has equally attributed to the secreted liver form\textsuperscript{160} and GPI-linked form expressed by astrocytes in the brain\textsuperscript{161}. Third, Kaneko et al. have reported elevated protein carbonyl content in the brain of an aceruloplasminemic patient compared to a healthy control\textsuperscript{162} which implies that protein oxidation in Cp-null astrocytes might be an additional cause of neuronal damage in these patients besides pathologic iron accumulation.

Interestingly, another research group reported that aceruloplasminemic patients also have increased lipid peroxidation levels in cortex and putamen of the brain\textsuperscript{163}. Since elevated level of oxidation in brain of Cp-null patients were found independent of
inflammation, all of the evidence mentioned above indicate that higher levels of protein carbonylation in the colon of Cp-null mice with experimental colitis is likely to be the "cause", not the "result", of extensive epithelial damage and inflammation.

We also suggest that Cp plays a major anti-inflammatory role not at the interface of organism's immunity and environment, e.g., mucosa, but during the inflammation per se, once the destructive defense mechanisms have been activated. This is supported by the fact that Cp-null animals, just as the aceruloplasminemic and Wilson's disease patients with very low level of Cp, do not spontaneously develop frequent inflammatory abnormalities, but when the disease is triggered by some other factor, it turns into a severe type of inflammation. Cp may also be acting as a "fire-proof" component of macrophage exterior as they crawl through the sites of inflammation loaded with highly toxic reactive oxygen species, which leads to a speculation that this major plasma ferroxidase found on the surface of macrophages may be necessary for their survival in highly destructive inflammatory environment.

Since macrophages play an important either pro- or anti-inflammatory role not only in innate immune responses but also in the adaptive immunity, patients and animals with Cp-deficient macrophages may also be under risk of developing severe autoimmune diseases. However, this speculation has to be confirmed by more vigorous statistical and epidemiological studies on Wilson's disease and aceruloplasminemic patients as well as trying experimental autoimmune diseases in Cp-null mice.
Another correlation between our animals study and the related human disease data in the literature is in individuals and mice heterozygous for Cp deficiency. Mice heterozygous for Cp deletion have approximately half the normal plasma levels of Cp. We found that these animals are more resistant to DSS treatment than their homozygous littermates and display symptoms close to WT controls. This corresponds to heterozygous aceruloplasminemia patients with half the normal level of blood Cp who believed to be asymptomatic or develop substantially milder neurological diseases\textsuperscript{164}.

The role of T cells in experimental models of IBD varies depending on whether the disease is acute or chronic. Some evidence indicate that T cells regulate immunological response to luminal antigens in the intestines\textsuperscript{165} and chronic experimental colitis induced by alternating DSS administration with water is characterized by both Th1 and Th2 cytokines\textsuperscript{166}. On the other hand, the acute type of the same animal model could be induced in severe combined immunodeficiency (SCID) mice that lack T and B cells which implies that acute experimental colitis is T cell-independent\textsuperscript{167}. Since the model used in our study was based on the acute form of the disease, it is unlikely that the anti-inflammatory Cp may perform its function via modulating T cell response.

The immunohistochemical staining for CD3+ T cells did not reveal excessive T cell infiltration in colon of Cp-/- mice compared to the colon of control animals throughout the acute phase of DSS-induced colitis (data not shown). Moreover, the macrophage injection experiment, e.g., injection of Cp-null macrophages into WT mice, also supports this conclusion because the receivers were still resistant to DSS. If Cp-null macrophages
were able to hyperactivate T cells, or any other effector cells, the WT mice with excess numbers of injected Cp-/- F4/80+ cells would develop severe colitis similarly to the Cp-/- animals. Finally, there is no evidence that Cp may have any effect on T cell function at normal homeostatic condition or during inflammation. The fact that inflammatory signaling-deficient mice such as those with a null mutation in MHC class II, T cell receptor-α chain, interleukin-2, or interleukin-10, spontaneously develop chronic intestinal inflammation, also denies Cp's interference with or involvement in inflammatory signaling because neither Cp-null mice nor aceruloplasminemic patients have frequent and spontaneous IBD.

Persistent DSS-induced colonic inflammation in Cp-null mice following antibiotic treatment and depletion of more 99.5% commensal microflora correlates with the function of alternatively-activated macrophage, that is, tissue repair. Cp-deficient anti-inflammatory macrophages are likely to be less capable of cleaning the DSS induced damage and suppressing the resulted oxidant activity which triggers the onset of colitis even in the absence of gut microbiota.

Since the discovery of Cp-MPO interaction there has been no in vivo study that would demonstrate its significance. Although the MPO deficiency in mice may have consequences far beyond Cp-MPO interaction it is still unlikely that this interaction plays a major role in the colitis phenotype of Cp-/- animals because of the surprising protective role of MPO in inflammatory disease such as atherosclerosis and experimental autoimmune encephalomyelitis.
Membrane-bound, alternatively spliced from of Cp was discovered in the brain astrocytes\textsuperscript{20}. Because these glial cells has been the only known major source of GPI-linked Cp, its anti-inflammatory potential was limited to neurological disorders. Because Cp expressed on the surface of macrophages may localize to virtually any inflammatory site in the body, our study ascribes a wider role to the minor membrane-bound form of abundant plasma ferroxidase in inflammatory diseases.

Interestingly, a trace amount of GPI-linked Cp is also found in liver, a major well-known source of the secretory form\textsuperscript{21}. Since our study indicates that the entire macrophage/monocytes lineage may be a potential source of membrane-bound Cp, Kupffer cells, the resident macrophages of the liver, might be the origin of Cp's GPI-linked message detected in liver.

The amino acid sequence of canonical secreted Cp differs from the GPI-linked form by a very short C-terminal sequence; 5 residues of the secreted form are replaced with 30 amino acids of the GPI signal\textsuperscript{21}. Since this is the only known difference between membrane-bound and secreted forms of Cp, how can the latter lack the anti-inflammatory function? Because the rest of the amino acid sequence (more than one thousand) is the same, it is unlikely for the two forms to have distinct catalytic activities. Therefore, we hypothesize that the local accumulation of Cp around activated macrophages sustained in part by the GPI-linked form is crucial for the anti-inflammatory function of macrophages. Although such a role is attributed to the alternatively-activated macrophages, it is yet to be determined whether the anti-inflammatory function of Cp is specific to a particular
subpopulation of these inflammatory cells. We also speculate that the source of protective 
Cp in neurodegenerative diseases\textsuperscript{170} is also monocyte/macrophage lineage and not the 
liver. Therefore, we propose that creation of tissue-specific expression of ceruloplasmin 
will elucidate the role of this abundant and complex enzyme in multiple mechanisms. 
Further investigation is also required to confirm that the two alternatively spliced forms 
have the same enzymatic activity and that anti-inflammatory function of Cp expressed by 
macrophages is GPI-linked. This could be achieved by creating macrophages capable of 
expressing only one form of the protein.

As a conclusion, we propose that both alternatively spliced forms of Cp synthesized 
by macrophages play important anti-inflammatory role. The major duties of Cp's secreted 
form produced by hepatocytes and abundantly present in the plasma, are facilitating iron 
transport, neutralize antioxidants entering bloodstream, and keep blood MPO inactive. 
The GPI-linked Cp expressed by activated macrophages recruited to the sites of 
inflammation, where the plasma form has very limited access, may be crucial for 
neutralization of certain reactive oxidant species in the close vicinity to macrophages and, 
thereby, protecting and/or assisting them in the tissue repair.
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