Mechanisms Underlying Opioid Modulation of Gut Immunity

A DISSERTATION
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Jingjing Meng

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Advisor: Sabita Roy, PhD

August 2014

ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge my advisor Dr. Sabita Roy. Completion of my thesis work would have been impossible without Dr. Roy's endless inspiration, encouragement and guidance through my whole graduate training. I feel extremely fortunate to have had the opportunity to work with such a patient, warm-hearted, and intelligent instructor and scientist. Her invaluable advises on research, presentation, and writing has taught me numerous lessons and insights on the workings of academic research. My thanks also go to the members of my thesis committee Dr. Sundaram Ramakrishnan, Dr. Ping-Yee Law, Dr. Thomas Molitor, and Dr. Sabita Roy. I'd like to give my sincere appreciation for many insightful comments and ideas that improved the contents of this dissertation.

I also would like to thank all the past and present members of Dr. Roy's Lab: Santanu Banerjee, Jing Ma, Raini Dutta, Umakant Sharma, Shamsudheen Moidunny, Greg Sindberg, Fuyuan Wang, Anuj Saluja Dan Li, Vidhu Anand, Haidong Yu, Jinghua Wang, Jana Ninkovic Lisa Koodie, and Richard Charboneau. Thank you all for the generous support, assistance, and suggestions.

Thanks to the Department of Pharmacology staff and faculty, as well as Directors of Graduate studies Dr. Jonathan Marchant, Dr. Gibson Wood, and Dr. Collin Campbell. Thanks to the graduate school of University of Minnesota for doctoral dissertation fellowship. Finally, I would like to thank my parents and my grandparents who always encouraged me to pursue my dream. Thank you so much for your unconditional support and love. Thanks to all my friends for all the support and all the fun times over the years.

DEDICATION

This thesis is dedicated to my parents and my grandparents for their sacrifice, tolerance, never ending love and continuous support.

ABSTRACT

Opioids are used widely by clinicians due to their potent analgesic activities and sedative properties. However, opioid use or abuse is associated with multiple adverse gastrointestinal (GI) symptoms and higher susceptibility to infection caused by pathogens with gut origin. Both clinical and laboratory studies implied that opioids showed suppressive effects on gut immunity and predisposed critically sick patients to infections while the mechanism underlying this defect is still unknown. In the present study we investigated how opioids modulate gut epithelial barrier function and immune responses of gut associated lymphoid tissue (GALT). We demonstrated significant bacterial translocation from gut lumen to mesenteric lymph node (MLN) and liver following morphine treatment in wild-type (WT) animals that was significantly attenuated in Toll-like receptor (TLR2 and 4) knockout mice. We further observed significant disruption of tight junction protein organization only in the ileum but not in the colon of morphine treated WT animals. Inhibition of myosin light chain kinase (MLCK) blocked the effects of both morphine and TLR ligands, suggesting the role of MLCK in tight junction modulation by TLR. Additionally we determined the immune responses of GALT to polymicrobial sepsis in the presence and absence of opioids by using a murine cecal ligation and puncture model. The results showed that opioids accelerated the mortality rate of polymicrobial sepsis. During sepsis progression, morphine treatment altered gut microbiome and subsequently promoted gram-positive bacterial dissemination, which induced excess IL-17A production in a TLR2-dependent manner, resulting in increased gut permeability, sustained inflammation and higher mortality. This study improved our

understanding of the role of morphine in modulating gut barrier functions and the roles of GALT in infection susceptibility, which may provide the potential therapeutic targets for novel drug development and lead to more powerful strategy to control or prevent severe infectious diseases like sepsis especially in the opioid using and abusing population.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1 BACKGROUND-LITERATURE REVIEW

CHAPTER 2 OPIOID TREATMENT MODULATES GUT EPITHELIAL BARRIER FUNCTION

CHAPTER 3 OPIOID TREATMENT MODULATES IMMUNE RESPONSES OF GALT

CHAPTER 1

BACKGROUND-LITERATURE REVIEW

1. OPIOIDS AND ADVERSE GASTROINTESTINAL (GI) SYMPTOMS

A. Clinical applications of opioids

Opioids are the drugs of choice for pain management in different clinical conditions due to their strong analgesia activities and sedative properties (Devlin and Roberts 2009). Opioid-induced analgesia is mediated by activation of opioid receptors, which are widely distributed within the central nervous system and throughout the peripheral tissues(Devlin and Roberts 2009; Baldo and Pham 2013).

To reduce pain and anxiety before and during the surgical procedure, high doses of morphine (40mg) or fentanyl (600μg) are used especially for cardiac surgery(Murphy et al. 2009). Furthermore, opioids are also a mainstay of therapy for postoperative pain and other forms of acute pain in hospitalized patients(Viscusi and Pappagallo 2012). The use of patient-controlled analgesia with intravenous opioid like morphine and the use of oral opioid like oxycodone are the main strategy for the management of acute postoperative pain. For ICU patients, morphine, fentanyl, and hydromorphone are the most frequently administered opioids for alleviating pain and facilitating mechanical ventilation(Devlin and Roberts 2009).

Besides acute pain management, long-term opioids are also the most common means of treatment for chronic pain. Today, 15% to 20% of office visits in the United States

now include the prescription of an opioid, and 4 million Americans per year are prescribed a long-acting opioid(Manchikanti et al. 2012). Most patients eventually take opioid dosages equivalent to more than 100 mg of morphine per day for many years(States 2014). Opioids including morphine, hydromorphone, oxycodone, hydrocodone, codeine, fentanyl, and methadone are generally used to relieve moderate to severe pain in cancer patients(Plante and VanItallie 2010). In clinical studies combining methadone with another opioid showed improved pain control and less adverse effects. Recently various novel formulations of opioids like fentanyl pectin nasal spray and transdermal fentanyl matrix patch are showing great promise for the treatment of cancer pain(Davis and Walsh 2014; Kress et al. 2008).

Unfortunately, the liberalization of laws governing opioids prescriptions for the treatment of chronic non-cancer pain by the state medical boards has led to dramatic increases in opioid use over the past 20 years. Patients with inflammatory bowl disease, rheumatoid arthritis, and pancreatitis usually get opioid prescription for pain management. However there is debate about whether opioids are appropriate for the treatment of chronic non-cancer pain due to a substantial risk for abuse potential and other adverse events(Manchikanti et al. 2012; Dunn et al. 2010). Therefore, more studies are still needed for assessment of the benefits and risks of opioid treatment in various clinical conditions.

B Opioids and immunosuppression

Although opioids are widely used for pain management, their adverse effects like abuse potential, respiratory suppression and immunosuppression limit their long term use.

Over the last century, opioid treatment has been shown to modulate both innate and adaptive immunity via direct and indirect pathways(Sabita Roy et al. 2011). Human and animal studies have provided a large amount of evidences supporting that opioids can modulate the functions of innate immune cells such as neutrophils, macrophages, and dendritic cells. Neutrophils are the first line of defense against invading pathogens. Their recruitment to infection sites is crucial for effective pathogen clearance. Recent studies have demonstrated that chronic morphine treatment results in significant delays and reduction in neutrophil recruitment by altering expression of chemokine like keratinocyte derived cytokine(Martin et al. 2010). Morphine has been shown to impair bactericidal function, leading to inadequate bacterial clearance and thereby increased bacterial persistence in the host. The mechanisms underlying this impairment has been shown to be due to inhibition of superoxide production(Simpkins et al. 1986; Sharp et al. 1985). Like neutrophils, macrophages are another member of innate immune cells responsible for bacterial clearance via their phagocytic and bactericidal activities. Morphine treatment has been shown to inhibit phagocytosis by inhibiting actin polymerization and to attenuate bacterial killing by inhibiting reactive oxygen and NO release(Ninković and Roy 2012; Ninkovic 2011). In addition to direct suppressive effects on phagocytic and bactericidal functions, morphine treatment also reduces the number of macrophages by decreasing the proliferative capacity of macrophage progenitor cells and enhancing macrophage apoptosis(Singhal et al. 1998; Sabita Roy et al. 1991). Dendritic cells are one of the most important antigen presentation cells responsible for the initiation and control of the adaptive immune response. Studies show that morphine inhibits IL-23 production

by murine dendritic cells resulting in compromised pulmonary mucosal host defense against *Streptococcus pneumonia*e infection(Ma et al. 2010).

Additionally, adaptive immune cells like T cells and B cells are also the targets of opioid treatment. In early publications, a decrease in splenic and thymic weight following morphine treatment has been observed, implying impaired lymphocyte function(LeVier et al. 1994). Morphine has been shown to modulate different aspects of adaptive immune response, including antigen presentation, T cell activation and lymphocyte migration(Beagles, Wellstein, and Bayer 2004; Wang et al. 2001). Our laboratory has shown that morphine inhibits IL-2 transcription in activated T cells in both mouse models and human cells(Wang, Barke, and Roy 2007) and that morphine skews T helper lineage towards Th2 phenotype (Roy et al. 2001).

The global effects of opioids on various innate and adaptive immune cells have been well studied over the last century. Recently, pioneering researches on the mucosal immune system have revealed distinct features of gut associated lymphoid tissues that are unique when compared with other traditional lymphoid organs. However, the effects of opioid treatment on these specialized mucosal associated lymphoid tissues have not been well characterized yet and remain a significant gap in the knowledge. There is an urgent need to fill the gaps in our understanding of the functions of GALT and its roles in infection susceptibility, which may lead to more powerful strategy to control or prevent severe infectious diseases like AIDS and sepsis in the opioid using and abusing population.

C Opioid receptor distribution in gut

Opioids exerts their actions by binding to several subtypes of opioid receptors, including (i) μ-receptors, (ii) δ-receptors, (iii) κ-receptors and (iv) non-classical opioid receptors(Waldhoer, Bartlett, and Whistler 2004). Originally, opioid receptors were thought to express only in central nervous system. However, more and more studies provide evidence that opioid receptors are also expressed widely in peripheral tissues such as GI tract. Several groups have attempted to characterize opioid receptor expression in peripheral tissues using quantitative real-time RT-PCR. Their results showed that all μ-, δ-, and κ-receptors have low expression levels in the small intestine(Peng, Sarkar, and Chang 2012). Using a similar approach in rats further validated the occurrence of μ-, δ-, and κ-receptors in both small and large intestine(Wittert, Hope, and Pyle 1996). In gut tissues, opioid receptors are mainly expressed in the enteric nervous system, which comprises of the myenteric and submucosal plexus. By binding to the opioid receptors in enteric nervous system, various endogenous and exogenous opioids are able to modulate GI motility and secretion. Besides ENS, opioid receptors expressed on immune cells have been shown to play a role in intestinal inflammation(Philippe et al. 2003; McCarthy et al. 2001). More recent studies report that the enterocytes isolated from the crypt epithelium of guineapigs have both u- and δ- opioid receptors(Lang et al. 1996). Opioid receptor expression in intestinal epithelial cells is further supported by Rousseaus's study, in which they showed that opioid receptors in intestinal epithelial cells could be up-regulated by *lactobacillus*,

which may provide novel approaches for the treatment of abdominal pain(Rousseaux et al. 2007).

By activating these opioid receptors in intestinal tissues (Figure 1.1), opioids exert their pharmacological and adverse effects in gut, including alleviating abdominal pain, modulating GI motility, and suppressing intestinal immune functions.

FIGURE 1.1

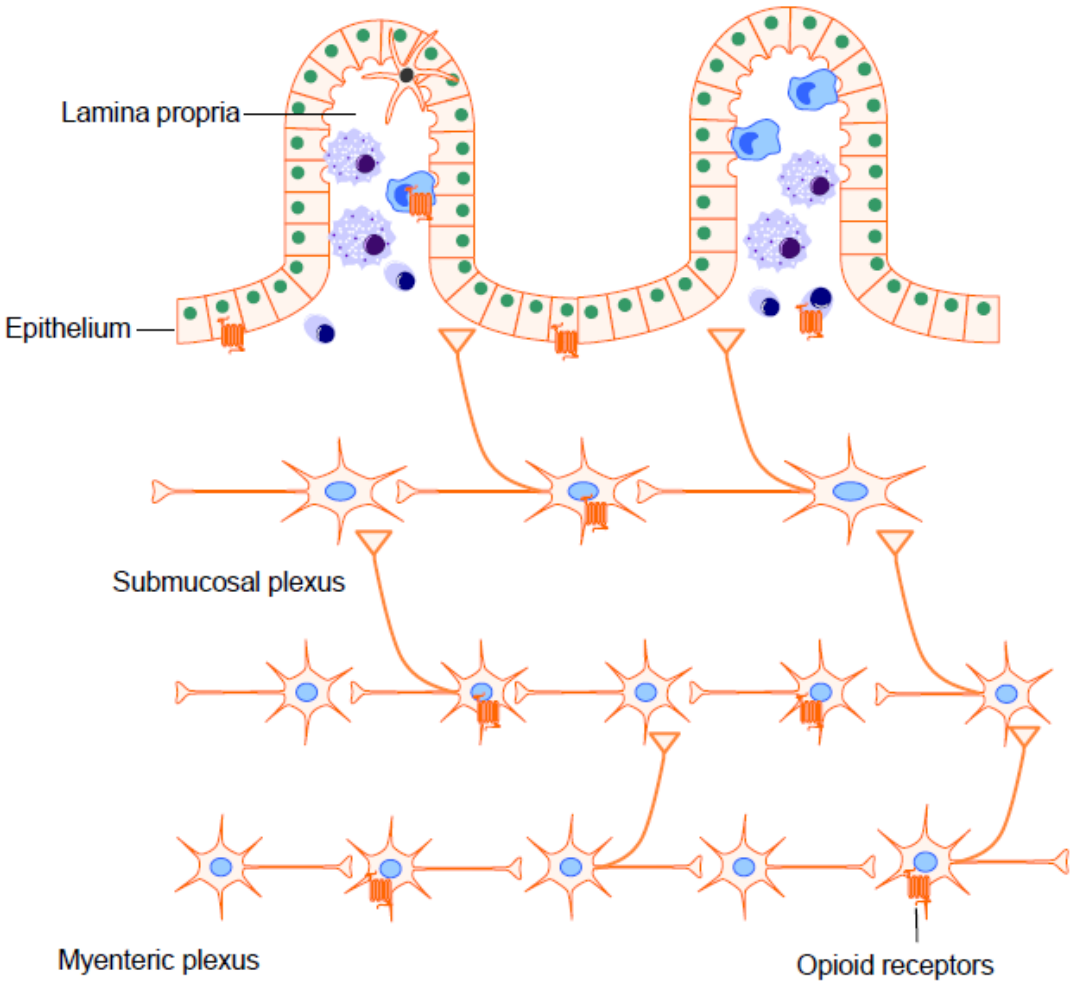


FIGURE 1.1 Opioid receptor distributions in intestines. Opioid receptors are not only expressed on neutrons in the myenteric plexus and submucosal plexus, which are involved in modulating gut motility and secretion but are also expressed on intestinal epithelial cells and immune cells, implying that opioids could impact the gut barrier function and immune responses as well.

D Adverse GI effects associated with opioid administration

Adverse GI symptoms like nausea, vomiting, constipation, and bloating are the major reasons for discontinuation of opioid use (Figure 1.2), leading to inadequate pain control, poor quality of life of patients, and bad clinical outcomes(Miaskowski 2009; Tuteja et al. 2010; Khansari, Sohrabi, and Zamani 2013). Nausea and vomiting are one of the common side effects associated with opioid analgesics. Multiple mechanisms are involved in opioid-induced nausea and vomiting, including direct stimulation of the chemoreceptor trigger zone (CTZ) for vomiting, inhibition of gut motility, and stimulation of the vestibular apparatus. Opioids are small molecules, which can cross blood-brain barrier and stimulate CTZ via the activation of μ and δ receptors. Opioid inhibition of gut motility results in gut distension, extended GI emptying time, and constipation, which stimulates visceral mechanoreceptors and chemoreceptors to induce nausea and vomiting. Other studies suggest that opioids bind to μ receptors in the vestibular apparatus directly and the stimulation of vestibular apparatus results in nausea and vomiting. Today there are several classes of dopamine blockers, anticholinergic agents, serotonin antagonists, and prokinetic agents available for treating nausea and vomiting. However, since the emetogenic mechanism involved in specific opioids is usually complex due to their different specificity for opioid receptors and various pharmacokinetic properties, it is important to identify the underlying cause of nausea and vomiting from among the multiple causative mechanisms for each patient so that effective treatment can be chosen(Porreca and Ossipov 2009).

Another adverse symptom associated with opioid treatment is a decrease in GI

motility. The normal motor functions of the GI tract are crucial for mixing and propelling food particles at rates that allow absorption of nutrients, cleaning the proximal intestine of residual food and bacteria, and enabling mass movement. Thus, the bowel dysfunction induced by opioid treatment may lead to serious consequences in patients(Ukleja 2010). There are multiple mechanisms contributing to opioid-induced decreased GI motility: Opioids are able to interact with the opioid receptors on presynaptic nerve terminals in the myenteric plexus to initiate signals that increase intestinal resting tone to the point of spasm while decreasing propulsive peristaltic waves. Moreover, by binding to μ receptors within the ENS, opioids treatment could increase activity of the sympathetic nervous system and inhibit vasoactive intestinal peptide release thus affecting gut secretion and absorption. The overall decreased gut secretion causes a delay in digestion, increased water and sodium reabsorption and formation of dryer and harder stools, which can contribute to prolonged transit of the intestinal contents through the GI tract. There are two major types of GI motility disorder limiting the clinical application of opioids: postoperative ileus and constipation. Prolonged postoperative ileus associated with opioid treatment might lead to increased morbidity, increased risk of postoperative nausea and vomiting, and delayed return to enteral feeding(Miaskowski 2009). Constipation is another type of GI motility disorder occurring with chronic opioid treatment. Although laxatives or opioid antagonists can alleviate constipation, their efficacy is still insufficient(Tuteja et al. 2010).

Despite the observation that opioid receptors are expressed on immune cells within the intestinal lamina propria, the effects of opioids on intestinal immune function and

inflammation are relatively sparce. Studies using animal models show that both chronic morphine and morphine withdrawal can lower host defense to enteric bacteria such as *Salmonella enterica* and *Pseudomonas aeruginosa*. Opioids can induce bacterial translocation into the systemic system resulting in sepsis in mice. Recent study show that opioids induced susceptibility to *Acinetobacter baumannii* or following LPS exposure sensitized mice to septic shock and accelerated mortality (LPS)(Hilburger et al. 1997; Babrowski et al. 2012; Feng et al. 2006; Ocasio et al. 2004; Breslow, Monroy, Daly, Meissler, Adler, et al. 2011). In addition to bacterial translocation, morphine has been documented to increase pro-inflammatory cytokine production in rats and accelerate the progression of LPS-induced sepsis to septic shock(Greeneltch et al. 2004a; Ocasio et al. 2004; S Roy, Charboneau, and Barke 1999). In clinical studies, higher circulating morphine levels were observed in patients with sepsis, severe sepsis, and septic shock compared with healthy controls, implying the role of opioids in the development of different infections(Glattard et al. 2010). Overall, both clinical and laboratory studies provide evidence that μ-opioid receptors are involved in the development and progression of various infectious diseases related to gut pathogens. However, the mechanisms underlying compromised gut immune function and increased susceptibility to infections after opioid treatment have not been well studied yet. The disrupted GI motility may contribute to increased susceptibility to infections with gut origin considering that impaired peristalsis results in accumulation of residual food and bacteria in gut lumen. Clinical studies imply that the early enteral feeding could enhance immune function and decrease the risk of infections in postoperative patients, so delayed enteral feeding

because of opioid treatment and subsequent ileus may be responsible for increased risk of infection. Besides the indirect effects mediated by GI peristalsis, other direct effects of opioids on epithelial cells and immune cells in intestinal lamina propria and gut associated lymphoid tissues also play important roles in impaired gut immune function. Opioid modulation of GALT will be discussed in the following chapter.

FIGURE 1.2

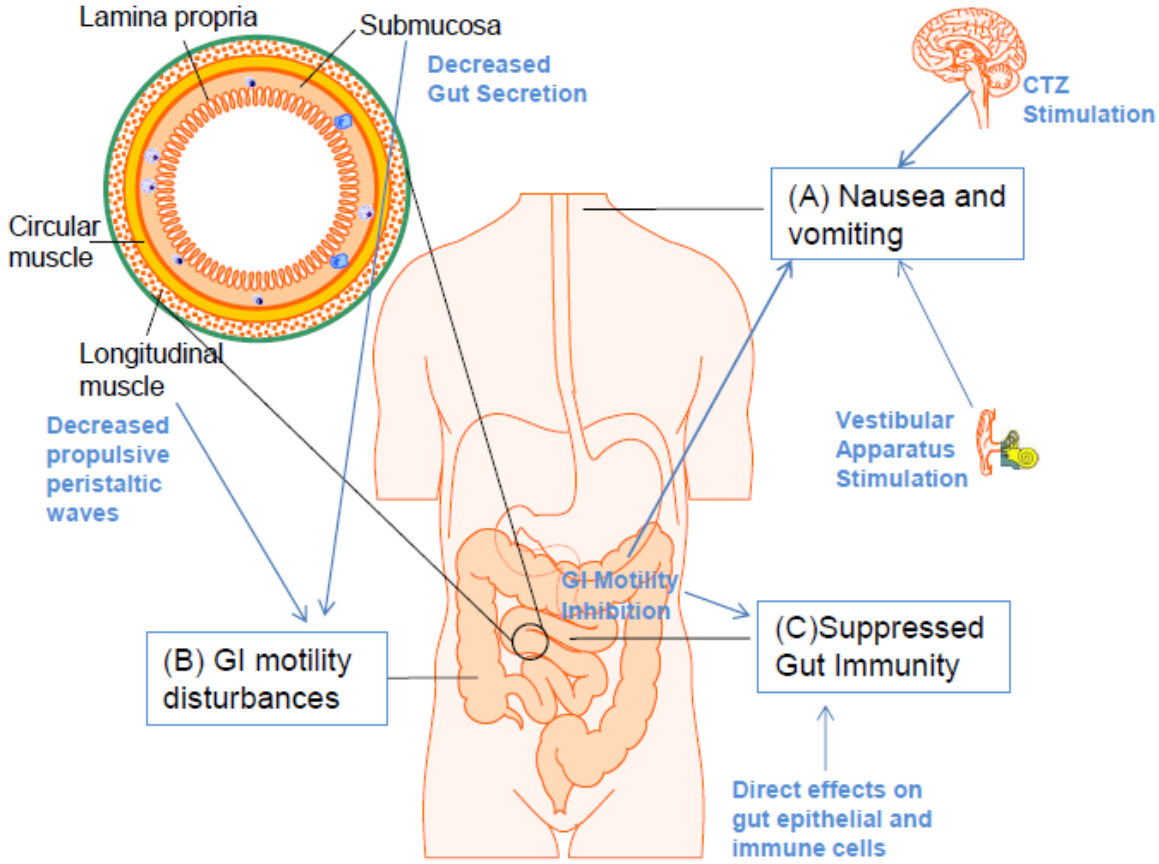


FIGURE 1.2 Adverse GI effects associated with opioid administration. (A) Opioid treatment induces nausea and vomiting via CTZ stimulation, vestibular stimulation, and GI motility inhibition. (B) Opioid treatment induces GI motility disturbances by decreasing propulsive peristaltic waves and inhibiting GI secretion. (C) Opioid treatment suppresses gut immunity by influencing gut epithelial cells and immune cells directly or by inhibiting GI motility.

2. OVERVIEW OF GUT IMMUNOLOGY

To understand the mechanism underlying opioid-induced immunosuppression in gut, the functions of intestinal epithelium, immune cells, and the microorganisms resident in gut lumen should be considered.

A The structure of gut epithelium

The intestinal epithelium is the first line of defense in the gut luminal environment. The well-organized intestinal epithelial cells not only provide physical barrier preventing potential pathogen or antigen invasion, but also play an important role in supporting nutrient and water transport and maintaining the homeostasis of the whole organism(Marchiando, Graham, and Turner 2010). The small intestine is where most chemical digestion takes place and responsible for absorption of most of the nutrients from ingested food. Besides digestion and absorption, the small intestine also plays an important role in immune surveillance and defense. To fulfill the multiple functions, the stem cells located in the crypt will differentiates into four cell types: i) absorptive enterocytes, ii) enteroendocrine cells, iii) goblet cells, and iv) paneth cells (Figure1.3)(Barker et al. 2007; Bullen et al. 2006). Absorptive enterocytes and enteroendocrine cells in the villus are responsible for digestion and absorption. The goblet cells will migrate to the villus from crypt after differentiation and produce large amounts of mucins, which is the major component of the mucus layer lining the intestinal epithelium(Linden et al. 2008). Paneth cell stays in the base of the crypt after differentiation and is the major source of anti-microbial peptides like α-defensins. The α-

defensins are cysteine-rich cationic peptides with antibiotic activity against a wide range of bacteria and other microbes. Studies of transgenic and knockout mice have supported a pivotal role of Paneth cell α-defensins in protection from bacterial pathogens(Porter et al. 2002). Another specialized epithelial cell occurring in small intestine is called the M cell (or microfold cell). M cells are found in the follicle-associated epithelium overlaying the peyer's patches. Unlike other intestinal epithelial cells, there is no mucus covering M cells, thus it plays a unique role in antigen presentation(Mabbott et al. 2013). In addition to epithelial cells differentiated from the crypt epithelial stem cells, some immune cells are also interspersed between epithelial cells. These include the dendritic cells and intraepithelial lymphocytes. In the intestines, dendritic cells can penetrate the epithelium to actively sample the antigens from the mucus layer(Rescigno and Sabatino 2009). The intraepithelial lymphocytes are mainly involved in insuring the integrity of gut epithelium and maintaining the balance between normal immune responses and excessive inflammation(Cheroutre 2005).

The major function of the colon is reabsorption of water and any remaining soluble nutrients from the food. There is no villus in the colon because it doesn't need as much surface area as the small intestine. Therefore, the epithelial stem cells are usually located in the lower parts of gut crypts and the differentiated cells migrate to the higher position and populate the colonic epithelial surface(Barker et al. 2007). To avoid paracellular leak of bacteria from gut lumen, the intercellular junctions known as tight junctions join adjacent cells together. These will be discussed in the following chapter.

FIGURE 1.3

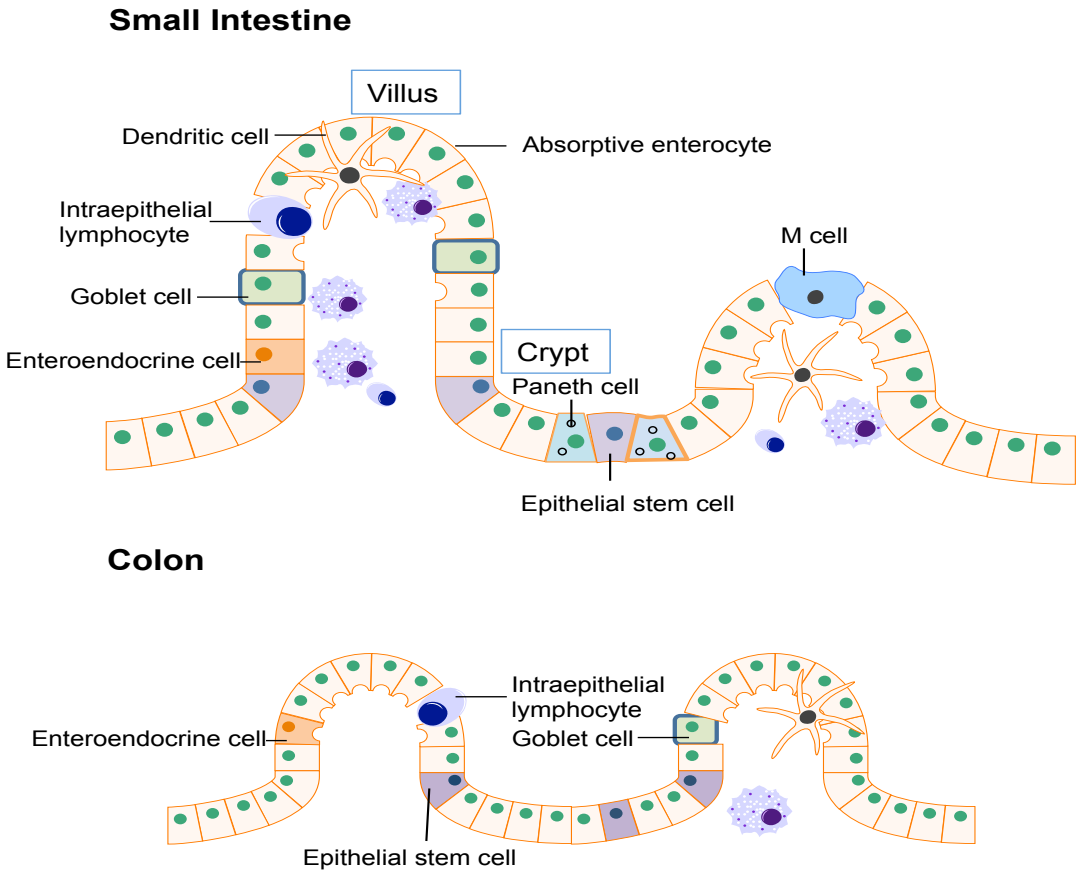


FIGURE 1.3 Structure of gut epithelium. In the small intestine, the epithelial stem cells located in the crypt will differentiate into four cell types: the absorptive enterocytes, enteroendocrine cells, goblet cells, and paneth cells. In the colon, the epithelial stem cells are usually located in the lower parts of gut crypts and the differentiated cells migrate to the higher position and populate the colonic epithelial surface.

B Immune cells in lamina propria, GALT, and MLN

Different types of immune cells are distributed in lamina propria, mesenteric lymph node, or gut associated lymphoid tissues (GALT) like Peyer's patches (PPs), and isolated lymphoid follicles. The major immune cell population within gut tissues includes macrophages, dendritic cells, mucosal mast cells, neutrophilic granulocyte, eosinophilic granulocyte, T lymphocytes, and plasma cells. These cells can modulate the intestinal microenvironment by secreting antibodies, cytokines, or chemokines as well as interaction with ENS.

In both humans and rodents, Peyer's patches are organized lymphoid nodules usually found in the ileum and less frequently in the jejunum. PPs are usually covered by follicle associated epithelium which contains special M cell. M cell can sample antigen directly from the gut lumen and deliver it to antigen-presenting cells like dendritic cells. T cells, B-cells and memory cells in PPs are stimulated upon encountering antigen. These cells then pass to the mesenteric lymph nodes to amplify the immune response. PPs are not present in the colon. Isolated lymphoid follicles provide immune surveillance and protection against the potential pathogen instead of PPs (Lycke and Bemark 2012; Suzuki et al. 2010; Koboziev, Karlsson, and Grisham 2010).

In the lamina propria, antigen presenting cells including dendritic cells, macrophages, and neutrophils. These cells recognize the antigens from the gut lumen and present them to adaptive lymphocytes (T cells and B cells) to initiate the subsequent responses. Various cytokines and chemokines produced by these APCs are also involved in the regulation of intestinal inflammation (Farache et al. 2013). The major populations

of T cells in the lamina propria are the CD4 T Cells, predominated by Th1 and Th17 population. Th1 cells produce IFN-γ, a cytokine important for the control of virus infection and responsible for the pathogenesis of inflammatory bowel disease. Th17 cells produce Th17 cytokines including IL-17A, IL-17F, and IL-22(Shale, Schiering, and Powrie 2013). Th17 cytokines play an important role in host defense against extracellular pathogens, which will be discussed in the following part. IgA+ plasma cells are the dominant population in the lamina propria. The secretory IgA produced by plasma cells can neutralize toxins and mediate ingestion of pathogen via opsonization(Suzuki et al. 2010).

The mesenteric lymph node (MLN) drains PPs and isolated lymphoid follicles via afferent lymphatics. By receiving the signals presented by dendritic cells, MLN amplify the immune responses. In this way, MLN plays a key role in tolerance induction to food proteins and in host defense against the pathogens from the gut lumen(Mason et al. 2008).

FIGURE 1.4

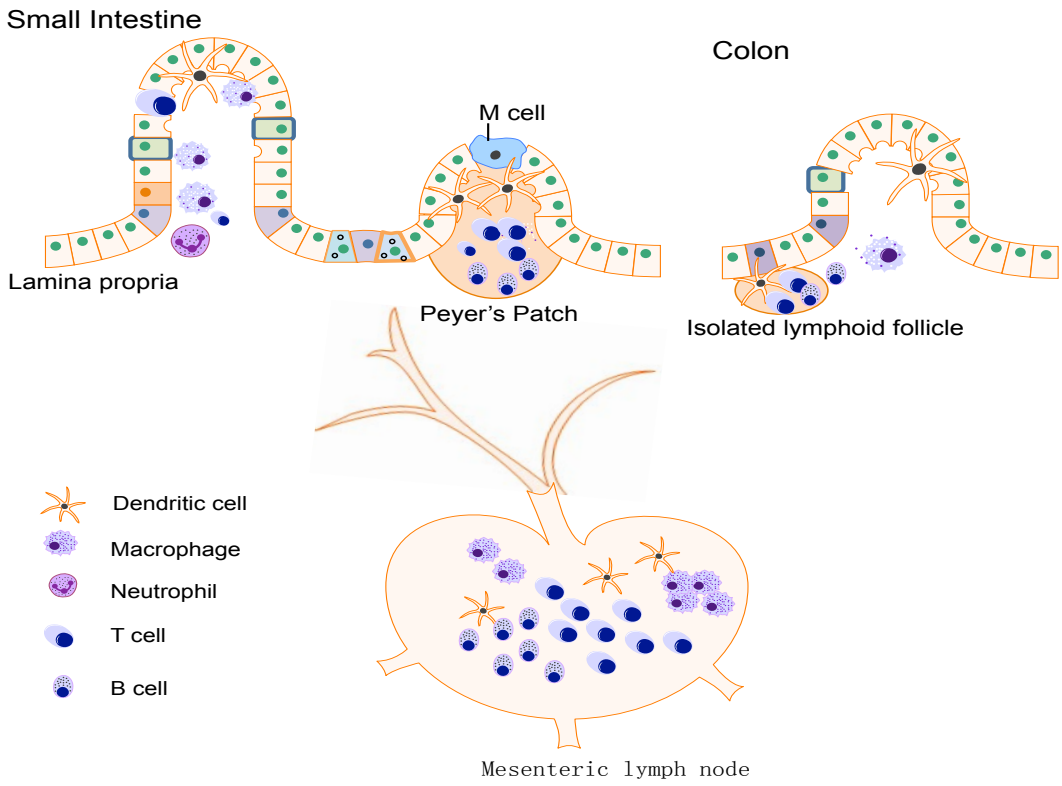


FIGURE 1.4 Composition of gut immune system. The major immune cell population within gut tissues includes macrophages, dendritic cells, neutrophilic granulocyte, T lymphocytes, and B-lymphocytes. The Peyer's patches in the small intestine and the isolated lymphoid follicle in the colon are composed of dendritic cells, B cells and T cells, which are crucial for antigen presentation from gut lumen.

C Gut microbiota in gut immunity

Gut commensal microbiota is an important compartment of gut immune system usually neglected previously. They play an important role in maintaining the intestinal homeostasis (Figure 1.5). Firstly, they are able to metabolize the toxic xenobiotic agents into harmless metabolite. They are also able to exclude the potential pathogens in the gut lumen(Sekirov et al. 2010). Secondly, they are involved in formation of mucus layer outside the epithelium. Previous studies have shown that microbial flora influences the number of goblet cell, their mucin production, and the glycosylation of mucins(Gaskins 2001). Moreover, they are also essential for the development of lymphoid tissues like PPs and modulate T cell and B cell responses(Lee and Mazmanian 2010). For example, most commensal bacteria have no direct contact with intestinal epithelial cells except for the segmented filamentous bacteria (SFB), which are able to adhere to epithelial cells especially to those in PPs. Interestingly, recent studies show that the mice without SFB have significantly less IL-17 production in their intestines, implying the important role of SFB in Th17 differentiation(Ivanov et al. 2009). Not surprisingly, lack of gut commensal microbiota will result in abnormalities such as increased susceptibility to infectious diseases. For example, germ-free mice showed increased susceptibility to *Salmonella* due to impaired IgA production and diminished T cell response(Hapfelmeier et al. 2010).

FIGURE 1.5

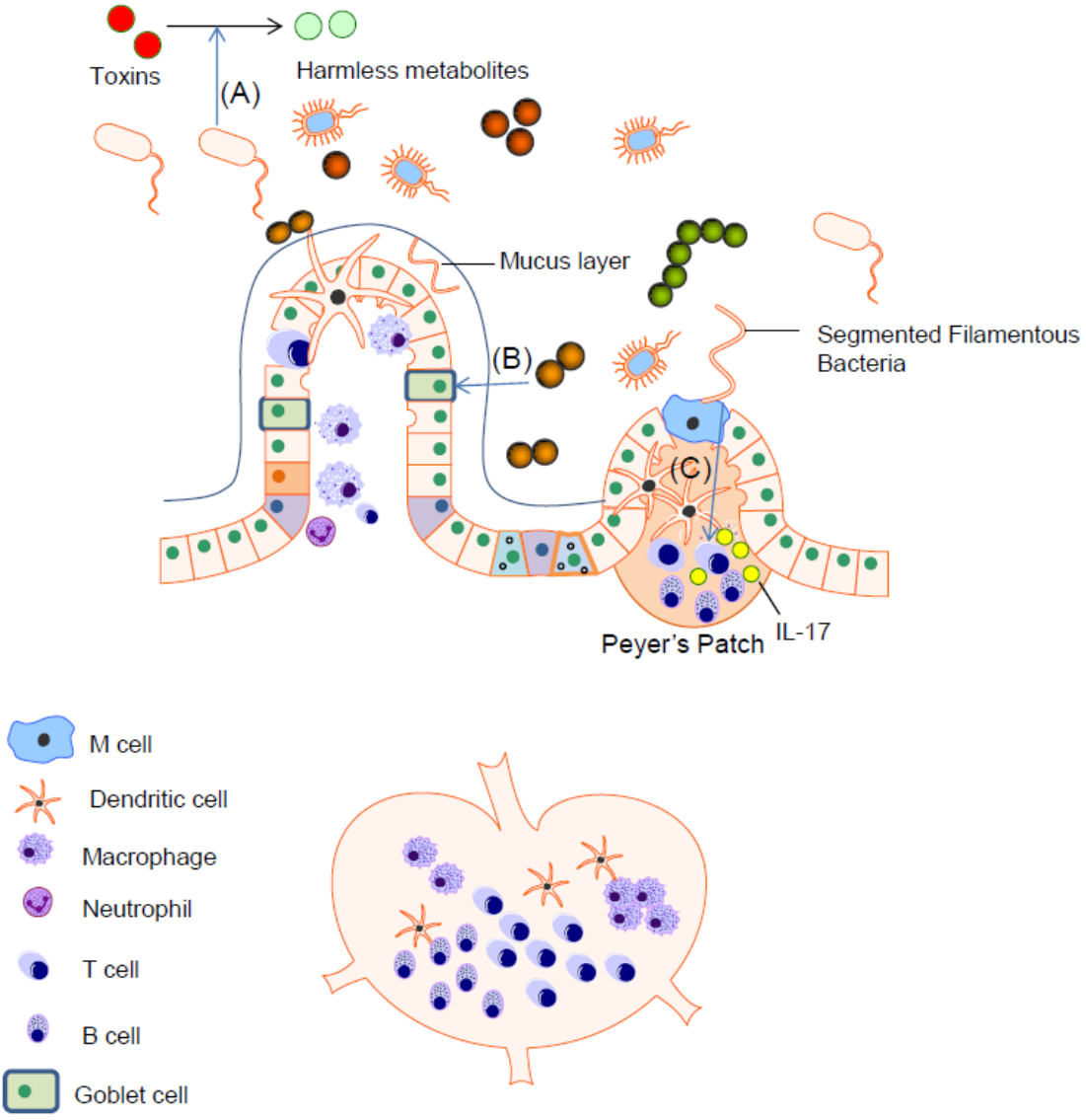


FIGURE 1.5 Gut microbiota in gut immunity. (A) Gut microbiota is able to metabolize the toxic xenobiotic agents into harmless metabolite. (B) Microbial flora influences mucin production by goblet cells. (C) Microbial flora influence lymphocyte responses.

3. TIGHT JUNCTIONS IN GUT EPITHELIUM

A The structure and function of TJ in gut epithelium

As discussed previously, tight junction proteins are located between the gut epithelial cells and their functions are to allow the transport of small solutes, water, and some macromolecules through the paracellular pathway selectively and exclude the potentially harmful molecules. Tight junction proteins in the intestinal epithelium includes transmembrane proteins such as the occludin and the claudin family members, which seal the paracellular pathway between the epithelial cells. In addition to transmembrane molecules, the paracellular proteins such as zona occludens-1 (ZO-1) and zona occludens-2 (ZO-2), play an important role in supporting the organization of TJ proteins(Peterson and Artis 2014). The permeability of tight junction is dynamic and determined by the isoforms, quantity, and organizations of these proteins. Disruption of gut tight junction protein results in barrier defects and is associated with various intestinal diseases. For example, the genetic polymorphisms of the tight junction protein have been linked to celiac disease, ulcerative colitis, and Crohn's disease(Schulzke et al. 2009).

B Toll like receptor and TJ in gut epithelium

The Toll-like receptor (TLR) family plays an important role in innate immunity and TLR signaling has to be regulated tightly in the intestines to maintain the balance between normal and over-exuberant activation due to the presence of large amount of commensal bacteria in the lumen of the gastrointestinal tract(Abreu 2010). Among all

TLRs in the gut, TLR2 and TLR4 play a dominant role in physiological and pathological processes, and are both involved in intestinal permeability regulation. TLR2 and TLR4 have been shown to regulate the gate-keeping functions of the intestinal follicle-associated epithelium(Chabot et al. 2006). In contrast, activation of TLR4 by LPS increases the permeability of intestinal cell monolayer by disrupting the complex consisting of tight junction proteins, myosin, and F-actins. This process is mediated by myosin light chain kinase (MLCK) (Figure 1.6)(Forsythe et al. 2002). Besides the direct effects on tight junction organization, TLR signaling is also involved in barrier modulation indirectly since TLR activation leads to production of various pro-inflammatory cytokines. Studies from different groups reported that interferon-γ, tumor necrosis factor-α, and interleukin-1β could increase the intestinal cell permeability by changing the expression and localization of tight junction proteins(Tazuke et al. 2003; Utech et al. 2005; Bruewer et al. 2003; Desai et al. 2002). IL-17 is pro-inflammatory cytokine associated with different autoimmune diseases especially inflammatory bowel disease like Crohn's disease and colitis(Gu, Wu, and Li 2013). In contrast, one study correlated the compromised gastrointestinal integrity in pigtail macaques with high levels of IL-17 production(Klatt et al. 2010). The exact effects of IL-17 on intestinal tight junction are still elusive. Interestingly, other researches focusing on tight junctions in endothelial cells that make up the blood brain barrier provide the potential mechanism by which IL-17 modulates tight junction proteins and the barrier functions. In these studies, IL-17 treatment resulted in reduced expression levels of tight junction protein and tight junction disorganization in MLCK-dependent manner(Huppert et al. 2010; Kebir et al.

2007).

C Opioids and TJ functions

The effects of opioid treatment on intestinal barrier function still remain to be determined while researches investigating tight junctions in blood brain barrier (BBB) imply the role of opioids in tight junction protein modulation and function. For example, morphine treatment has been shown to induce release of pro-inflammatory cytokines TNF- α, which significantly decreased both ZO-1 and occludin gene expression in brain microvascular endothelial cells, resulting in impaired barrier function of BBB(Mahajan et al. 2008).

There is abundant evidence demonstrating that intracellular cross talk between MOR signaling and TLR signaling in various kinds of cells results in alteration of immune responses and progress of inflammation. For example, our lab has recently shown that chronic morphine treatment can synergistically increase and activate TLRs in the presence of HIV-1 protein TAT and *S. pneumonia* in microglial cells, which induced a significant increase in pro-inflammatory cytokines (IL-6, TNF-α) levels. Therefore the crosstalk between MOR signaling and TLR signaling might provide the potential mechanism by which opioid treatment modulate intestinal tight junction protein function (Dutta et al. 2012).

FIGURE 1.6

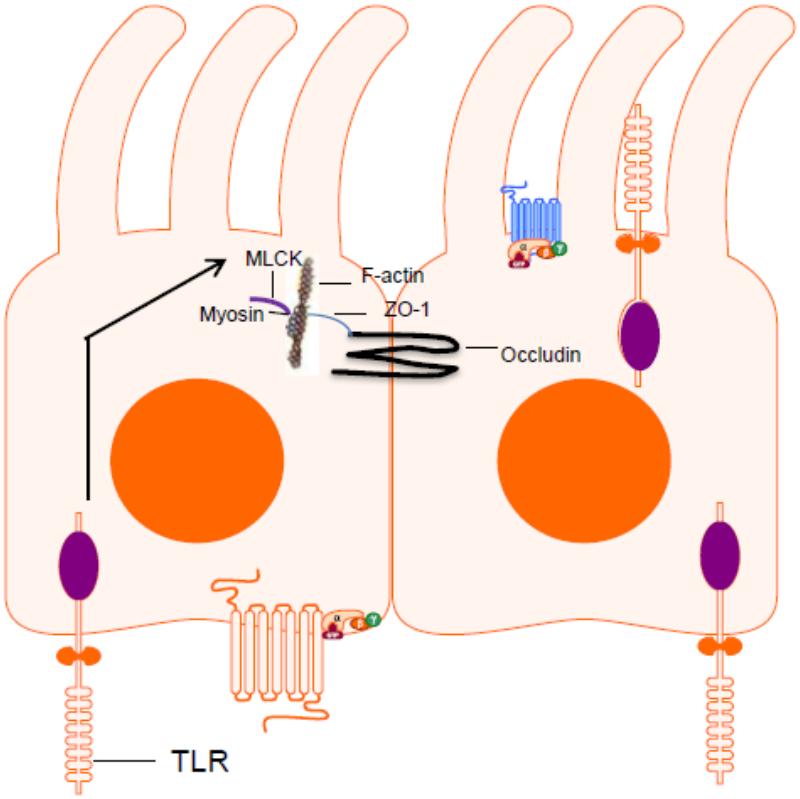


FIGURE 1.6 TLR activation modulates TJ organization in a MLCK-dependent pathway. Activation of TLRs by TLR ligands increases the permeability of intestinal epithelial cells by disrupting the complex consisting of tight junction proteins, myosin, and F-actins in a MLCK-dependent manner.

4. IL-17 CYTOKINES AND GUT IMMUNITY

A IL-17 cytokines and their functions

The IL-17 family consists of six members, which include IL-17A-F. By binding to IL-17 receptors in various tissues, IL-17 cytokines participate in acute and chronic inflammatory responses, playing a crucial role in host defense against microbial infections and acting as pro-inflammatory cytokines involved in pathogenesis of autoimmune diseases like IBD. The IL-17 receptor family now consists of five members, which are IL-17RA, RB, RC, RD and RE. IL-17RA is a common receptor forming heterodimers with IL-17RB, IL-17RC, and IL-17RE and all of these receptors needs AKT as the adapter molecule to initiate the downstream signaling pathways (Figure 1.7)(Gu, Wu, and Li 2013).

Among all IL-17 cytokines, IL-17A and IL17F are the most widely investigated in the recent studies. All IL-17A and IL-17F homodimers and IL-17AF heterodimers bind to heterodimeric receptor complex composed of IL-17RA and IL-17RC. IL-17A shares 56% sequence homology with IL-17F and has 100–1000 times higher affinity for IL-17RA than does IL-17F whereas the binding affinities for IL-17RC is comparable between the two cytokines. IL-17A is able to synergize with other molecules like TNF-α to enhance pro- inflammatory responses via enhancing the chemokine expression through stabilizing these mRNAs(Chang and Dong 2011; Gu, Wu, and Li 2013). Depletion of IL-17RA or IL-17A in murine models resulting in increased host susceptibility to a variety of pathogen infection such as *Salmonella enterica*, *Streptococcus pneumoniae*,

Staphylococcus aureus, *Helicobacter pylori*, and *Citrobacter rondentium* etc., thus demonstrating the essential role of IL-17 in host defense(Chang and Dong 2011; Gu, Wu, and Li 2013; Rubino, Geddes, and Girardin 2012). However, IL-17A is also identified as the major driver for several inflammatory and autoimmune diseases like multiple sclerosis (MS), psoriasis, asthma, Crohn's disease and rheumatoid arthritis(Akdis et al. 2012; Chang and Dong 2011). The controversial roles of IL-17 in the gut have attracted more attention recently. On one hand, IL-17A, IL-17F, and Th17 cells are abundantly up-regulated in the intestinal mucosa of Crohn's disease and ulcerative colitis patients and both IL-17R-deficiency and IL17F-difficiency protected mice from acute trinitrobenzenesulfonic acid (TNBS) - or dextran sodium sulfate (DSS)-induced colitis. On the other hand, several studies showed that the protective role of IL-17A in T-cell mediated colitis by inhibiting Th1 polarization for IFN-γ dependent inflammation(Akdis et al. 2012; Sanchez-Muñoz 2008; Strober and Fuss 2011; O'Connor et al. 2009). Therefore, more investigations are needed to understand the exact functions of IL-17A and F in the intestinal tissues. The function of IL-17C is very similar to IL-17A. By binding to the IL-17RA-RE complex, it mediates host defense responses and contributes to the pathogenesis of autoimmune diseases(Gu, Wu, and Li 2013).

IL-17E, known as IL-25 as well, exerts very different functions from IL-17A. Its signaling through the IL-17RA-RB receptor complex induces Th2 responses by activating MAPK NF-κB pathways. Due to high levels of IL-17-RB in lung, IL-17E can induce lung inflammation by promoting the differentiation of naïve T cells to effector Th2 cells. In human asthmatic tissue, both IL-17E and IL-17RB expression were shown

to be elevated compared to healthy controls, implying that IL-17E is involved in allergic airway inflammation. The targets and activities of IL-17B and IL-17D still need future investigation(O'Connor et al. 2009; Swaidani et al. 2009; Swaidani et al. 2011).

FIGURE 1.7

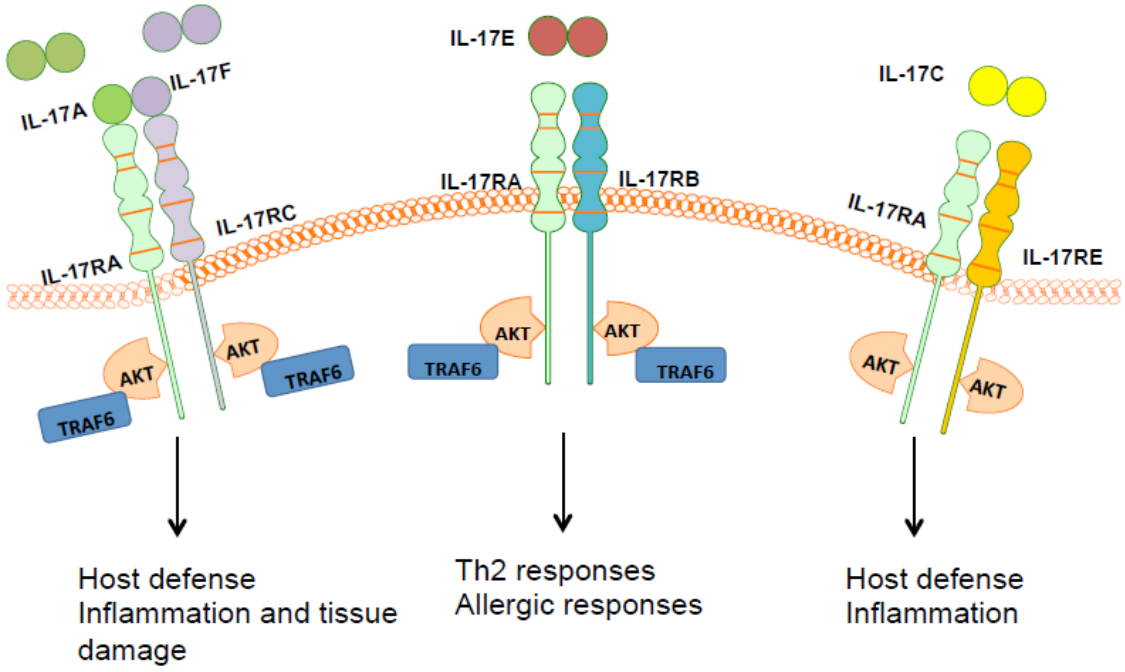


FIGURE 1.7 IL-17 cytokine family and their functions. IL-17A and IL-17F bind to complexes of IL-17RA and IL-17RC to initiate the signaling pathway involved in host defense and tissue inflammation. IL-17C binds to complexes of IL-17RA and IL-17RE and shows the similar activity to IL-17A. IL-17E binds to complexes of IL-17RA and IL-17RB to initiate Th2 and allergic responses.

B IL-17 responses in immunity and inflammation

In response to bacteria stimulation, different types of intestinal immune cells including CD4+ Th17 cells, CD8+ T cells, γδT cells, natural killer (NK) cells, innate lymphoid cells, and neutrophils, are able to produce IL-17 to mediate the defense mechanism and induce the inflammatory progress(Rubino, Geddes, and Girardin 2012). Th17 cells were the first cell population reported to produce IL-17A and IL-22. However, the differentiation pathways that lead to Th17 cells are not clearly defined. In early studies, it was postulated that Th17 cells were derived form naïve precursors in the presence of IL-6 and TGF-β and that IL-23 acts downstream of IL-6 and TGF-β to sustain the Th17 phenotype. However, subsequent studies show that IL-6, IL-1β, and IL-23 can also differentiate naïve T cells into Th17 cells in the absence of TGF-β. Interestingly, the effector cytokines produced by Th17 cells from these two differentiating pathways are different. The cells derived via the TGF-β dependent pathway are identified as more regulated classical Th17 cells characterized by the production of more IL-21, IL-9, and IL-10, which may play a more protective role in maintenance of barrier integrity in intestinal mucosal sites. Whereas the cells derived through TGF-β independent pathway produce high levels of IL-22, GM-CSF, and IFN-γ, which may play a more prominent role in pathogenic inflammation in infectious or autoimmune diseases (Figure 1.8)(Akdis et al. 2012).

In addition to CD4+ Th17 cells, many other innate immune cells also produce IL-17 as well. For example, neutrophils have been shown to induce the expression of IL-17A and IL-17RC following stimulation with IL-6 and IL-23, which probably contribute to

the etiology of microbial and inflammatory diseases(Taylor et al. 2014). Furthermore, pattern recognition receptors (PRRs) such as TLR2 also mediate rapid IL-17 production in response to bacteria encounter in γδ T cells. More recently, it was discovered that there exists a group of innate lymphoid cells (ILCs) belonging to the lymphoid lineage. So far, three groups of ILCs has been characterized, among which the group three ILCs are the important sources of IL-17 and IL-22 during early phases of infection(Rubino, Geddes, and Girardin 2012).

FIGURE 1.8

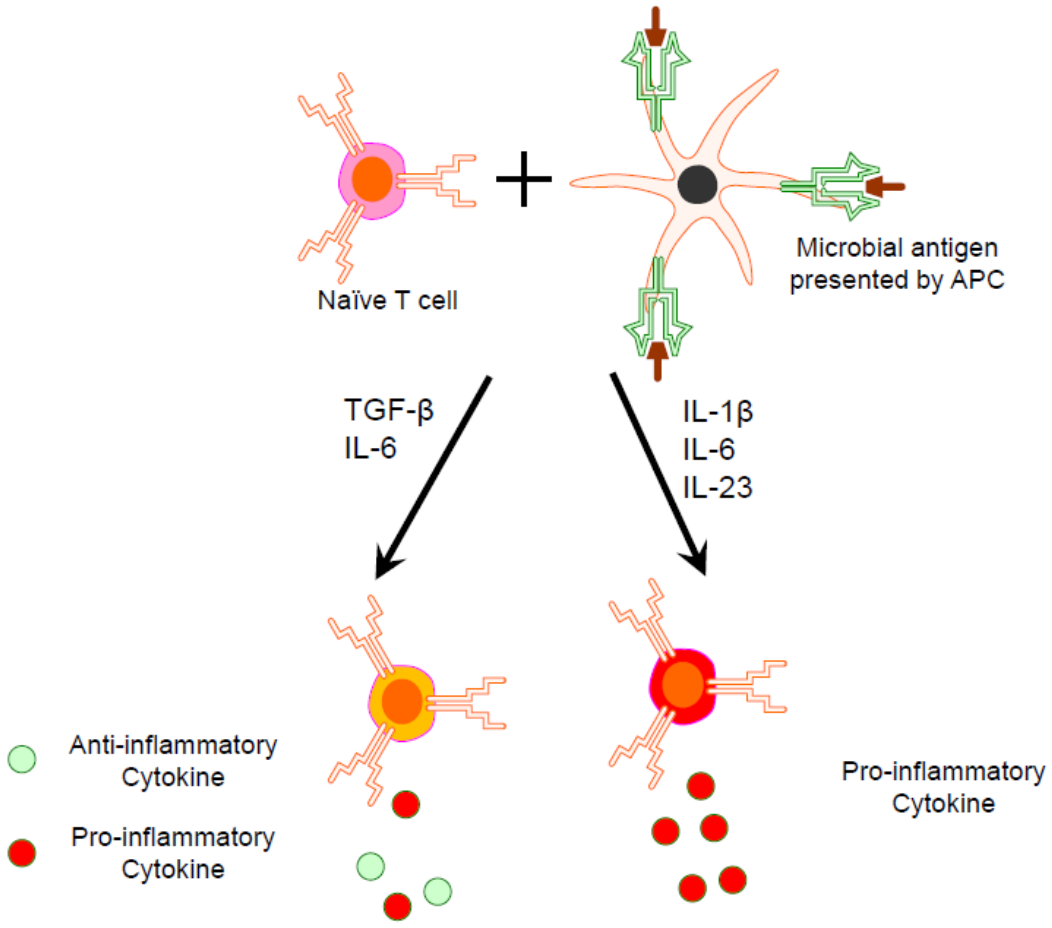


FIGURE 1.8 Th17 cells derived via two different pathways. The Th17 cells derived via the TGF-β dependent pathway are identified as more regulated classical Th17 cells which may play a more protective role in maintenance of barrier integrity in intestinal mucosal sites. Whereas Th17 cells derived through the TGF-β independent pathway produce high levels of pro-inflammatory cytokine, which may play a more prominent role in pathogenic inflammation in infectious or autoimmune diseases.

C Opioid treatment and IL-17 responses

Since IL-17 is a cytokine discovered pretty recently, not many studies have been performed to understand the influence of opioid treatment on IL-17 responses and the results of these studies are confusing. Some studies imply the inhibitory effects of opioids on IL-17 production. For example, in an intranasal *S Pneumonia* infection model, our lab reported that morphine administration reduced dendritic cell IL-23 expression and impaired IL-17A production by γδ T cell, resulting in increased susceptibility to Pneumonia(Ma et al. 2010). Another group reported the similar results: morphine treatment suppressed the expressions of IL-17A and other chemokines, which are crucial for neutrophil recruitment. The impaired neutrophil recruitment sensitized mice to *Acintobacter* infection and led to increased mortality of mice during infection(Breslow, Monroy, Daly, Meissler, Gaughan, et al. 2011). Whereas other group showed that circulating Th17 numbers were not significantly altered but the production of IL-17A was significantly increased by chronic morphine treatment(Cornwell et al. 2013). All these results suggest that multiple mechanisms are involved in IL-17 regulation and that more investigations are required to understand the role of opioids in IL-17 responses.

.

CHAPTER 2

OPIOID TREATMENT MODULATES GUT EPITHELIAL BARRIER FUNCTION

INTRODUCTION

Morphine is the most widely used analgesic worldwide for the management of pain. Morphine use is especially prevalent in patients undergoing invasive procedures that are associated with long operative times and extended hospitalization(Regan L, Chapman AR, Celnik A, Lumsden L, Al-Soufi R 2013; Ripamonti and Bruera 1991). Clinically, morphine use has been shown to be an independent risk factor for infection and infection-related morbidity in burn patients(Alexander et al. 2005; Sabita Roy et al. 2011). Furthermore, clinical studies have reported that patients with sepsis, severe sepsis, and septic shock had significant higher circulating morphine levels than patients with systemic inflammatory response syndrome and healthy controls(Glattard et al. 2010), while the opioid antagonist naltrexone has been shown to block acute endotoxic shock by inhibiting tumor necrosis factor-α production(Greeneltch et al. 2004a). Studies using animal models show that both chronic morphine and morphine withdrawal can lower host defense to enteric bacteria such as Salmonella enterica and Pseudomonas aeruginosa, induce spontaneous sepsis in mice, and sensitize mice to mortality induced by Acinetobacter baumannii infection or lipopolysaccharide (LPS) (Greeneltch et al. 2004a; Ocasio et al. 2004; S Roy, Charboneau, and Barke 1999). In addition to bacterial

translocation, morphine has been documented to sustain high serum IL-6 levels and accelerate the progression of LPS-induced sepsis to septic shock(Banerjee et al. 2013; Ocasio et al. 2004). Overall, both clinical and laboratory studies provide evidence that μ-opioid receptors are involved in the development and progression of various infectious diseases related to gut pathogens. However, the mechanisms underlying compromised gut immune function and increased susceptibility to infections after morphine treatment have not been well characterized. Therefore, the objective of the present study was to understand the correlation between morphine treatment and compromised gut barrier function, in order to support the development of novel strategies to treat or prevent gut bacterial infection in opioid-using or -abusing populations.

Epithelium is one of the most important components of intestinal mucosal immunity, which is required for prevention of potential pathogen invasion. The intestinal epithelium, as the first line of defense in the gut luminal environment, is not only a simple physical barrier but also plays an essential role in supporting nutrient and water transport and maintaining the homeostasis of the whole organism. Not surprisingly, compromised barrier function allows the intestinal microbiota to translocate through the epithelium, leading to increased susceptibility to infection by gut pathogens, and faster progression of infectious disease(Turner 2009). Gut epithelial cells play an important role in recognizing and preventing potential pathogen or antigen invasion. To accomplish these complicated functions, well-organized transmembrane and paracellular tight junction proteins are expressed in these polarized cells. Tight junction proteins in intestinal epithelium include transmembrane proteins such as occludin and claudin family

members, which seal the paracellular pathway between the epithelial cells, as well as paracellular proteins such as zona occludens-1 (ZO-1) and zona occludens-2 (ZO-2), acting as scaffolding molecules. Disruption of gut tight junction barrier function has severe consequences including bacterial translocation from the gut leading to immune activation and inflammation(Schulzke et al. 2009).

Toll-like receptor (TLR) signaling is one of the most important components of innate immunity and has to be regulated tightly in gut epithelium to maintain the balance between normal and over-exuberant activation due to the presence of large amount of commensal bacteria in the lumen of the gastrointestinal tract(Abreu 2010). Among all TLRs in the gut, TLR2 and TLR4 play important roles in physiological and pathological processes, and are both involved in intestinal permeability regulation. TLR2 and TLR4 have been shown to regulate the gate-keeping functions of the intestinal follicle-associated epithelium. Paradoxically, activation of TLR4 by LPS increases intestinal monolayer permeability in a myosin light chain kinase (MLCK)-dependent manner (Moriez et al. 2005). Meanwhile, there is evidence showing intracellular cross talk between MOR signaling and TLR signaling in various kinds of cells(Sabita Roy et al. 2011). For example, morphine significantly inhibits tumor necrosis factor-α (TNF- α), but not interleukin-6 (IL-6) production, in a MOR-independent manner in polyglycan-stimulated peripheral blood mononuclear cells(Bonnet et al. 2008). However, the intracellular mechanism underlying how morphine compromises epithelial barrier function via modulating TLRs is still not defined. In the present study, we hypothesize that morphine disrupts the barrier function of gut epithelium by increasing the sensitivity

of gut epithelial cells to TLR activation, resulting in bacterial translocation from the gut lumen. We investigated the effects of morphine on gut barrier function in wild type (WT), TLR2 knockout, TLR4 knockout, and TLR2/4 double knockout mice. The direct effects of morphine on gut epithelial cells were further studied with rodent small intestinal and colonic epithelial cell lines, IEC-6 and CMT-93, respectively. Our results from in vivo and in vitro studies indicate that morphine treatment compromises gut barrier function in a TLR-dependent manner.

METHODS

Experimental animals

Pathogen-free B6129PF2, C57BL/6J, B6.129$^{Tlr2tm1Kir/J}$ (TLR2 knockout) and C57BL/10ScNJ$^{Tlr4lps-del}$ (TLR4 knockout) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). We crossed TLR2 knockout withTLR4 knockout mice to generate TLR2/4 double knockout mice. MOR knockout (MORKO) mice (C57BL/6129/Ola genetic background) were generated by Loh and his colleagues(S Roy, Barke, and Loh 1998). Briefly, a XhoI/XbaIfragment, which spans exons 2 and 3, was replaced with a Neor cassette, followed by the ligation of a thymidine kinase expression cassette to the 3′ end of this segment. All animals were housed in a specific-pathogen-free facility under barrier conditions. All animal experiments were done in accordance with the Institutional Animal Care and Use Committee's guidelines at the University of Minnesota. The protocol was approved by Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota (protocol# 0909A72719). All surgeries were performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Animal treatment

Mice received morphine and pellet implantation method as described. Using this method, plasma levels of morphine are in the 0.6 – 2.0-microg/ml range (range seen in opioid abusers and patients on opioids for moderate to severe pain). Furthermore, this model is commonly used in the study of opiate dependence and addiction(Bryant et al. 1988). Briefly, placebo or 75 mg morphine pellets (National Institutes of Health

[NIH]/National Institute on Drug Abuse [NIDA], Bethesda, MD) were inserted in a small pocket created by a small skin incision on the animal's dorsal side; incisions were closed using surgical wound clips (Stoelting, 9 mm Stainless Steel, Wooddale, IL). Animals were injected with MLCK inhibitor ML-7 (2 mg/kg) overnight before LPS or Lipoteichoic acid (LTA) treatment. At this dose, ML-7 successfully inhibited activity of myosin light chain kinase and protected the barrier function of endothelial cells in mice(Huppert et al. 2010).

Intestinal permeability

All animals were gavaged with ampicillin-resistant *E. coli* (2×107 CFU suspended in 400 µl of sterile saline) or FITC-dextran (600 mg/kg body weight in 20 mg/ml concentration) utilizing a 4-cm long, curved needle with a plastic ball at the tip. After sacrifice, MLN and liver were collected and cultured on LB plates containing 100 µg/ml of ampicillin to measure bacterial translocation. Whole blood FITC-dextran concentration was determined by fluorometry based on a standard curve.

Immunofluorescence

Sections of small intestinal and colonic tissue from all mice sacrificed for tight junction staining were frozen in TFM™ tissue freezing medium (TBS, Durham, NC). At least five sections from each of three animals for each condition were analyzed by immunofluorescence microscopy. Representative images are shown. For immunostaining, 5 µm frozen sections were fixed with 1% paraformaldehyde in PBS for

10 min at room temperature. After washing in PBS and blocking of nonspecific binding sites with 5% bovine serum albumin (BSA), tissues were incubated with polyclonal rabbit anti-occludin or rabbit anti-ZO-1 (both used at 5 μg/ml, Invitrogen) in PBS with 5% bovine serum albumin (BSA) for 120 min at room temperature. After washing, sections were incubated with rhodamine phalloidin (Invitrogen) and DyLightTM 488-conjugated AffiniPure Donkey anti-rabbit IgG (0.075 mg/ml, Jackson Lab, WestGrove, PA) for 60 min. Sections were then washed and mounted under coverslips using ProLong Gold antifade reagent with DAPI (Invitrogen). Sections were imaged using a confocal microscope (Nikon). Image J RG2B co-localization software was used to quantify the intensity of yellow fluorescence (indicating co-localization of green and red) and normalized to blue fluorescence (DAPI).

Western blots

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Sigma). Lysates (80 μg protein per lane) were separated by SDS-PAGE, and proteins were electrotransferred from gel onto nitrocellulose membrane. Membranes were blocked in Tris-buffered saline, 0.1% Tween 20, 5% BLOT-QuickBlockerTM (G-Biosciences, St Louis, MO), and incubated with primary and secondary IRDye® anti-IgG Abs (LI-COR Biosciences). Protein bands were visualized using Odyssey infrared imaging system (LI-COR Biosciences).

Realtime PCR

Total cellular RNA was extracted using TRIzol (Invitrogen), and cDNA was synthesized with the M-MLV Reverse Transcription Kit (Promega). Primers for TLR2, TLR4, and 18S ribosomal RNA were purchased from IDT. Quantitative real-time polymerase chain reaction (PCR) was performed on an Applied Biosystems 7500 Realtime PCR Detection system. All samples were run in triplicate, and relative mRNA expression levels were determined after normalizing all values to 18S RNA. Primer sequence: 18S: 5-GTAACCCGTTGAACCCCATT-3; 5-CCATCCAATCGGTAGTAGCG-3; TLR2: 5-CGCCTAAGAGCAGGATCAAC-3; 5-GGAGACTCTGGAAGCAGGTG-3; TLR4: 5-CCAGAGCCGTTGGTGTATCT-3; 5-TCAAGGCTTTTCCATCCAAC-3.

Epithelial cell isolation

Epithelial cells were isolated as described previously(Roulis et al. 2011). Small intestine was excised from mice, flushed with HBSS/2% FBS, opened longitudinally, and cut into 0.5-cm pieces. The tissue was further washed and incubated in HBSS/2% FBS, 0.5 mM EDTA, and 1 mM DTT, at 37° C in shaking water bath for 45 min. The cell suspension released upon vigorous shaking was layered on a discontinuous 25%/40% Percoll gradient (Sigma) and centrifuged at 600×g for 10 min. Intestinal epithelial cells (IEC) were collected from the interphase and incubated with anti-cytokeratin antibody (BD Pharmingen), anti-TLR2 and anti-TLR4 antibodies (eBiosciences).

Cell culture and treatment

IEC-6 and CMT-93 cell lines were purchased from American Type Culture Collection (Manassas, VA) and cultured as recommended by the supplier. IEC-6 and CMT-93 cells are rodent small intestinal and colonic epithelial cell lines. Cells were pretreated with MLCK inhibitor ML-7 before LPS (1 μg/ml, Sigma) or LTA (5 μg/ml, Sigma) stimulation. Inactivation of MLCK by ML-7 has been shown to protect barrier function in various endothelial and epithelial cell lines(Huppert et al. 2010).

Measurement of trans-epithelial resistance

ECIS 1600R (Applied BioPhysics, Troy, NY) was used to measure trans-epithelial resistance (TER) of epithelial monolayers as described previously [29]. Epithelial cells were seeded in the wells of the electrode array and grown to confluence as indicated below. Then medium was exchanged, and baseline TER was measured for 60 min to equilibrate monolayers. Afterward, 400 μl of medium containing ML-7 (10 μM), LPS (1 μg/ml), or LTA ((5 μg/ml) was applied to the wells.

RESULTS

Chronic Morphine compromises the barrier function of gut epithelium and promotes bacterial translocation

To determine whether chronic morphine treatment modulates bacterial dissemination, we determined spontaneous gut bacterial translocation following morphine treatment. B6129PF2 wild type mice were implanted with 75 mg morphine or placebo pellet subcutaneously. Mesenteric lymph node (MLN) (n = 9) and liver (n = 10) suspensions were collected after 24 hours, cultured on blood agar plates (BD Biosciences) overnight and the colony forming units (CFUs) were quantified. Placebo-implanted mice showed no colonies growing on the plates, indicating no bacterial translocation. Conversely, mice receiving morphine revealed an increased number of CFUs, indicating bacterial dissemination to MLN and liver following 24 hours of morphine treatment (Figure 2.1A). At 48 hours, morphine-induced bacterial translocation into liver and MLN persisted (Figure 2.1E). To determine the role of μ-opioid receptors (MOR) in morphine modulation of bacterial translocation, we implanted MOR knockout (MORKO) mice with morphine pellets, as described above. Morphine-induced bacterial translocation was completely abolished in MORKO mice (Figure 2.1B), suggesting that MOR mediated morphine's effects on bacterial translocation. To further confirm that the disseminated bacteria were from the gut lumen rather than opportunistic infections, we gavaged WT mice with ampicillin-resistant E.coli and quantified bacterial translocation with Lysogeny broth (LB) plates containing ampicillin. Morphine-treated mice showed ampicillin-resistant E.coli dissemination into MLN and liver (Figure 2.1C), indicating

that morphine treatment promotes bacterial translocation of commensal bacteria from the gut lumen. In addition, morphine treatment promoted fluorescein isothiocyanate (FITC)-conjugated dextran translocation from gut lumen to blood (Figure2.1D), suggesting that morphine increased the permeability of the gut epithelium. Serotyping of the disseminated bacteria (Veterinary Diagnostic Laboratory, University of Minnesota) revealed a prevalence of Staphylococcus, Enterococcus, and Bacillus sp., which are commensal bacteria in the gut lumen.

Chronic morphine induces inflammation and disrupts organization of tight junction proteins between epithelial cells in small intestine

To investigate the effects of morphine on the morphology of the intestinal epithelium, small intestine and colon were excised and fixed in a formalin solution for hematoxylin and eosin (H&E) staining. Histological analysis showed injured epithelium and increased inflammatory infiltrates in small intestinal villi of morphine-treated mice (Figure 2.2). In contrast, no morphological change was observed in the colon of morphine-treated mice, suggesting a differential effect by morphine on small intestinal and colonic epithelium. Our findings of morphine-induced microbial translocation and barrier compromise in the small intestine of mice prompted us to study the tight-junction organization of the intestinal epithelium. Wild-type mice were implanted with placebo or 75 mg morphine pellet for 24 hours. Then parts of the small intestine were excised, frozen and 5 μm sections were cut. The sections were stained for occludin and zona occludens 1 (ZO-1), two proteins integral to the formation of epithelial tight-junction. In

placebo treated mice, the trans-membrane protein occludin localized to the apical side of epithelium with a continuous and intact organization (Figure 2.3A). Images showed that occludin co-localized with the well-organized F-actin on the membrane of epithelial cells of placebo-treated mice (Figure 2.3A). In contrast, morphine treated mice showed disrupted localization of occludin, suggesting impaired recruitment of the protein to the membrane (Figure 2.3B). Similar to occludin, the paracellular tight junction protein ZO-1 also localized with F-actin on the apical side of the membrane in placebo-treated mice, and its organization was seen to be disrupted following 24 hours of morphine treatment (Figure 2.3C). Morphine treatment did not change the expression levels of occludin or ZO-1 (Figure 2.4), suggesting that morphine modulated the distribution of tight junction proteins, resulting in increased intestinal permeability. Quantification of yellow fluorescence (indicating the co-localization of red and green) also showed significant reduction in the co-localization of tight junction and F-actin in morphine-treated mice (Figure 2.3B and D). In MORKO mice, consistent with our bacterial translocation data, morphine did not have any effect on occludin and ZO-1 organization in the small intestine, indicating that morphine's effect on intestinal tight junction were mediated by MOR (Figure 2.3E). Interestingly, morphine did not have an effect on either occludin or ZO-1 organization in the colonic epithelium, where both placebo- and morphine-treated mice showed intact and continuous localization of occludin and ZO-1 (Figure 2.3F). This finding suggests the differential regulation of barrier functions in different compartments of the gastrointestinal epithelium.

Morphine treatment up-regulates TLR expression in epithelial cells of small intestine.

As we have discussed previously, there is a clear correlation between TLR activation and tight junction disruption in intestinal mucosa, consistent with instances recently described in the literature(Sheth et al. 2007; Shifflett et al. 2005). To determine whether TLR expression on gut epithelial cells is one mechanism by which morphine modulates barrier function, we implanted mice with placebo or morphine pellets for 24 hours and isolated epithelial cells from the small intestines as described previously. Total RNA was isolated from these cells and processed for qPCR. For flow cytometery, the isolated cells were gated by cytokeratin as an epithelial marker(Quinlan et al. 2006) (Figure 2.5A).Results showed 24 hours of morphine treatment up-regulated both mRNA (Figure 2.5B) and protein levels (Figure 2.5C – F) of TLR2 and TLR4. In addition, the messenger RNA levels of TLR2 and TLR4 in colonic epithelial cells following morphine treatment was determined by gel-based PCR. The results showed that neither TLR2 nor TLR4 was significantly up-regulated by morphine in the colonic epithelium in contrast to the observation in the small intestinal epithelium (Figure 2.6).

Morphine-induced bacterial translocation is attenuated in TLR2/TLR4 knockout mice

To further determine roles of TLR2 and TLR4 in morphine-induced bacterial translocation, we implanted C57BL/6 J WT (n = 9), TLR2 knockout (n = 9), TLR4 knockout (n = 9), and TLR2/4double knockout (n = 9) mice with morphine pellets to

determine bacterial load in MLN and liver as described previously. Placebo-treated TLR 2, 4 KO mice showed very low basal levels of bacterial load in MLN and liver. Morphine-treated WT mice still showed significant bacterial translocation to MLN and liver. In contrast, morphine-treated TLR2, 4 knockout mice showed lower bacterial translocation into MLN and liver than did WT mice (Figure 2.7) although TLRKO did not show any effects on morphine-induced constipation, suggesting that constipation is not the only dominant factor causing bacterial translocation following morphine treatment and other TLR-dependent mechanisms also contribute to the process of TJ disorganization and barrier dysfunction (Figure 2.8). These findings indicated that both TLR2 and TLR4 are involved in morphine modulation of intestinal barrier function.

TLR2/TLR4 knockout protects tight junction organization from morphine-induced disruption

To further determine the role of TLRs in morphine's modulation of intestinal tight junction proteins, we isolated the small intestine from WT, TLR2 knockout, TLR4 knockout, and TLR2/4 double knockout mice to assess the organization of tight junction proteins, as described previously. In TLR2KO and TLR2/4KO mice, the occludin and ZO-1 staining were continuous and intact following morphine treatment (Figure 2.9A and B). In TLR4KO mice, some degree of tight junction disruption was observed following morphine treatment; however, the disruption was not as dramatic as that observed with morphine treatment in WT mice, suggesting a dominant role of TLR2 in morphine modulation of intestinal tight junction organization, which was consistent with our in

vitro study: small intestinal cell IEC-6 and colonic epithelial cell CMT-93 were stained for tight junction proteins ZO-1(Figure 2.10A). LPS and LTA but not morphine induced ZO-1 internalization. And morphine enhanced LTA's effects on IEC-6 cells, further validating that TLR2 plays a more dominant role in TJ modulation in gut epithelial cells following morphine treatment. In contrast, neither LPS nor LTA showed any effect on TJ distribution in colonic CMT-93 cells, consistent with our in vivo data (Figure 2.10B).

TLR signaling modulates intestinal tight junction organization in a MLCK-dependent manner

Since our data (Figure 2.4) show that TLR ligands have no effect on tight junction protein expression levels, the increased permeability of epithelial cells by TLR activation may involve post-translation mechanisms. Recent studies showed that myosin light chain kinase (MLCK) regulates the contraction of tight junctions by phosphorylating myosin light chains(Utech et al. 2005; Moriez et al. 2005; Marchiando, Graham, and Turner 2010). Activation of MLCK induces phosphorylation of the myosin light chains, resulting in the contraction of cytoskeleton proteins such as F-actin and thus inducing the internalization of associated tight junction proteins such as occludin and ZO-1. To determine whether MLCK is responsible, we determined the barrier function of IEC-6 cells by electrical cell impedance sensing (ECIS) arrays. The cells were grown to confluence in ECIS arrays, and the trans-epithelial resistance (TER) values were measured to test whether morphine would affect epithelial barrier integrity. The baseline TER of each experiment was normalized to 1.0 to enable comparison and statistical

analysis of TER changes over time following different treatments. IEC-6 cells were treated with MLCK inhibitor ML-7, and the TER values were measured in the presence of LTA (Figure 2.11A) and LPS (Figure 2.11B). Inhibition of MLCK restored the TER values to the control levels, indicating that the effects of TLR agonists on epithelial cells are dependent on MLCK. To further validate the role of MLCK in tight junction modulation, WT mice were injected with 2 mg of ML-7/kg body weight prior to morphine treatment as described previously. ML-7 inhibited morphine-induced bacterial translocation to MLN and liver (Figure 2.11C), and protected occludin and ZO-1 organization from morphine-induced disruption (Figure 2.11D), although it did not block constipation caused by morphine treatment (Figure 2.8).

DISSCUSSION

In the current study, we show that morphine mediated signaling by μ-opioid receptors 1) induced bacterial dissemination into MLN and liver of WT mice; 2) compromised intestinal barrier function; and 3) disrupted tight junction organization in gut epithelial cells through a TLR- dependent mechanism.

Our studies show significant bacterial translocation to the mesenteric lymph node and liver of WT mice that are morphine treated (Figure 2.1A and Figure 2.2). Over the past two decades, a large amount of studies have been conducted to investigate the effects of morphine on bacterial translocation and intestinal permeability using various rodent models. Consistently these studies demonstrate that morphine alters intestinal transit and promote bacterial translocation in rodents(Runkel et al. 1993; Kueppers et al. 1993) although in one study morphine only in the presence of TNF was able to increase intestinal permeability. Bacterial translocation was not measured in these studies (Leslie et al. 1994). It is not clear why there is a discrepancy between this study and the majority of other studies but the differences in the results may be attributed to differences in the doses of morphine used, the route of administration or the sensitivity of the permeability experiments. However, most recent studies clearly establish that morphine treatment in doses that are clinically relevant results in bacterial translocation in both rats and mice. In addition, we rule out the possibility that the bacteria detected in liver and lymph node is not a consequence of opportunistic infections due to suppressed immune function by morphine by measuring ampicillin-resistant E. coli and FITC-conjugated dextran

translocation (Figure 2.1C and D), validating that the observed bacterial translocation is a consequence of disrupted intestinal barrier function following chronic morphine treatment. We further show that morphine's effects were abolished in the MOR knockout mice (Figure 2.1B), indicating that morphine's modulatory effect on intestinal barrier function were mediated by MOR.

We then demonstrated through morphological evaluation of the gut that morphine potentiated inflammation in small intestine. Histological analysis showed injured epithelium and increased inflammatory infiltrates in the villi of the small intestines in morphine-treated mice (Figure 2.3), which was usually associated with disrupted intestinal barrier function. Interestingly, we failed to observe any effect of morphine on colonic epithelium (Figure 2.3), suggesting a differential effects of morphine on small intestinal and colonic derived epithelium, despite the observation that MOR expression is similar in the colon and in the small intestine. These observations are consistent with the previous studies where it was demonstrated that tolerance to morphine is differentially regulated in the ileum versus the colon(Ross et al. 2008). Although, in this study, the cellular basis for the differential expression of morphine tolerance in the ileum versus the colon was not defined, it is conceivable that signaling downstream of MOR activation may contribute to the differential effect.

Our studies also demonstrated that the organization of tight junction proteins in small intestines were disrupted following morphine treatment (Figure 2.3A to D), suggesting paracellular translocation of bacteria from the gut lumen. Tight junction

proteins have been shown to seal the gap between gut epithelial cells and play an important role in preventing potential pathogen invasion. Interestingly, morphine did not affect tight junction proteins' expression levels in intestinal epithelial cells (Figure 2.4), implying that it is their distribution that is involved in modulating intestinal permeability. To understand the cellular mechanism underlying tight junction modulation by morphine, we used IEC-6 cells as an in vitro model and determined its tight junction distribution following morphine treatment. To our surprise, morphine alone showed no effect on tight junction of epithelial cells. However, we observed that TLR2 and TLR4 ligands disrupted the tight junction organization of monolayers formed by small intestinal epithelial cells (IEC-6). Morphine modulated TJ organization of IEC-6 cells only in the presence of TLR2 ligand, suggesting that morphine's effects were mediated by TLRs. On the other hand, neither morphine nor TLR ligands showed any effect on barrier function of colonic epithelial cells (Figure 2.10), implying differential regulation of TJ in the ileum and colon by TLRS.

Historically, many studies have investigated the role of TLRs in modulating tight junctions in various epithelial cells: invasive bacterial pathogens *S. pneumoniae* and *H. influenzae* were observed to translocate across the epithelium through TLR-dependent down-regulation of tight junction components(Clarke TB, Francella N, Huegel A 2011). LPS also has been reported to disrupt tight junction of cholangiocytes-the epithelial cells of the bile duct by a TLR4-dependent mechanism(Sheth et al. 2007). Our in vivo studies support the role of TLRs in tight junction modulation in gut epithelial cells. Protein levels of TLR2 and TLR4 were increased in small intestine following morphine treatment

(Figure 2.5). Bacterial translocation and tight junction disruption were significantly attenuated in TLR2KO, TLR4KO, and TLR2/4 double knockout mice following morphine treatment (Figure 2.7 and 2.9), demonstrating that both TLR2 and TLR4 contribute to morphine-induced intestinal barrier disruption. Interestingly, TLR4 signaling was not involved in morphine modulation of epithelial barrier function in IEC-6 cells (Figure 2.11), which was contradictory to our *in vivo* study, where we show significant protection of tight junction from morphine-induced disruption in TLR4 knockout. These results suggest that activation of TLR4 in other cell types and not on the epithelial cells may play a more dominant role in morphine modulation of epithelial barrier function. TLR4 has been shown to play an important role in cytokine production in gut associated lymphoid tissue (GALT), which plays crucial roles in maintaining intact intestinal barrier function and defense against potential pathogen invasion. We postulate that TLR4 activation in the GALT, but not in epithelial cells, is involved in gut barrier modulation. In support of this hypothesis, it has been demonstrated that abnormal pro-inflammatory cytokine production induced by translocated bacteria causes disruption of tight junction proteins in gut epithelium. This feed-forward vicious cycle contributes to serious gut inflammatory disease and even sepsis. Therefore, it is conceivable that other factors activated by TLR4 may play a role in disrupting intestinal barrier function by modulating pro-inflammatory cytokines TNF-alpha and IL-6(Bruewer et al. 2003).

In addition, both *in vitro* and *in vivo* studies demonstrated that the distribution of tight junction was modulated by myosin light chain kinase (MLCK). MLCK inhibition completely blocked LTA- and LPS- induced barrier dysfunction in IEC-6 cells and

morphine-induced bacterial dissemination in mice (Figure 2.11), which confirmed that the impaired barrier function of epithelial cells following TLR activation is due to MLCK-induced redistribution of tight junction proteins rather than decreased tight junction protein expression levels.

In summary, our studies demonstrate that morphine treatment up-regulates TLR expression levels in small intestinal epithelial cells and sensitized small intestinal epithelial cells to TLR stimulation, which induced disruption of tight junctions between epithelial cells, increased gut permeability, and resulted in increased bacterial translocation and inflammation in the small intestine (Figure 2.12). In contrast, colonic epithelium did not show any response to morphine treatment, suggesting differential effects of morphine on small intestinal and colonic barrier function. Currently, opiates are among the most prescribed drugs for pain management. However, they induce multiple adverse gastrointestinal symptoms including dysfunction of the gut immune system, which may lead to a higher risk of gut bacterial infection as well as faster progression of infectious diseases such as sepsis. These adverse effects seriously affect patients' quality of life and limit the prolonged use of opiates for pain management. These studies contribute to the urgent need to understand the mechanism through which morphine modulates intestinal barrier function, enhancing our ability to develop novel strategies for treating or preventing gut bacterial infection in opiate-using or -abusing populations.

FIGURE 2.1

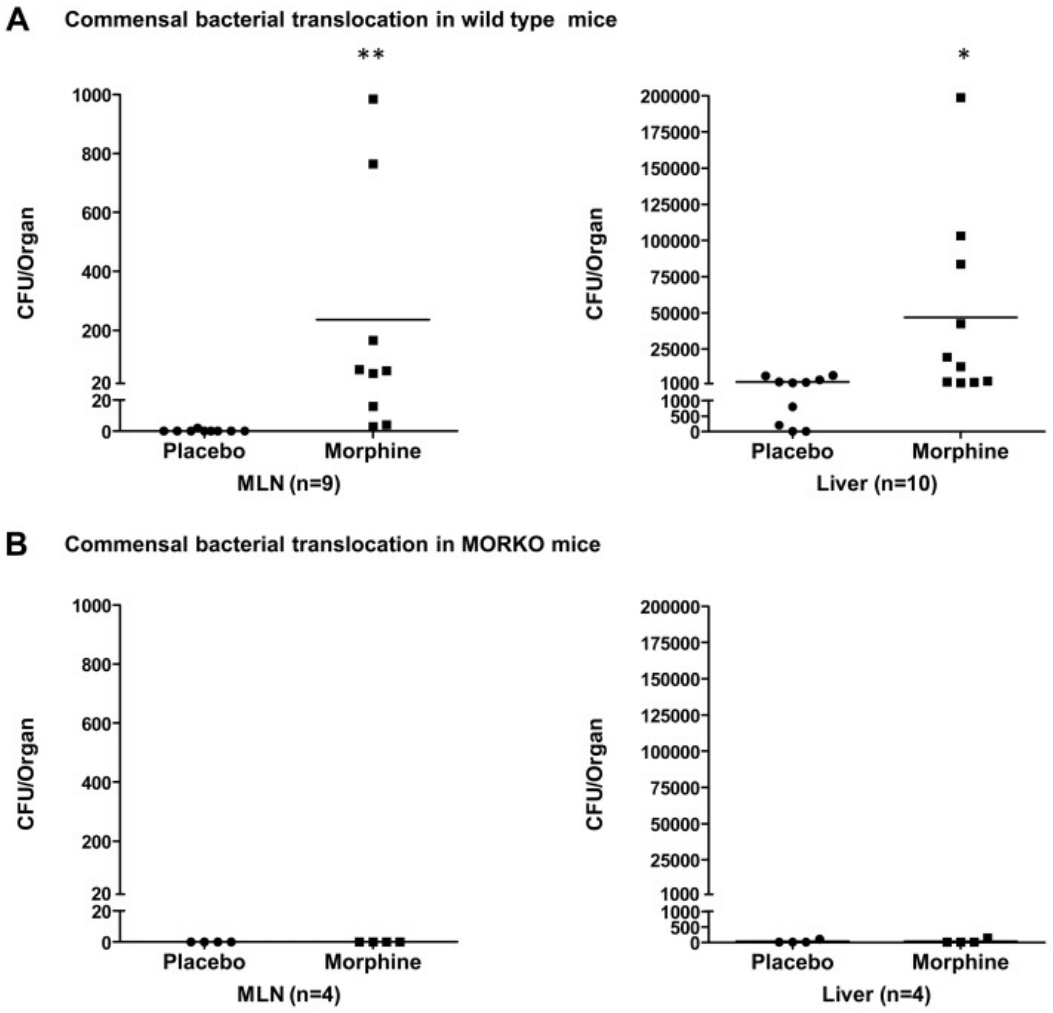


FIGURE 2.1 Chronic morphine compromises barrier function of gut epithelium and promotes bacterial translocation. Wild type (A) and MORKO (B) mice were treated with 75 mg morphine pellets for 24 hours, MLN and liver homogenates were cultured on blood agar plate overnight. Bacterial colonies were quantified and described as colony forming units (CFU). ** $p<0.01$ *$P<0.05$ by Mann-Whitney test

FIGURE 2.1

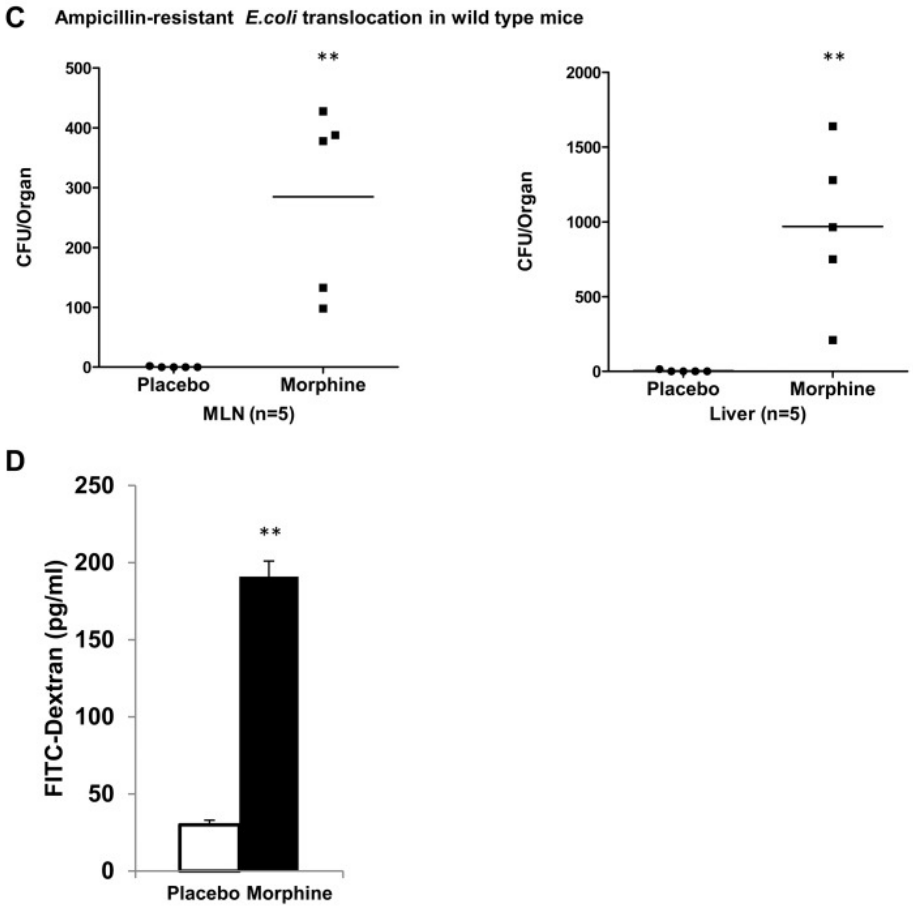


FIGURE 2.1 Chronic morphine compromises barrier function of gut epithelium and promotes bacterial translocation. (C) WT mice were gavaged with ampicillin -resistant E. coli after morphine treatment, and the number of E. coli in MLN and liver were quantified using an LB agar plate containing ampicillin. (D) The permeability of gut epithelium increased after morphine treatment as determined by measuring the whole blood FITC-dextran concentration. – Median of CFU; (C)** $p<0.01$ *$P<0.05$ by Mann – Whitney test. (D) **$P<0.01$ by Student's t-test.

FIGURE 2.1

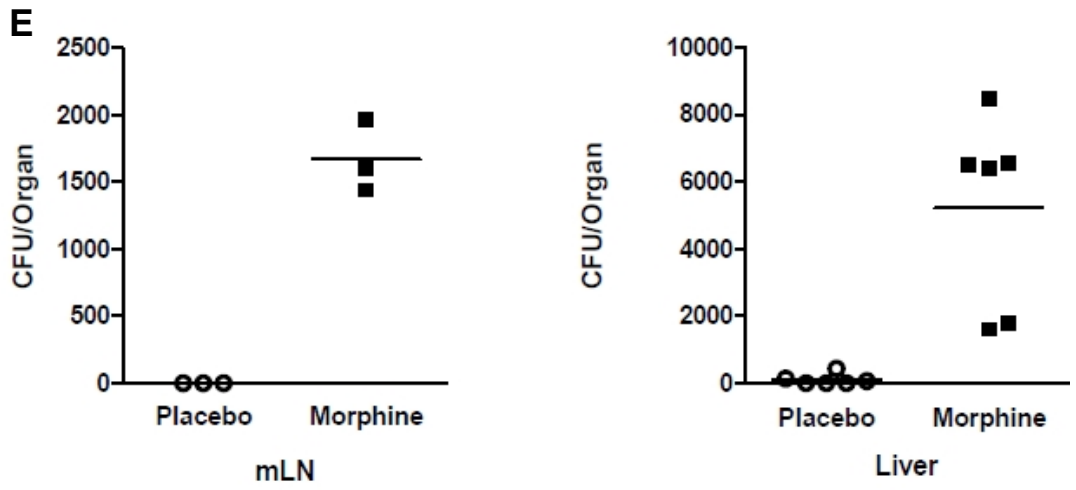


FIGURE 2.1 Chronic morphine compromises barrier function of gut epithelium and promotes bacterial translocation. (E) Wild type mice were treated with 75 mg morphine pellets for 24 hours, MLN and liver homogenates were cultured on blood agar plate overnight. Bacterial colonies were quantified and described as colony forming units (CFU).

FIGURE 2.2

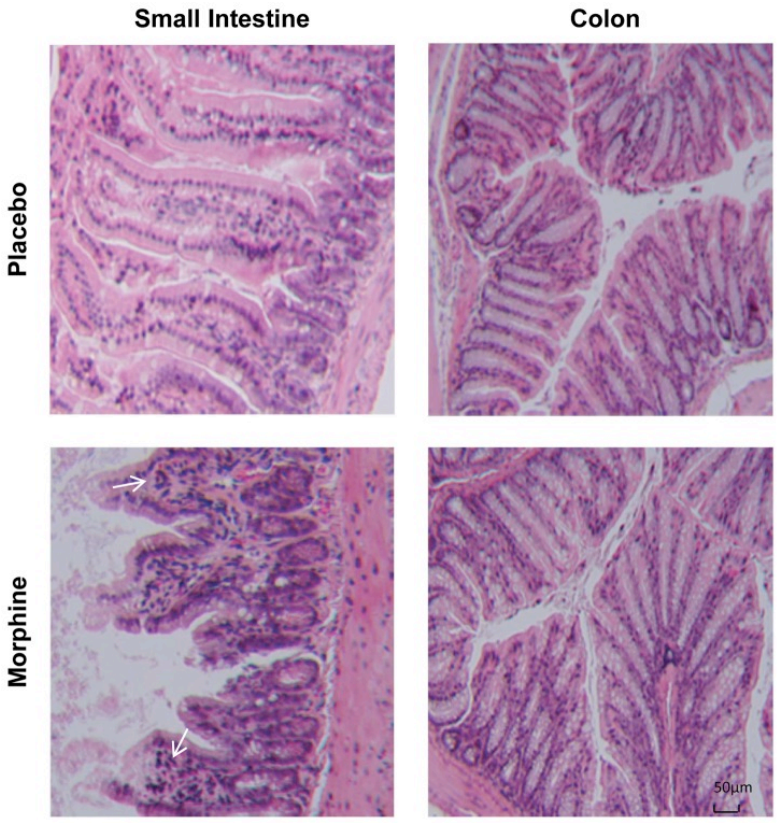


FIGURE 2.2 Chronic morphine induces inflammation in small intestine. Representative hematoxylin and eosin (H&E)-stained sections from the small intestine and colon of placebo- and morphine- treated WT mice. White arrow indicates inflammatory cell infiltration.

FIGURE 2.3

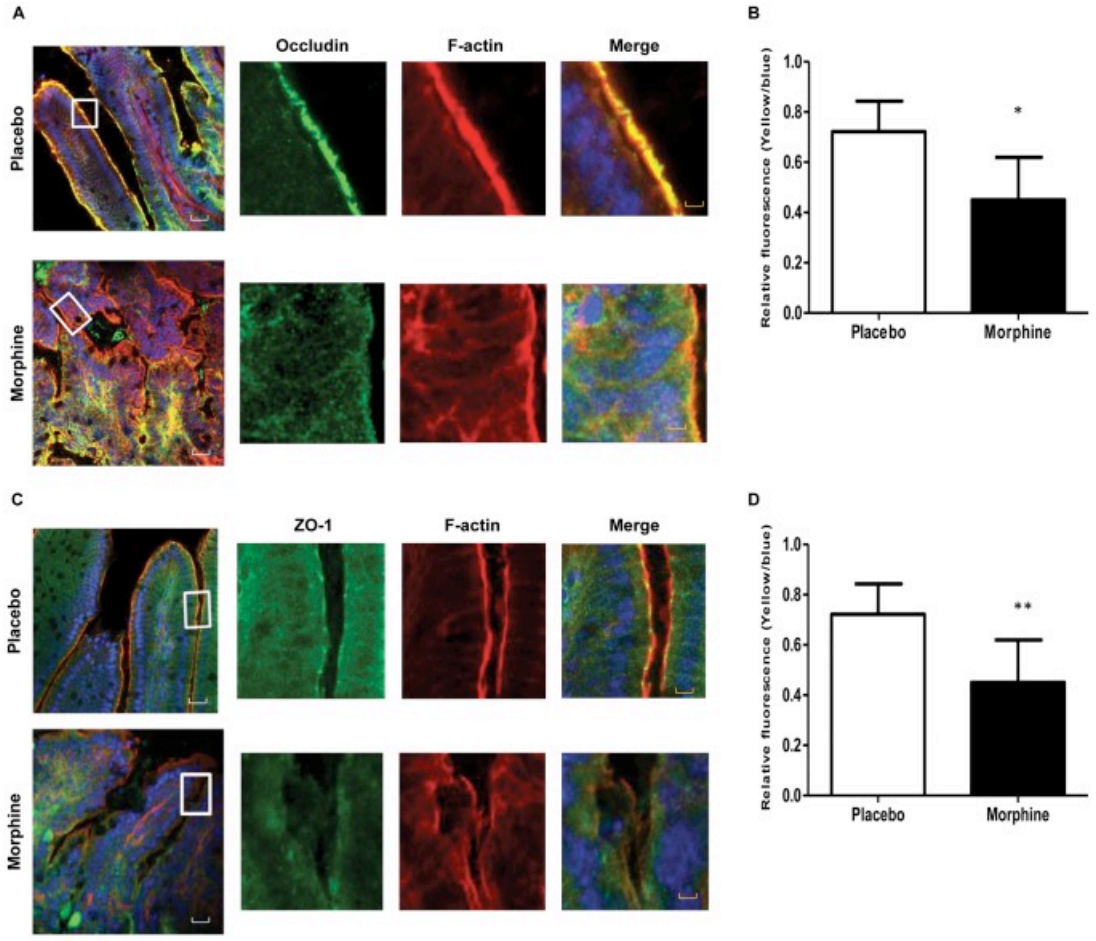


FIGURE 2.3 Chronic morphine disrupts tight junction organization between small intestinal epithelial cells. (A) Occludin organization in small intestine of WT mice. (C) ZO-1 organization in small intestine of WT mice. Quantification of co-localization of occludin (B) or ZO-1 (D) with F-actin are showed as relative intensity of yellow fluorescence normalized to blue fluorescence (DAPI) The same parts of small intestines and colons were excised and fixed. Images were analyzed by confocal scanning microscope. (n = 5) Scale bar: white 50 μm; yellow 10 μm * $P<0.05$, ** $P<0.01$ by Student's t-test.

FIGURE 2.3

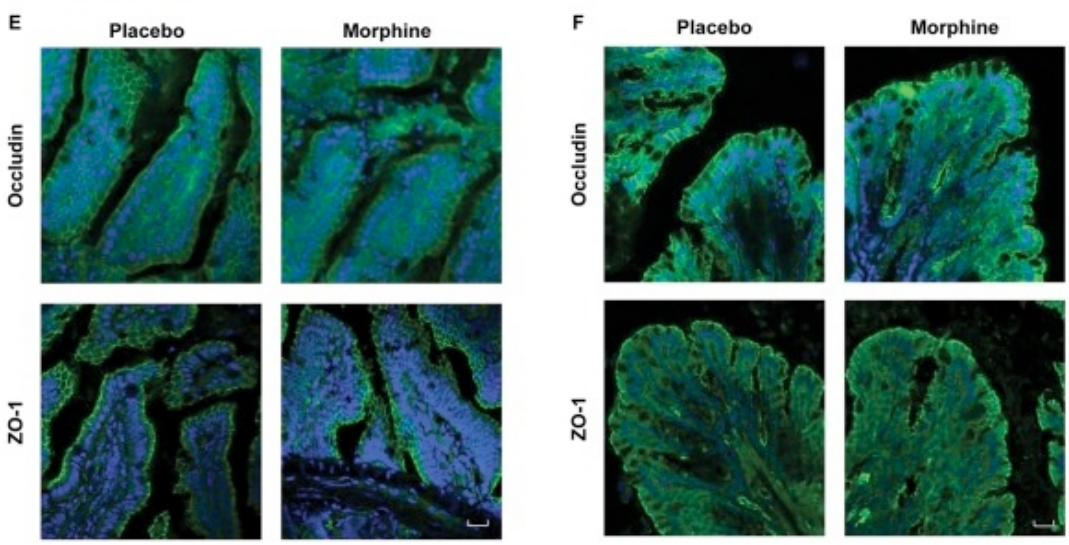


FIGURE 2.3 Chronic morphine disrupts tight junction organization between small intestinal epithelial cells. (E) Occludin and ZO-1 organization in small intestine of MORKO mice. (F) Occludin and ZO-1 organization of colon in WT mice. WT and MORKO mice were treated with 75 mg morphine pellet for 24 hours. The same parts of small intestines and colons were excised and fixed. Images were analyzed by confocal scanning microscope.

FIGURE 2.4

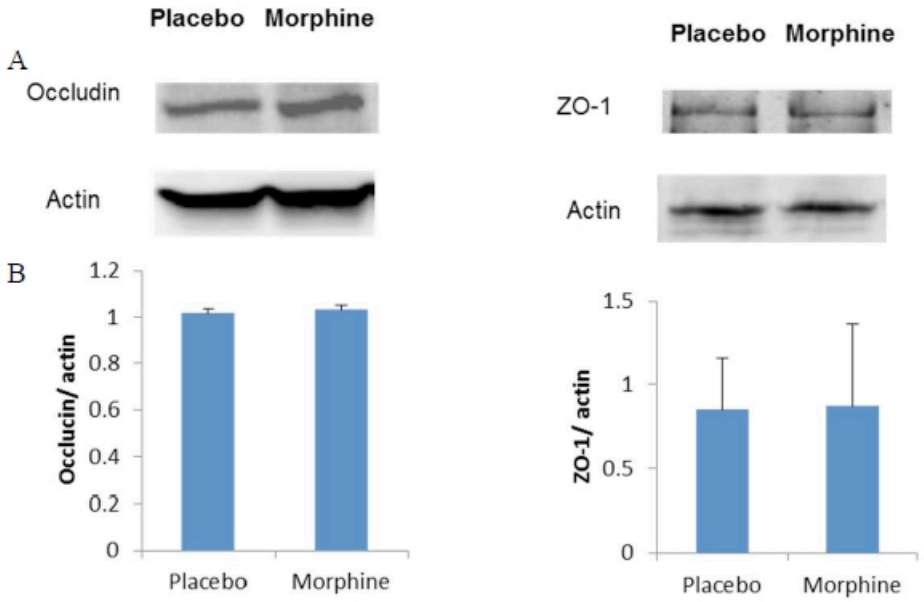


FIGURE 2.4 Occludin and ZO-1 expression of total small intestinal epithelial cells. Small intestinal epithelial cells were isolated from placebo and morphine-treated mice and lysed with RIPA buffer. The sample was used for WB. Figure B is the quantification of 3-time experiments.

FIGURE 2.5

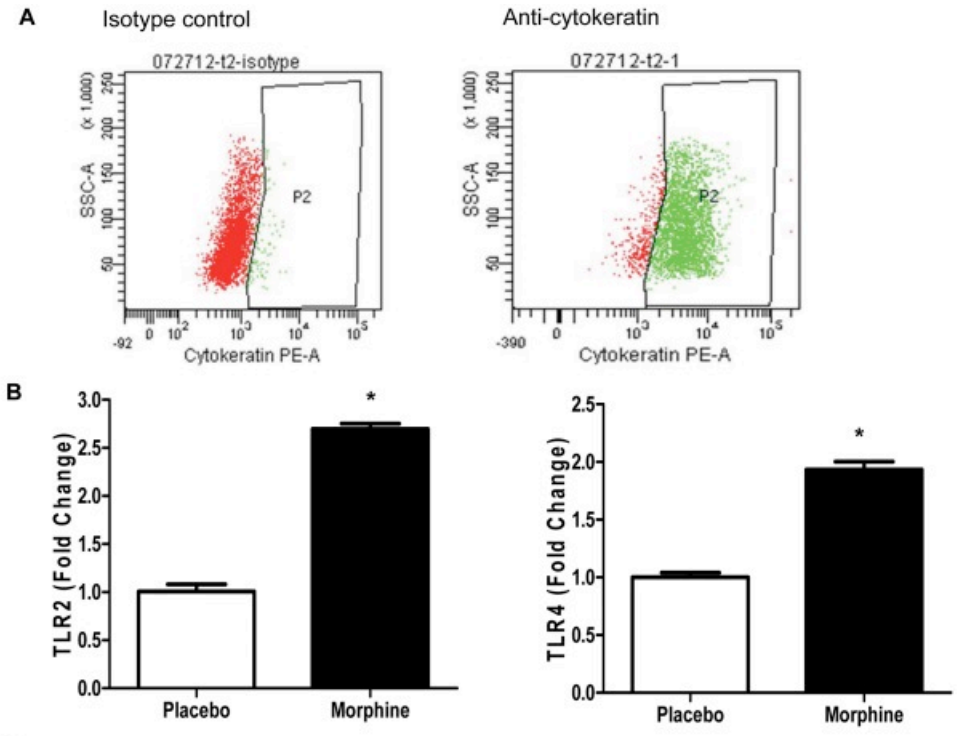


FIGURE 2.5 Morphine treatment upregulates TLR expression in small intestinal epithelial cells.(A) Isolated cells were fixed using eBioscience Fixation and Permeabilization Kit and then incubated with anti-cytokeratin antibody or isotype control. Cytokeratin positive cells were gated in P2 according to isotype control. (B) Real-time PCR analysis of mRNA levels of TLR2 and TLR4 in epithelial cells of small intestine after 24 hour morphine treatment. * $P<0.05$ by Student's t-test.

FIGURE 2.5

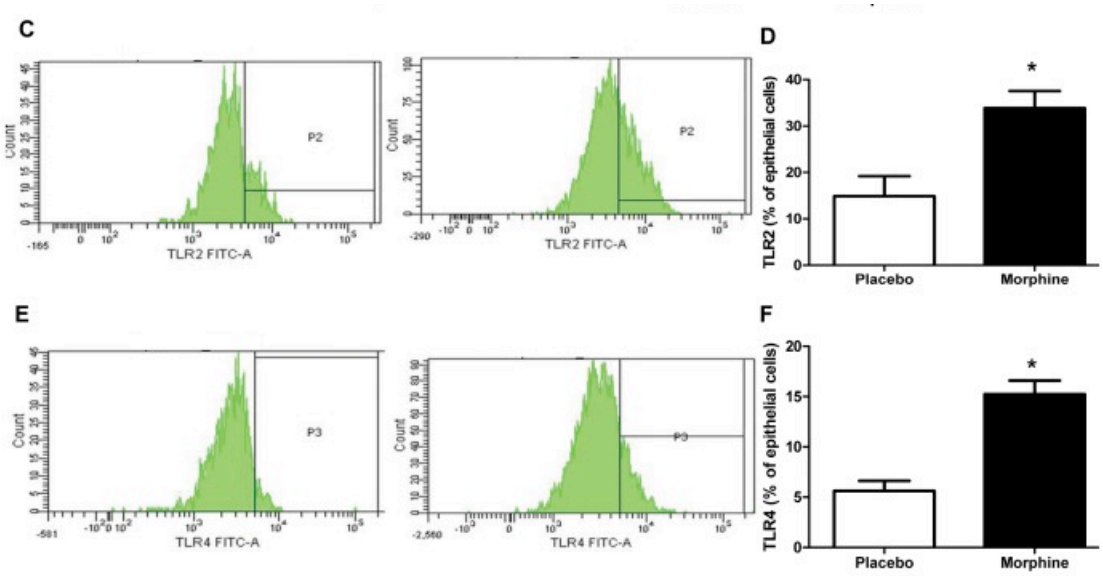


FIGURE 2.5 Morphine treatment upregulates TLR expression in small intestinal epithelial cells. (C) and (E) Representative expression of TLR2 and TLR4 in epithelial cells of small intestine after 24 hour morphine treatment from 3-time experiments. (D) and (F) Frequencies of TLR2 and TLR4 positive cells within cytokeratin positive cells. * $P<0.05$ by Student's t-test.

FIGURE 2.6

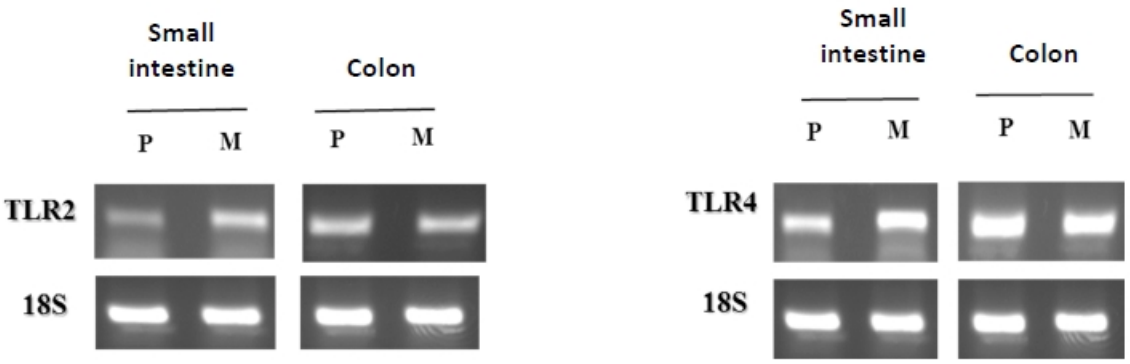


FIGURE 2.6 MOR expressions in small intestinal and colonic epithelial cells. Gel-based PCR analysis of mRNA levels of MOR in epithelial cells of small intestinal and colonic epithelial cells. SI: Small intestine; C: Colon.

FIGURE 2.7

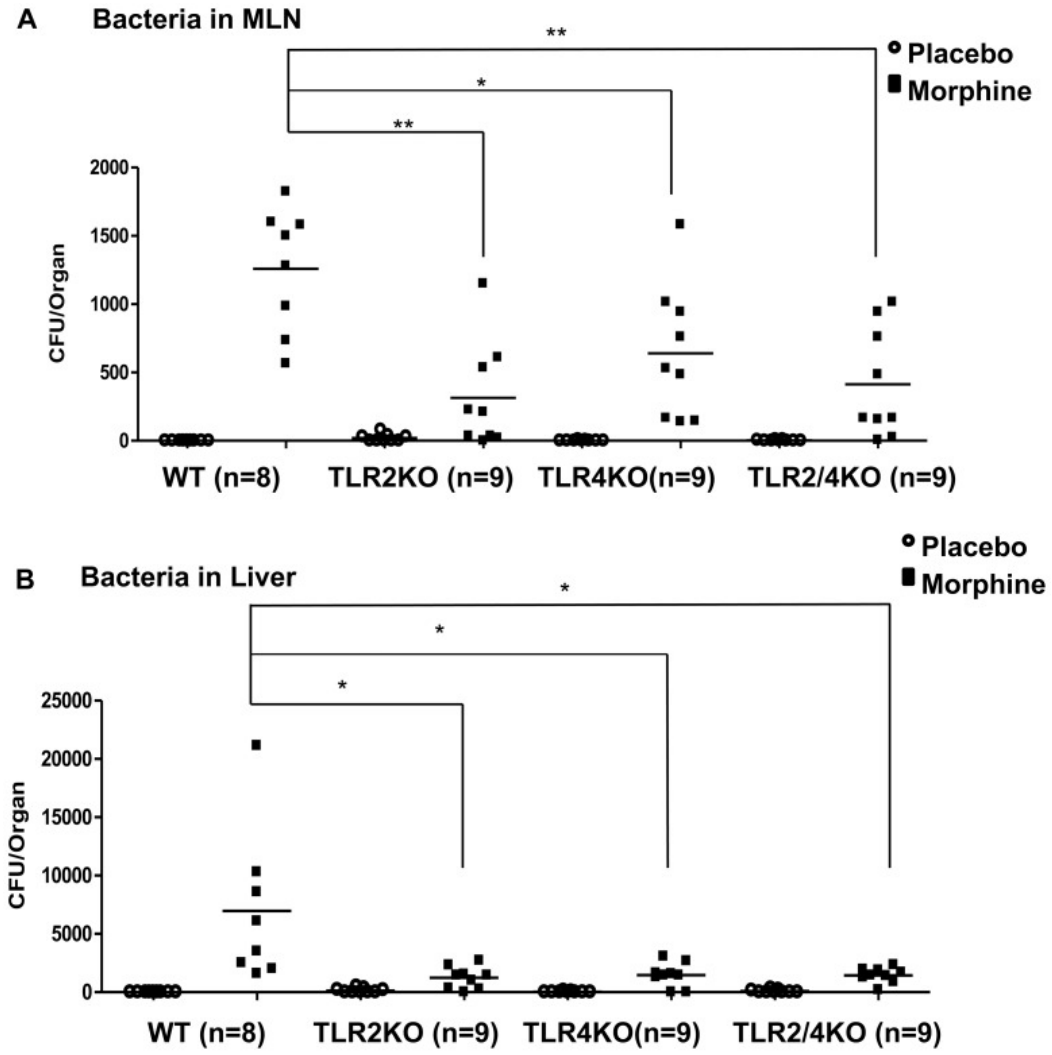


Figure 2.7 Morphine-induced bacterial translocation is attenuated in TLR2/TLR4 knockout mice. WT, TLR2 knockout, TLR4 knockout, and TLR2/4 double knockout mice were implanted with 75 mg morphine pellet for 24 hours; MLN(A), liver (B) were cultured on blood agar plates overnight. Bacterial colonies were quantified and described as CFU. – Mean of CFU *P<0.05, **P<0.01 by ANOVA one-way analysis, followed by Bonferroni post-test (n = 9).

FIGURE 2.8

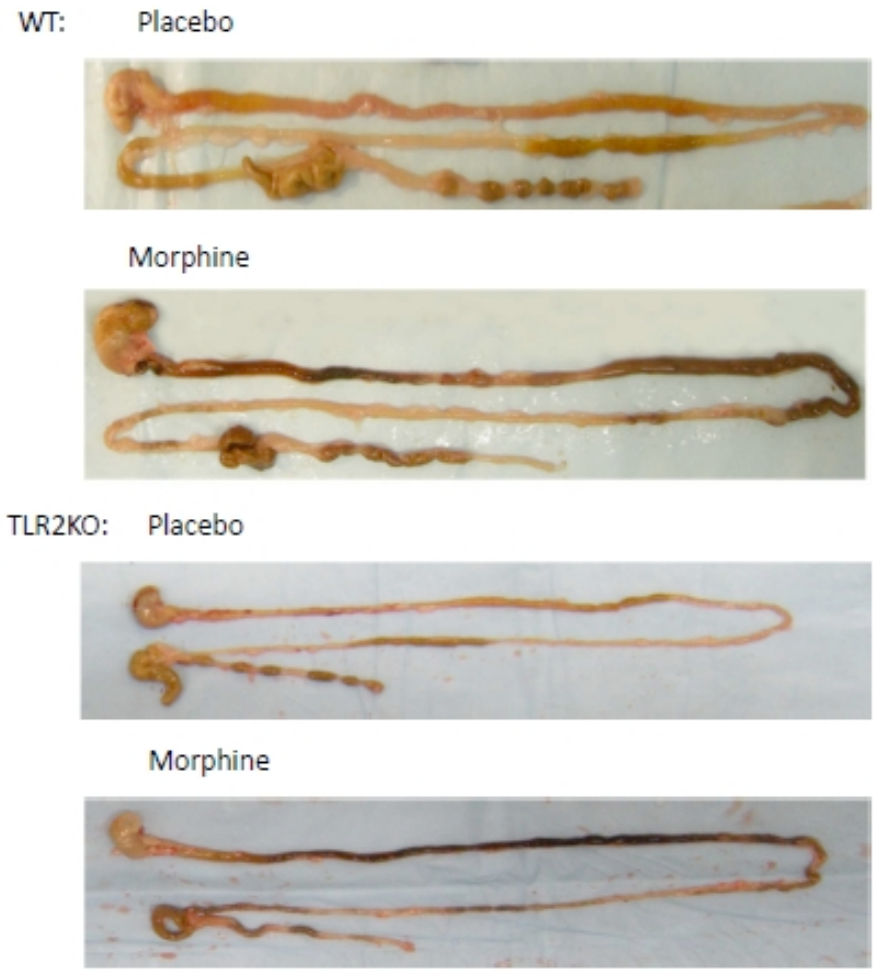
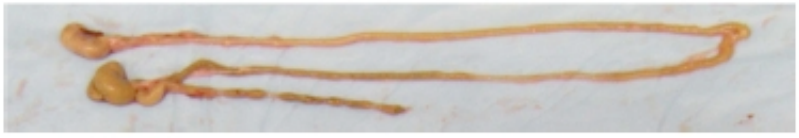


FIGURE 2.8 Morphine induces constipation in mice. Pictures of intestines from placebo- and morphine-treated WT, TLR2KO, TLR4KO, TLR2/4KO mice and mice treated with ML-7 or vehicle.

FIGURE 2.8

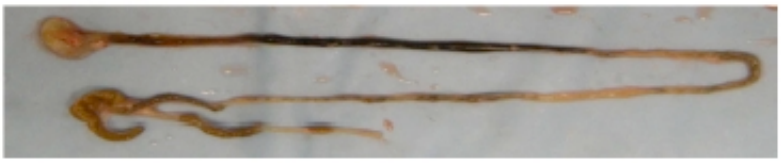
TLR4KO: Placebo



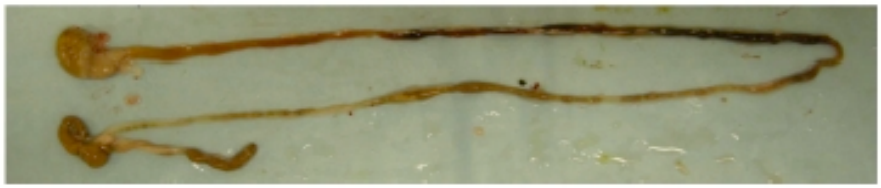
Morphine

TLR2/4KO: Placebo

Morphine



WT: Morphine +Saline



Morphine +ML-7

FIGURE 2.9

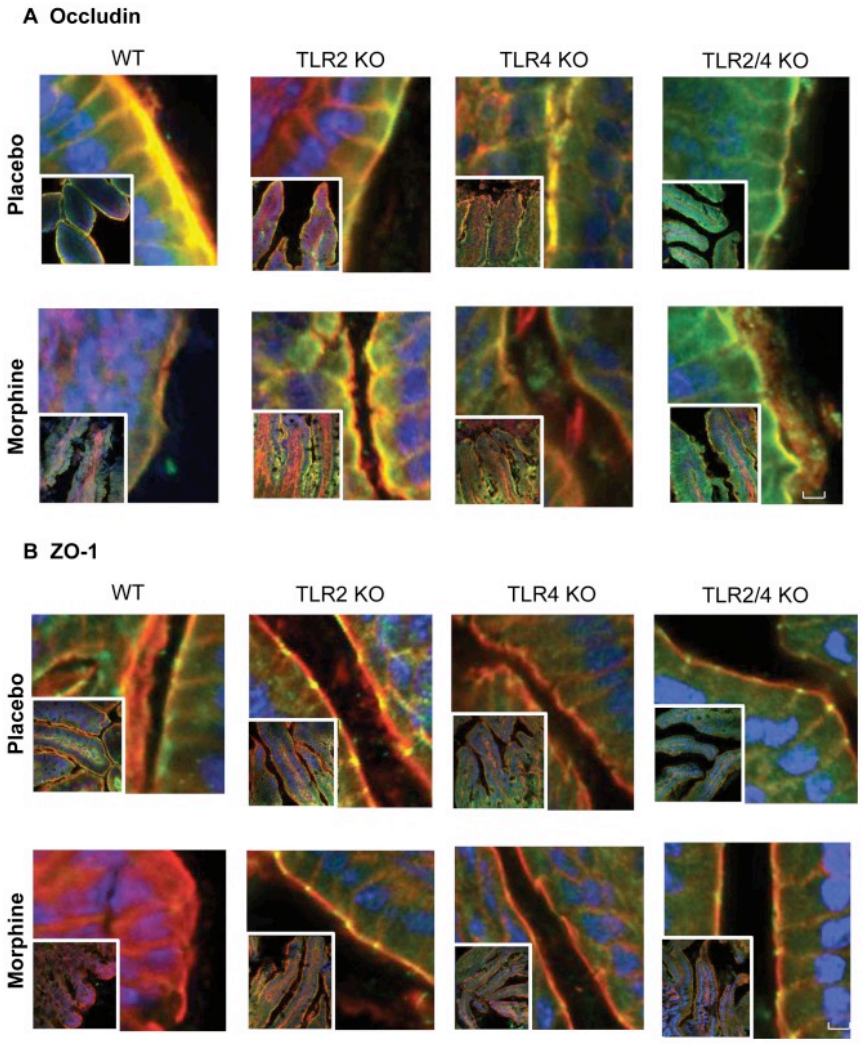


FIGURE 2.9 TLR2/TLR4 double knockout protects tight junction organization from morphine-induced disruption. (A) Occludin organization in small intestine of WT and TLRKO mice. (B) ZO-1 organization in small intestine of WT and TLRKO mice. WT, TLR2 knockout, TLR4 knockout, and TLR2/4 double knockout mice were implanted with 75 mg morphine pellet for 24 hours. The similar parts of small intestines were excised and fixed. Images were analyzed by confocal scanning microscope. (n = 5) Scale bar: 10 μm.

FIGURE 2.10

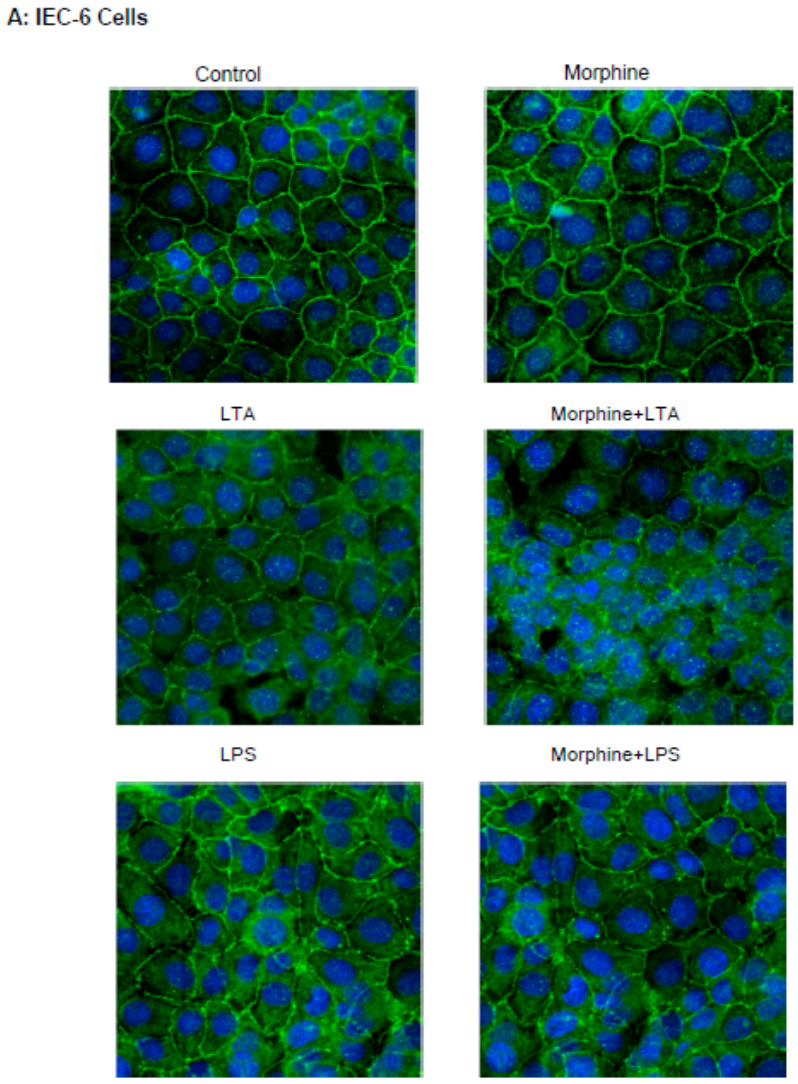


FIGURE 2.10 Morphine's effects on tight junction of IEC-6 and CMT-93 cells. IEC-6 and CMT-93 Cells were fixed and incubated with anti-zo-1 antibody, followed by FITC-labeled secondary antibody. Magnification ×600.

FIGURE 2.10

B: CMT-93Cells

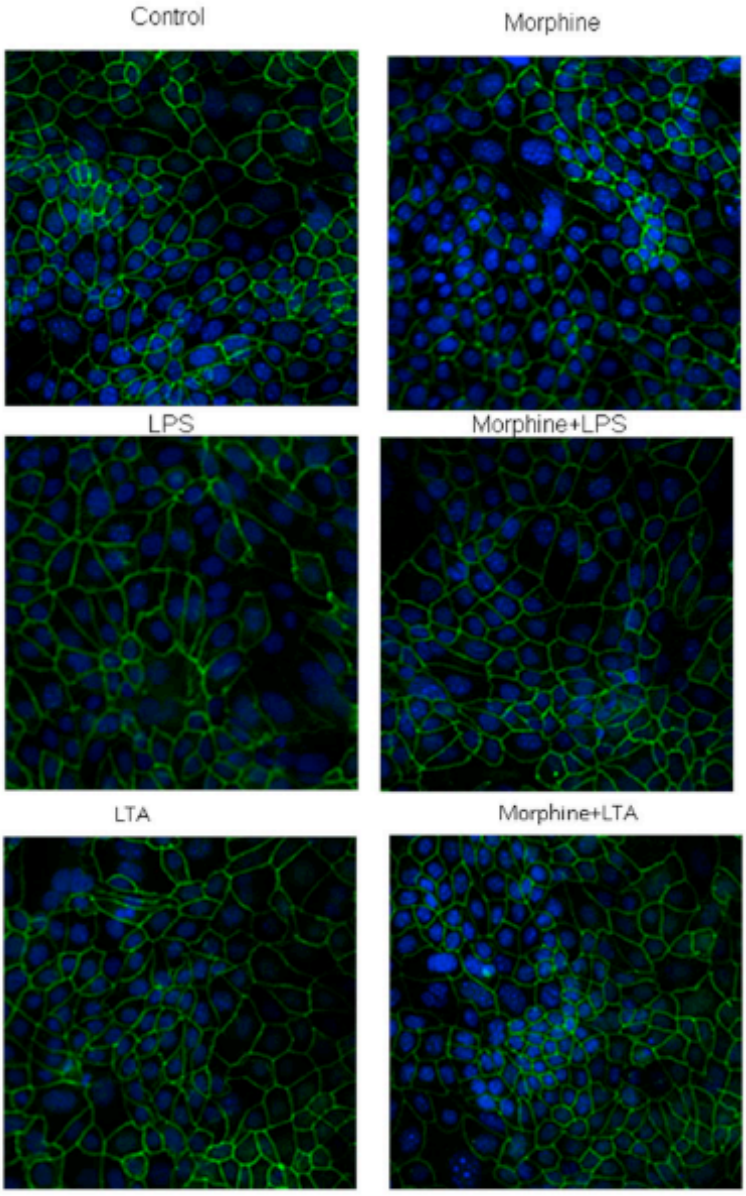


FIGURE 2.11

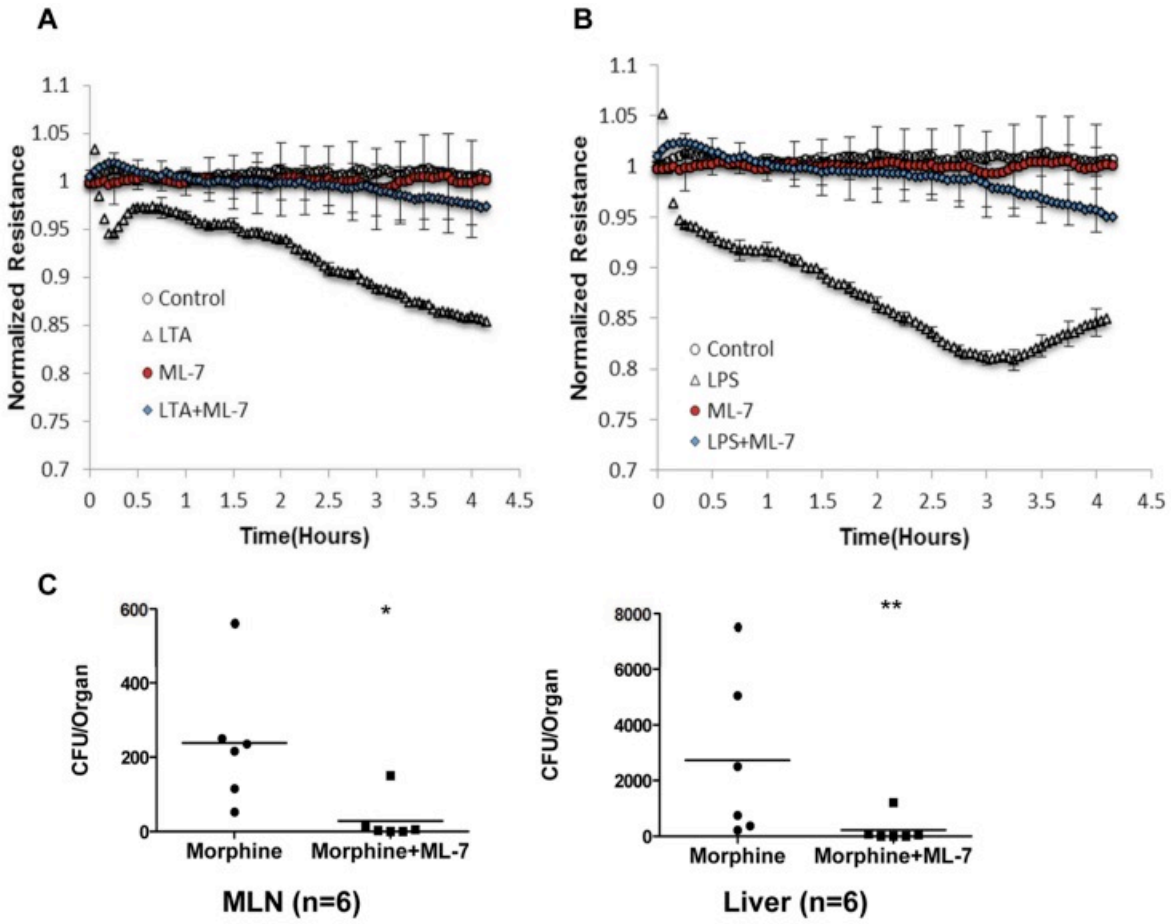


FIGURE 2.11 TLR signaling modulates intestinal tight junction organization in a MLCK-dependent manner.(A) Effects of LTA on TER of IEC-6 cells are blocked by MLCK inhibition. (B) Effects of LPS on TER of IEC-6 cells are blocked by MLCK inhibition. (C) Bacterial translocation to MLN and liver are blocked by MLCK inhibition. ** $p<0.01$ *$P<0.05$ by Mann-Whitney test.

FIGURE 2.11

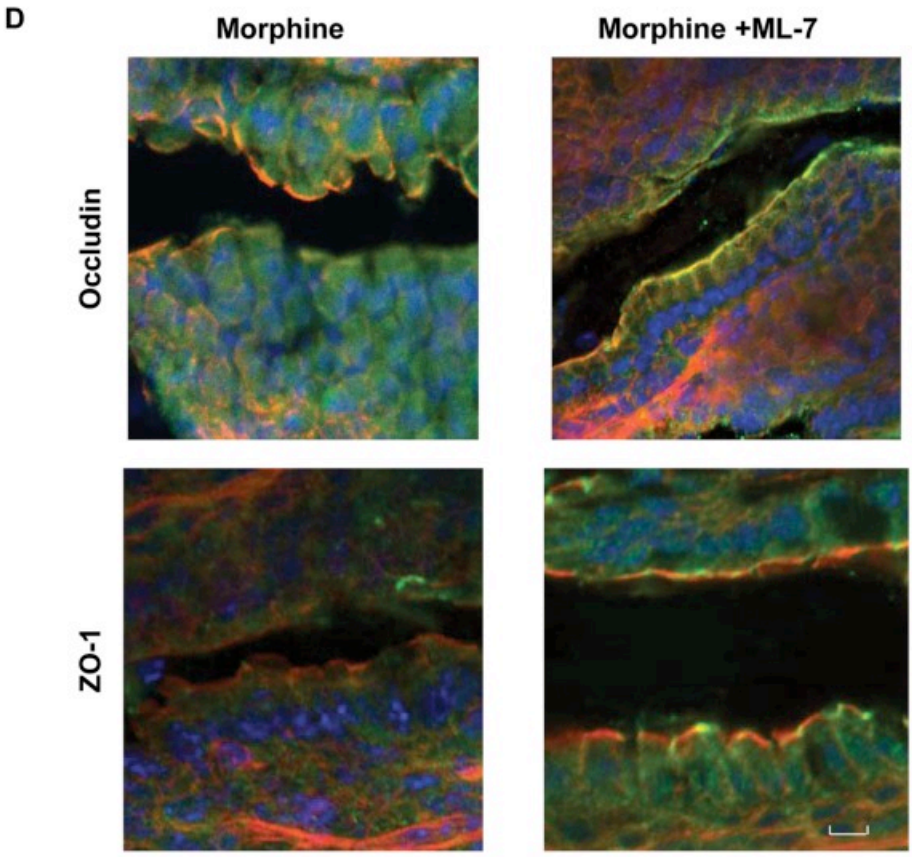


FIGURE 2.11 TLR signaling modulates intestinal tight junction organization in a MLCK-dependent manner. (D) MLCK inhibition protects tight junction organization following morphine treatment. (n = 6) Scale bar: 10 μm.

FIGURE 2.12

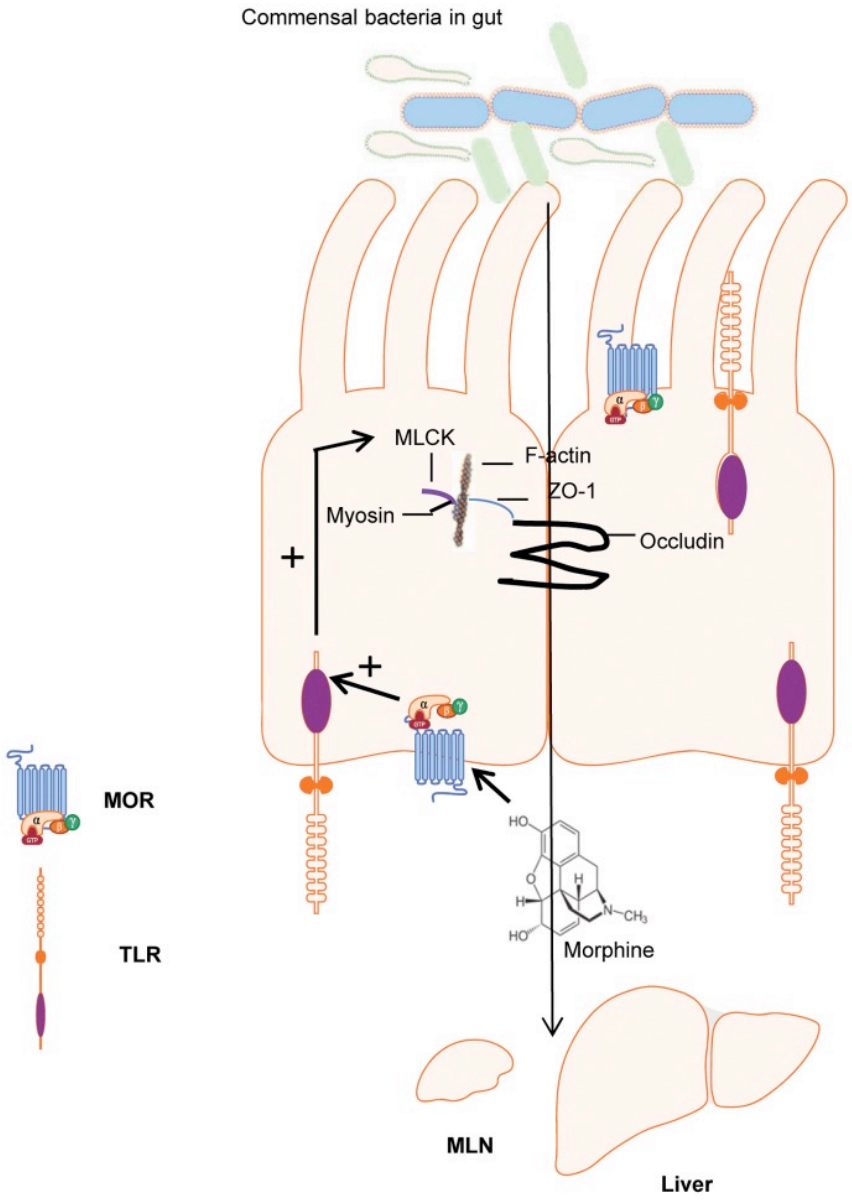


FIGURE 2.12 Model of morphine-induced disruption of gut epithelial barrier function. Morphine treatments up-regulate TLR expression levels in small intestinal epithelial cells. Activated TLR signaling induces tight junction disruption between epithelial cells and increases gut permeability, resulting in increased bacterial translocation.

CHAPTER 3

OPIOID TREATMENT MODULATES IMMUNE RESPONSES OF GALT

INTRODUCTION

Sepsis is a life threatening complication of infection. Infection-induced systemic inflammation contributes to multi-organ dysfunctions, resulting in high mortality(Angus and van der Poll 2013). International epidemiological studies show that the incidence of sepsis is increasing and half of the cases are occurring in the intensive care units (ICUs). Today, sepsis has become the leading cause of death in the ICUs with mortality rate as high as 60%, and accounts for approximately 40% of total ICU expenditures(Vincent et al. 2009).

Opioids are widely used in ICU patients for pain management and to optimize patient comfort and facilitate mechanical ventilation due to their potent analgesic activities and sedative properties(Devlin and Roberts 2009). However, the adverse effects associated with opioid therapy raise safety issues especially in ICU patients. The immunosuppressive effects of opioids are well documented (Sabita Roy et al. 2011). In clinical studies, higher circulating morphine levels were observed in patients with sepsis, severe sepsis, and septic shock compared with healthy controls(Glattard et al. 2010). Animal studies also showed that morphine treatment induced bacterial translocation from gut lumen into peritoneal organs including mesenteric lymph nodes (MLN), liver, and subsequently, the circulatory system(Hilburger et al. 1997; Meng et al. 2013). In addition,

morphine has been shown to accelerate the progression of LPS-induced sepsis by interfering with endotoxin tolerance(Banerjee et al. 2013). However, the clear mechanisms by which opioids modulate sepsis progression still remain largely elusive. In the present study, the cecal ligation and puncture procedure (CLP) was used to induce polymicrobial sepsis in C57BL/6J mice treated with opioids or placebo. The responses and the survival rate of mice were analyzed to investigate the effects of opioids on sepsis progression. Our results demonstrated that both morphine and methadone increased mortality of animals following CLP. And morphine treatment increased bacterial dissemination and pro-inflammatory cytokine interleukin-17A (IL-17A) and interleukin-6 (IL-6) production during sepsis progression.

IL-17A is a member of the interleukin-17 (IL-17) family consisting of a subset of cytokines that participate in both acute and chronic inflammatory responses. In various infectious diseases, IL-17A is involved in neutrophil recruitment and host defense and implicated in excessive inflammation and overt tissue damage(Gu, Wu, and Li 2013). To date, the roles of IL-17A in the sepsis progression have not been well characterized. Our study show that overexpression of IL-17A following morphine treatment resulted in increased gut permeability, higher bacterial load in different organs, sustained inflammation, and subsequently higher mortality. More importantly, neutralization of IL-17A protected morphine-treated animals from sepsis-induced mortality.

In addition, we also show that morphine modulation of IL-17A response was mediated by the gut microbiome. Morphine treatment induced enrichment of gram-positive bacteria *Staphylococcus* and *Enterococcus* in the gut lumen. Subsequently, more

Staphylococcus and *Enterococcus* disseminated to various organs and were recognized by Toll like receptor 2 (TLR2). TLR2 is a member of the pattern recognition receptor (PRR) family, specially recognizing cell-wall components such as peptidoglycan, lipoteichoic acid and lipoprotein from gram-positive bacteria(Takeda, Kaisho, and Akira 2003). Activation of TLR2 led to overexpression of IL-17A, resulting in excessive inflammation, and consequently accelerated progression of sepsis and higher mortality compared with placebo-treated animals. These results are consistent with an interesting clinical observation that the most common gram-positive isolates from patients with sepsis are *Staphylococcus aureus*. Additionally, infection with *Enterococcus* species has been considered as one independent factor associated with greater risk of hospital death(Vincent et al. 2009). Therefore, our studies provide insights into the influence of opioids on sepsis progression; implying that the therapeutic window for the dose of opioids used in ICU patients is narrow and that IL-17A may be the potential therapeutic targets to treat sepsis caused by gram-positive infection, especially in ICU patients who are on a moderate to severe pain management regimen.

METHODS

Experimental animals and cell lines

Male C57BL/6 and TLR2KO mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All animals were maintained in pathogen-free facilities and all procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee. Typically, 8–10 week old animals were used for our studies. IEC-6 cell lines was obtained from American Type Culture Collection and cultured as recommended by the supplier. IEC-6 cells are rodent small intestinal epithelial cell lines, which have been used for studying intestinal barrier and integrity in previous publications.

Induction of sepsis using CLP model and opioid treatment

Polymicrobial sepsis was induced as previously described(Toscano, Ganea, and Gamero 2011). Mice were anesthetized with 3% isoflurane, a 1-cm midline incision was made on the anterior abdomen. The cecum was exposed and ligated and the distance from the distal end of cecum to ligation point was approximately 1 cm. A double puncture was made with 22-gauge needle to induce sepsis. The cecum was squeezed to allow cecum contents to be expressed through the punctures. The cecum was placed back in the abdominal cavity and the peritoneal wall and skin incision were closed. All animals received 1 ml of saline by subcutaneous injection immediately after the surgery. Sham-operated animals (controls) underwent identical laparotomy but without cecum ligation or puncture. The survival rates of animals were observed every 12 h up to 7 days after

surgery. For morphine treatment, a small incision was made at the dorsal torso of the mice. The appropriate pellet was inserted into the small pocket created during incision and the wound was closed using stainless steel wound-clips. For methadone treatment, saline or 15mg/kg of methadone was given by intraperitoneal injection. For IL-17A neutralization, 100μg of LEAF^{TM} anti-mouse IL-17A antibodies (BioLegend) were administered to mice by intraperitoneal injection every other day.

Bacterial counts in blood, peritoneal lavage, MLN, liver and spleen

The bacterial counts were determined 6, 24, 72, and 168 hours after CLP surgery in the peritoneal lavage, MLN, liver, spleen and blood. Peritoneal lavage, blood, and homogenates of liver, MLN, and spleen were cultured on blood agar plate overnight. Bacterial colonies were quantified and described as colony forming units (CFU).

Cytokine measurements

The cytokine levels were detected in peritoneal lavage, serum, and supernatant of MLN cells. The following mouse ELISA kits were used: IL-1β, IL-6, IL-23, IL-17A, (eBiosciences). All experiments were performed according to the manufacturer's protocol.

Flow cytometry analysis

MLN was homogenized and single cell suspension was incubated with anti-CD3, anti-CD4, and anti-IL-17A antibodies following manufacturer's intracellular staining

protocol (eBiosciences).

Intestinal permeability

Animals were gavaged with 4kD FITC-dextran (500 mg/kg body weight in 50mg/ml concentration) utilizing a 4-cm long, curved needle with a plastic ball at the tip. The images of animals were analyzed using Xenogen Spectrum system. After sacrifice, blood and peritoneal lavage were collected and the intensity of FITC was determined with a fluorometer using an excitation wavelength of 488 nm, and detecting emission at 520 nm.

Dendritic cell isolation

Dendritic cells from blood of wide type or TLR2KO were purified by magnetic separation (Miltenyi Biotec) according to the manufacturer's instruction.

Measurement of trans-epithelial resistance

ECIS 1600R (Applied BioPhysics, Troy, NY) was used to measure trans-epithelial resistance (TER) of epithelial monolayers as described previously(Schlegel et al. 2010). Epithelial cells were seeded in the wells of the electrode array and grown to confluence as indicated below. Then medium was changed, and baseline TER was measured for 60 min to equilibrate monolayers. Afterward, 400 ml of medium containing different concentrations of IL-17A was applied to each well. The baseline TER of each experiment was normalized to 1.0 to enable comparison and statistical analysis of TER changes over

time following different treatments.

Transwell assays

Transwell assay was performed as described previously(Wójciak-Stothard et al. 2001).IEC-6 cells were cultured in the top chamber of transwell system and 5mg/ml 4kD FITC-dextran was added to the top chamber after 24 hours of IL-17A (100ng/ml) stimulation. The medium in the lower chamber was taken after 6 hours. The amounts of FITC-dextran in the lower chambers were determined with a fluorometer using an excitation wavelength of 488 nm, and detecting emission at 520 nm.

Immunofluorescence

IEC-6 cells in the chamber slides were fixed with 1% paraformaldehyde in PBS for 10 min at room temperature. After washing in PBS and blocking of nonspecific binding sites with 5% bovine serum albumin (BSA), slides were incubated with polyclonal rabbit anti-ZO-1 (both used at 5 mg/ml, Invitrogen) in PBS with 5% bovine serum albumin (BSA) for 120 min at room temperature. After washing, slides were incubated with rhodamine phalloidin (Invitrogen) and DyLightTM 488-conjugated AffiniPure Donkey anti-rabbit IgG (0.075 mg/ml, Jackson Lab, WestGrove, PA) for 60 min. Slides were then washed and mounted under coverslips using ProLong Gold antifade reagent with DAPI (Invitrogen). Slides were imaged using a confocal microscope (Nikon).

Gut microbiome analysis

Fecal content was collected from gut region encompassing distal cecum and approximately one inch of the colon. The gut tissue was washed and stored separately. The fecal matter was lysed using glass beads in MagnaLyser tissue disruptor (Roche) and total DNA isolated using Power-soil/fecal DNA isolation kit (Mo-Bio) as per manufacturer's specifications. All samples was quantified via the Qubit® Quant-iT dsDNA Broad-Range Kit (Invitrogen, Life Technologies, Grand Island, NY) to ensure that they met minimum concentration and mass of DNA and submitted to Second genome Inc. for microbiome analysis as follows: To enrich the sample for the bacterial 16S V4 rDNA region, DNA was amplified utilizing fusion primers designed against the surrounding conserved regions which are tailed with sequences to incorporate Illumina (San Diego, CA) flow cell adapters and indexing barcodes. Each sample was PCR amplified with two differently bar coded V4 fusion primers and were advanced for pooling and sequencing. For each sample, amplified products were concentrated using a solid-phase reversible immobilization method for the purification of PCR products and quantified by electrophoresis using an Agilent 2100 Bioanalyzer®.The pooled 16S V4 enriched, amplified, barcoded samples were loaded into the MiSeq® reagent cartridge, and then onto the instrument along with the flow cell. After cluster formation on the MiSeq instrument, the amplicons were sequenced for 250 cycles with custom primers designed for paired-end sequencing. Using QIIME and custom scripts, sequences were quality filtered and demultiplexed using exact matches to the supplied DNA barcodes. Resulting sequences were then searched against the Greengenes reference database of 16S sequences, clustered at 97% by uclust (closed-reference OTU picking). The longest

sequence from each Operation Taxonomic Unit (OTU) thus formed was then used as the OTU representative sequence, and assigned taxonomic classification via mothur's bayesian classifier, trained against the Greengenes database clustered at 98%.

Statistical analysis

The data (except for the survival curves and scatter plots) were reported as the means $\pm$ SEM of values of triplicates. The means of different treatments were compared by student t test or ANOVA followed by Bonferroni's t test (GraphPad Prism Software). Bacterial counts were reported as means of CFU and were analyzed by the Mann-Whitney U test (GraphPad Prism Software). The survival rate was expressed as the percentage of live animals, and the Mantel-Cox log rank test was used to determine differences between survival curves (GraphPad Prism Software version 3). A p value of 0.05 or less was considered significant. For gut microbiome analysis, the Adonis test was utilized for finding significant whole microbiome differences among discrete categorical or continuous variables. In this randomization/Monte Carlo permutation test, the samples were randomly reassigned to the various sample categories, and the mean normalized cross-category differences from each permutation were compared to the true cross-category differences. The fraction of permutations with greater distinction among categories (larger cross-category differences) than that observed with the non-permuted data reported as the p-value for the Adonis test.

RESULTS

Opioids increased mortality rates in a polymicrobial sepsis model induced by CLP.

To determine the effects of opioids on the outcome of polymicrobial sepsis, C57BL/6J wild type (WT) mice were subjected to polymicrobial sepsis using the CLP model and implanted with placebo or 25mg morphine pellet subcutaneously. There was a significant decrease in the weight of the animals from Day1 to day3 following CLP due to diarrhea. However from Day 4, no weight loss was observed and the animals showed signs of recovery (Figure 3.1A). In contrast, morphine's anti-diarrhea effects attenuated weight loss in mice following CLP (Figure 3.1A). Interestingly, the survival rates of mice after CLP were not associated with the weight loss. As shown in Figure 3.2A, morphine-treated mice showed significant reduction in survival rate following CLP. At 24h after CLP, 100% of placebo-treated mice were alive compared with only 66.67% in morphine-treated mice. None of the morphine treated mice survived beyond 96 hours while 78.57% of placebo-treated mice survived for the whole period of the observation ($p < 0.01$ by Mantel-Cox log rank test). All the sham-operated mice treated with placebo pellet survived for the whole period of observation while 80% of sham-operated mice treated with morphine pellet survived. To investigate the influence of other prescription opioids on outcome of CLP-induced sepsis, we also injected methadone or saline to mice after the CLP procedure. Methadone showed the similar effects on survival rates of mice following CLP (Figure 3.2B). 66.67% of saline-treated mice were alive for the whole period of observation (7days) whereas no methadone-treated mice survived on the fifth day after CLP ($p<0.05$ by Mantel-Cox log rank test). All the sham-operated mice injected

with saline or methadone survived for 7 days after the procedure. Moreover, morphine and methadone-induced mortality following CLP was significantly reduced by the opioid receptor antagonist naltrexone (Figure 3.2C and D), indicating that opioid treatment influenced the outcome of polymicrobial sepsis in an opioid receptor-dependent manner.

Morphine promoted bacterial dissemination and inhibited bacterial clearance during sepsis.

To understand the mechanism underlying the higher mortality in our sepsis model associated with opioid treatment, we determined the bacterial load in the peritoneal lavage, mesenteric lymph node (MLN), liver, spleen, and blood at different time points following CLP in the presence or absence of morphine treatment.

As shown in Figure 3.3, at 24 hours, morphine treatment alone induced bacterial translocation into the peritoneal cavity, MLN, liver, and spleen, implying that morphine itself could compromise gut epithelial barrier function. In the placebo-treated CLP animals, the amount of bacterial in peritoneal lavage, MLN, liver, spleen, and blood reached the highest levels at 24 hours, and decreased at 72 hours. 168 hours after CLP, almost all bacteria disseminated into peritoneal lavage, MLN, liver, spleen, and blood were cleared in the placebo-treated animals. In the morphine-treated mice that were subjected to CLP, a significant increase in the amount of bacteria in peritoneal lavage, MLN, liver, spleen, and blood was observed when compared to the placebo-treated mice at both 24 and 72 hours, indicating that morphine treatment promoted bacterial dissemination and inhibited bacterial clearance. Since none of the animals survived

beyond 168 hours in the morphine-treated group, data on bacterial load was not available for that time point for morphine-treated animals.

Morphine treatment modulated microbiota in the gut lumen.

We next serotyped the bacterial species disseminated into the MLN, liver, and spleen following CLP to identify the bacterial species. In placebo-treated CLP animals, the most common bacterial species detected in MLN, Spleen, and liver were non-haemolytic *Escherichia coli* (Figure. 3.4A) which were common gram negative commensal bacteria resident in gut lumen, and only small amount of Enterococcus were detected in MLN and spleen. However all MLN, spleen, and liver isolates from morphine-treated animals with or without CLP procedure revealed a prevalence of the gram-positive families Staphylococcus and Enterococcus (Fig. 3.4A).

Interestingly, analysis of the gut microbiome showed that morphine treatment induced enrichments of mostly the Firmicutes phylum and specifically the gram positive bacterial species *staphylococcus sciuri, staphylococcus cohnii, and staphylococcus aureus* as well as *enterococcus durans, enterococcus casseliflavus, enterococcus faecium,* and *enterococcus faecalis* in the gut lumen (Fig. 3.4B and C), all of which belonged to the gram positive family of bacteria. Interestingly, these were also the same species that were observed to translocate to various organs following morphine treatment. Morphine-induced alterations of gut microbiome was antagonized by the opioid receptor antagonist naltrexone, further validating that morphine treatment modulated gut microbiome and thereby influenced the outcome of polymicrobial sepsis in an opioid receptor-dependent manner.

Morphine up-regulated IL-17A production during sepsis

To determine the effects of morphine on the immune responses in the CLP-induced sepsis model, we collected serum, peritoneal lavage and MLN to measure IL-17A production at different time points. The results show that IL17A levels in peritoneal lavage and serum increased at 6 hours after CLP in both placebo and morphine-treated mice. In placebo-treated mice, IL-17A concentration reduced to baseline level at 24 hours while in the morphine group a sustained high level of IL-17A was observed (Figure 3.5A and 3B). Previous studies showed that there were two sources of IL-17A in response to infections: the T helper 17 cells and type three innate lymphoid cells(Walker, Barlow, and McKenzie 2013). To determine which types of cells are producing IL-17A following CLP procedure, we used anti-CD3 and anti-CD4 antibodies to separate MLN cells into two populations: CD3+CD4+ T helper cells and CD3- non-T Cells. The flow cytometry analysis showed that placebo-treated animals did not show significant up-regulation of IL-17A at 24 hours alter CLP (Figure 3.4C and 3.4E). In contrast, a significant increase in IL-17A production was observed in the morphine treated animals. Further characterization revealed that the major source in MLN of IL-17A was from the CD3+CD4+ T helper cells which increased significantly (Figure 3.5C) but not CD3- non-T Cells (Figure 3.5E).

Neutralization of IL-17A improved survival rate and attenuated sustained inflammation in CLP mice treated with morphine

To investigate the role of IL-17A in sepsis progression following morphine

treatment, a neutralizing monoclonal antibody to IL-17A or isotype control IgG was administered to morphine-treated mice by intraperitoneal injection after the CLP procedure. As shown in Figure. 3.6A, when morphine-treated mice were administered with anti-IL-17A, the survival rate was significantly improved (65.6% survival) at 96 hours after CLP, whereas none of isotype injected control mice survived. For the whole period of observation (7days), the survival rate of mice treated with anti-IL-17A was 52.5% (Figure 3.6A). Additionally, mice treated with anti-IL-17A showed significantly reduced bacterial disseminations to peritoneal lavage, MLN, and liver at 24 hours following CLP compared with isotype-control-treated animals (Figure 3.6B-D), implying that neutralization of IL-17A following CLP improved gut barrier functions. It has been reported that serum levels of IL-6 is a good marker for severity during sepsis12. Thus, we determined the IL-6 levels in serum at different time points following CLP. ELISA results showed that in placebo-treated animals, serum IL-6 peaked to 1700pg/ml at 24 hours after CLP and then reduced to baseline levels at 72 hours while morphine-treated mice showed sustained high levels of IL-6 in serum even at 72 hours following CLP (Figure 3.6E). Neutralization of IL-17A significantly decreased IL-6 serum level in the morphine-treated animals at 72 hour after CLP (Figure 3.6F), validating the pro-inflammatory role of IL-17A during sepsis progression.

High levels of IL-17A compromised gut epithelial barrier function and increased gut permeability.

To determine the gut permeability during sepsis progression, we gavaged the mice

with fluorescein isothiocyanate (FITC) labeled dextran and tracked the diffusion of dextran using Xenogen Spectrum imaging system. As shown in Figure 3.7A, morphine treatment resulted in an increase in FITC-dextran diffusion across the gut epithelium, indicating that morphine treatment increased gut permeability during sepsis. To further validate that gut permeability was increased by morphine treatment, we quantified FITC-dextran in the peritoneal lavage and blood. In morphine-treated animals, FITC intensities were significantly higher in both peritoneal lavage and blood compared with placebo-treated animals (Figure 3.7B). In morphine treated animals that were injected with anti-IL-17A antibodies, we observed a decrease in FITC-dextran diffusion across the gut epithelium into the peritoneal cavity and blood (Figure 3.7C and D), demonstrating that neutralization of IL-17A restored gut barrier function in morphine-treated CLP animals. To investigate the effects of morphine on the morphology of the intestinal epithelium in CLP animals, small intestine and colon were excised and fixed in a formalin solution for hematoxylin and eosin (H&E) staining. Histological analysis indicated severe epithelial injury in small intestinal villi of morphine-treated mice after CLP compared with the appearance of continuous epithelial cells lining gut mucosal surfaces in placebo-treated mice (Fig 3.7E). Interestingly, the morphology of the colonic epithelium were not affected by morphine treatment (Figure 3.8), suggesting differential sensitivities of colonic and small intestinal epithelial cells to inflammatory stimulation. Neutralization of IL-17A also protected the epithelial structure of small intestines in morphine-treated mice following CLP (Figure 3.7F).To determine the direct effects of IL-17A on small intestinal epithelial cells, we determined the barrier function of IEC- 6 cells, which are rat small

intestine epithelial cells, by electrical cell impedance sensing (ECIS) arrays. The cells were grown to confluence in ECIS arrays, and the trans-epithelial resistance (TER) values were measured to test whether IL-17A would affect epithelial barrier integrity. The baseline TER of each experiment was normalized to 1.0 to enable comparison and statistical analysis of TER changes over time following different treatments. IEC-6 cells were treated with different concentrations of IL-17A (Figure 3.7G). TER values were significantly reduced following IL-17A treatment and the duration of the effects of IL-17A persisted for a longer time as its concentration increased. We further determined the barrier function of IEC-6 cells using a trans-well system. Tans-well assay showed that both apical and basolateral stimulation with 100ng/ml of IL-17A increased permeability of IEC-6 monolayer (Figure 3.7H). Next, we investigated the organization of tight junction proteins between the epithelial cells which plays an important role in modulation of the epithelial barrier function. Staining of tight junction protein zona occludens 1 (ZO-1) in IEC-6 cells also indicated the disruptive effects of IL-17A on IEC-6 monolayer (Figure 3.7I). As shown in Figure 3.6I, ZO-1 (green color) localized with F-actin (red color) on the apical side of the membrane in vehicle-treated cells, and its organization was seen to be disrupted following 6 hours of IL-17A stimulation.

Gram Positive Bacteria Stimulated MLN to produce IL-17A in a TLR2-dependent manner.

To investigate the mechanism by which morphine modulated IL-17A production in CLP animals, we cultured the immune cells from MLN in vitro and stimulated the cells

with either a gram negative bacteria species (*E. coli*) or the gram positive species that translocated into the MLN following morphine treatment, which were mixture of *Enterococcus* and *Staphylococcus*. The results showed that *E. coli* was able to induce higher levels of IL-6 production when compared with mixture of Enterococcus and Staphylococcus however high levels of IL-17A were only observed when MLN cells were stimulated by *Enterococcus* and *Staphylococcus* (Figure 3.9A and B). Meanwhile MLN cell were treated with morphine and IL-17A concentrations in the supernatant were measured by ELISA at different time points following gram positive bacteria stimulation. Interestingly, morphine treatment alone did not show any direct effects on IL-17A production by MLN cells (Figure 3.9C). TLR2 has been shown to play an important role in recognizing cell-wall components from gram-positive bacteria and initiating the immune responses to pathogen stimulation. To understand the role of TLR2 in IL-17A production by MLN, we measured IL-17A production in serum, peritoneal lavage, and MLN in TLR2KO mice following CLP procedure. No IL-17A induction was observed in placebo or morphine-treated CLP TLR2KO animals, strongly implying the role of TLR2 in IL-17A responses during CLP-induced sepsis (Figure 3.9D to F).

To understand the different roles of specific immune cells in IL-17A response, we separate the adherent cell and non-adherent cell as described previously(Weinberger et al. 1980) and stimulated the cells with gram positive bacterial mixture separately. No IL-17A induction were observed (Figure 3.9G), suggesting that antigen presenting cells were required for an IL-17A response. Since dendritic cells have been shown to initiate IL-17 responses(Akdis et al. 2012), we isolated non-adherent cells from MLNs of WT or

TLR2-knockout (TLR2KO) mice and co-cultured them with dendritic cells purified from the blood of WT or TLR2KO mice. The flow-cytometic analysis indicated that approximately 80% of non-adherent cells in MLN were CD3+ T cells (Figure 3.10). In response to bacterial stimulation, WT and TLR2KO T cells which were co-cultured with WT dendritic cells were able to produce high levels of IL-17A. Conversely, when TLR2KO T cells and WT T cells were co-cultured with TLR2KO dendritic cells, no significant IL-17A induction was observed (Figure 3.9H), indicating that TLR2 expressed on dendritic cells was an essential requirement for IL-17A production. Interestingly, deficiency of TLR2s in T cells attenuated the IL-17A production significantly (Figure 3.9H), implying that TLR2s on T cells were also involved in maximal IL-17A response.

IL-1β and IL-23 promoted IL-17A production by MLN Cells

Previous studies have shown that IL-17A production was mediated by IL-6, TGF-β, IL-1β or IL-23 in different cell types(Kim et al. 2013; Reynolds et al. 2010; Taylor et al. 2014; Rubino, Geddes, and Girardin 2012). To determine the role of each cytokine in IL17A production by MLN, we measured the concentrations of IL-6, TGF-β, IL-1β, or IL-23 in MLN adherent cell supernatant at different time points following bacterial stimulation. We observed significant up-regulation of IL-6, IL-1β, and IL-23 at 24 hours which trended towards reduction after 48 hours. In contrast, no TGF-β up-regulation was observed until 48 hours after bacterial stimulation (Figure 3.11 A to D). We next determined the role of these cytokines in IL-17A production by using cytokine neutralization antibodies (5μg/ml) (Figure 3.11 E to I). As shown in Fig. 3.11E and F, neutralization of IL-6 or TGF-β showed no effects on IL-17A production. In contrast,

neutralizing IL-1β and IL-23 inhibited IL-17A production and IL-1β showed a more dominant role in IL-17A modulation (Figure 3.11 G to I). Additionally, IL-1β alone was sufficient to induce IL-17A production and the mixture of IL-1β with IL-23 showed synergistic effects on IL-17A induction in MLN cells (Figure 3.11J), further validating the roles of IL-1β and IL-23 in IL-17A responses.

DISCUSSION

Sepsis is a more complex and variable syndrome than normal infections, in which pro-inflammatory mechanism contributes to pathogen clearance as well as tissue damage and multiple organ failure. To date, the Surviving Sepsis Campaign has issued the third iteration of clinical guidelines for the management of severe sepsis and septic shock while the mortality rate of sepsis still remains high(Angus and van der Poll 2013; Investigators 2014; Vincent et al. 2009). Previous studies have identified many factors independently associated with greater risks of death in septic cases including comorbid cancer, heart failure, immunosuppression, cirrhosis to name a few(Vincent et al. 2009). Several studies indicate that morphine treatment results in spontaneous sepsis in mice(Hilburger et al. 1997) and that opioid antagonist naltrexone block acute endotoxic shock(Greeneltch et al. 2004b), however the underlying mechanism that contributes to sepsis progression and outcome following opioid treatment has not been completely elucidated. In the present study, two commonly prescribed opioids morphine and methadone were administered to mice in the context of polymicrobial sepsis. Both opioids significantly increased mortality rates of septic mice, suggesting that the therapeutic window of opioids for pain management might be narrower in ICU patients who are septic. A large amount of clinical and laboratory studies have provided strong evidences that multiple mechanisms are involved in opioid-induced immunosuppression (Ninković and Roy 2013; Wang et al. 2005; Sabita Roy et al. 2011; Breslow et al. 2010; Hilburger et al. 1997; Börner and Kraus 2013), which are consistent with our observations. We also demonstrate that morphine treatment inhibited neutrophil

recruitment into the peritoneal cavity in the early stages of sepsis (Figure 3.12) and also induced splenic atrophy (Figure 3.13), implying that the defensive functions of immune cells and organs against infections during sepsis were compromised by morphine treatment. The compromised immune function led to delayed bacterial clearance following CLP in morphine-treated animals, resulting in sustained high bacterial load in different organs and circulatory systems. In addition to higher bacterial load, we also found that gram-positive bacterial families Staphylococcus and Enterococcus showed higher potential for translocating across the gut epithelium following morphine treatment. Even morphine treatment alone induced the translocation of these two bacteria families, suggesting that opioid treatment might be an independent risk factor for gram-positive infections with gut origin. The imbalance of gut microbiota and increased prevalence of staphylococcus and Enterococcus in the gut lumen following morphine treatment might be responsible for bacterial translocation. Interaction between gut microbiota and intestinal epithelial surface play important roles in the prevention of pathogenic bacterial outgrowth and maintaining gastrointestinal homeostasis(Hill and Artis 2010). Changes in composition or density of the microbiota may lead to higher susceptibility to a variety of pathogens and abnormal mucosal immune responses(Kamada et al. 2013). In our sepsis model, the overgrowth of staphylococcus and enterococcus in the gut lumen following morphine treatment resulted in gram positive bacterial dissemination and excessive systemic inflammation. The overexpression of pro-inflammatory cytokine IL-17A following specific gram positive bacteria stimulation induced gut epithelial damage and promoted further bacterial translocation. This vicious cycle contributes to accelerated

progression of infection and high mortality following the CLP procedure. The present study is an example of how alterations in gut microbiota determine the immune responses of the host and predict the outcome of infectious diseases, which support microbiota manipulation strategies to control or treat infectious diseases.

Up-regulation of IL-17A associated with morphine treatment in our sepsis model prompted us to investigate the roles of IL-17A in sepsis progression. IL-17A is a pro-inflammatory cytokine involved in the initiation and maintenance of several autoimmune disorders such as encephalomyelitis, and inflammatory bowel disease(Akdis et al. 2012; Towne et al. 2012). And one of the most important mechanisms involved in excess inflammation associated with high levels of IL-17A is that IL-17A is able to modulate tight junction protein organization in endothelial cells and thereby promoting blood-brain barrier disruption and central nervous system inflammation(Huppert et al. 2010; Kebir et al. 2007). Increased IL-17 production has been shown to be associated with compromised gastrointestinal integrity in pigtail macaques(Klatt et al. 2010). Our stuides similarly show that overexpression of IL-17A during sepsis induced epithelial barrier dysfunction by disrupting the organization of tight junction proteins in gut epithelial cells. The role of IL-17A in these defects are supported by the observation that neutralization of IL-17A controlled the inflammation and protected morphine-treated animals from death following the CLP procedure, which was consistent with Flierl's observation that IL-17A promoted inflammation and showed adverse functions in adult mice with severe sepsis(Flierl et al. 2008). Studies in contrary however show that IL-17 receptor signaling was required to control sepsis(Deshmukh et al. 2014; Freitas et al. 2009). This

inconsistency may be explained by the differences in experimental approaches used: Deshmukh and Freitas used IL17R knockout mice or neutralized IL-17A in neonatal mice. Lack of IL-17 signaling in infant mice might result in compromised development of neutrophils and other immune cells, which may have contributed to the increased host susceptibility to infections.

The major sources of IL-17A in our study are CD4+ helper T (TH) cells. To date, scientists have identified two different population of TH17 cells: one being the inducible TH17 (iTH17) cells that arise from naive CD4+ T cells in response to antigen and cytokine stimulation and the other the natural TH17(nTH17) cells that acquire the capacity to produce IL-17 during development in the thymus(Kim et al. 2013). The nTH17 cells are poised to rapidly produce IL-17 upon stimulation without further differentiation in the peripheral tissues(Kim et al. 2011) and show great recruitment to the gut Peyer's patches and lamina propria(Marks et al. 2009). The presence of nTH17 cells may explain the quick IL-17A responses to bacterial stimulation in our study. Another unexpected observation we have made is that IL-17A production by MLN following gram positive bacterial stimulation was independent of IL-6 and TGF-β, the key cytokines driving TH17 differentiation in previous publications(Chang and Dong 2011; Gu, Wu, and Li 2013; Taylor et al. 2014). In our sepsis model, dendritic cells expressing TLR2 were required for a robust IL-17A response, which was mediated by IL-1β and IL-23. Recently many researchers suggest that the CD4+ T cells will differentiate into more regulated classical TH17 cells in the presence of TGF-β. In contrast, the TGF-β independent pathway will generate more pathogenic alternative TH17 cells, which are

responsible for the excessive inflammation in many autoimmune disorders(Akdis et al. 2012). Our results support this theory. We show that IL-17A induction is not dependent on IL-6 or TGF-β, instead requires IL-1β and IL-23 to produce pro-inflammatory and pathogenic IL-17A. The induction of IL-17A contributes to gut epithelial damage, compromised barrier function, leading to continuous bacterial dissemination and sustained high levels of inflammation. In addition, we also observed that TLR2 on T cells were involved in the IL-17A response, which was consistent with Joseph M's study: TLR2 agonists activated TLR2 signaling in CD4+ T cells and led to more robust proliferation and TH17 cytokine production, resulting in more severe pathology in autoimmune disease like EAE(Reynolds et al. 2010). The present study further lends support to the pro-inflammatory role of IL-17A in the progression of sepsis. Today, fast development of antibiotic resistance in various pathogens is a growing threat to the world and researchers have paid more attentions to developing novel anti-cytokine strategies for controlling infectious diseases rather than rely on antibiotics alone. However, no current large-scale trials of anti-cytokine molecules in the treatment of sepsis has achieved satisfying efficacy(Angus and van der Poll 2013). Therefore, our study may provide novel therapeutic targets to develop anti-cytokine strategies to control specific gram positive infections especially in the context of opioid pain management.

To summarize, we report that during CLP-induced sepsis, morphine treatment induced enrichment of gram positive bacteria Staphylococcus and Enterococcus in the gut lumen and promote gram positive bacterial dissemination. Disseminated gram positive bacteria induced IL-17A overexpression in a TLR2-dependent manner. Excess

IL-17A increased gut permeability and contributed to higher mortality rate associated with opioid treatment, implying that opioid administration might contribute to higher risk of death for ICU patients and neutralization of IL-17A might be a novel strategy to control excess inflammation and to improve survival during sepsis.

FIGURES

FIGURE 3.1

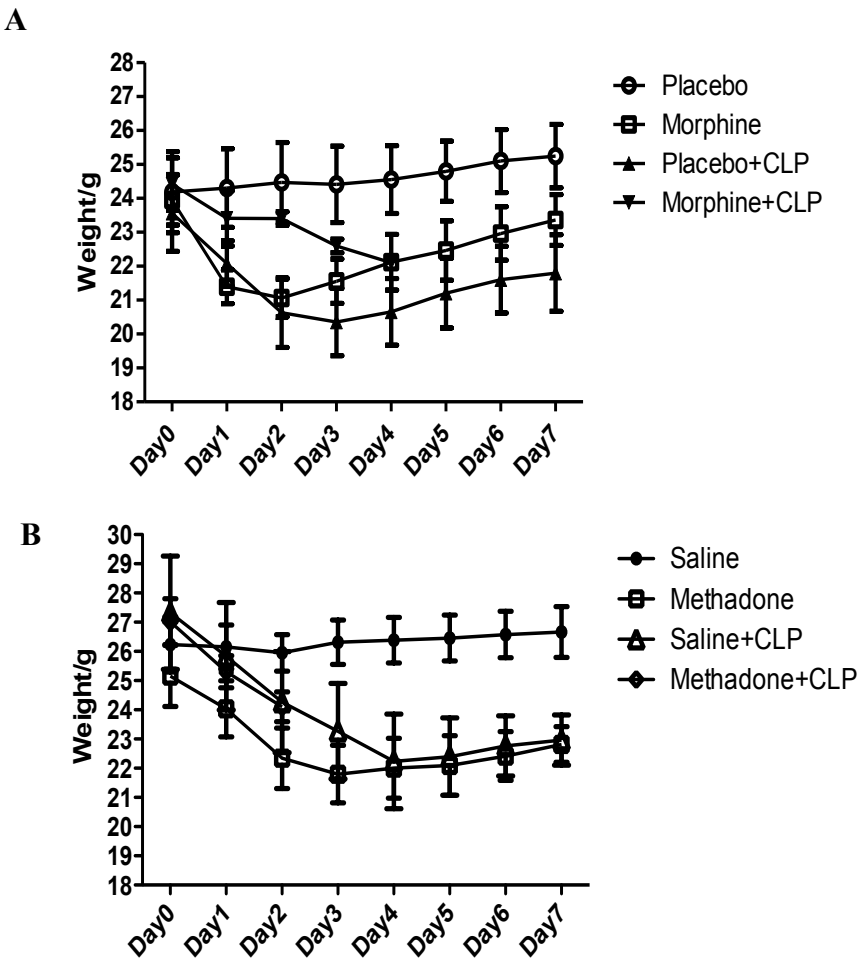


FIGURE 3.1 Weight loss induced by sepsis was attenuated by morphine treatment. (A) Weight curves of sham-operated or CLP mice treated with placebo or 25mg morphine pellet.(B) Weight curves of sham-operated or CLP mice injected with saline or 15mg/kg methadone.

FIGURE 3.2

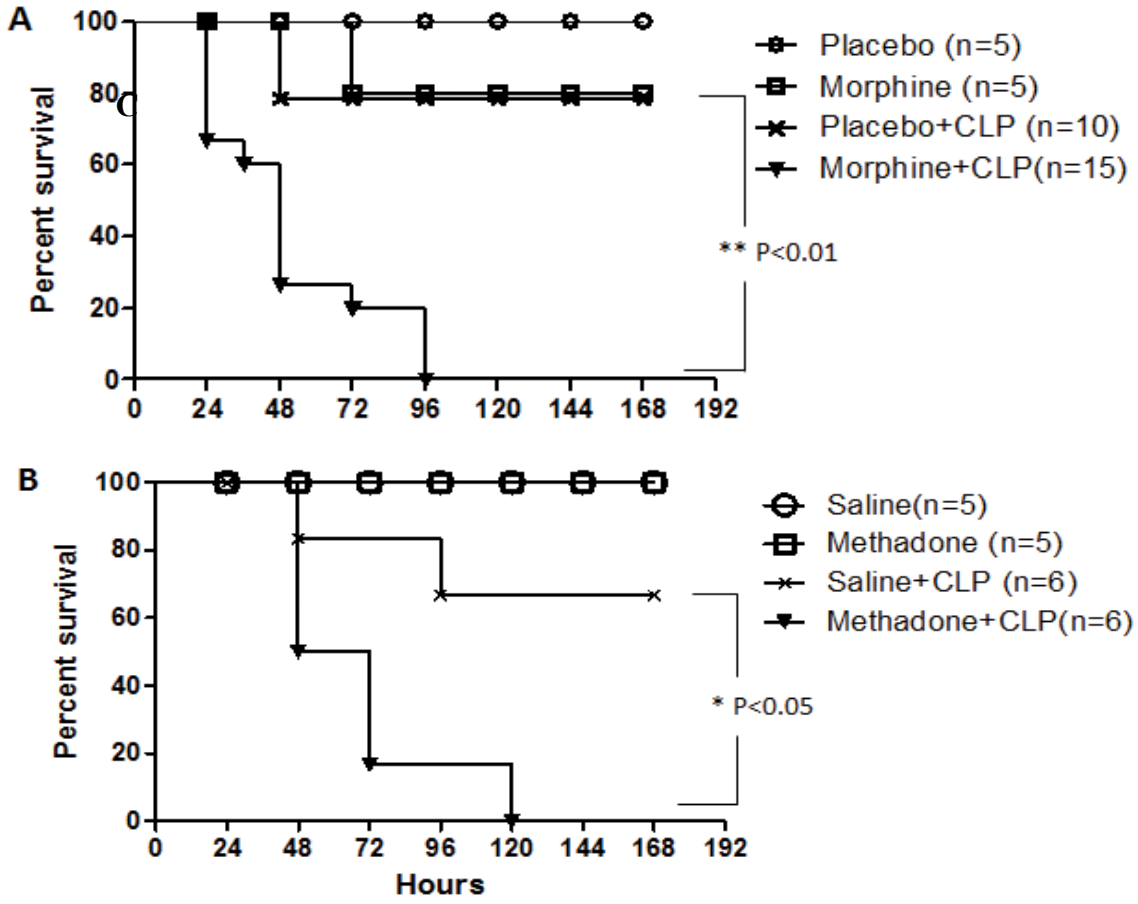


FIGURE 3.2 Opioids increased mortality rates of polymicrobial sepsis induced by CLP. (A) Kaplan–Meier plots of sham-operated or CLP mice treated with placebo or 25mg morphine pellet. ** $p<0.01$ compared with placebo-treated mice subjected to CLP (Mantel-Cox log rank test) (B) Kaplan–Meier plots of sham-operated or CLP mice injected with saline or 15mg/kg methadone. Numbers of mice used for each condition are shown in the frame. * $p< 0.05$ compared with saline-treated mice subjected to CLP (Mantel-Cox log rank test)

FIGURE 3.2

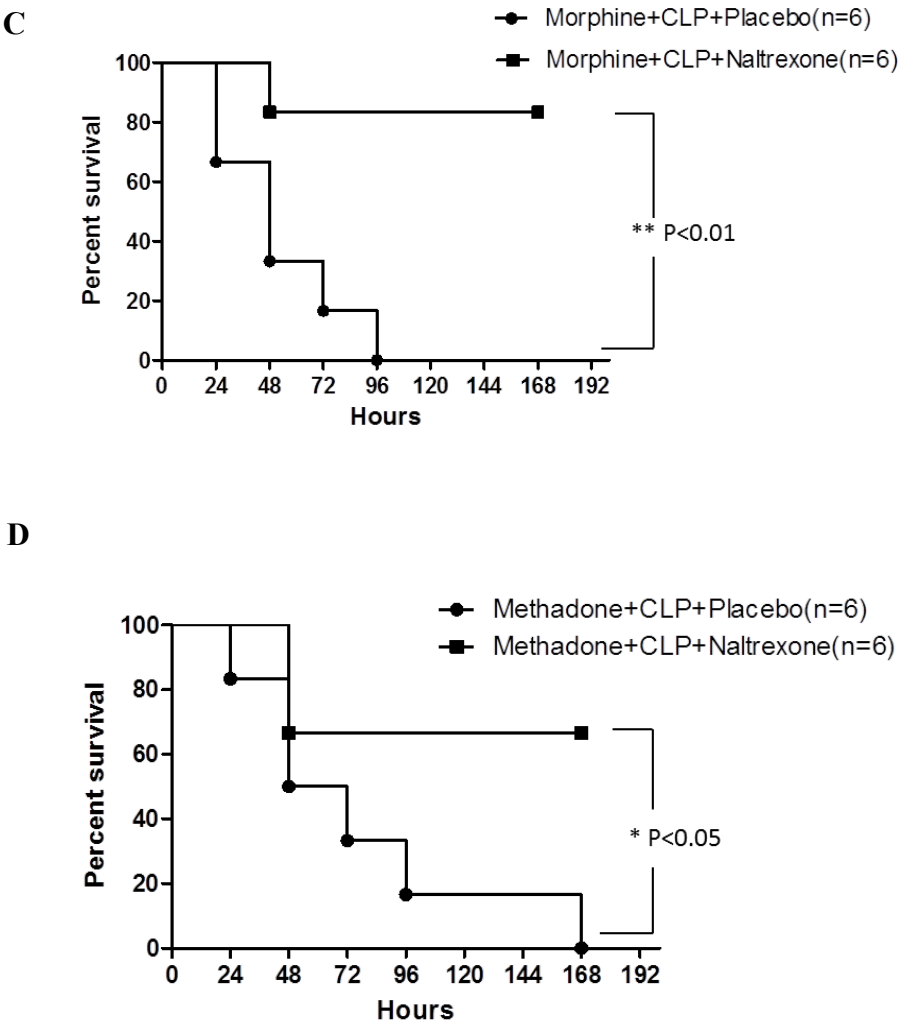


FIGURE 3.2 Opioids increased mortality rates of polymicrobial sepsis induced by CLP. (C) Kaplan–Meier plots of morphine-treated CLP mice treated with placebo or 30mg naltrexone pellet. ** $p<0.01$ compared with placebo-treated mice (Mantel-Cox log rank test) (D) Kaplan–Meier plots of methadone-treated CLP mice treated with placebo or 30mg naltrexone pellet. * $p<0.05$ compared with placebo-treated mice (Mantel-Cox log rank test) Numbers of mice used for each condition are shown in the frame.

FIGURE 3.3

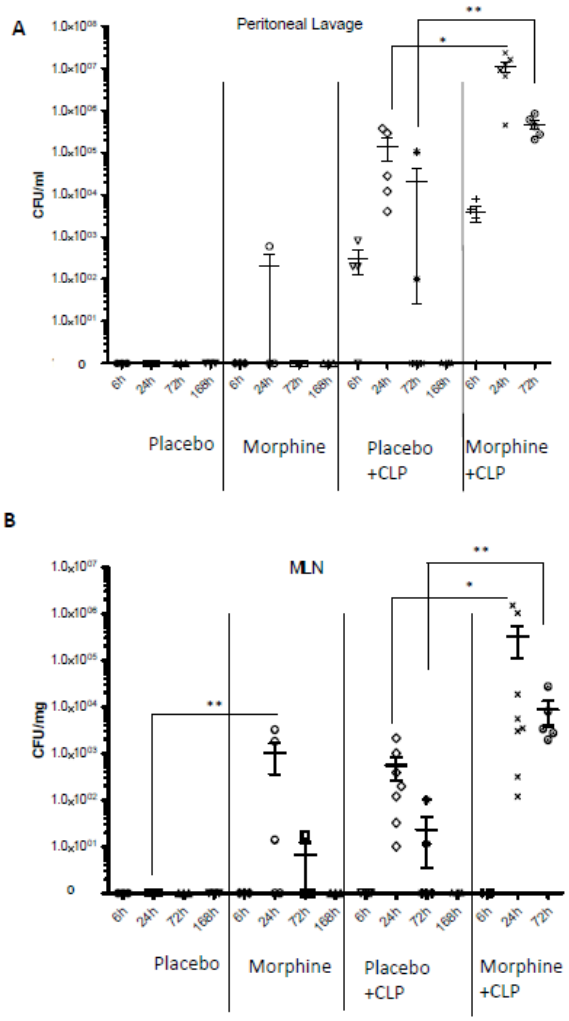


FIGURE 3.3 Morphine Inhibited Bacterial Clearance and Promoted Bacterial Dissemination during Sepsis. (A) Peritoneal lavage was collected at different points and cultured on blood agar plate overnight. Bacterial colonies were quantified and described as CFU. (B) Bacterial colonies of MLN homogenates. *P<0.05 compared with placebo-treated animals ** p<0.01 compared with placebo-treated animals (Mann-Whitney U test)

FIGURE 3.3

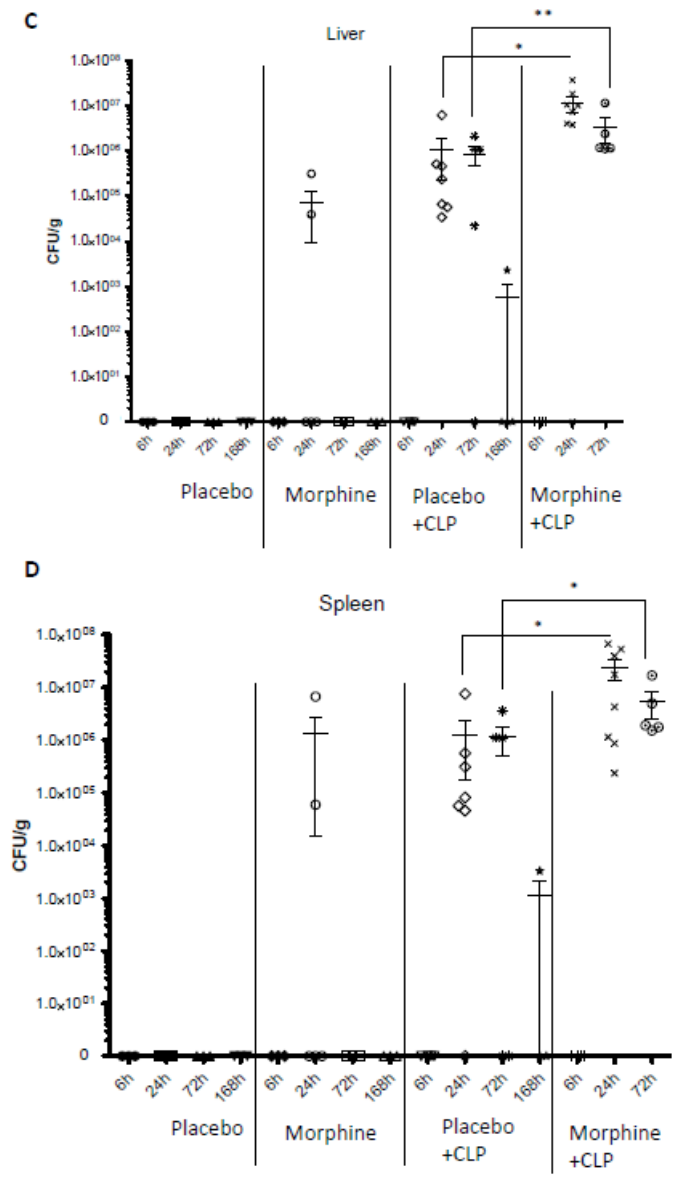


FIGURE 3.3 Morphine Inhibited Bacterial Clearance and Promoted Bacterial Dissemination during Sepsis. (C) Bacterial colonies of liver homogenates. (D) Bacterial colonies of spleen homogenates. *P<0.05 compared with placebo-treated animals ** p<0.01 compared with placebo-treated animals (Mann-Whitney U test)

FIGURE 3.3

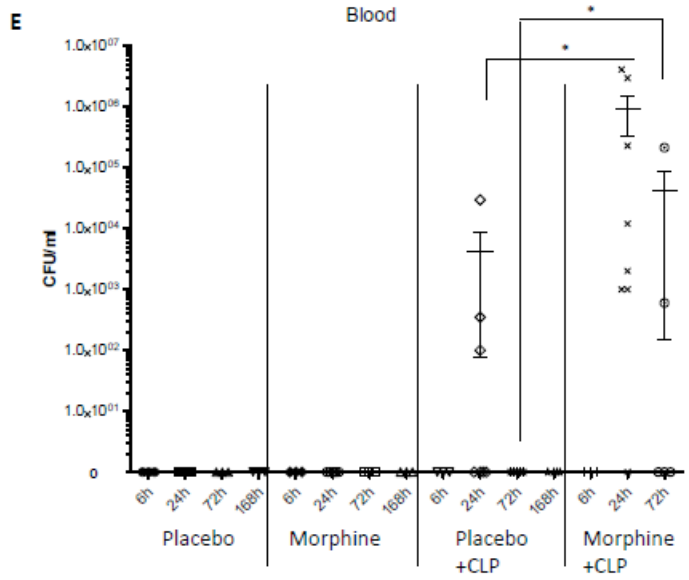


FIGURE 3.3 Morphine Inhibited Bacterial Clearance and Promoted Bacterial Dissemination during Sepsis. Wild type mice were treated with 25mg morphine pellets following CLP procedure. (E) Bacterial colonies of whole blood.*P<0.05 compared with placebo-treated animals (Mann-Whitney U test)

FIGURE 3.4

A Bacteria detected in MLN, Spleen,and Liver

	MLN	Spleen	Liver
Placebo	None	None	None
Morphine	Enterococcus sp.(G+) Staphylococcus sp. (G+)	Enterococcus sp. (G+)	Enterococcus sp.(G+) Staphylococcus sp. (G+) Bacillus sp. (G+)
Placebo+CLP	Escherichia coli (Non-haemolytic) (G-) Enterococcus sp. (G+)	Escherichia coli (Non-haemolytic) (G-) Enterococcus sp. (G+)	Escherichia coli (Non-haemolytic) (G-)
Morphine+CLP	Staphylococcus sp. (G+) Enterococcus sp. (G+) Escherichia coli (Non-haemolytic) (G-)	Staphylococcus sp.(G+) Enterococcus sp. (G+) Escherichia coli (Non-haemolytic) (G-)	Staphylococcus sp.(G+) Enterococcus sp. (G+) Escherichia coli (Non-haemolytic) (G-)

G+: Gram positive bacteria G- : Gram negative bacteria

FIGURE 3.4 Morphine treatment modulated microbiome in gut lumen. (A) Serotyping of the disseminated bacteria in MLN, spleen, and liver from morphine and placebo-treated animals (Veterinary Diagnostic Laboratory, University of Minnesota).

FIGURE 3.4

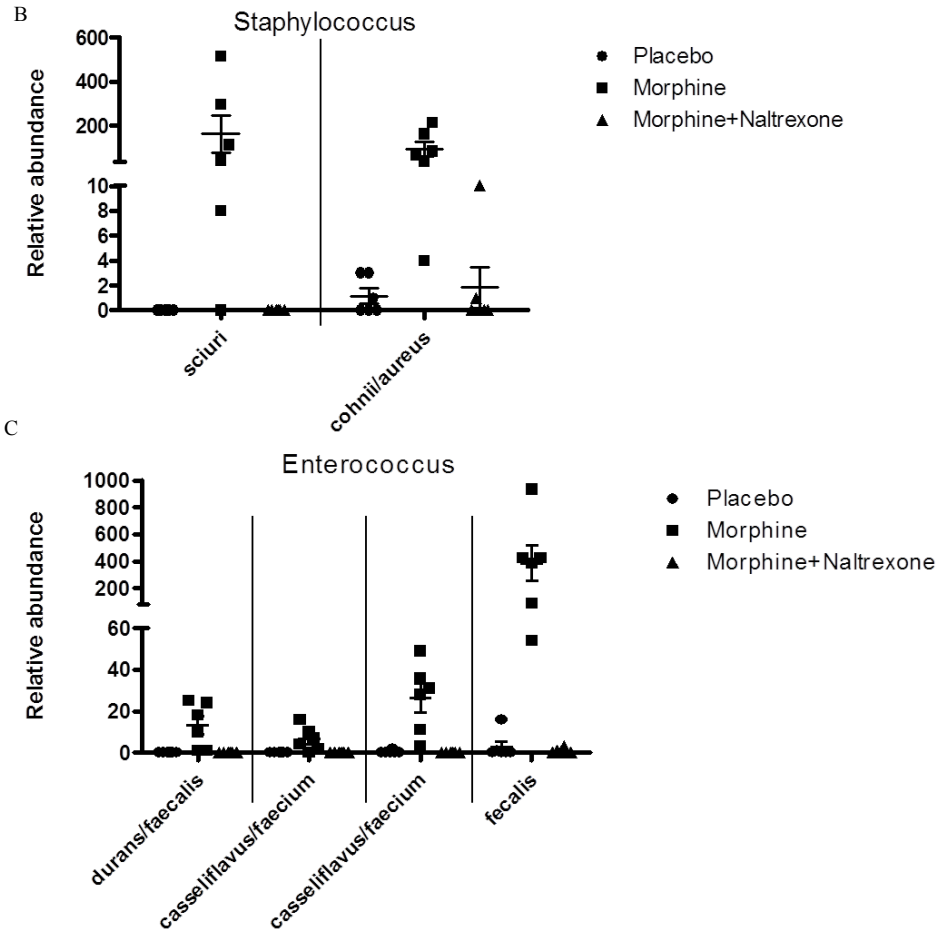


FIGURE 3.4 Morphine treatment modulated microbiome in gut lumen. (B)-(C) Bacterial species identified in fecal contents from placebo or 25mg morphine pellet-treated mice.

FIGURE 3.5

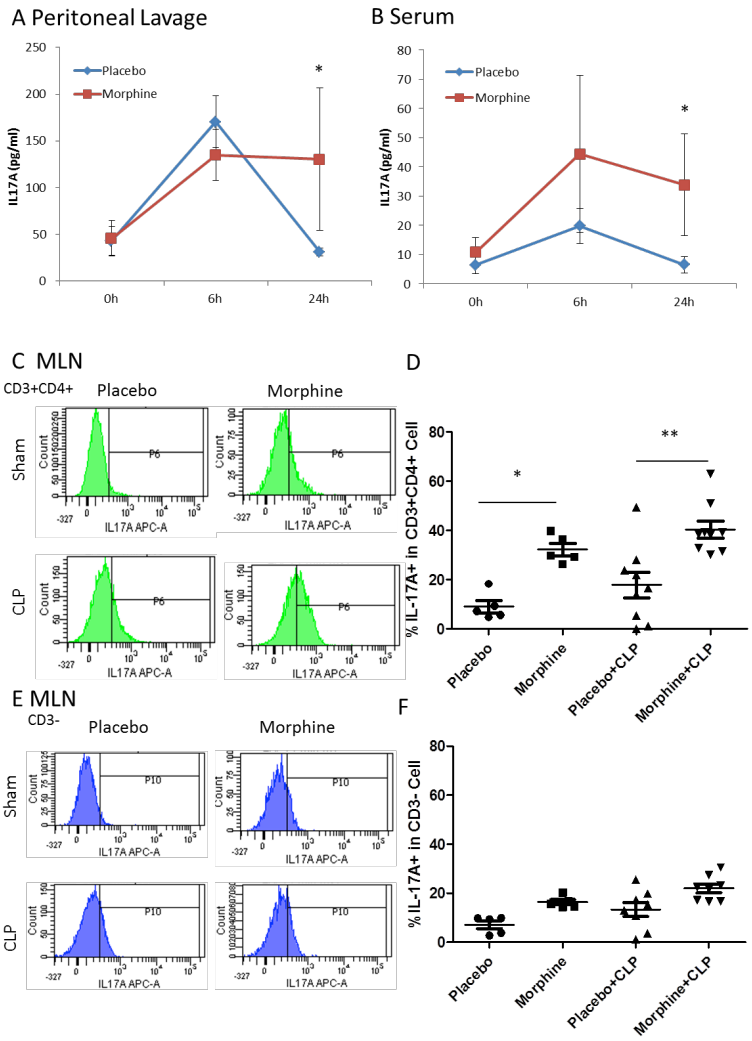


FIGURE 3.5 Morphine modulated pro-inflammatory cytokine IL-17A production during sepsis. (A) IL-17A concentrations in peritoneal lavage at different time points following CLP. *p<0.05 (Student t test) (B) IL-17A concentrations in serum at different time points following CLP. *p<0.05 (Student t test) (C) IL-17A expression in MLN cells which were CD3+CD4+. (D) Frequencies of IL-17A positive cells in CD3+CD4+ cells. *p<0.05，**p<0.01 (ANOVA followed by Bonferroni's t test) (E) IL-17A expression in MLN cells which were CD3- (F) Frequencies of IL-17A positive cells in CD3- cells

FIGURE 3.6

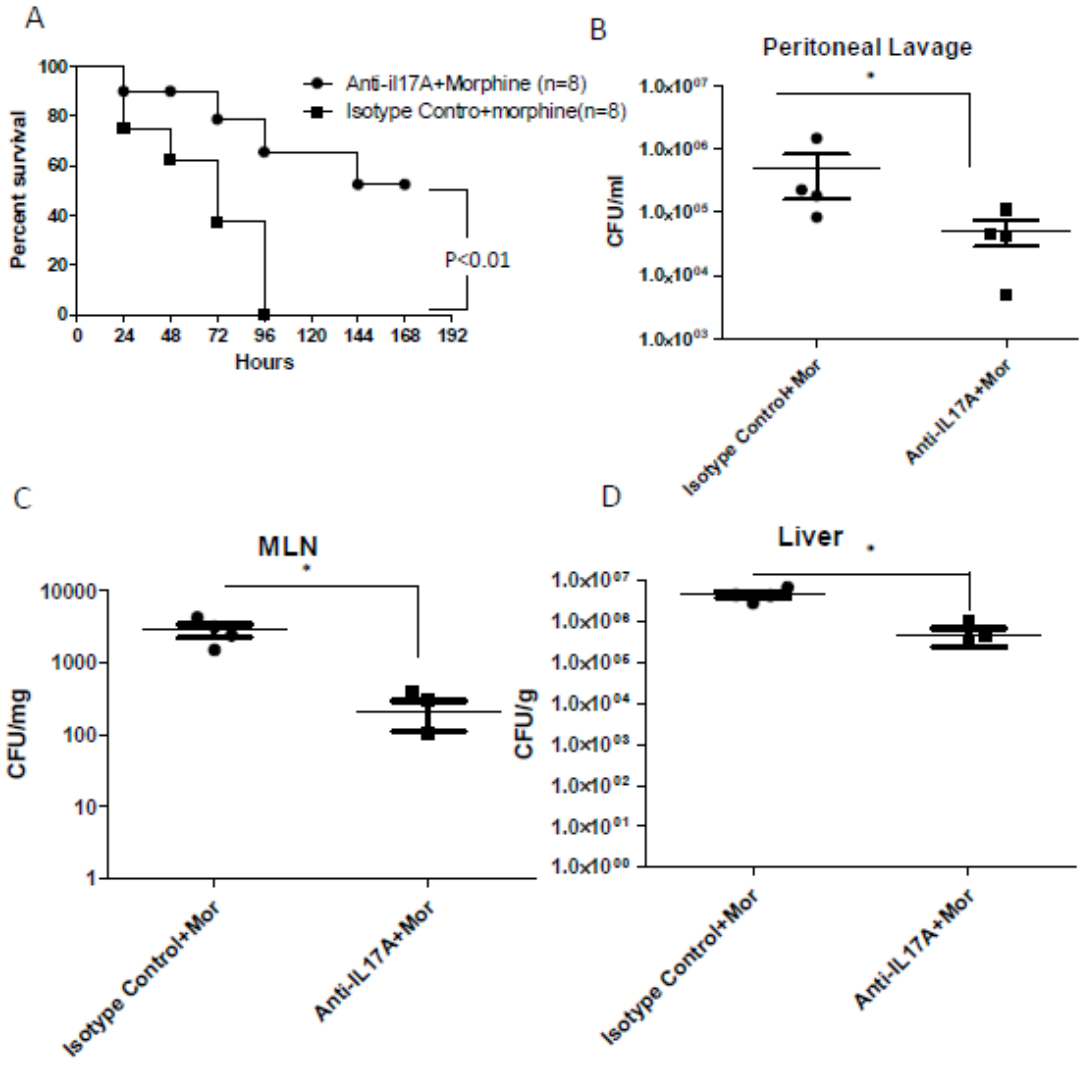


FIGURE 3.6 Neutralization of IL-17A improved survival rate and attenuated sustained inflammation in CLP mice treated with morphine. (A) Kaplan–Meier plots of morphine-treated CLP mice injected with isotype control or anti-IL17A antibody $p<0.01$ compared with anti-IL17A-treated mice subjected to CLP (Mantel-Cox log rank test) (B) Bacterial colonies of peritoneal lavage (C) Bacterial colonies of MLN (D) Bacterial colonies of liver *$p<0.05$ (Mann-Whitney U test)

FIGURE 3.6

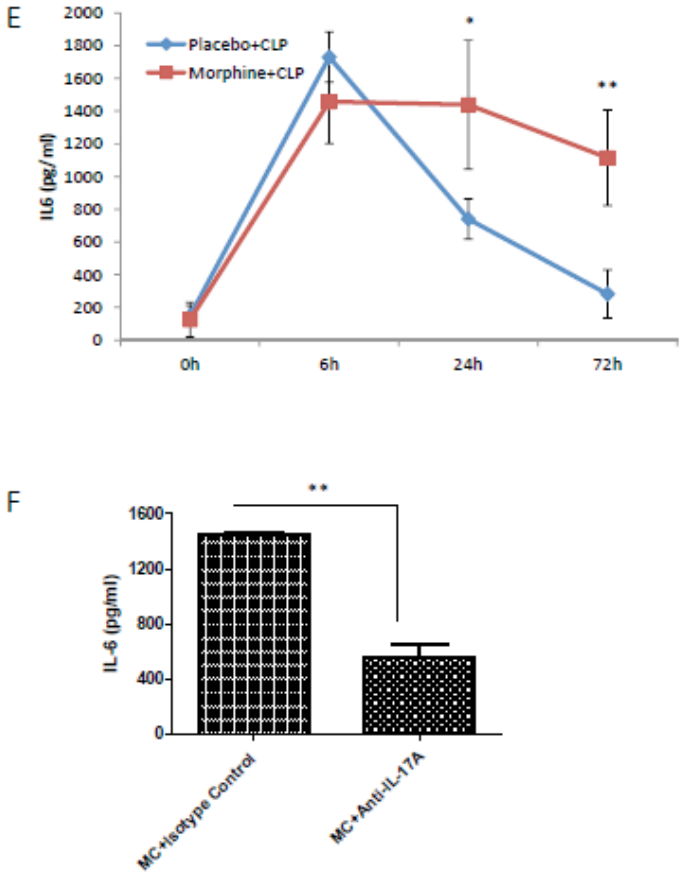


FIGURE 3.6 Neutralization of IL-17A improved survival rate and attenuated sustained inflammation in CLP mice treated with morphine. (E) IL-6 concentrations in serum at different time points following CLP. *p<0.05 **p<0.01 (Student t test) (F) IL-6 concentrations in serum in morphine-treated CLP mice injected with isotype control or anti-IL-17A antibody. **p<0.01 (Student t test)

FIGURE 3.7

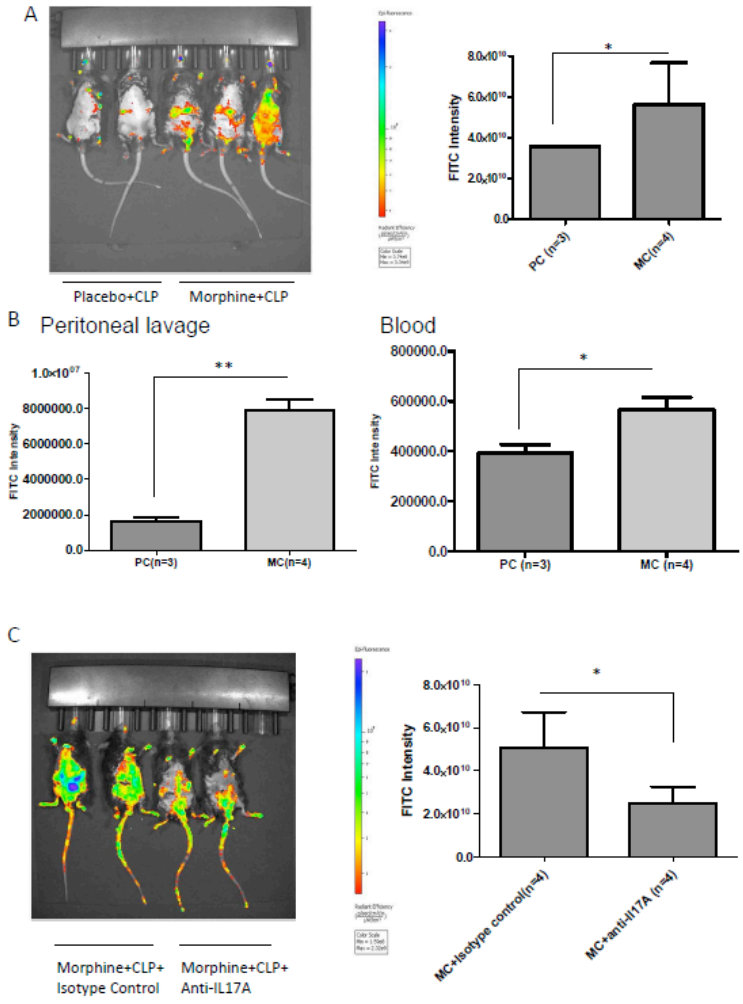


FIGURE 3.7 High levels of IL-17A compromised gut epithelial barrier function and increased gut permeability. (A) Morphine increased FITC-dextran diffusion across the gut epithelium. FITC-dextran diffusion across the gut epithelium in morphine-treated CLP animals. The right panel was quantification of FITC intensity. (B) Quantification of FITC intensity in peritoneal lavage and whole blood (C) Anti-IL-17A injection reduced FITC-dextran diffusion across the gut epithelium in morphine-treated CLP animals. The right panel was quantification of FITC intensity. *p<0.05 **P<0.01(Student t test)

FIGURE 3.7

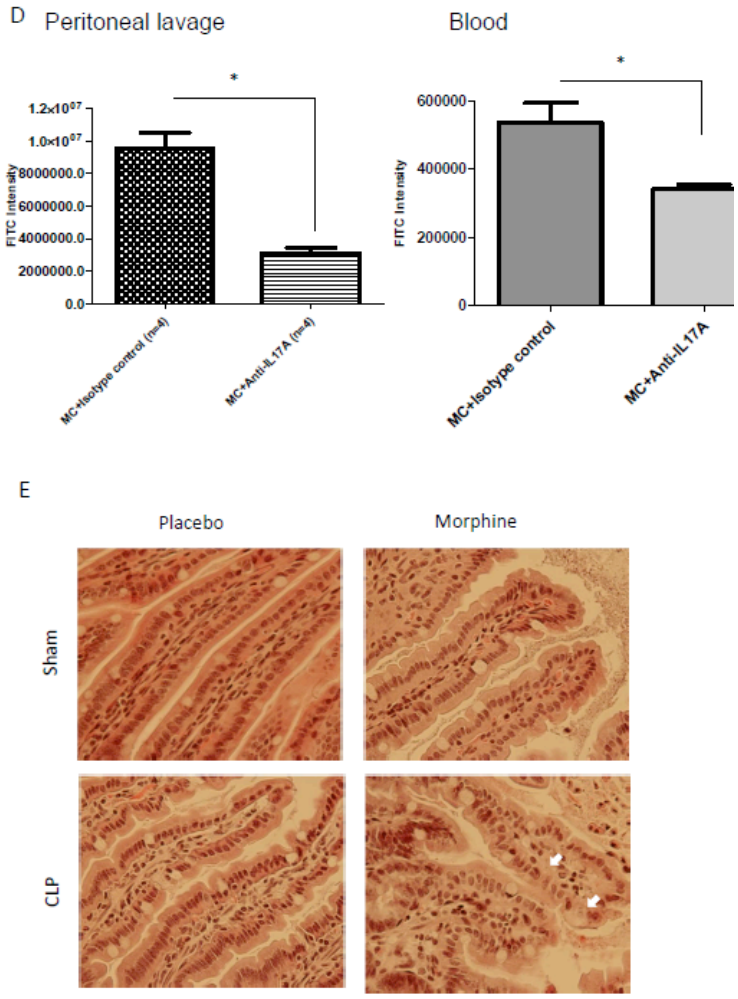


FIGURE 3.7 High levels of IL-17A compromised gut epithelial barrier function and increased gut permeability. (D) FITC intensity in peritoneal lavage and whole blood *p<0.05 (Student t test) (E) H&E sections of small intestines from sham-operated or CLP animals treated with morphine or placebo. White arrow indicates epithelial disruption.

FIGURE 3.7

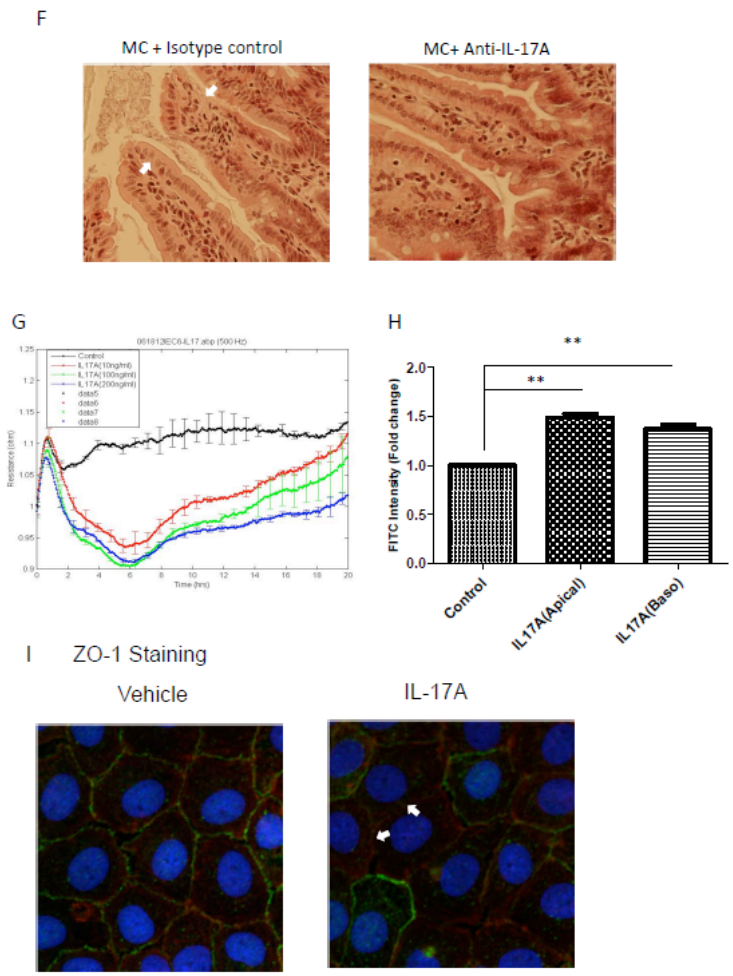


FIGURE 3.7 High levels of IL-17A compromised gut epithelial barrier function and increased gut permeability. (F) H&E sections of small intestines form morphine-treated CLP animals injected with isotype control and anti-IL-17A. White arrow indicates epithelial disruption (G) TER was decreased by IL-17A in IEC-6 cell monolayer (H) The permeability of IEC-6 cell monolayer was increased in transwell system **p<0.01 (ANOVA followed by Bonferroni's t test) (I) ZO-1 organization in IEC-6 cell monolayer treated by vehicle or 100ng/ml IL-17A. Blue:DAPI Red:F-actin Green:ZO-1 White arrow indicates ZO-1 disruption

FIGURE 3.8

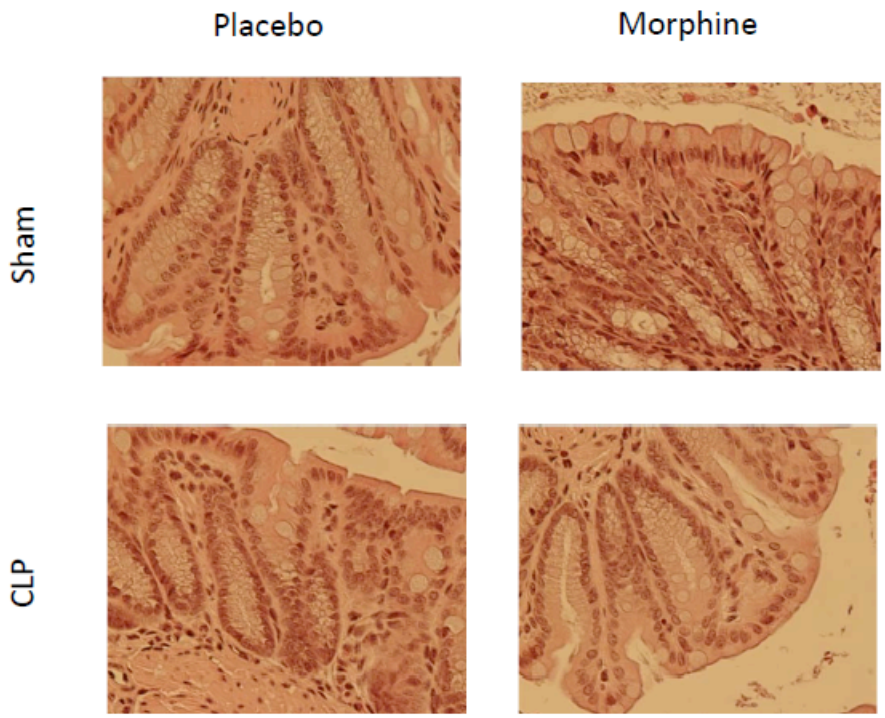


FIGURE 3.8 Colonic epithelium was not affected by high levels of IL-17A. H&E sections of colons from sham-operated or CLP animals treated with morphine or placebo.

FIGURE 3.9

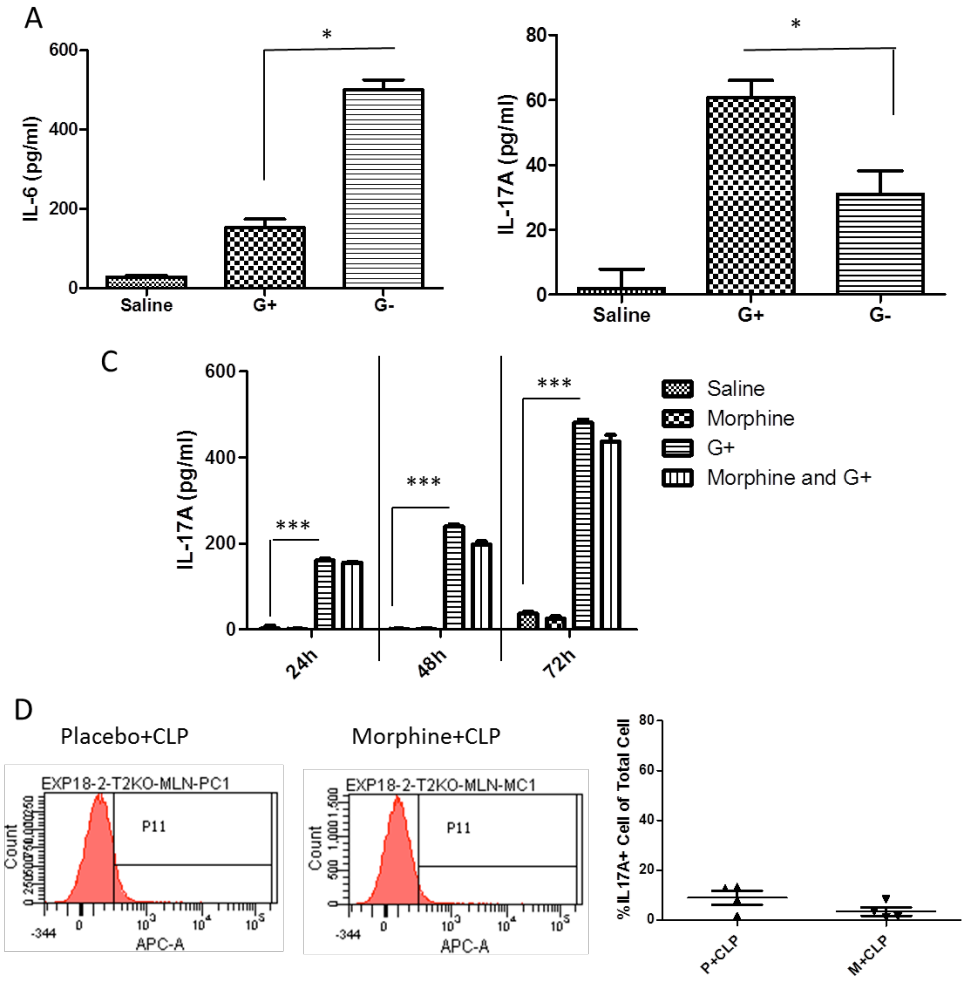


FIGURE 3.9 Gram Positive Bacteria Stimulated MLN to produce IL-17A in a TLR2-dependent manner. IL-6 (A) and IL-17A (B) concentrations of MLN cell supernatant stimulated by gram-positive or gram-negative bacteria. *$p<0.05$ (ANOVA followed by Bonferroni's t test) (C) IL-17A concentrations of MLN supernatant treated with morphine or gram-positive bacteria ***$p<0.001$ (ANOVA followed by Bonferroni's t test) (D) IL-17A expression in MLN cells from TLR2KO mice. The right panel is the frequencies of IL-17A positive cells in MLN from TLR2KO mice.

FIGURE 3.9

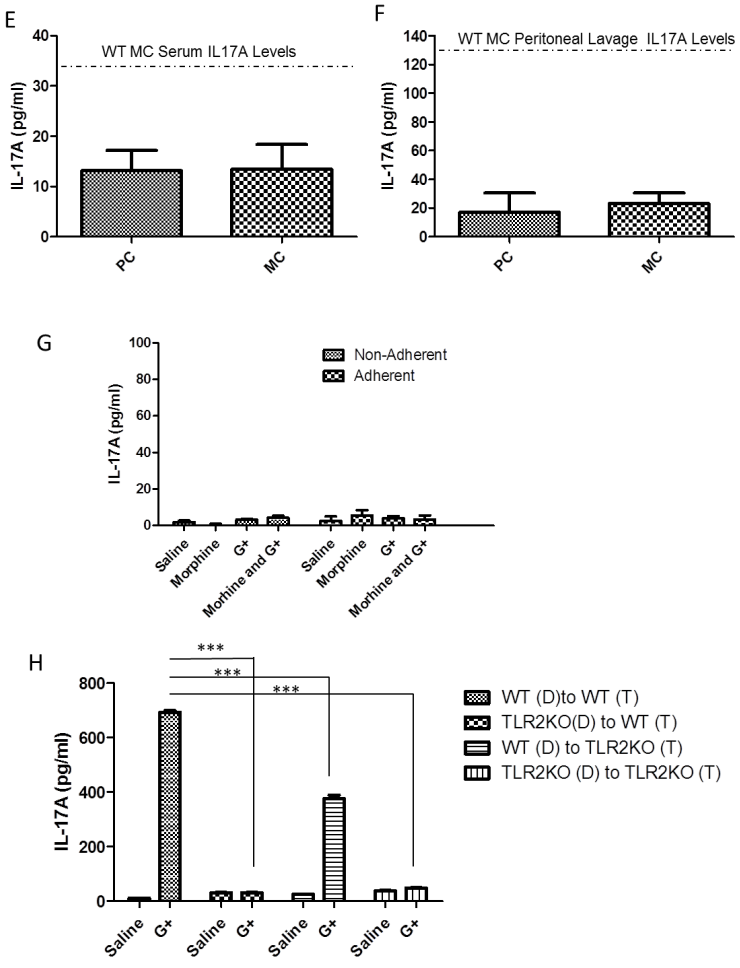


FIGURE 3.9 Gram Positive Bacteria Stimulated MLN to produce IL-17A in a TLR2-dependent manner. (E) IL-17A concentrations in serum of TLR2KO mice. PC: placebo+CLP MC: morphine+CLP (F) IL-17A concentrations in peritoneal lavage of TLR2KO mice. PC: placebo+CLP MC: morphine+CLP (G) IL-17A concentrations in supernatant of adherent and non-adherent cells from MLN following gram-positive bacterial stimulation. (H) IL-17A concentrations in supernatant of non-adherent cells from MLN of WT or TLR2KO mice co-cultured with dendritic cells from blood of WT of TLR2KO mice ***$p<0.001$ (Two-way ANOVA followed by Bonferroni's t test)

FIGURE 3.10

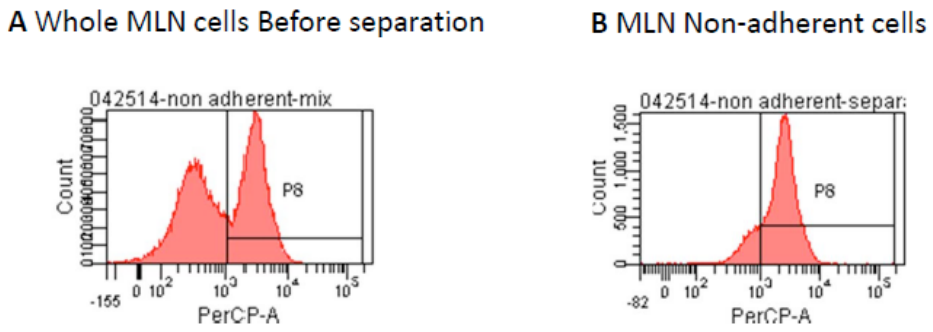


FIGURE 3.10 MLN cells were fixed and incubated with anti-CD3 antibodies and separated as two populations: CD3+ Cells and CD3- Cells. After separation, more than 80% of non-adherent cells were CD3+ T cells.

FIGURE 3.11

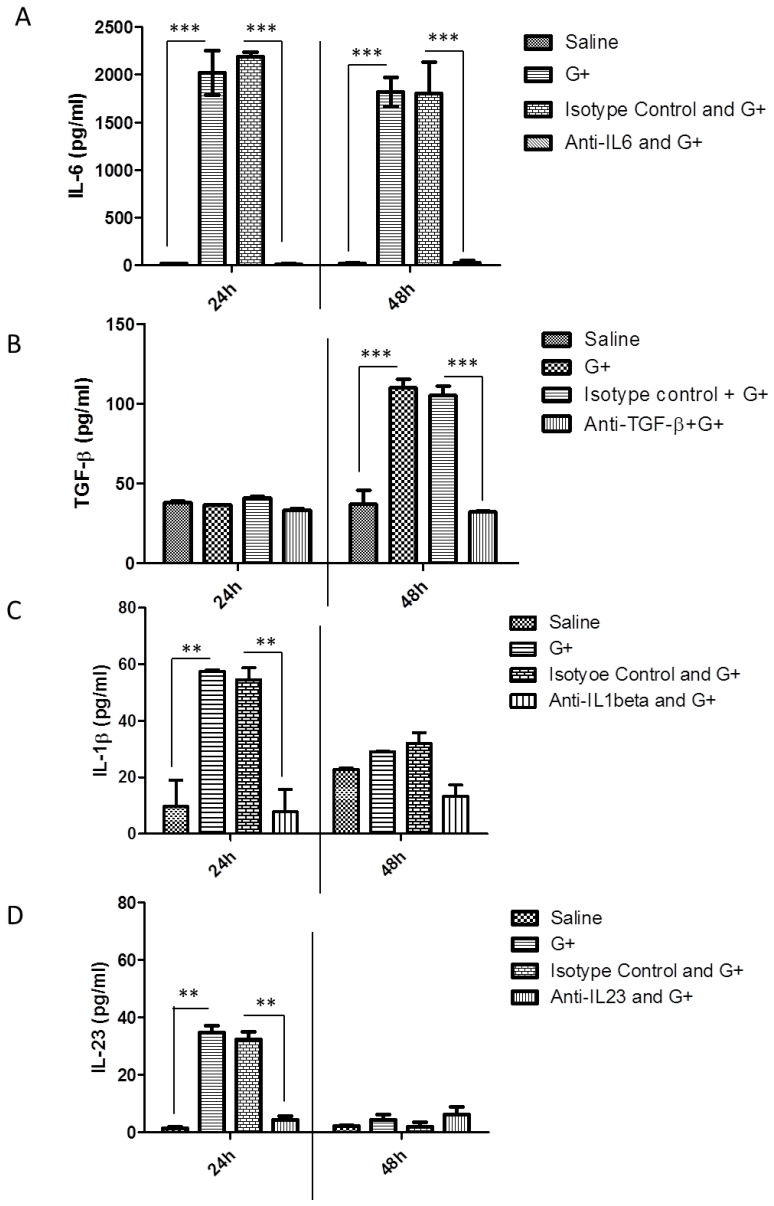


Figure 3.11 IL-1β and IL-23 promoted IL-17A production by MLN Cells (A)-(D) IL-6 , TGF-β , IL-1β, and IL-23 concentrations of MLN adherent cell supernatant following G+ bacterial stimulation ***$p<0.001$ **$p<0.01$ (Two-way ANOVA followed by Bonferroni's t test).

FIGURE 3.11

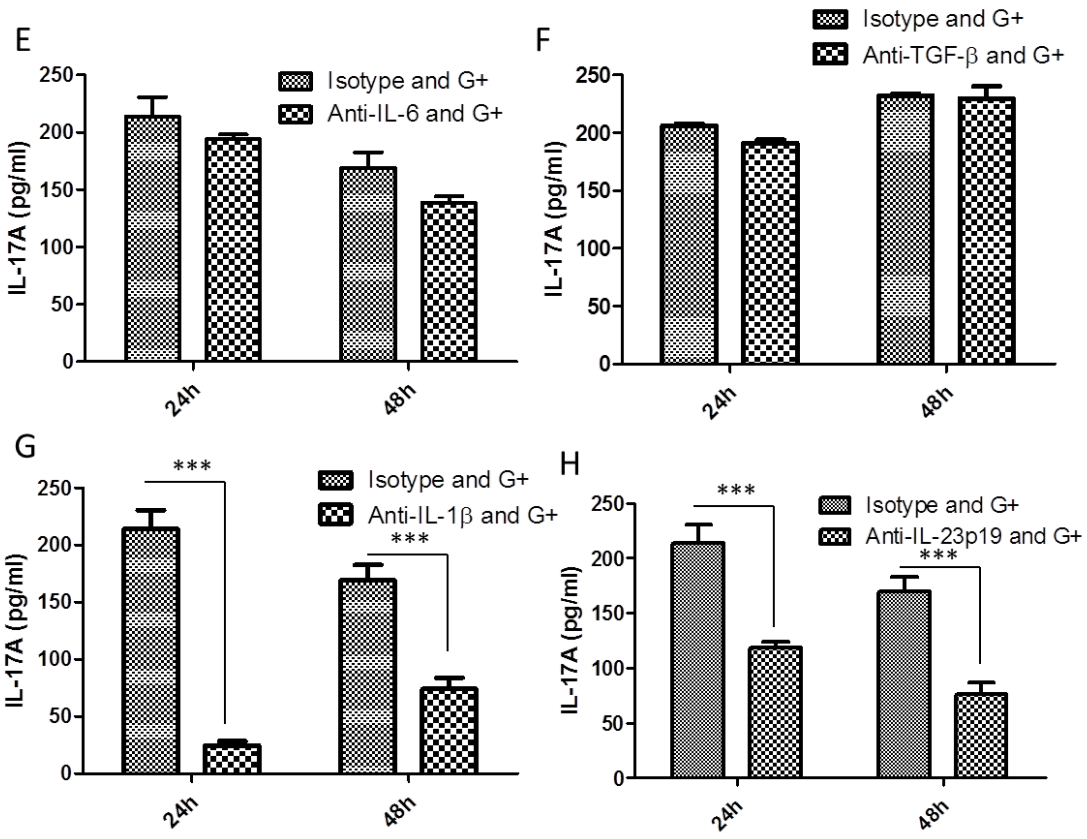


Figure 3.11 IL-1β and IL-23 promoted IL-17A production by MLN Cells. (E)-(H) IL-17A concentrations of MLN supernatant following G+ bacterial stimulation in the presence of isotype control, anti-IL-6, anti-TGF-β,anti-IL-1β or anti-IL-23p19 antibodies. ***p<0.001 (ANOVA followed by Bonferroni's t test). ***p<0.001 (ANOVA followed by Bonferroni's t test)

FIGURE 3.11

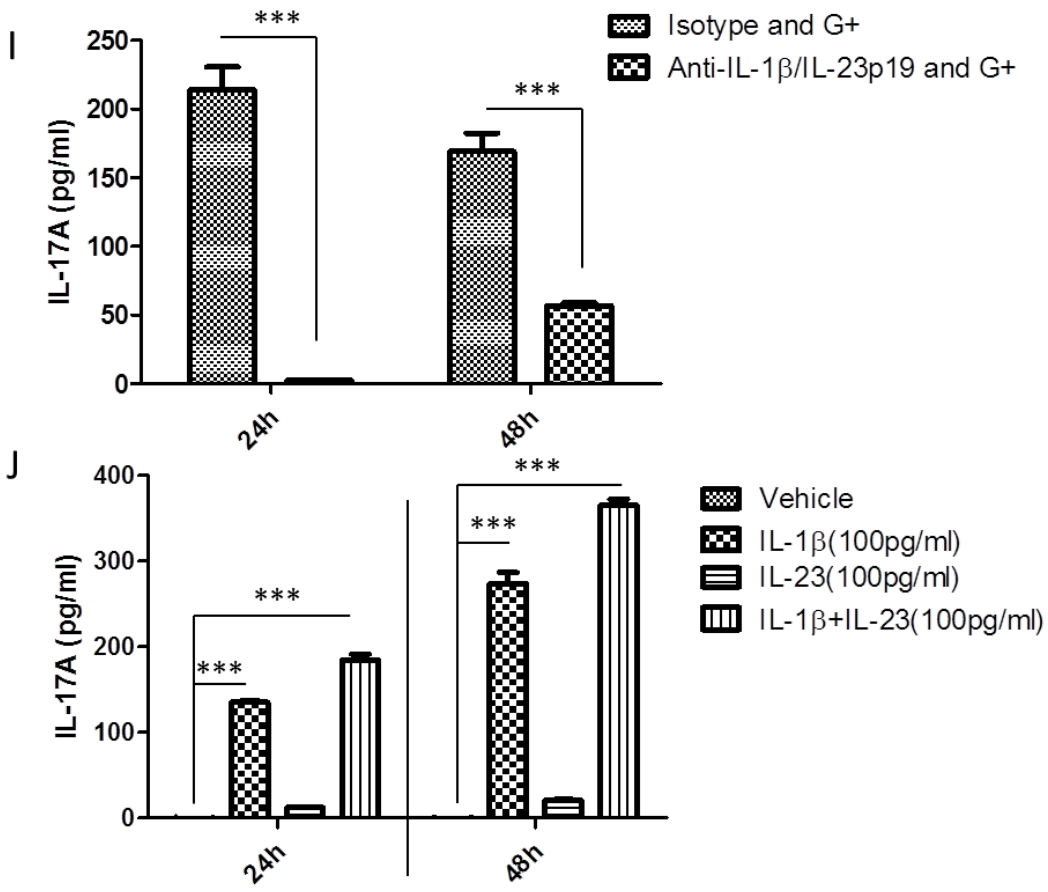


FIGURE 3.11 IL-1β and IL-23 promoted IL-17A production by MLN Cells. (I) IL-17A concentrations of MLN supernatant following G+ bacterial stimulation in the presence of isotype control, or anti-IL-1β and anti-IL-23p19 antibodies. ***$p<0.001$ (ANOVA followed by Bonferroni's t test) (j) IL-17A concentrations of MLN cell supernatant following IL-1β or IL-23 stimulation. ***$p<0.001$ (ANOVA followed by Bonferroni's t test)

FIGURE 3.12

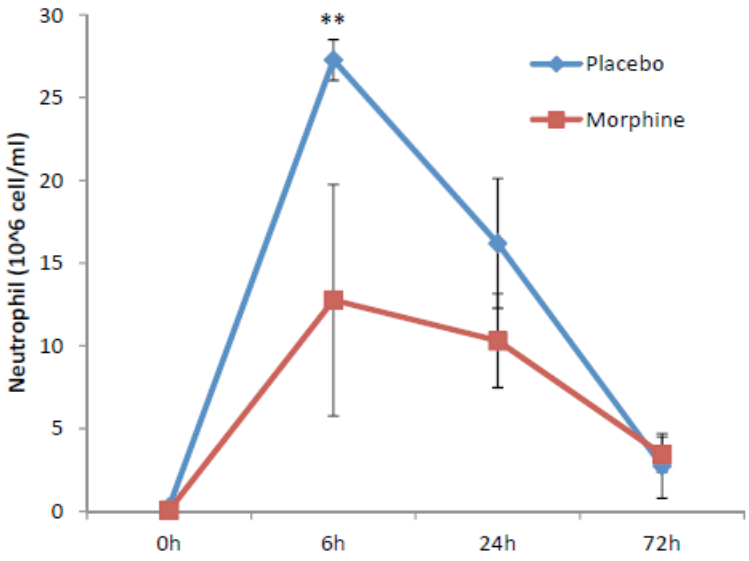


FIGURE 3.12 Morphine inhibited neutrophil recruitment at early stage of sepsis. The numbers of neutrophils in peritoneal lavage at different time points were determined by anti-neutrophil antibodies (Abcam) **p<0.01 (Student t test)

FIGURE 3.13

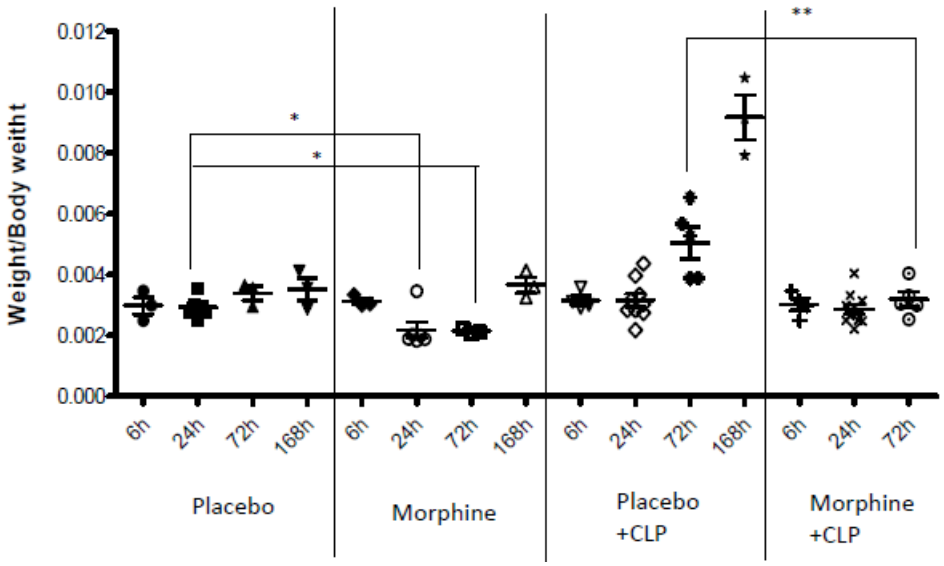


FIGURE 3.13 Morphine induced splenic atrophy. The weight of spleen were normalized to the whole body weight of mice .* $p<0.05$ **$p<0.01$ (Mann-Whitney U test test)

FIGURE 3.14

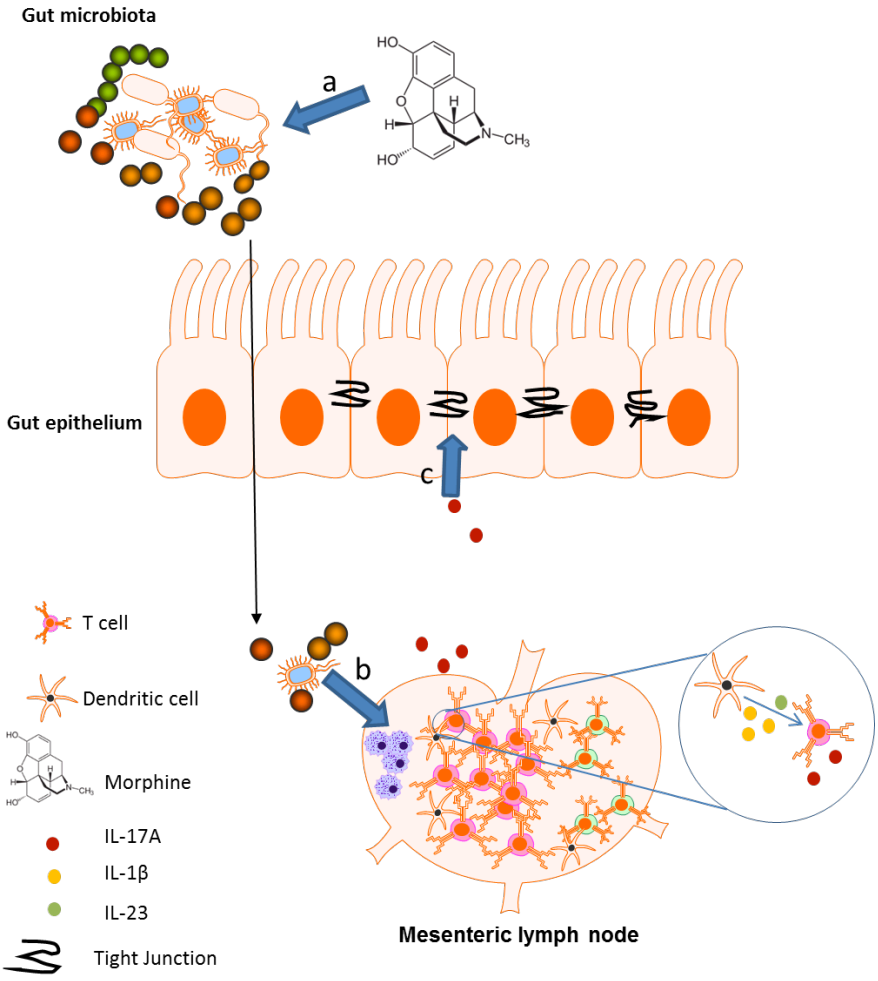


Figure 3.14 Model of opioid modulation of sepsis progression. (a) Morphine treatment induces enrichments of gram-positive bacteria *Staphylococcus* and *Enterococcus* in the gut lumen and promotes gram-positive bacterial dissemination. (b) Disseminated gram-positive bacteria induced IL-17A overexpression in a TLR2-dependdent manner. (c) Excess IL-17A disrupted tight junction organization and increased gut permeability, which contributed to sustained inflammation and higher mortality rate associated with opioid treatment.

CONCLUSION

Over centuries, opioid analgesics are considered the "gold standard" for pain relief. However, the adverse GI effects associated with opioid treatment especially their suppressive effects on gut immunity have become a growing concern for both physicians and patients. In our studies, we investigated the mechanisms underlying impaired gut immune function following opioid treatment, which provide insight on our understanding of the complicated mechanisms by which opioid treatment influence intestinal homeostasis and leading to alternate therapeutic strategies to control or prevent severe infectious diseases like AIDS and sepsis in the opioid using or abusing population.

Firstly, we observed that opioid treatment alone could induce bacterial translocation from the gut lumen through a paracellular pathway. The following study validated that the increased gut permeability following morphine treatment results from disrupted organization of tight junction proteins between the epithelial cells. However, morphine alone didn't show any direct effects on intestinal epithelial cells in our *in vitro* studies. The results from TLRKO mice explained this inconsistency. Morphine-induced bacterial translation and tight junction disorganization were attenuated in TLR2KO, TLR4KO, and TLR2/4KO mice and both TLR2 and TLR4 expression levels were increased following morphine treatment, suggesting that morphine's effects were mediated by TLR signaling. Finally, we showed that morphine by up-regulating TLR2 and TLR4 sensitizes gut epithelial cells to TLR ligands in the gut lumen. The over-activation of TLRs in intestines activated MLCK and subsequent induction and delocalization of F-actins, myosin, and other tight junction proteins, resulting in impaired

barrier function.

In addition to modulating TLRs in gut epithelial cells, we also showed that opioid treatment could modulate the immune responses during infection in a cecal ligation puncture (CLP) model, which could induce polymicrobial sepsis in mice. Following CLP procedure, we showed that opioid treatment could induce sustained bacterial dissemination and high levels of IL-17A responses via alteration of microbiota resident in the gut lumen. The overexpression of IL-17A led to impaired intestinal barrier function and prolonged inflammation, which may contribute to higher mortality of sepsis associated with opioid treatment. Neutralization of IL-17A improved survival rates and protected intestinal barrier function in the morphine-treated CLP animals, implying that IL-17A is a potential therapeutic target to treat sepsis in opioid use or abuse population.

Our discoveries have provided novel insights into the mechanisms by which opioids compromise gut immune system and increase host susceptibility to gut infections. However, limitations might attenuate its clinical relevance. The first limitation of our study is that we primarily used subcutaneous morphine pellets in our animal model. Today, the most common prescription opioids used for pain management are hydromorphone, oxycodone, and hydrocodone. Oxycodone and hydrocodone are especially popular since they can be taken orally. The gut epithelia of patients who are taking oxycodone or hydrocodone are exposed to high concentrations of opioids, which may exert distinct effects on the gut epithelial cells. Therefore, it will be worthwhile to investigate the specific effects of various prescription opioids and their route of administration on gut immunity. Second limitation is that we injected IL-17A

neutralization antibodies prior to CLP procedure, which reduced morphine-induced higher mortality rates and protected gut epithelial barrier function in our CLP model. Based on this observation, we believe that IL-17A will be a potential therapeutic target to control inflammation during sepsis progression. However, it is impossible for physicians to consider anti-cytokine treatment before clear diagnosis of sepsis. It will be important to determine whether delayed injection of anti-IL-17A could still protect mice from morphine-induced excessive inflammation in our sepsis model. Therefore, treatment of IL-17A neutralization antibodies at different time points after CLP procedure will provide a better clue whether IL-17A neutralization will benefit the septic patients and improve the prognosis of sepsis or septic shock. The third limitation is that we only show morphine treatment altered gut microbiota and thereby exacerbated the outcome of sepsis in mice model. The gut microbiota in humans is very different from those in mice. Therefore, to further validate the effects of opioids on human gut microbiota, it will be interesting to establish humanized mice model in a future study. By transplanting human immune cells into immunocompromised mice and gavaging these mice with human feces, we can mimic the interactions between human gut microbiota and human gut immune system in a humanized mice model and investigate the effects of opioids on human gut immune system.

In summary, the present study demonstrates that opioids treatment compromises gut epithelial barrier function by modulating TLR signaling and modulates immune responses of gut immune system by altering gut microbiota. It is important to note that the mechanisms by which opioids modulate gut immune system and thereby influence the

outcome of infectious diseases are very complicated. In order to provide a translational perspective to this study future studies are still needed to elucidate the mechanisms underlying increased host susceptibility to gut infections associated with opioid treatment.

LITERATURE CITED

Abreu, Maria T. 2010. “Toll-like Receptor Signalling in the Intestinal Epithelium: How Bacterial Recognition Shapes Intestinal Function.” *Nature Reviews. Immunology* 10 (2) (February): 131–44. doi:10.1038/nri2707. http://www.ncbi.nlm.nih.gov/pubmed/20098461.

Akdis, Mübeccel, Oscar Palomares, Willem van de Veen, Marloes van Splunter, and Cezmi a Akdis. 2012. “TH17 and TH22 Cells: A Confusion of Antimicrobial Response with Tissue Inflammation versus Protection.” *The Journal of Allergy and Clinical Immunology* 129 (6) (June): 1438–49; quiz1450–1. doi:10.1016/j.jaci.2012.05.003. http://www.ncbi.nlm.nih.gov/pubmed/22657405.

Alexander, Michelle, TanJanika Daniel, Irshad H Chaudry, and Martin G Schwacha. 2005. “Opiate Analgesics Contribute to the Development of Post-Injury Immunosuppression.” *The Journal of Surgical Research* 129 (1) (November): 161–8. doi:10.1016/j.jss.2005.04.028. http://www.ncbi.nlm.nih.gov/pubmed/16139307.

Angus, Derek C, and Tom van der Poll. 2013. “Severe Sepsis and Septic Shock.” *The New England Journal of Medicine* 369 (9) (August 29): 840–51. doi:10.1056/NEJMra1208623. http://www.ncbi.nlm.nih.gov/pubmed/23984731.

Babrowski, Trissa, Christopher Holbrook, Jonathan Moss, Lawrence Gottlieb, Vesta Valuckaite, Alexander Zaborin, Valeriy Poroyko, Donald C Liu, Olga Zaborina, and John C Alverdy. 2012. “Pseudomonas Aeruginosa Virulence Expression Is Directly Activated by Morphine and Is Capable of Causing Lethal Gut-Derived Sepsis in Mice during Chronic Morphine Administration.” *Annals of Surgery* 255 (2) (February): 386–93. doi:10.1097/SLA.0b013e3182331870. http://www.ncbi.nlm.nih.gov/pubmed/21989372.

Baldo, Brian a., and Nghia H Pham. 2013. “Opioid Analgesic Drugs.” *Drug Allergy: Clinical Aspects, Diagnosis, Mechanisms, Structure-Activity Relationships,*: 295–317. doi:10.1007/978-1-4614-7261-2. http://link.springer.com/10.1007/978-1-4614-7261-2.

Banerjee, Santanu, Jingjing Meng, Subhas Das, Anitha Krishnan, Justin Haworth, Richard Charboneau, Yan Zeng, Sundaram Ramakrishnan, and Sabita Roy. 2013. “Morphine Induced Exacerbation of Sepsis Is Mediated by Tempering Endotoxin Tolerance through Modulation of miR-146a.” *Scientific Reports* 3 (January): 1977. doi:10.1038/srep01977. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3679508&tool=pmcentrez&rendertype=abstract.

Barker, Nick, Johan H van Es, Jeroen Kuipers, Pekka Kujala, Maaike van den Born, Miranda Cozijnsen, Andrea Haegebarth, et al. 2007. “Identification of Stem Cells in Small Intestine and Colon by Marker Gene Lgr5.” *Nature* 449 (7165) (October 25): 1003–7. doi:10.1038/nature06196. http://www.ncbi.nlm.nih.gov/pubmed/17934449.

Beagles, Karen, Anton Wellstein, and Barbara Bayer. 2004. “Systemic Morphine Administration Suppresses Genes Involved in Antigen Presentation.” *Molecular Pharmacology* 65 (2) (February): 437–42. doi:10.1124/mol.65.2.437. http://www.ncbi.nlm.nih.gov/pubmed/14742686.

Bonnet, Marie-Pierre, Hélène Beloeil, Dan Benhamou, Jean-Xavier Mazoit, and Karim Asehnoune. 2008. “The Mu Opioid Receptor Mediates Morphine-Induced Tumor Necrosis Factor and Interleukin-6 Inhibition in Toll-like Receptor 2-Stimulated Monocytes.” *Anesthesia and Analgesia* 106 (4) (April): 1142–9, table of contents. doi:10.1213/ane.0b013e318165de89. http://www.ncbi.nlm.nih.gov/pubmed/18349186.

Börner, Christine, and Jürgen Kraus. 2013. “Inhibition of NF-κB by Opioids in T Cells.” *Journal of Immunology (Baltimore, Md. : 1950)* 191 (9) (November 1): 4640–7. doi:10.4049/jimmunol.1300320. http://www.ncbi.nlm.nih.gov/pubmed/24068670.

Breslow, Jessica M, Pu Feng, Joseph J Meissler, John E Pintar, John Gaughan, Martin W Adler, and Toby K Eisenstein. 2010. “Potentiating Effect of Morphine on Oral Salmonella Enterica Serovar Typhimurium Infection Is M-Opioid Receptor-Dependent.” *Microbial Pathogenesis* 49 (6) (December): 330–5. doi:10.1016/j.micpath.2010.07.006. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2956788&tool=pmcentrez&rendertype=abstract.

Breslow, Jessica M, M Alexandra Monroy, John M Daly, Joseph J Meissler, Martin W Adler, and Toby K Eisenstein. 2011. “Morphine, but Not Trauma, Sensitizes to Systemic Acinetobacter Baumannii Infection.” *J Neuroimmune Pharmaco* 6 (4): 551–565. doi:10.1007/s11481-011-9303-6.Morphine.

Breslow, Jessica M, M Alexandra Monroy, John M Daly, Joseph J Meissler, John Gaughan, Martin W Adler, and Toby K Eisenstein. 2011. “Morphine, but Not Trauma, Sensitizes to Systemic Acinetobacter Baumannii Infection.” *Journal of Neuroimmune Pharmacology : The Official Journal of the Society on NeuroImmune Pharmacology* 6 (4) (December): 551–65. doi:10.1007/s11481-011-9303-6. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3973428&tool=pmcentrez&rendertype=abstract.

Bruewer, Matthias, Andreas Luegering, Torsten Kucharzik, Charles a Parkos, James L Madara, Ann M Hopkins, and Asma Nusrat. 2003. “Proinflammatory Cytokines

Disrupt Epithelial Barrier Function by Apoptosis-Independent Mechanisms." *Journal of Immunology (Baltimore, Md. : 1950)* 171 (11) (December 1): 6164–72. http://www.ncbi.nlm.nih.gov/pubmed/14634132.

Bryant, H U, B C Yoburn, C E Inturrisi, E W Bernton, and J W Holaday. 1988. "Morphine-Induced Immunomodulation Is Not Related to Serum Morphine Concentrations." *European Journal of Pharmacology* 149 (1-2) (April 27): 165–9. http://www.ncbi.nlm.nih.gov/pubmed/3260867.

Bullen, Tim F, Sharon Forrest, Fiona Campbell, Andrew R Dodson, Michael J Hershman, D Mark Pritchard, Jerrold R Turner, Marshall H Montrose, and Alastair J M Watson. 2006. "Characterization of Epithelial Cell Shedding from Human Small Intestine." *Laboratory Investigation; a Journal of Technical Methods and Pathology* 86 (10) (October): 1052–63. doi:10.1038/labinvest.3700464. http://www.ncbi.nlm.nih.gov/pubmed/16909128.

Chabot, S., J. S. Wagner, S. Farrant, and M. R. Neutra. 2006. "TLRs Regulate the Gatekeeping Functions of the Intestinal Follicle-Associated Epithelium." *The Journal of Immunology* 176 (7) (March 17): 4275–4283. doi:10.4049/jimmunol.176.7.4275. http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.176.7.4275.

Chang, Seon Hee, and Chen Dong. 2011. "Signaling of Interleukin-17 Family Cytokines in Immunity and Inflammation." *Cellular Signalling* 23 (7) (July): 1069–75. doi:10.1016/j.cellsig.2010.11.022. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3078175&tool=pmcentrez&rendertype=abstract.

Cheroutre, Hilde. 2005. "IELs: Enforcing Law and Order in the Court of the Intestinal Epithelium." *Immunological Reviews* 206 (August): 114–31. doi:10.1111/j.0105-2896.2005.00284.x. http://www.ncbi.nlm.nih.gov/pubmed/16048545.

Clarke TB, Francella N, Huegel A, Weiser JN. 2011. "Invasive Bacterial Pathogens Exploit TLR-Mediated Downregulation of Tight Junction Components to Facilitate Translocation across the Epithelium. Clarke TB1, Francella N, Huegel A, Weiser JN. Author Information." *Cell Host Microbe* 9 (5): 404–14.

Cornwell, William D, Mark G Lewis, Xiaoxuan Fan, Jay Rappaport, and Thomas J Rogers. 2013. "Effect of Chronic Morphine Administration on Circulating T Cell Population Dynamics in Rhesus Macaques." *Journal of Neuroimmunology* 265 (1-2) (December 15): 43–50. doi:10.1016/j.jneuroim.2013.09.013. http://www.ncbi.nlm.nih.gov/pubmed/24090653.

Davis, Mellar P., and Declan Walsh. 2014. “Methadone for Relief of Cancer Pain: A Review of Pharmacokinetics, Pharmacodynamics, Drug Interactions and Protocols of Administration.” *Supportive Care in Cancer* 9 (2) (February 9): 73–83. doi:10.1007/s005200000180. http://link.springer.com/10.1007/s005200000180.

Desai, Tina R, Nicholas J Leeper, Karen L Hynes, and Bruce L Gewertz. 2002. “Interleukin-6 Causes Endothelial Barrier Dysfunction via the Protein Kinase C Pathway.” *The Journal of Surgical Research* 104 (2) (May 15): 118–23. doi:10.1006/jsre.2002.6415. http://www.ncbi.nlm.nih.gov/pubmed/12020130.

Deshmukh, Hitesh S, Yuhong Liu, Ogechukwu R Menkiti, Junjie Mei, Ning Dai, Claire E O’Leary, Paula M Oliver, Jay K Kolls, Jeffrey N Weiser, and G Scott Worthen. 2014. “The Microbiota Regulates Neutrophil Homeostasis and Host Resistance to Escherichia Coli K1 Sepsis in Neonatal Mice.” *Nature Medicine* (April) (April 20): 1–9. doi:10.1038/nm.3542. http://www.nature.com/doifinder/10.1038/nm.3542.

Devlin, John W, and Russel J Roberts. 2009. “Pharmacology of Commonly Used Analgesics and Sedatives in the ICU: Benzodiazepines, Propofol, and Opioids.” *Critical Care Clinics* 25 (3) (July): 431–49, vii. doi:10.1016/j.ccc.2009.03.003. http://www.ncbi.nlm.nih.gov/pubmed/19576523.

Dunn, Kate M, Kathleen W Saunders, Carolyn M Rutter, Caleb J Banta-green, Joseph O Merrill, Mark D Sullivan, Constance M Weisner, and Michael J Silverberg. 2010. “Annals of Internal Medicine Article Opioid Prescriptions for Chronic Pain and Overdose A Cohort Study.”

Dutta, Raini, Anitha Krishnan, Jingjing Meng, Subash Das, Jing Ma, Santanu Banerjee, Jinghua Wang, et al. 2012. “Morphine Modulation of Toll-like Receptors in Microglial Cells Potentiates Neuropathogenesis in a HIV-1 Model of Coinfection with Pneumococcal Pneumoniae.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 32 (29) (July 18): 9917–30. doi:10.1523/JNEUROSCI.0870-12.2012. http://www.ncbi.nlm.nih.gov/pubmed/22815507.

Farache, Julia, Ehud Zigmond, Guy Shakhar, and Steffen Jung. 2013. “Contributions of Dendritic Cells and Macrophages to Intestinal Homeostasis and Immune Defense.” *Immunology and Cell Biology* 91 (3) (March): 232–9. doi:10.1038/icb.2012.79. http://www.ncbi.nlm.nih.gov/pubmed/23399695.

Feng, Pu, Allan L Truant, Joseph J Meissler, John P Gaughan, Martin W Adler, and Toby K Eisenstein. 2006. “Morphine Withdrawal Lowers Host Defense to Enteric Bacteria: Spontaneous Sepsis and Increased Sensitivity to Oral Salmonella Enterica Serovar Typhimurium Infection.” *Infection and Immunity* 74 (9) (September): 5221–6. doi:10.1128/IAI.00208-06.

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1594820&tool=pmcentrez&rendertype=abstract.

Flierl, Michael a, Daniel Rittirsch, Hongwei Gao, Laszlo M Hoesel, Brian a Nadeau, Danielle E Day, Firas S Zetoune, et al. 2008. “Adverse Functions of IL-17A in Experimental Sepsis.” *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* 22 (7) (July): 2198–205. doi:10.1096/fj.07-105221. http://www.ncbi.nlm.nih.gov/pubmed/18299333.

Forsythe, Raquel M, Da-zhong Xu, Qi Lu, and Edwin A Deitch. 2002. “OXIDE INDUCES INTESTINAL MONOLAYER PERMEABILITY IN AN AUTOCRINE FASHION.” *Shock* 17 (3): 180–184.

Freitas, Andressa, José C Alves-Filho, Tatiana Victoni, Thomas Secher, Henrique P Lemos, Fabiane Sônego, Fernando Q Cunha, and Bernhard Ryffel. 2009. “IL-17 Receptor Signaling Is Required to Control Polymicrobial Sepsis.” *Journal of Immunology (Baltimore, Md. : 1950)* 182 (12) (June 15): 7846–54. doi:10.4049/jimmunol.0803039. http://www.ncbi.nlm.nih.gov/pubmed/19494309.

Gaskins, H Rex. 2001. “Microbial Modulation of Innate Defense : Goblet Cells and the” 73 (April 2000).

Glattard, Elise, Ingeborg D Welters, Thomas Lavaux, Arnaud H Muller, Alexis Laux, Dan Zhang, Alexander R Schmidt, et al. 2010. “Endogenous Morphine Levels Are Increased in Sepsis: A Partial Implication of Neutrophils.” *PloS One* 5 (1) (January): e8791. doi:10.1371/journal.pone.0008791. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2808358&tool=pmcentrez&rendertype=abstract.

Greeneltch, Kristy M, Christian C Haudenschild, Achsah D Keegan, and Yufang Shi. 2004a. “The Opioid Antagonist Naltrexone Blocks Acute Endotoxic Shock by Inhibiting Tumor Necrosis Factor-Alpha Production.” *Brain, Behavior, and Immunity* 18 (5) (September): 476–84. doi:10.1016/j.bbi.2003.12.001. http://www.ncbi.nlm.nih.gov/pubmed/15265541.

———. 2004b. “The Opioid Antagonist Naltrexone Blocks Acute Endotoxic Shock by Inhibiting Tumor Necrosis Factor-Alpha Production.” *Brain, Behavior, and Immunity* 18 (5) (September): 476–84. doi:10.1016/j.bbi.2003.12.001. http://www.ncbi.nlm.nih.gov/pubmed/15265541.

Gu, Chunfang, Ling Wu, and Xiaoxia Li. 2013. “IL-17 Family: Cytokines, Receptors and Signaling.” *Cytokine* 64 (2) (November): 477–85. doi:10.1016/j.cyto.2013.07.022. http://www.ncbi.nlm.nih.gov/pubmed/24011563.

Hapfelmeier, Siegfried, Melissa a E Lawson, Emma Slack, Jorum K Kirundi, Maaike Stoel, Mathias Heikenwalder, Julia Cahenzli, et al. 2010. “Reversible Microbial Colonization of Germ-Free Mice Reveals the Dynamics of IgA Immune Responses.” *Science (New York, N.Y.)* 328 (5986) (June 25): 1705–9. doi:10.1126/science.1188454. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3923373&tool=pmcentrez&rendertype=abstract.

Hilburger, M E, M W Adler, a L Truant, J J Meissler, V Satishchandran, T J Rogers, and T K Eisenstein. 1997. “Morphine Induces Sepsis in Mice.” *The Journal of Infectious Diseases* 176 (1) (July): 183–8. http://www.ncbi.nlm.nih.gov/pubmed/9207365.

Hill, David a, and David Artis. 2010. “Intestinal Bacteria and the Regulation of Immune Cell Homeostasis.” *Annual Review of Immunology* 28 (January): 623–67. doi:10.1146/annurev-immunol-030409-101330. http://www.ncbi.nlm.nih.gov/pubmed/20192812.

Huppert, Jula, Dorothea Closhen, Andrew Croxford, Robin White, Paulina Kulig, Eweline Pietrowski, Ingo Bechmann, et al. 2010. “Cellular Mechanisms of IL-17-Induced Blood-Brain Barrier Disruption.” *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* 24 (4) (April): 1023–34. doi:10.1096/fj.09-141978. http://www.ncbi.nlm.nih.gov/pubmed/19940258.

Investigators, The Process. 2014. “A Randomized Trial of Protocol-Based Care for Early Septic Shock.” *The New England Journal of Medicine* (March 18): 1–11. doi:10.1056/NEJMoa1401602. http://www.ncbi.nlm.nih.gov/pubmed/24635773.

Ivanov, Ivaylo I, Koji Atarashi, Nicolas Manel, Eoin L Brodie, Tatsuichiro Shima, Ulas Karaoz, Dongguang Wei, et al. 2009. “Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria.” *Cell* 139 (3) (October 30): 485–98. doi:10.1016/j.cell.2009.09.033. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2796826&tool=pmcentrez&rendertype=abstract.

Jr, Theodore J Sanford, N Ty Smith, Walter K Harrison, and Holly Dec-silver. 1986. “A Comparison of Morphine, Fentanyl, and Sufentanil Anesthesia for Cardiac Surgery:” 259–266.

Kamada, Nobuhiko, Sang-Uk Seo, Grace Y Chen, and Gabriel Núñez. 2013. “Role of the Gut Microbiota in Immunity and Inflammatory Disease.” *Nature Reviews. Immunology* 13 (5) (May): 321–35. doi:10.1038/nri3430. http://www.ncbi.nlm.nih.gov/pubmed/23618829.

Kebir, Hania, Katharina Kreymborg, Igal Ifergan, Aurore Dodelet-Devillers, Romain Cayrol, Monique Bernard, Fabrizio Giuliani, Nathalie Arbour, Burkhard Becher, and Alexandre Prat. 2007. "Human TH17 Lymphocytes Promote Blood-Brain Barrier Disruption and Central Nervous System Inflammation." *Nature Medicine* 13 (10) (October): 1173–5. doi:10.1038/nm1651. http://www.ncbi.nlm.nih.gov/pubmed/17828272.

Khansari, MahmoudReza, MasourReza Sohrabi, and Farhad Zamani. 2013. "The Useage of Opioids and Their Adverse Effects in Gastrointestinal Practice: A Review." *Middle East Journal of Digestive Diseases* 5 (1) (January): 5–16. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3990131&tool=pmcentrez&rendertype=abstract.

Kim, Jiyeon S, Tammarah Sklarz, Lauren B Banks, Mercy Gohil, Adam T Waickman, Nicolas Skuli, Bryan L Krock, et al. 2013. "Natural and Inducible TH17 Cells Are Regulated Differently by Akt and mTOR Pathways." *Nature Immunology* (May) (May 5): 1–9. doi:10.1038/ni.2607. http://www.ncbi.nlm.nih.gov/pubmed/23644504.

Kim, Jiyeon S, Jennifer E Smith-Garvin, Gary a Koretzky, and Martha S Jordan. 2011. "The Requirements for Natural Th17 Cell Development Are Distinct from Those of Conventional Th17 Cells." *The Journal of Experimental Medicine* 208 (11) (October 24): 2201–7. doi:10.1084/jem.20110680. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3201206&tool=pmcentrez&rendertype=abstract.

Klatt, N R, L D Harris, C L Vinton, H Sung, J a Briant, B Tabb, D Morcock, et al. 2010. "Compromised Gastrointestinal Integrity in Pigtail Macaques Is Associated with Increased Microbial Translocation, Immune Activation, and IL-17 Production in the Absence of SIV Infection." *Mucosal Immunology* 3 (4) (July): 387–98. doi:10.1038/mi.2010.14. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2891168&tool=pmcentrez&rendertype=abstract.

Koboziev, Iurii, Fridrik Karlsson, and Matthew B. Grisham. 2010. "Gut-Associated Lymphoid Tissue, T Cell Trafficking, and Chronic Intestinal Inflammation." *Ann N Y Acad Sci* 1207 (Suppl 1): 1–11. doi:10.1111/j.1749-6632.2010.05711.x.Gut-associated.

Kress, Hans G, Dorothea Von der Laage, Klaus H Hoerauf, Thomas Nolte, Tarja Heiskanen, Rasmus Petersen, Lena Lundorff, et al. 2008. "A Randomized, Open, Parallel Group, Multicenter Trial to Investigate Analgesic Efficacy and Safety of a New Transdermal Fentanyl Patch Compared to Standard Opioid Treatment in Cancer Pain." *Journal of Pain and Symptom Management* 36 (3) (September): 268–

79. doi:10.1016/j.jpainsymman.2007.10.023. http://www.ncbi.nlm.nih.gov/pubmed/18538974.

Kueppers, P M, T a Miller, C Y Chen, G S Smith, L F Rodriguez, and F G Moody. 1993. "Effect of Total Parenteral Nutrition plus Morphine on Bacterial Translocation in Rats." *Annals of Surgery* 217 (3) (March): 286–92. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1242782&tool=pmcentrez&rendertype=abstract.

Lang, M E, J S Davison, S L Bates, and J B Meddings. 1996. "Opioid Receptors on Guinea-Pig Intestinal Crypt Epithelial Cells." *The Journal of Physiology* 497 (Pt 1 (1996) (November 15): 161–74. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1160920&tool=pmcentrez&rendertype=abstract.

Lee, Yun Kyung, and Sarkis K Mazmanian. 2010. "Has the Microbiota Played a Critical Role in the Evolution of the Adaptive Immune System?" *Science (New York, N.Y.)* 330 (6012) (December 24): 1768–73. doi:10.1126/science.1195568. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3159383&tool=pmcentrez&rendertype=abstract.

Leslie, KA, R Behme, A Clift, S Martin, D Grant, and Duff Jh. 1994. "Synergistic Effects of Tumour Necrosis Factor and Morphine on Gut Barrier Function. Leslie KA1, Behme R, Clift A, Martin S, Grant D, Duff JH. Author Information." *Can J Surg* 37 (2): 143–7.

LeVier, D G, J a McCay, M L Stern, L S Harris, D Page, R D Brown, D L Musgrove, L F Butterworth, K L White, and a E Munson. 1994. "Immunotoxicological Profile of Morphine Sulfate in B6C3F1 Female Mice." *Fundamental and Applied Toxicology : Official Journal of the Society of Toxicology* 22 (4) (May): 525–42. http://www.ncbi.nlm.nih.gov/pubmed/8056200.

Linden, S K, P Sutton, N G Karlsson, V Korolik, and M a McGuckin. 2008. "Mucins in the Mucosal Barrier to Infection." *Mucosal Immunology* 1 (3) (May): 183–97. doi:10.1038/mi.2008.5. http://www.ncbi.nlm.nih.gov/pubmed/19079178.

Lycke, Nils Y, and Mats Bemark. 2012. "The Role of Peyer's Patches in Synchronizing Gut IgA Responses." *Frontiers in Immunology* 3 (November) (January): 329. doi:10.3389/fimmu.2012.00329. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3500999&tool=pmcentrez&rendertype=abstract.

Ma, Jing, Jinghua Wang, Jing Wan, Richard Charboneau, Yaping Chang, Roderick a Barke, and Sabita Roy. 2010. "Morphine Disrupts Interleukin-23 (IL-23)/IL-17-

Mediated Pulmonary Mucosal Host Defense against Streptococcus Pneumoniae Infection." *Infection and Immunity* 78 (2) (February): 830–7. doi:10.1128/IAI.00914-09. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2812200&tool=pmcentrez&rendertype=abstract.

Mabbott, Neil A, David S Donaldson, Hiroshi Ohno, and Ifor R Williams. 2013. "Microfold (M) Cells : Important Immunosurveillance Posts in the Intestinal Epithelium." *Nature* 6 (4): 666–677. doi:10.1038/mi.2013.30.Microfold.

Mahajan, Supriya D, Ravikumar Aalinkeel, Donald E Sykes, Jessica L Reynolds, B Bindukumar, Stanley F Fernandez, Ramnik Chawda, Thomas C Shanahan, and Stanley a Schwartz. 2008. "Tight Junction Regulation by Morphine and HIV-1 Tat Modulates Blood-Brain Barrier Permeability." *Journal of Clinical Immunology* 28 (5) (September): 528–41. doi:10.1007/s10875-008-9208-1. http://www.ncbi.nlm.nih.gov/pubmed/18574677.

Manchikanti, Laxmaiah, Standiford Helm, Bert Fellows, Jeffrey W Janata, Vidyasagar Pampati, Jay S Grider, and Mark V Boswell. 2012. "Opioid Epidemic in the United States." *Pain Physician* 15 (3 Suppl) (July): ES9–38. http://www.ncbi.nlm.nih.gov/pubmed/22851109.

Marchiando, Amanda M, W Vallen Graham, and Jerrold R Turner. 2010. "Epithelial Barriers in Homeostasis and Disease." *Annual Review of Pathology* 5 (January): 119–44. doi:10.1146/annurev.pathol.4.110807.092135. http://www.ncbi.nlm.nih.gov/pubmed/20078218.

Marks, Benjamin R, Heba N Nowyhed, Jin-Young Choi, Amanda C Poholek, Jared M Odegard, Richard a Flavell, and Joe Craft. 2009. "Thymic Self-Reactivity Selects Natural Interleukin 17-Producing T Cells That Can Regulate Peripheral Inflammation." *Nature Immunology* 10 (10) (October): 1125–32. doi:10.1038/ni.1783. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2751862&tool=pmcentrez&rendertype=abstract.

Martin, Josephine L, Lisa Koodie, Anitha G Krishnan, Richard Charboneau, Roderick a Barke, and Sabita Roy. 2010. "Chronic Morphine Administration Delays Wound Healing by Inhibiting Immune Cell Recruitment to the Wound Site." *The American Journal of Pathology* 176 (2) (February): 786–99. doi:10.2353/ajpath.2010.090457. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2808085&tool=pmcentrez&rendertype=abstract.

Mason, Katie Lynn, Gary B Huffnagle, Mairi C Noverr, and John Y Kao. 2008. "Overview of Gut Immunology." *Advances in Experimental Medicine and Biology*

635 (January): 1–14. doi:10.1007/978-0-387-09550-9_1. http://www.ncbi.nlm.nih.gov/pubmed/24629727.

McCarthy, L, I Szabo, J F Nitsche, J E Pintar, and T J Rogers. 2001. "Expression of Functional Mu-Opioid Receptors during T Cell Development." *Journal of Neuroimmunology* 114 (1-2) (March 1): 173–80. http://www.ncbi.nlm.nih.gov/pubmed/11240029.

Meng, Jingjing, Haidong Yu, Jing Ma, Jinghua Wang, Santanu Banerjee, Rick Charboneau, Roderick a. Barke, and Sabita Roy. 2013. "Morphine Induces Bacterial Translocation in Mice by Compromising Intestinal Barrier Function in a TLR-Dependent Manner." Edited by Shilpa J. Buch. *PLoS ONE* 8 (1) (January 18): e54040. doi:10.1371/journal.pone.0054040. http://dx.plos.org/10.1371/journal.pone.0054040.

Miaskowski, Christine. 2009. "A Review of the Incidence, Causes, Consequences, and Management of Gastrointestinal Effects Associated With Postoperative Opioid Administration." *Journal of PeriAnesthesia Nursing* 24 (4 (August)): 222–228.

Moriez, Raphaël, Christel Salvador-Cartier, Vassilia Theodorou, Jean Fioramonti, Helene Eutamene, and Lionel Bueno. 2005. "Myosin Light Chain Kinase Is Involved in Lipopolysaccharide-Induced Disruption of Colonic Epithelial Barrier and Bacterial Translocation in Rats." *The American Journal of Pathology* 167 (4) (October): 1071–9. doi:10.1016/S0002-9440(10)61196-0. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1603678&tool=pmcentrez&rendertype=abstract.

Murphy, Glenn S, Joseph W Szokol, Jesse H Marymont, Steven B Greenberg, Michael J Avram, Jeffery S Vender, Saadia S Sherwani, Margarita Nisman, and Victoria Doroski. 2009. "Morphine-Based Cardiac Anesthesia Provides Superior Early Recovery Compared with Fentanyl in Elective Cardiac Surgery Patients." *Anesthesia and Analgesia* 109 (2) (August): 311–9. doi:10.1213/ane.0b013e3181a90adc. http://www.ncbi.nlm.nih.gov/pubmed/19608797.

Ninkovic, Jana. 2011. "Morphine Inhibition of Macrophage Phagocytosis and Bactericidal Functions" (January).

Ninković, Jana, and Sabita Roy. 2012. "Morphine Decreases Bacterial Phagocytosis by Inhibiting Actin Polymerization through cAMP-, Rac-1-, and p38 MAPK-Dependent Mechanisms." *The American Journal of Pathology* 180 (3) (March): 1068–79. doi:10.1016/j.ajpath.2011.11.034. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3349892&tool=pmcentrez&rendertype=abstract.

———. 2013. “Role of the Mu-Opioid Receptor in Opioid Modulation of Immune Function.” *Amino Acids* 45 (1) (July): 9–24. doi:10.1007/s00726-011-1163-0. http://www.ncbi.nlm.nih.gov/pubmed/22170499.

O’Connor, William, Masahito Kamanaka, Carmen J Booth, Terrence Town, Susumu Nakae, Yoichiro Iwakura, Jay K Kolls, and Richard a Flavell. 2009. “A Protective Function for Interleukin 17A in T Cell-Mediated Intestinal Inflammation.” *Nature Immunology* 10 (6) (June): 603–9. doi:10.1038/ni.1736. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2709990&tool=pmcentrez&rendertype=abstract.

Ocasio, Frank M, Yuhui Jiang, Steven D House, and Sulie L Chang. 2004. “Chronic Morphine Accelerates the Progression of Lipopolysaccharide-Induced Sepsis to Septic Shock.” *Journal of Neuroimmunology* 149 (1-2) (April): 90–100. doi:10.1016/j.jneuroim.2003.12.016. http://www.ncbi.nlm.nih.gov/pubmed/15020069.

Peng, Jinsong, Sraboni Sarkar, and Sulie L Chang. 2012. “Opioid Receptor Expression in Human Brain and Peripheral Tissues Using Absolute Quantitative Real-Time RT-PCR.” *Drug and Alcohol Dependence* 124 (3) (August 1): 223–8. doi:10.1016/j.drugalcdep.2012.01.013. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3366045&tool=pmcentrez&rendertype=abstract.

Peterson, Lance W, and David Artis. 2014. “Intestinal Epithelial Cells: Regulators of Barrier Function and Immune Homeostasis.” *Nature Reviews. Immunology* 14 (3) (February 25): 141–53. doi:10.1038/nri3608. http://www.ncbi.nlm.nih.gov/pubmed/24566914.

Philippe, David, Laurent Dubuquoy, Hervé Groux, Valérie Brun, Myriam Tran Van Chuoï-mariot, Claire Gaveriaux-ruff, Jean-frédéric Colombel, Brigitte L Kieffer, and Pierre Desreumaux. 2003. “Anti-Inflammatory Properties of the M Opioid Receptor Support Its Use in the Treatment of Colon Inflammation” 111 (9): 1329–1338. doi:10.1172/JCI200316750.Introduction.

Plante, Gérard E, and Theodore B VanItallie. 2010. “Opioids for Cancer Pain: The Challenge of Optimizing Treatment.” *Metabolism: Clinical and Experimental* 59 Suppl 1 (Suppl 1) (October): S47–52. doi:10.1016/j.metabol.2010.07.010. http://www.ncbi.nlm.nih.gov/pubmed/20837194.

Porreca, Frank, and Michael H Ossipov. 2009. “Nausea and Vomiting Side Effects with Opioid Analgesics during Treatment of Chronic Pain: Mechanisms, Implications, and Management Options.” *Pain Medicine (Malden, Mass.)* 10 (4): 654–62.

doi:10.1111/j.1526-4637.2009.00583.x.
http://www.ncbi.nlm.nih.gov/pubmed/19302436.

Porter, E M, C L Bevins, D Ghosh, and T Ganz. 2002. "Cellular and Molecular Life Sciences The Multifaceted Paneth Cell" 59: 156–170.

Quinlan, Jonathan M, Wei-Yuan Yu, Mark a Hornsey, David Tosh, and Jonathan M W Slack. 2006. "In Vitro Culture of Embryonic Mouse Intestinal Epithelium: Cell Differentiation and Introduction of Reporter Genes." *BMC Developmental Biology* 6 (January): 24. doi:10.1186/1471-213X-6-24.
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1489925&tool=pmcentrez&rendertype=abstract.

Regan L, Chapman AR, Celnik A, Lumsden L, Al-Soufi R, McCullough NP. 2013. "Nose and Vein , Speed and Pain : Comparing the Use of Intranasal Diamorphine and Intravenous Morphine in a Scottish Paediatric Emergency Department ." *Emerg Med J* 30 (1): 200970. doi:10.1136/emermed-2011-200970.

Rescigno, Maria, and Antonio Di Sabatino. 2009. "Science in Medicine Dendritic Cells in Intestinal Homeostasis and Disease" 119 (9): 2441–2450. doi:10.1172/JCI39134.of.

Reynolds, Joseph M, Bhanu P Pappu, Juan Peng, Gustavo J Martinez, Yongliang Zhang, Yeonseok Chung, Li Ma, et al. 2010. "Toll-like Receptor 2 Signaling in CD4(+) T Lymphocytes Promotes T Helper 17 Responses and Regulates the Pathogenesis of Autoimmune Disease." *Immunity* 32 (5) (May 28): 692–702. doi:10.1016/j.immuni.2010.04.010.
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2878917&tool=pmcentrez&rendertype=abstract.

Ripamonti, C, and E Bruera. 1991. "Rectal, Buccal, and Sublingual Narcotics for the Management of Cancer Pain." *J Palliat Care* 7 (1): 1991.

Ross, Gracious R, Bichoy H Gabra, William L Dewey, and Hamid I Akbarali. 2008. "Morphine Tolerance in the Mouse Ileum and Colon" 327 (2): 561–572. doi:10.1124/jpet.108.143438.and.

Roulis, Manolis, Maria Armaka, Menelaos Manoloukos, Maria Apostolaki, and George Kollias. 2011. "Intestinal Epithelial Cells as Producers but Not Targets of Chronic TNF Suffice to Cause Murine Crohn-like Pathology." *Proceedings of the National Academy of Sciences of the United States of America* 108 (13) (March 29): 5396–401. doi:10.1073/pnas.1007811108.
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3069201&tool=pmcentrez&rendertype=abstract.

Rousseaux, Christel, Xavier Thuru, Agathe Gelot, Nicolas Barnich, Christel Neut, Laurent Dubuquoy, Caroline Dubuquoy, et al. 2007. “Lactobacillus Acidophilus Modulates Intestinal Pain and Induces Opioid and Cannabinoid Receptors.” *Nature Medicine* 13 (1) (January): 35–7. doi:10.1038/nm1521. http://www.ncbi.nlm.nih.gov/pubmed/17159985.

Roy, S, S Balasubramanian, S Sumandeep, R Charboneau, J Wang, D Melnyk, G J Beilman, R Vatassery, and R a Barke. 2001. “Morphine Directs T Cells toward T(H2) Differentiation.” *Surgery* 130 (2) (August): 304–9. doi:10.1067/msy.2001.116033. http://www.ncbi.nlm.nih.gov/pubmed/11490364.

Roy, S, R a Barke, and H H Loh. 1998. “MU-Opioid Receptor-Knockout Mice: Role of Mu-Opioid Receptor in Morphine Mediated Immune Functions.” *Brain Research. Molecular Brain Research* 61 (1-2) (October 30): 190–4. http://www.ncbi.nlm.nih.gov/pubmed/9795212.

Roy, S, R G Charboneau, and R a Barke. 1999. “Morphine Synergizes with Lipopolysaccharide in a Chronic Endotoxemia Model.” *Journal of Neuroimmunology* 95 (1-2) (March 1): 107–14. http://www.ncbi.nlm.nih.gov/pubmed/10229120.

Roy, Sabita, Jana Ninkovic, Santanu Banerjee, Richard Gene Charboneau, Subhas Das, Raini Dutta, Varvara a Kirchner, et al. 2011. “Opioid Drug Abuse and Modulation of Immune Function: Consequences in the Susceptibility to Opportunistic Infections.” *Journal of Neuroimmune Pharmacology : The Official Journal of the Society on NeuroImmune Pharmacology* 6 (4) (December): 442–65. doi:10.1007/s11481-011-9292-5. http://www.ncbi.nlm.nih.gov/pubmed/21789507.

Roy, Sabita, S Ramakrishnan, Horace H Loh, and Nancy M Lee. 1991. “Chronic Morphine Treatment Selectively Suppresses Macrophage Colony Formation in Bone Marrow.” *European Journal of Pharmacology* 195: 359–363.

Rubino, Stephen J, Kaoru Geddes, and Stephen E Girardin. 2012. “Innate IL-17 and IL-22 Responses to Enteric Bacterial Pathogens.” *Trends in Immunology* 33 (3) (March): 112–8. doi:10.1016/j.it.2012.01.003. http://www.ncbi.nlm.nih.gov/pubmed/22342740.

Runkel, N S, F G Moody, G S Smith, L F Rodriguez, Y Chen, M T Larocco, and T a Miller. 1993. “Alterations in Rat Intestinal Transit by Morphine Promote Bacterial Translocation.” *Digestive Diseases and Sciences* 38 (8) (August): 1530–6. http://www.ncbi.nlm.nih.gov/pubmed/8344112.

Sanchez-Muñoz, Fausto. 2008. “Role of Cytokines in Inflammatory Bowel Disease.” *World Journal of Gastroenterology* 14 (27): 4280. doi:10.3748/wjg.14.4280. http://www.wjgnet.com/1007-9327/14/4280.asp.

Schlegel, Nicolas, Michael Meir, Wolfgang-moritz Heupel, Bastian Holthöfer, Rudolf E Leube, and Jens Waschke. 2010. “Desmoglein 2-Mediated Adhesion Is Required for Intestinal Epithelial Barrier Integrity”: 774–783. doi:10.1152/ajpgi.00239.2009.

Schulzke, Joerg D, Svenja Ploeger, Maren Amasheh, Anja Fromm, Sebastian Zeissig, Hanno Troeger, Jan Richter, Christian Bojarski, Michael Schumann, and Michael Fromm. 2009. “Epithelial Tight Junctions in Intestinal Inflammation.” *Annals of the New York Academy of Sciences* 1165 (May): 294–300. doi:10.1111/j.1749-6632.2009.04062.x. http://www.ncbi.nlm.nih.gov/pubmed/19538319.

Sekirov, Inna, Shannon L Russell, L Caetano M Antunes, and B Brett Finlay. 2010. “Gut Microbiota in Health and Disease.” *Physiological Reviews* 90 (3) (July 1): 859–904. doi:10.1152/physrev.00045.2009. http://physrev.physiology.org/content/90/3/859.

Shale, Matthew, Chris Schiering, and Fiona Powrie. 2013. “CD4(+) T-Cell Subsets in Intestinal Inflammation.” *Immunological Reviews* 252 (1) (March): 164–82. doi:10.1111/imr.12039. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3736165&tool=pmcentrez&rendertype=abstract.

Sharp, Burt M, William F Keane, Hwan J Suh, Genya Gekker, Dean Tsukayama, and Phillip K Peterson. 1985. “OPIOID PEPTIDES RAPIDLY STIMULATE SUPEROXIDE PRODUCTION BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES AND MACROPHAGES.” *Endocrinology* 117 (2): 793–795.

Sheth, P, N Delos Santos, A Seth, N F Larusso, and R K Rao. 2007. “Lipopolysaccharide Disrupts Tight Junctions in Cholangiocyte Monolayers by a c-Src- , TLR4- , and LBP-Dependent Mechanism” 38163: 308–318. doi:10.1152/ajpgi.00582.2006.

Shifflett, Donnie E, Daniel R Clayburgh, Athanasia Koutsouris, Jerrold R Turner, and Gail a Hecht. 2005. “Enteropathogenic E. Coli Disrupts Tight Junction Barrier Function and Structure in Vivo.” *Laboratory Investigation; a Journal of Technical Methods and Pathology* 85 (10) (October): 1308–24. doi:10.1038/labinvest.3700330. http://www.ncbi.nlm.nih.gov/pubmed/16127426.

Simpkins, C O, S T Alailima, E a Tate, and M Johnson. 1986. “The Effect of Enkephalins and Prostaglandins on O-2 Release by Neutrophils.” *The Journal of Surgical Research* 41 (6) (December): 645–52. http://www.ncbi.nlm.nih.gov/pubmed/3023754.

Singhal, P C, P Sharma, a a Kapasi, K Reddy, N Franki, and N Gibbons. 1998. "Morphine Enhances Macrophage Apoptosis." *Journal of Immunology (Baltimore, Md. : 1950)* 160 (4) (February 15): 1886–93. http://www.ncbi.nlm.nih.gov/pubmed/9469450.

States, United. 2014. "Opioids for Chronic Pain" 171 (16): 1426–1427.

Strober, Warren, and Ivan J Fuss. 2011. "Proinflammatory Cytokines in the Pathogenesis of Inflammatory Bowel Diseases." *Gastroenterology* 140 (6) (May): 1756–67. doi:10.1053/j.gastro.2011.02.016. http://www.ncbi.nlm.nih.gov/pubmed/21530742.

Suzuki, Keiichiro, Shimpei Kawamoto, Mikako Maruya, and Sidonia Fagarasan. 2010. *GALT: Organization and Dynamics Leading to IgA Synthesis. Advances in Immunology*. 1st ed. Vol. 107. Elsevier Inc. doi:10.1016/B978-0-12-381300-8.00006-X. http://www.ncbi.nlm.nih.gov/pubmed/21034974.

Swaidani, Shadi, Katarzyna Bulek, Zizhen Kang, Muhammet Fatih Gulen, Caini Liu, Weiguo Yin, Amina Abbadi, Mark Aronica, and Xiaoxia Li. 2011. "T Cell-Derived Act1 Is Necessary for IL-25-Mediated Th2 Responses and Allergic Airway Inflammation." *Journal of Immunology (Baltimore, Md. : 1950)* 187 (6) (September 15): 3155–64. doi:10.4049/jimmunol.1002790. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3666175&tool=pmcentrez&rendertype=abstract.

Swaidani, Shadi, Katarzyna Bulek, Zizhen Kang, Caini Liu, Yi Lu, Weiguo Yin, Mark Aronica, and Xiaoxia Li. 2009. "The Critical Role of Epithelial-Derived Act1 in IL-17- and IL-25-Mediated Pulmonary Inflammation." *Journal of Immunology (Baltimore, Md. : 1950)* 182 (3) (February 1): 1631–40. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3015148&tool=pmcentrez&rendertype=abstract.

Takeda, Kiyoshi, Tsuneyasu Kaisho, and Shizuo Akira. 2003. "Toll-like Receptors." *Annual Review of Immunology* 21 (1) (January): 335–76. doi:10.1146/annurev.immunol.21.120601.141126. http://www.ncbi.nlm.nih.gov/pubmed/12524386.

Taylor, Patricia R, Sanhita Roy, Sixto M Leal, Yan Sun, Scott J Howell, Brian a Cobb, Xiaoxia Li, and Eric Pearlman. 2014. "Activation of Neutrophils by Autocrine IL-17A-IL-17RC Interactions during Fungal Infection Is Regulated by IL-6, IL-23, RORγt and Dectin-2." *Nature Immunology* 15 (2) (February): 143–51. doi:10.1038/ni.2797. http://www.ncbi.nlm.nih.gov/pubmed/24362892.

Tazuke, Y, R a Drongowski, D H Teitelbaum, and a G Coran. 2003. "Interleukin-6 Changes Tight Junction Permeability and Intracellular Phospholipid Content in a

Human Enterocyte Cell Culture Model." *Pediatric Surgery International* 19 (5) (July): 321–5. doi:10.1007/s00383-003-1003-8. http://www.ncbi.nlm.nih.gov/pubmed/12732938.

Toscano, Miguel G, Doina Ganea, and Ana M Gamero. 2011. "Cecal Ligation Puncture Procedure." *Journal of Visualized Experiments : JoVE* (51) (January): 1–5. doi:10.3791/2860. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3339843&tool=pmcentrez&rendertype=abstract.

Towne, J.E., J.R. Maxwell, Y. Zhang, W.a. Brown, J.B. Rottman, and A.L. Budelsky. 2012. "Differential Roles for IL-23 and IL-17 in Inflammatory Bowel Disease." *Cytokine* 59 (3) (September): 515. doi:10.1016/j.cyto.2012.06.074. http://linkinghub.elsevier.com/retrieve/pii/S1043466612003195.

Turner, Jerrold R. 2009. "Intestinal Mucosal Barrier Function in Health and Disease." *Nature Reviews. Immunology* 9 (11) (November): 799–809. doi:10.1038/nri2653. http://www.ncbi.nlm.nih.gov/pubmed/19855405.

Tuteja, a K, J Biskupiak, G J Stoddard, and a G Lipman. 2010. "Opioid-Induced Bowel Disorders and Narcotic Bowel Syndrome in Patients with Chronic Non-Cancer Pain." *Neurogastroenterology and Motility : The Official Journal of the European Gastrointestinal Motility Society* 22 (4) (April): 424–30, e96. doi:10.1111/j.1365-2982.2009.01458.x. http://www.ncbi.nlm.nih.gov/pubmed/20100280.

Ukleja, Andrew. 2010. "Altered GI Motility in Critically Ill Patients: Current Understanding of Pathophysiology, Clinical Impact, and Diagnostic Approach." *Nutrition in Clinical Practice : Official Publication of the American Society for Parenteral and Enteral Nutrition* 25 (1) (February): 16–25. doi:10.1177/0884533609357568. http://www.ncbi.nlm.nih.gov/pubmed/20130154.

Utech, Markus, Andrei I Ivanov, Stanislav N Samarin, Matthias Bruewer, Jerrold R Turner, Randall J Mrsny, Charles A Parkos, and Asma Nusrat. 2005. "Mechanism of IFN- □ -Induced Endocytosis of Tight Junction Proteins : Myosin II-Dependent Vacuolarization of the Apical Plasma Membrane" 16 (October): 5040–5052. doi:10.1091/mbc.E05.

Vincent, Jean-louis, John Marshall, Antonio Anzueto, Claude D Martin, and Charles Gomersall. 2009. "International Study of the Prevalence and Outcomes of Infection in Intensive Care Units." *CARING FOR THE CRITICALLY ILL PATIENT* 302 (21): 2323–2329.

Viscusi, Eugene R, and Marco Pappagallo. 2012. “A Review of Opioids for in-Hospital Pain Management.” *Hospital Practice (1995)* 40 (1) (February): 149–59. doi:10.3810/hp.2012.02.955. http://www.ncbi.nlm.nih.gov/pubmed/22406890.

Waldhoer, Maria, Selena E Bartlett, and Jennifer L Whistler. 2004. “Opioid Receptors.” *Annual Review of Biochemistry* 73 (January): 953–90. doi:10.1146/annurev.biochem.73.011303.073940. http://www.ncbi.nlm.nih.gov/pubmed/15189164.

Walker, Jennifer a, Jillian L Barlow, and Andrew N J McKenzie. 2013. “Innate Lymphoid Cells--How Did We Miss Them?” *Nature Reviews. Immunology* 13 (2) (February): 75–87. doi:10.1038/nri3349. http://www.ncbi.nlm.nih.gov/pubmed/23292121.

Wang, Jinghua, Roderick a Barke, Richard Charboneau, and Sabita Roy. 2005. “Morphine Impairs Host Innate Immune Response and Increases Susceptibility to Streptococcus Pneumoniae Lung Infection.” *Journal of Immunology (Baltimore, Md. : 1950)* 174 (1) (January 1): 426–34. http://www.ncbi.nlm.nih.gov/pubmed/15611267.

Wang, Jinghua, Roderick a Barke, and Sabita Roy. 2007. “Transcriptional and Epigenetic Regulation of Interleukin-2 Gene in Activated T Cells by Morphine.” *The Journal of Biological Chemistry* 282 (10) (March 9): 7164–71. doi:10.1074/jbc.M604367200. http://www.ncbi.nlm.nih.gov/pubmed/17227776.

Wang, Jinghua, Richard Charboneau, Sudha Balasubramanian, Roderick A Barke, Horace H Loh, and Sabita Roy. 2001. “Morphine Modulates Lymph Node-Derived T Lymphocyte Function : Role of Caspase-3 , -8 , and Nitric Oxide Experiment Animal” 70 (October): 527–536.

Weinberger, O, S H Herrmann, M F Mescher, B Benacerraf, and S J Burakoff. 1980. “Cellular Interactions in the Generation of Cytolytic T Lymphocyte Responses: Role of Ia-Positive Splenic Adherent Cells in Presentation in H-2 Antigen.” *Proceedings of the National Academy of Sciences of the United States of America* 77 (10) (October): 6091–5. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=350219&tool=pmcentrez&rendertype=abstract.

Wittert, G, P Hope, and D Pyle. 1996. “Tissue Distribution of Opioid Receptor Gene Expression in the Rat.” *Biochemical and Biophysical Research Communications* 218 (3) (January 26): 877–81. doi:10.1006/bbrc.1996.0156. http://www.ncbi.nlm.nih.gov/pubmed/8579608.

Wójciak-Stothard, B, S Potempa, T Eichholtz, and a J Ridley. 2001. “Rho and Rac but Not Cdc42 Regulate Endothelial Cell Permeability.” *Journal of Cell Science* 114 (Pt 7) (April): 1343–55. http://www.ncbi.nlm.nih.gov/pubmed/11257000.