AMERICAN UNIVERSITY OF BEIRUT

TET2 EXPRESSION IN A MOUSE MODEL OF DSS-INDUCED COLITIS

by
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ABSTRACT
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Inflammatory bowel disease (IBD) is characterized by the infiltration of inflammatory cells culminating in non-functional intestinal barrier. Despite the exponential increase of IBD prevalence worldwide, there is no cure yet. Previous studies reported that inflammatory milieu in human colon augments the expression of intercellular complexes called connexins to facilitate homocellular and heterocellular communication. Moreover, in the same context, epigenetic key players such as TET2 have shown to be upregulated in order to facilitate DNA demethylation process of different genes involved. In this study, we investigated the variation of the expression of Cx43 and TET2 in a DSS-induced colitis mouse model and after gap junction blockade. Under inflammatory conditions, Cx43 and TET2 expression levels were increased to be then reduced back to normal when followed by gap junctions' inhibition. Furthermore, TET2 function does not seem to be affected since 5-hmc accumulation does not vary significantly upon inflammation nor GJ blockade. These results show that Cx43 and TET2 may have a potential role in IBD pathogenesis. Finally, Cx43 can be a potential therapeutic target for IBD treatments.

Keywords: Inflammatory Bowel Disease, DNA demethylation, gap junctions, TET2, Cx43

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ABBREVIATIONS

- 5-caC: 5-carboxylcytosine
- 5-fC: 5-formylcytosine
- 5-hmC: 5-hydroxymethylcytosine
- 5-mC: 5-methylcytosine
- AJ: adherens junctions
- AML: Acute Myeloid Leukemia
- CBX: carbenoxolone
- CD: Crohn's Disease
- Cx43: connexin 43
- DNMT: DNA methyltransferase
- DSBH: double-stranded β-helix
- dsDNA: double-stranded DNA
- DSS: Dextran Sulphate Sodium
- ESCs: embryonic stem cells
- GIP: gastric inhibitory polypeptide
- GJ: Gap junctions
- GJIC: Gap junctions intercellular communication
- H&E: Hematoxylin-eosin staining
- IBD: Inflammatory Bowel Disease
- IECs: intestinal epithelial cells
- IFN-γ: interferon-gamma
- IL-1β: interleukin 1 Beta
- JAMs: junctional adhesion molecules
- kDa: Kilo Dalton
- KO: knockout mouse
- M cells: Microfold cells

- MALT: mucosa-associated lymphoid tissue
- MLL: Mixed-Lineage Leukemia
- PFA: Paraformaldehyde
- RT-qPCR: Real-time – quantitative Polymerase Chain Reaction
- TDG: thymine DNA glycosylase
- TET: Ten-Eleven Translocation
- TJ: tight junctions
- TNF-α: tumor necrosis factor-alpha
- UC: Ulcerative colitis
- ZO-1 and ZO-2: zonula occludins proteins 1 and 2

CHAPTER I

INTRODUCTION

Inflammatory Bowel Disease (IBD) prevalence is exponentially increasing worldwide, particularly in the newly developing countries of Asia, South America, and Africa in addition to the middle-east area [1]. Chronic inflammation is a result of an inappropriate immune response attacking the intestinal tract. While the causes behind this attack remain unclear, it is well assumed to be tightly linked to a combination of genes susceptibility, environmental factors, and immune system disturbance. In other words, it is the result of the disruption of the communication between the internal environment of the intestinal epithelial barrier and the external environment of the gut. So far, IBD treatments can improve only the patient's lifestyle but do not cure it. Knowing the importance of epigenetic regulation in many diseases, the role of epigenetic regulators and specifically of Ten-Eleven Translocation (TET) proteins in the context of inflammatory diseases including IBD, is not well assessed yet. Therefore, new treatments and therapeutic strategies based on epigenetic regulation are still under investigation.

A. Physiology and physiopathology of intestinal epithelial barrier

1. Physiology of intestinal epithelial barrier

Structure

A large part of the gastrointestinal (GI) tract is lined by a monolayer of non-ciliated simple columnar epithelial cells forming what is known as the intestinal epithelial barrier. The essential role of this barrier is to control the communication between the intestinal cells and gut lumen and is characterized by a balanced local response against pathogens.

The epithelial monolayer is a highly folded and polarized structure organized into invaginations called crypts of Lieberkühn, and finger-like protrusions called villi. While both structures are present in the small intestine, only crypts are present in the colon. These crypts contain four types of intestinal epithelial cells (IECs) including mainly absorptive cells in addition to Paneth, goblet, and enteroendocrine cells that altogether originate from pluripotent intestinal stem cells, responsible for the rapid self-renewing (every 4-5 days). These cell types are expressed differently in the crypt: the base of the crypt is rich with Paneth cells that secrete microbicidal peptides and immunomodulatory proteins like α-defensins, C-type lectins, and lysozymes while the other three types of cells migrate apically towards the lumen where their apoptosis takes place spontaneously, few days after their differentiation [2]. Moreover, cell types differ between proximal, intermediate and distal colon. Moving from the cecum to the colon, Paneth cell's number decreases. While goblet cells are normally at a higher density in the descending left colon. Goblet cells

secrete high-molecular-weight glycosylated proteins called mucins that form the mucus layer protecting the mucosa from pathogens overgrowth and penetration. Similarly, enteroendocrine cells are specialized endocrine and secretory cells in the GI tract that secrete a wide range of hormones including secretin, ghrelin, and gastric inhibitory polypeptide (GIP) controlling different physiological functions of the digestive tract such as stomach emptying, food intake, and glucose homeostasis [3]. Enterocytes form the absorptive lineage within the epithelial layer and occupy more than 80% of the total cells. They are highly polarized columnar cells joined together by junctional complexes forming a strong physical barrier capable of protecting against pathogens invasion and controlling the transport of water and electrolytes across the colonic epithelium (Figure 1).

Located between the mucosal layer and the submucosa, Peyer's patches, organized lymphoid follicles, and a critical part of the mucosa-associated lymphoid tissue (MALT) in the colon, are overlaid by specialized IECs called Microfold (M) cells. These cells induce the gut immune response against pathogens by the selective transcytosis of luminal antigens and activating the intraepithelial monocytes and lymphocytes [2].

The integrity of the epithelial barrier and the tight binding between its different epithelial cells is ensured by intercellular junctional complexes that are crucial for its paracellular permeability [4].

Junctional Complexes

The intestinal barrier is a physical barrier that separates the luminal contents of the gut from the lamina propria and controls the absorption and the transport of water, electrolytes, commensal microbes, and foreign antigens. This selective permeability of the barrier is due to the presence of a highly sealed epithelial lining. The junctional complexes involved are tight (TJ) and adherens junctions (AJ), gap junctions (GJ) and desmosomes. TJ and AJ together form the apical junctional complexes (AJC). AJC form a belt-like structure joining adjacent IECs and maintain the polarity and the integrity of the epithelium. In addition to adhesion, together with the subjacent desmosomes, they master the paracellular passive diffusion of ions and molecules of MW<600 Da across the intestinal mucosa [5] while gap junctions (GJ) ensure the intercellular communication.

Apical Junctional Complexes

Tight junctions are complex structures composed of transmembrane proteins like claudins, occludins, and junctional adhesion molecules (JAMs) and cytoplasmic adaptor proteins connected to the intracellular cytoskeleton like zonula occludins proteins (ZO-1 and ZO-2 connected to actomyosin ring).

Similarly, transmembrane proteins like E-cadherin and cytoplasmic scaffolding proteins like α- and β- catenin are linked to the actin cytoskeleton form adherens junctions, responsible for cell-cell adhesion.

Underlying these complexes are the desmosomes composed of transmembrane proteins like desmoglein and desmocollin and cytokeratin intermediate filaments linked proteins like desmoplakin [6].

Gap Junctions

GJ ensure the intercellular communication, linking directly the cytoplasm of two neighbouring cells. GJs are transmembrane proteins that permit the exchange of ions, second messengers, and low molecular weight metabolites between two adjacent cells. This direct communication regulates many intracellular signaling involved mainly in cellular differentiation and apoptosis in all the human body cells [7]. GJs are mainly composed of connexins. A hexamer of connexins form a connexon or a hemichannel that can be assembled end-to-end to another hexamer in the adjacent cell and in an aligned manner in the extracellular space to form a GJ channel. The permeability of the channel differs one from another according to different connexins arrays forming either homo or hetero-hexamers.

21 identified members of human connexin gene family are named according to their molecular weight. The most studied isoform in the context of intestinal inflammation is connexin 43 (Cx43); 43 kDa protein.

In addition to their role in transferring biologically important molecules between cells, connexins have proved to have gap junction intercellular communication-independent functions. These include the ability of connexins to influence cell

proliferation, apoptosis, migration, signaling, and tumorigenesis. Connexins have been recognized as tumor suppressors since they are significantly down-regulated in different solid cancers including breast, lung, liver and prostate cancer and their upregulation in in-vitro tumor cell line studies was correlated with an induction of apoptosis, decreased cell proliferation, reduced angiogenesis, and reversed EMT [7].

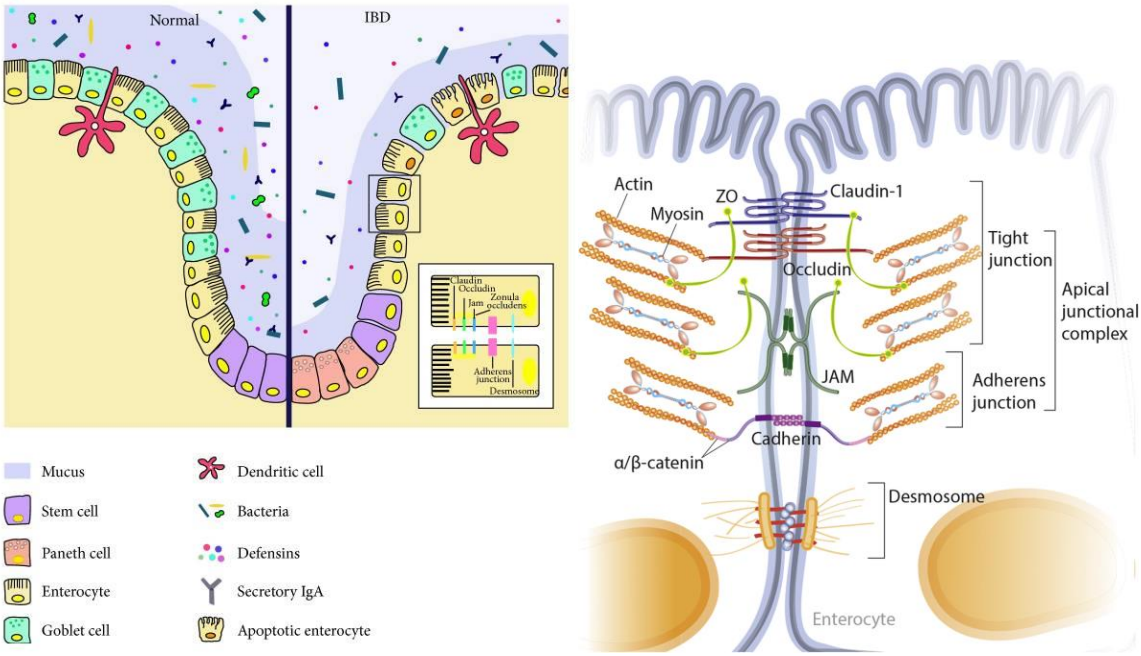

Figure 1: Intestinal cells in healthy gut (left) and inflamed gut (right). The components of the intestinal barrier and the basic structure of the junctional complexes that are comprised in it. [8, 9]

2. Pathogenesis of IBD and defect in intestinal epithelial barrier

IBD Pathogenesis

Inflammatory Bowel Disease (IBD) is a global idiopathic disease characterized by a chronic inflammation in the gastrointestinal (GI) tract. According to the inflammation localization, IBD disease is divided into two types: 1-Crohn's Disease (CD) where GIT could be affected in any part going from the mouth to the anus and 2- Ulcerative Colitis (UC) where the inflammation is limited only to the colon and intestine.

CD and UC are the result of a multifactorial pathogenesis driven by the interaction of different environmental, genetic, gut microbial, and immune-mediated factors. The environmental factors involved include lifestyle behaviors evolved during the late decades like smoking, reduction of fibers intake and replacing it with fatty diets, reduction of breastfeeding, increased exposure to pollutants, and the variation of basic daily routines to be characterized by an almost sedentary lifestyle with high-stress levels. Likewise, genetic factors are of high importance. The genome-wide association studies and the sequencing studies have keyed out over 240 genetic loci of IBD that control several pathways critical for intestinal cellular homeostasis [10]. Furthermore, the large pool of commensal microbes inhabiting the gut lumen must be kept under control and pathogens overgrowth must be also prevented by the immunological barrier [11].

Epithelial integrity disruption

Loss of integrity of the intestinal epithelium is one of the major factors in IBD pathogenesis [12]. Some studies suggest that permeability defect in IBD could be due to epithelial cell apoptosis but others have shown that apoptosis is insufficient. Indeed, a decrease of junctional protein levels including occludins, ZO-1, and E-cadherin was reported in IBD patients [13]. Moreover, the increase of cytokines upon inflammation contributes to the increase of intestinal permeability by changing the membranous localization of JAMs and AJs and redistribution of TJs to be internally localized in cultured T84 epithelial cells [14] (figure 2).

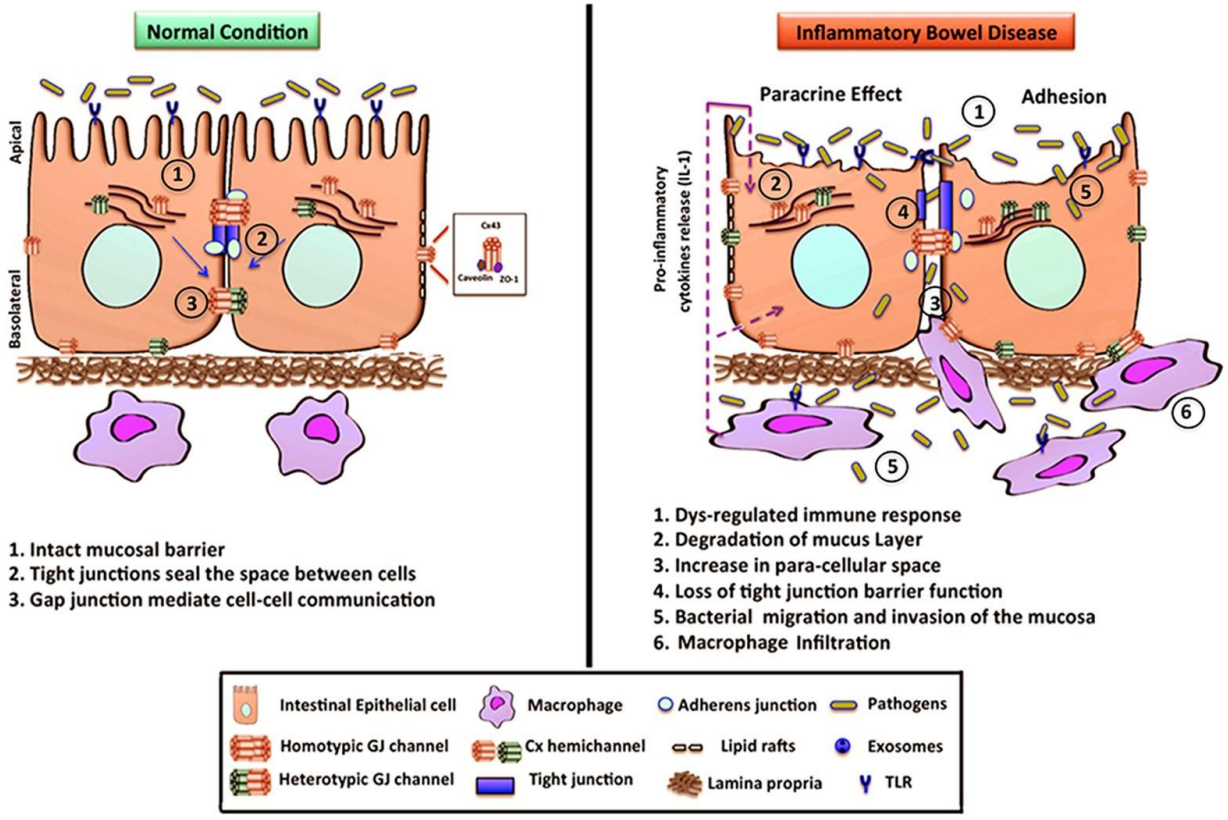

Figure 2: The dysregulation of the intestinal epithelial barrier in IBD [15].

Immune Response Dysregulation

The disturbance of the balance of the immune response inside the gut is a significant hallmark of inflammatory bowel disease. Defense against penetrating pathogens and toxins start by the innate immunity comprises the barrier function of the mucosa, innate immune cells like macrophages, neutrophils, dendritic cells, and natural killer cells that infiltrate the mucosa and the lamina propria, and innate cytokines like interleukin 1 Beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) known for their pro-inflammatory effect [16]. T and B cells are activated in the pathogen-specific adaptive immunity as a consequence of innate immunity failure in chronic inflammatory conditions [17].

The epithelial damage and its increased permeability are common manifestations of the dysregulated immunity in IBD. The milieu enriched with pro- and anti-inflammatory cytokines in the sub-epithelial space due to the leukocytes recruitment impairs the epithelial barrier function. Interestingly, pro-inflammatory mediators (IL-1β, TNF-α) have been shown to increase the intestinal paracellular permeability due to the decrease of junctional protein levels. However, anti-inflammatory cytokines (IL-10, IL-22) stimulate epithelial repair and barrier function. IL-10 has been reported to promote barrier repair after inhibiting the production of TNF-α and IFN-γ and suppressing the macrophages and T_H1 immune response [18, 19]. This contributes to acute and chronic inflammatory processes in IBD colitis

Gap junctions in epithelial cells in IBD

Under inflammatory conditions, cellular communication is impaired due to the activation of the immune system thus gap junctions expression is modulated depending on the tissue microenvironment. In IBD, Cx43 expression is augmented and it is associated with a redistribution of connexins to the basolateral surface of the IECs thus facilitating the hetero-cellular communication between IECs and infiltrating macrophages [15]. Other studies demonstrated the role of connexins hemichannels, specifically Cx43 hemichannels, to be permeable to ATP released from activated neutrophils and monocytes allowing their survival under inflammatory conditions [20]. On the contrary, connexins (Cx26 and Cx43) modulation can act on inducing or inhibiting programmed cell death by regulating pro- and anti-apoptotic genes in colorectal cancer cell lines [21]. However, less is known about the role of connexins in IBD and what occurs after their assembly disruption by gap junction blockers.

3. IBD treatment

Scientists believe that reducing the symptoms of the disease or keeping the disease at a fixed stage, avoiding surgical treatment, and preventing complications are the main objectives of the treatment of IBD. Thus, as a first step before initiating the treatment, it is necessary to examine the clinical symptoms of the patient and determine the severity of the disease [22]. The pharmaceutical medications are prescribed stage by stage to provide the patient with the desired relief. For instance, aminosalicylates, a group of medicines that

acts on inhibiting the inflammatory mediators from lipoxygenase and cyclooxygenase pathways, can improve the symptoms of mild to moderate UC [23]. Also, corticosteroids are proper selective drugs for severe chronic conditions. With these two conventional treatments come the immunosuppressive cytotoxic substances and antineoplastic purine analogs as reported UC drugs [22]. On the other hand, the inflammation in bowel disease, especially in CD, can't be managed without a high dose of antibiotics like ciprofloxacin, metronidazole, and azithromycin. The latter proved its effect by reducing the inflammatory response and ameliorating the extent of lesions. More recently, anti-TNF-α agents -such as infliximab, adalimumab, and golimumab for UC- are being prescribed by the physicians for the non-responding-patients to conventional drugs providing them safer long-term use than corticosteroids. Since all these drugs are generally not that effective in the long-term and may cause many complications, new treatments should be introduced.

4. Models of IBD and Gap Junctions in Research

Dextran Sulfate Sodium

To understand the inflammatory processes occurring in IBD and investigate new therapeutic modalities, many suitable models either in vitro or in vivo were adapted. IBD animal models can be categorized into 5 different groups: (1) genetic (transgenic/knockout) colitis, (2) chemical/immunological-induced colitis, (3) antigen-induced colitis, (4) adoptive cell transfer colitis, and (5) spontaneous colitis models. So far, despite the high number of models, none of them is considered to be perfect because each

has its particular genetic background and immunological response against inflammatory stimuli [24]. For instance, BALB/c mice differ genetically from other inbred strains and react differently to experimental colitis (DSS-induced colitis).

The frequently used model and the faster and easier is the DSS-induced colitis model. Studies have shown that Dextran Sulphate Sodium (DSS) is a simple polysaccharide that induces an injury to the epithelial barrier then stimulates a local inflammatory response mimicking clinical and histological hallmarks of UC. It is administered by drinking water to mice (usually C57BL6 or BALB/c) in different concentrations and for a specific period depending on the type of the targeted inflammation (acute or chronic). Animals surviving the acute colitis recover eventually after DSS removal. After investigating for the inflammatory cytokines (IL-1β, TNF-α, IFN-γ, etc…) and histological features, DSS-induced ulcerative colitis appears to be more severe in the distal colon of BALB/c mice [25].

Carbenoxolone

Many tools were used to assess the role of GJ, among them is a widely used inhibitor called carbenoxolone (CBX). CBX is a glycyrrhetinic acid derivative that induces the closure of GJ [26]. It was originally used to accelerate the healing from gastric ulcers [27]. Its proposed mechanism of action is that it can reversibly inhibit the aggregation of connexin subunits within the cellular niche [28]. Although it inhibits junctional transfer, carbenoxolone induces upregulation of Cx43 expression in aortic endothelial cells

suggesting a feedback mechanism to control connexin expression [29]. Moreover, in vitro studies have shown that it activates cellular apoptosis, decreases cell viability and inhibits cell growth of several cancer cells including thyroid [30], breast [31], lung [32], glioma [33] and leukemic cells [34]. However, CBX therapeutic role has not been yet investigated in ulcerative colitis.

B. TET2

1. Role in DNA methylation

DNA methylation is one of the well-established epigenetic DNA modifications that are tightly correlated to gene expression in the vertebrate genome. 70-80% of the DNA methylation occurs at CpG sites where a cytosine nucleotide is followed by a guanine nucleotide. Methyl group is added to the carbon atom at position 5 in the cytosine of CpG sites via DNA methyltransferase (DNMT) enzymes and form 5-methylcytosine (5-mC). Ten-Eleven Translocation (TET) family comprises three members of methylcytosine dioxygenases: TET1, TET2, and TET3 that catalyze the conversion of 5-mC into 5-hydroxymethylcytosine (5-hmC) as a first step of DNA demethylation. While passive DNA demethylation occurs when a methyl group is simply lost during replication, the intervention of the enzymatic process as for TET is considered as an active DNA demethylation process. It is an iterative oxidation process in which TET intervention leads to the formation of many intermediates in addition to 5-hmC including 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) to be excised by thymine DNA glycosylase (TDG)

and restore the unmodified cytosine (Figure 3) [35, 36].

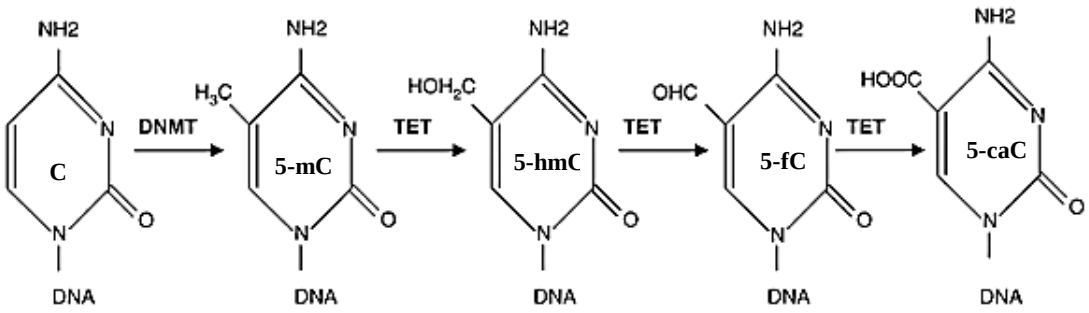

Figure 3: Iterative oxidation of 5-mC by TET enzymes. TET enzymes are responsible for the consecutive oxidation reactions of the DNA methylated cytosine nucleotide enabling the formation of 5-hmC, 5-fC, and 5-caC and restore the unmethylated cytosine. [35]

TET proteins are expressed in mammals. They are large proteins (180 - 230 kDa) with a C-terminal core catalytic domain that contains a double-stranded β-helix (DSBH) domain with Fe(II) and 2-oxoglutarate (2-OG) binding sites on both sides of a large low-complexity region of unknown function. Beside this DSBH domain, the catalytic domain contains a cysteine-rich domain toward the N-terminal domain. The latter consists of the CXXC zinc finger domain, in TET1 and TET3, which can bind directly to CpG sites on dsDNA and preferably to unmethylated substrates [37]. These dioxygenases vary between humans and mice by the length of the polypeptide but the domains of action are being stable in both species. TET1 and TET3 are longer in mice while TET2 protein constitutes of fewer amino acids than the human homolog (1912 versus 2002 amino acids in humans).

TET proteins were initially discovered as a part of the chromosomal ten-eleven translocation (t(10;11)(q22;q23)) in Acute Myeloid Leukemia (AML) patients [38]. Their function in reversing DNA methylation was recently revealed when TET1 was proved to have a potential role in epigenetic regulation and to be able to convert 5-mC into 5-hmC in vitro [39]. Many studies were done revealing some physiological roles for the TET protein family. In development, neither TET1 [40] nor TET2 [41] deletion in knockout mouse (KO) models show any developmental phenotypes whereas TET3 KO mouse models are lethal [42]. On the other side, the triple-KO (TET1/2/3) mouse embryonic stem cells (ESCs) showed that active DNA demethylation is important for embryonic development and differentiation [43]. Moreover, depletion of either TET1 or TET2 shows limited changes in DNA methylation in mouse ESCs. Hence, in some cases, the increased activity or expression of one of TET isoforms can, but not fully, compensate for the loss of one or two other TETs [44].

In addition to their role in embryogenesis and HSCs differentiation, TET proteins show a crucial role in neurogenesis [45], memory and learning processes, smooth muscle cells differentiation and plasticity [46], and induced pluripotent stem cells formation [47].

2. TET and Cancer

TET proteins were mainly studied in hematological malignancies. TET1 was characterized as a partner gene to Mixed-Lineage Leukemia (MLL) gene in AML then TET2 gene was excavated in a myelodysplastic syndrome (MDS) study [48]. Recent

investigations have proved that frequent point mutations in the TET2 gene can lead to its truncation and loss of enzymatic activity unlike TET1 and TET3 mutations. This makes TET2 the most important and studied protein of the TET family and that is why we assessed it in this work. TET2 mutations were shown in AML patients that present a reduced global 5-hmC level [49, 50]. However, despite the rare alterations in TET1 and TET3 in hematological cancer patients compared to that in TET2, TET1 was shown to play a tumor suppressor role in such malignancies especially B-cell lymphomas [51] while TET2 with TET3 suppress aggressive myeloid cancer in mice [52].

Decreased expression of TET proteins and their genetic disruption was also observed in a wide range of solid tumors. Combined with lower 5-hmC levels, decreased TET proteins expression is an important hallmark of different cancer types like colorectal, gastric, prostate, breast, lung cancer, melanoma and glioblastoma [53-55]. One of the proposed mechanisms behind lower 5-hmC levels is the cell cycle-dependent effect of cancer cells in which the high proliferation rate of these malignant cells causes the general loss of tissue-specific 5-hmC levels. Others propose that TET2-interacting proteins like IDAX/CXXC4, overexpressed in solid tumors, are negatively correlated to TET2 function and stability in breast and colorectal tissues [56]. Moreover, mutations in metabolic genes such as IDH1/2 and SDH showed an inhibition and impairment of all TET enzymes and thus cause an aberrant DNA hypermethylation pattern [57].

In conclusion, the epigenetic alteration of methylation and demethylation patterns is the result of mutation of TET proteins, high proliferation rate, and disruption of TET regulators.

3. TET2 and inflammation

The role of TET2 in inflammation was barely studied. A unique study done by Zhang *et. al* shows an anti-inflammatory property for TET2. Indeed, upon inflammation, TET2 repressed IL-6 transcription in dendritic cells and macrophages [58]. Moreover, TET2 deficient bone marrow-derived dendritic cells and macrophages had high mRNA levels of IL-6 at the late phase of LPS stimulation. This confirms the regulatory role of TET2 for IL-6. Moreover, another study shows the increase of TET2 mRNA levels in IBD patients [59].

4. TET2 and GJ

To date, there are no studies linking gap junctions with TET2 in any disease although both proved to be key regulators of cellular homeostasis. But what is known is that all TET proteins can act on the methylation/hypermethylation status of genes promoters thus regulating their corresponding expression.

CHAPTER II

AIMS

Epigenetic regulators represent important controllers of genes expression especially in disease state. Methyltransferases, such as TET2, showed a crucial role in regulating the methylation status of different gene promoters in IBD. Moreover, gap junctions are key regulators of inflammation in IBD. However, few studies have focused on the variation of the expression of TET2 and its correlation with Cx43 in IBD. That's why the specific aims of our work were as follows:

Specific aim 1: To assess the effect of gap junctions inhibition in DSS-induced colitis

Specific aim 2: To assess the variation of molecular level of the epigenetic regulator TET2 in DSS-induced colitis and investigate whether it is linked to gap junctions

CHAPTER III

MATERIALS AND METHODS

A. Experimental Design

This study was approved by the Institutional Animal Care and Utilization Committee of the American University of Beirut (IACUC# 18-03-476). Male Balb/c mice (6-8 weeks) were obtained from the Animal Care Facility (ACF) at the American University of Beirut. Mice were divided into 4 different groups (5 mice per group):
1- untreated control mice, 2- Dextran Sodium Sulphate (DSS) – treated mice, 3- Carbenoxolone (CBX) – treated mice, 4-double treated mice (DSS+CBX).
A 2.5% DSS drinking water was administered to mice for 10 days (fresh DSS solution was prepared every 2 days), while CBX was injected intraperitoneally every other day starting day 11 till day 19 at a 30 mg/kg concentration. Mice were sacrificed at day 21 by cervical dislocation after being anesthetized by isoflurane inhalation. Body weights were measured daily and colon length was measured during sacrifice from the cecum to the anus. Colon tissues were washed by flushing with PBS (1X) and collected for histological and molecular examinations.

B. Quantitative Polymerase Chain Reaction and Dot Blot

a) RNA extraction from tissues

RNA Extraction from tissues

For each 50 mg of distal colon tissue, 500 µL of TRI reagent (Sigma-Aldrich; cat#T9424) was added. The tissue was homogenized using a tissue homogenizer. Homogenate was then centrifuged at 12,000 G for 10 minutes to get rid of the residues. Supernatant was transferred to a new tube and 100 µL of chloroform was added to allow phase separation. After 15 minutes of centrifugation at 12,000 G at 4°C, three phases were

formed: a colorless upper aqueous phase containing the RNA, an interphase containing the DNA, and a red lower organic phase containing the proteins.

To isolate the RNA, the upper phase was transferred carefully into a labeled 1.5 mL tube and a volume of 0.5 mL of isopropanol per 1 mL of trizol was mixed to allow RNA precipitation. After 10 minutes of incubation at room temperature, samples were centrifuged at 12,000 G for 10 minutes at 4°C. An RNA pellet was formed. After removing the supernatant, the RNA pellet was washed with 1 mL of 75% ethanol per mL of trizol. Next, the samples were centrifuged at 7,500 G for 5 minutes at 4°C. Finally, the RNA pellet was re-suspended with an appropriate volume of nuclease-free water.

mRNA purification

According to Viennois *et al*., DSS treatment inhibits the mRNA amplification from tissues in RT-qPCR by inhibiting the activities of both polymerase and reverse transcriptase, therefore mRNA purification is crucial. mRNA purification was done before cDNA synthesis according to the mRNA purification protocol [60]. Briefly, 0.1 volume of 8 M lithium chloride (LiCl) solution was added to 1 volume of dissolved RNA with 2 volumes of 100% ethanol to be incubated on ice for 2 hours after pipetting up and down well. A centrifugation step at 14,000 G for 30 minutes at 4°C follows the incubation. Then, the supernatant was discarded and the RNA pellet was re-suspended in nuclease-free water. All the above steps were repeated twice to ensure the purification of RNA from the DSS contaminant. After that, 0.1 volume of 3 M sodium acetate solution (pH=5.2) was added to 1 volume of dissolved RNA with 2 volumes of 100% ethanol and mixed well. Mixture was left to precipitate well at -20°C for 30 minutes to 3 hours. The mixture was centrifuged at 14,000 G for 30 minutes at 4°C. The obtained pellet was washed with pre-chilled 70% ethanol to remove the traces of LiCl. Finally, the mixture was centrifuged at 14,000 G for 10 minutes at 4°C; the RNA pellet was re-suspended with nuclease-free water and quantified using a nucleic acid spectrophotometer.

It is important to mention that all RNA samples with different conditions were equally subjected to the LiCl purification process [60], to be treated the same way.

DNA extraction

To extract the DNA, it was first precipitated from the interphase layer by adding 150 µL of pure ethanol after removing the upper aqueous phase containing the RNA. Each tube was mixed by inversion and allowed to stand for 5 minutes before centrifugation at 2,000 G for 5 minutes at 4℃. The obtained DNA pellet was washed twice with 500 µL of 0.1M trisodium citrate prepared in 10% ethanol, periodically mixed, and allowed to stand for at least 30 minutes at room temperature. This step allows us to wash the DNA pellet from phenol residues stuck in it. Then, the samples were centrifuged at 2,000 G for 5 minutes at 4℃. The DNA pellet was then washed with 1 mL of 70-75% ethanol and allowed to stand for 15 minutes at room temperature. It was dried for 5 minutes at room temperature and dissolved in an appropriate volume of 8mM sodium hydroxide (NaOH) to ensure the hydrolysis of RNA.

b) Real-time – quantitative Polymerase Chain Reaction (RT-qPCR)

1 µg of total RNA was reverse-transcribed to cDNA using the iScript cDNA synthesis kit (Bio-rad Laboratories). Quantitative PCR (qPCR) was performed using the iQ SYBR GreenSupermix in a CFX96 system (Bio-Rad Laboratories). cDNA was amplified using forward and reverse primers that recognize mouse TET-2, Cx-43, IL-1β, TNF-α, IL-10, and GAPDH (Table 1). PCR parameters consist of a pre-cycle of 95 °C for 3 min followed by 40 cycles consisting of denaturation at 95 °C for 10 sec, annealing at the gene's annealing temperature (°C) for 30 sec, and extension at 72 °C for 30 sec with a final extension at 72 °C for 5 min. The fluorescence threshold cycle value (Ct) was obtained for each gene-each sample as duplicate- and normalized to its corresponding GAPDH. Experiments were independently performed at least four times.

c) Dot Blot

DNA samples were prepared by serial dilution (500 ng and 1000 ng in a final volume of 25 µL) in eppendorf tubes using nuclease-free water as a diluent. They were re-quantified by a nanodrop spectrophotometer and boiled at 95℃ for 10 minutes before loading on the slot-blot apparatus (PR600 Slot Blot Filtration Manifold, Amersham Biosciences) containing nylon membrane. The vacuum pump was turned on before applying the samples. After loading, samples were subjected twice to crosslinking by UV-cross linker at optimal power (1200*100µJ/min). The nylon membrane was washed with ddH_2O to remove all unlinked residues. Then, it was stained with methylene blue (1% in 0.5M sodium acetate, pH=5.2) for 5 minutes to ensure equal spotting of total DNA on the membrane. DNA blots were blocked for 1 hour at RT with 5% fat-free milk in PBS (1X) after washing well from MB. Primary rabbit 5-hmC antibody (ab-214728; RM236; 1:5000) was used on the membrane overnight at 4℃ and HRP-conjugated secondary antibody (1:5000) was used for 1 hour at RT before blots detection on the chemidoc machine (BioRad Laboratories) using enhanced chemiluminescence detection kit (BioRad Laboratories).

C. Western Blot

a) Protein Extraction

Colon tissues were lysed and homogenized on ice using RIPA Buffer composed of Tris-HCl (pH=7.5, C_f=50mM), NaCl (C_f=150mM), Nonidet; NP_{40} (1%), Na Deoxycholate (1%) and SDS (1%) with 2% protease inhibitor and 10% Phosphatase Inhibitor (ROCHE, Switzerland). Proteins were later quantified by DC Protein Assay II kit (BioRad Laboratories, cat#5000112) and absorbance was read using a spectrophotometer at 750nm.

b) ImmunoBlot

Protein samples were loaded on 8% SDS-polyacrylamide gel and exposed to electrophoresis at 30mA/gel at RT until GAPDH (37 kDa of the ladder) reaches the bottom of the gel for better separation of TET2 (224 kDa). The transfer of each gel was done on an activated PVDF membrane and assembled in the gel holder cassette overnight at 30V in the cold room (4℃). The next day, to make sure that all proteins were transferred from the gel to the membrane, gels and membranes were stained with Coomassie blue and Ponceau-S respectively. Membrane blocking was done by 5% fat-free milk (in PBS (1X)) for 1 hour at RT. They were then incubated with primary rabbit antibodies (Table 2) overnight at 4℃, washed with PBS-tween (0.05%) three times (10 minutes each), then incubated with HRP-conjugated secondary antibody (mouse anti-rabbit cat#: sc-2357, 1:5000). Proteins blots were detected by chemidoc machine (BioRad Laboratories) and by using enhanced chemiluminescence detection kit (BioRad Laboratories). The intensity of the detected bands was quantified by BioRad Image Lab Software.

D. Histology

a) Hematoxylin-eosin staining (H&E)

The colonic tissues were fixed with 10% paraformaldehyde (PFA) for 3 days then dehydrated with 95% ethanol overnight before being embedded in paraffin for histological purposes. 5µm thick sections were cut to proceed with hematoxylin-eosin staining. Images were taken using the Axio Olympus microscope.

b) Immuno-fluorescent Assay

Paraffin-embedded colon tissues, fixed by 10% paraformaldehyde, were cut into 5µm sections on confocal slides. These sections were de-paraffinized and rehydrated then washed with ddH_2O. Antigen retrieval step is necessary to unmask protein antigens on the surface of the tissue section. It was done by immersing the slides in Coplin jar containing citrate buffer (pH=6). After cooling down at RT and being washed with TBS (1X), the tissues were permeabilized by ice-cold methanol (for 5-hmC staining) or Triton-X 100 (for TET2 staining) for 10 minutes. Then, they were blocked with 5%NGS for 1 hour at RT and incubated with primary antibody (5-hmC (ab-214728; RM236), TET2 (ab-94580),

Cx43 (SAB-4501175), CD68 (ab-201340)) in final concentration 1 μg/mL overnight in a humidified chamber at 4℃. This was followed by a TBS-tween (0.05%) wash to be later incubated with IgG-conjugated secondary antibody (Alexa Flour 488; A11070; 1 μg/ml) for 2 hours at RT. Tissues were then incubated with DAPI (1 μg/ml) for 10 min to stain their nuclei, washed 3X with PBS, and finally mounted using Prolong Anti-fade kit and observed by confocal microscopy.

E. Statistical Analysis

Statistical significance in q-PCR and western blot histograms was determined by the unpaired Student's t-test. Error bar represents SEM of four independent experiments. Differences between groups were assessed by one-way analysis of variance (ANOVA).

CHAPTER IV

RESULTS

Part 1: Role of Gap junctions in DSS-induced colitis in mice

A. DSS-induced colitis mouse model characterization

Inhibiting gap junctions induces a continuous body loss and a recovered colon length after DSS-induced inflammation in mice

To mimic the IBD model, mice were treated with DSS. Furthermore, as gap junctions are suggested to play a role in IBD pathogenesis [15], a group of mice were injected with CBX, a gap junction blocker, to evaluate the role of gap junctions in DSS-induced colitis.

Weight loss index, one of the elements of the disease activity index (DAI), was evaluated in four groups of mice: the control, DSS, CBX, and DSS+CBX-treated mice along with the experimental protocol.

As expected, figure 4 B. shows that DSS-treated mice display a significant decrease in body weight starting from day 4 of DSS treatment (*P* value=0.02) till day 12 (the end of DSS treatment). This was also seen in the CBX and DSS+CBX-treated group but from the first day of treatment (day 11). While at day 13 (after the end of DSS treatment) DSS-treated mice seem to start recovering from DSS and gain weight again, double-treated group show a continuous decrease in body weight similar to that in CBX-treated group.

A shorten colon length is another indicator of DSS-induced colitis [61]. Interestingly colon length increases significantly with DSS administration and goes back to normal in double treated mice.

Altogether these data indicate that acute colitis was induced in BALB/c mice after DSS administration justified by weight loss and colon length variation and it seems to be

reversible 3 days after DSS-free water intake. Besides, blocking gap junctions may reduce DSS-induced colitis pathogenesis.

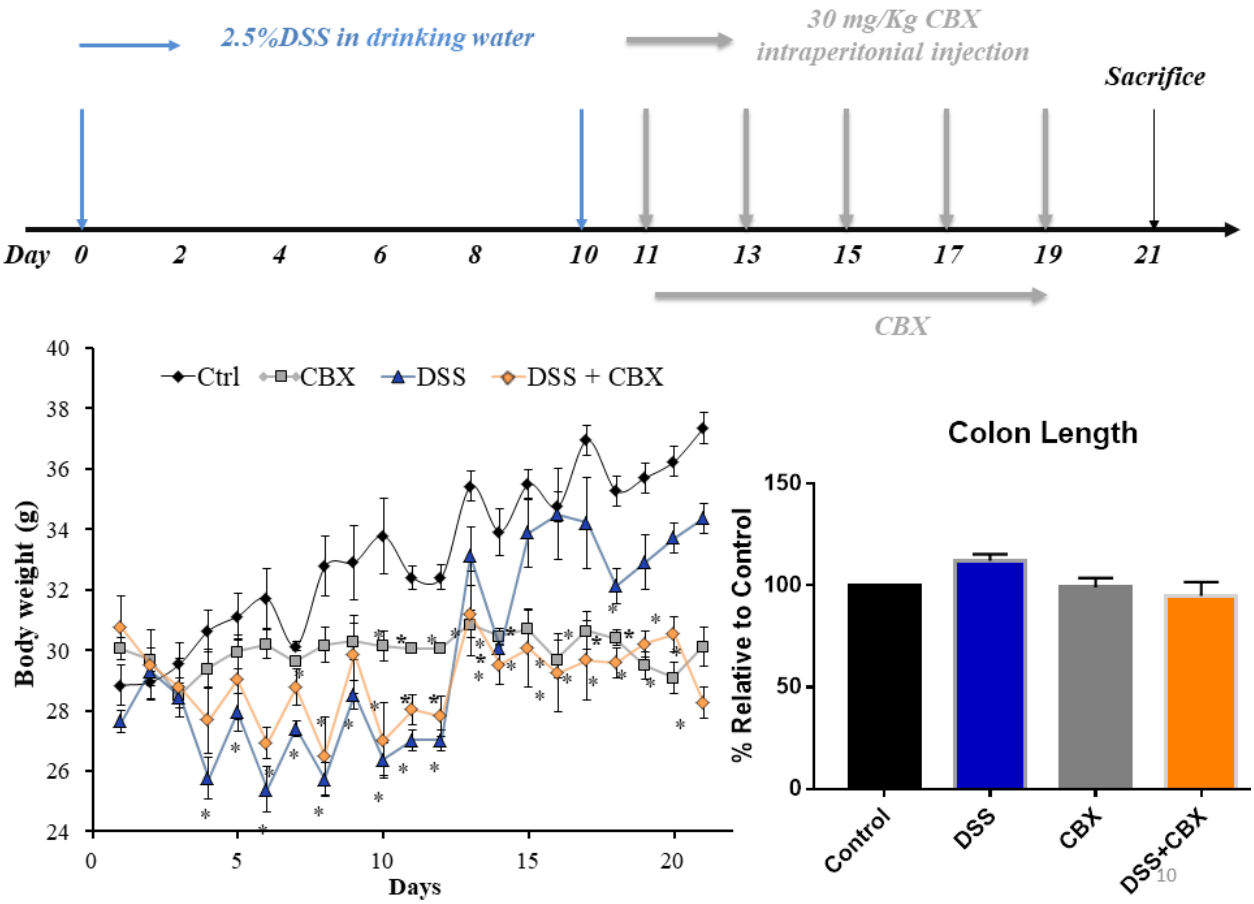

Figure 4. Mouse body weight and colon length variation of DSS, CBX, and with DSS +CBX-treated mice. A. Experimental timeline. Balb/C male mice were divided into four groups: control group (black), DSS-treated group (blue) 2.5% DSS drinking water was administered for ten days, CBX-treated group (grey); CBX was injected from day 11 to day 19 every other day, and DSS+ CBX treated group (orange); this group was exposed to DSS and injected by CBX in the same previous conditions. All mice were then sacrificed at day 21 and colon tissues were taken. Variation of **B.** mice body weight (g) and **C.** mice colon length (cm) of different mice groups. n=5 mice for each group. *: $P<0.05$ **: $P<0.005$ ***: $P< 0.0001$.

Inhibiting gap junctions rescues a partial DSS-induced inflammation in the distal colon

In IBD, the distal colon is the most affected part of the colon (previously mentioned). H&E staining was done on the distal colon to verify the inflammatory profile of our DSS-induced colitis model. The infiltration of inflammatory cells is noticed in the three treated conditions when compared to the control group. Moreover, an epithelial disruption is clear in the DSS-treated colon in agreement with the literature. This disruption was reduced in mice injected with CBX after DSS administration. Furthermore, goblet cell number increases with DSS administration and is decreased in DSS-CBX-treated tissues indicating a potent faster recovery with gap junctions' disruption.

Altogether these histopathological changes may indicate that blocking gap junctions may reduce both the disruption of the epithelial barrier integrity and the immune response in the acute colitis model.

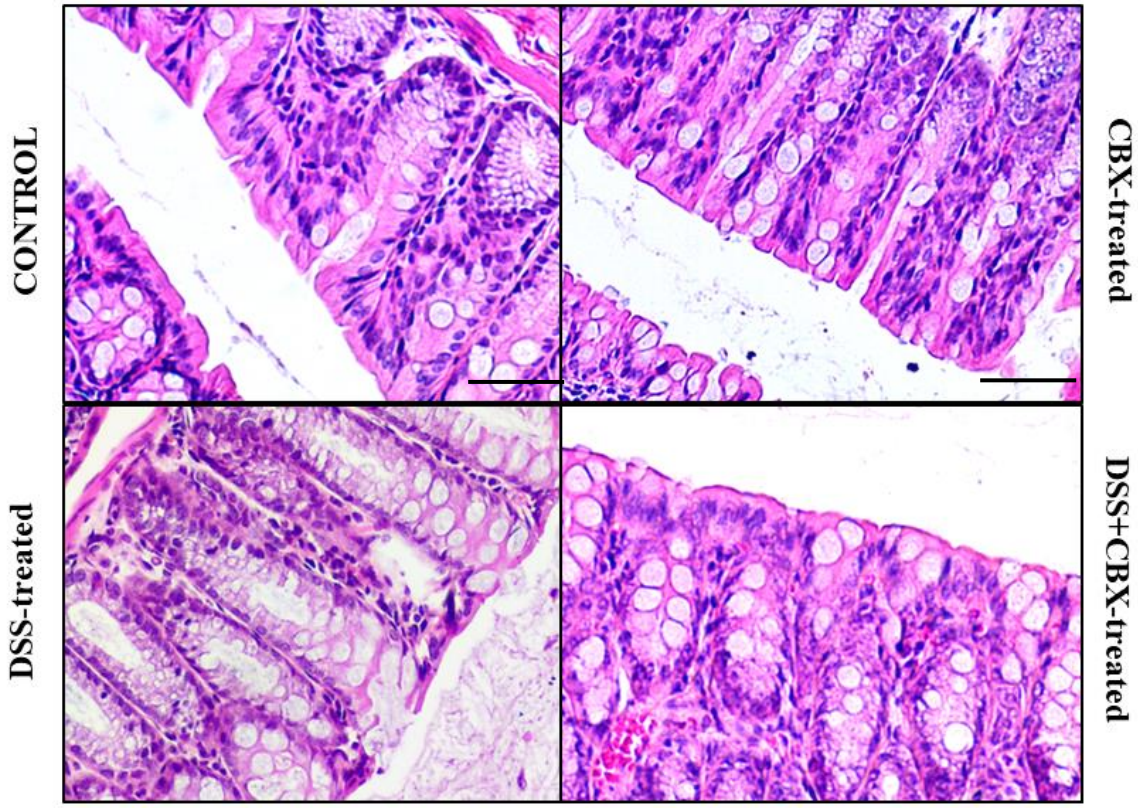

Figure 5. Hematoxylin and eosin staining on distal colon tissue of control, CBX, DSS, and CBX+DSS-treated groups. Representative images of Hematoxylin and eosin staining of 5 independent experiments of n=5 each. Scale bar: 100μm.

Inhibiting gap junctions reduces the number of activated macrophages after DSS-induced inflammation in the distal colon.

To be able to identify the activated macrophages (CD68 positive cells), one of the first mediators of inflammation, we performed CD68 staining on distal colon sections. As expected, macrophages are more infiltrated in the DSS-treated colon compared to the untreated group. Interestingly, when CBX was injected after DSS exposure, activated macrophages number was reduced but remains higher compared to control.

This result indicates that CBX reduces the immune response partially via the reduction of particularly the activated macrophages reducing then the onset of inflammation.

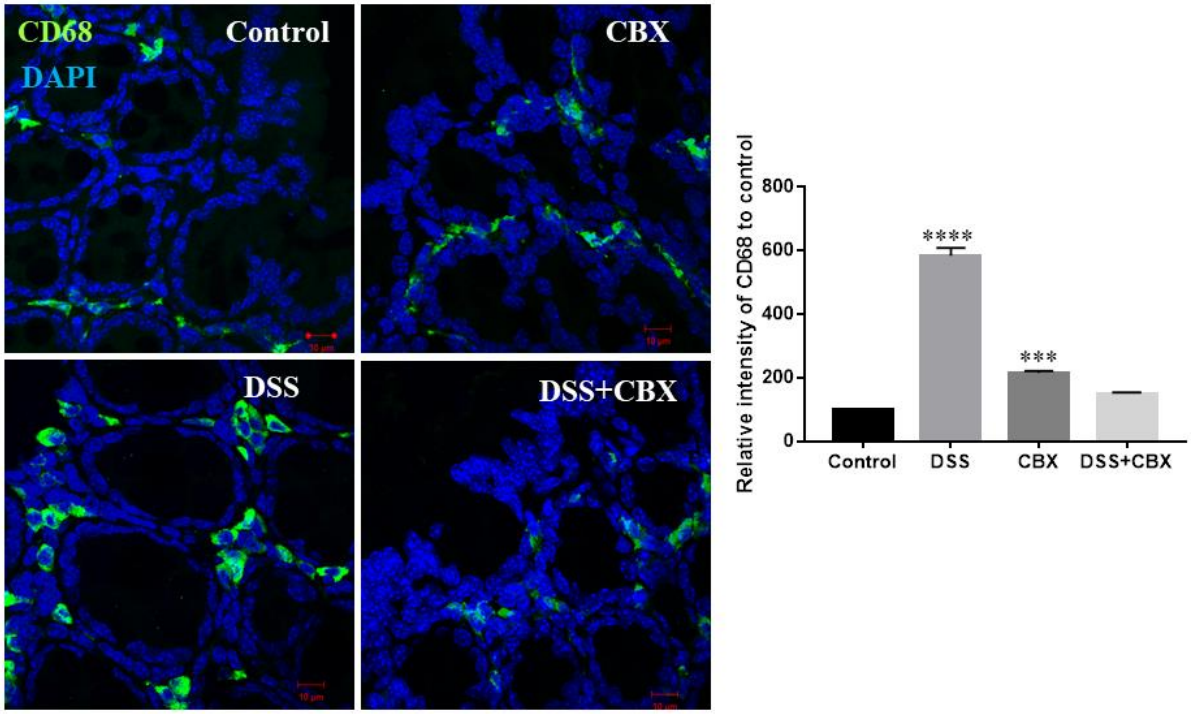

Figure 6. CD68 staining on distal colon tissue sections of control, CBX, DSS, and DSS+CBX-treated groups. Representative images of CD68 staining (green) of 3 independent experiments of n=3 each. The arrowheads indicate macrophages Images captured at a 63x/1.46 Oil Plan-Apochromatic objective. Scale bar: 10 μm.

Inhibiting gap junctions reduces the expression level of pro-inflammatory and anti-inflammatory cytokines after DSS-induced inflammation in the distal colon

As a last checkpoint for inflammation, pro-inflammatory cytokines expression level was assessed. As expected, pro-inflammatory cytokines IL-1β and TNF-α, important immune regulators in colitis [62], expression increases significantly (4-folds) in DSS condition. In the CBX group, IL1-β increases but to a lesser extent to the DSS group while TNF-α mRNA level increases even more than the DSS group (10-folds). Interestingly, the treatment of CBX with DSS together brings IL1-β and TNF-α levels back to normal.

IL-10, an anti-inflammatory agent, increases significantly in all treated conditions compared to the control but it is the higher in DSS-treated distal colon (6-fold increase compared to 4-fold).

These data indicate that DSS stimulates the production of pro-inflammatory and anti-inflammatory cytokines even after its discontinuity for 10 days. But interestingly, CBX significantly stops the pro-inflammatory response and decreases the anti-inflammatory response (IL-10) after DSS-induced colitis; thus facilitating recovery through gap junctions' blockage.

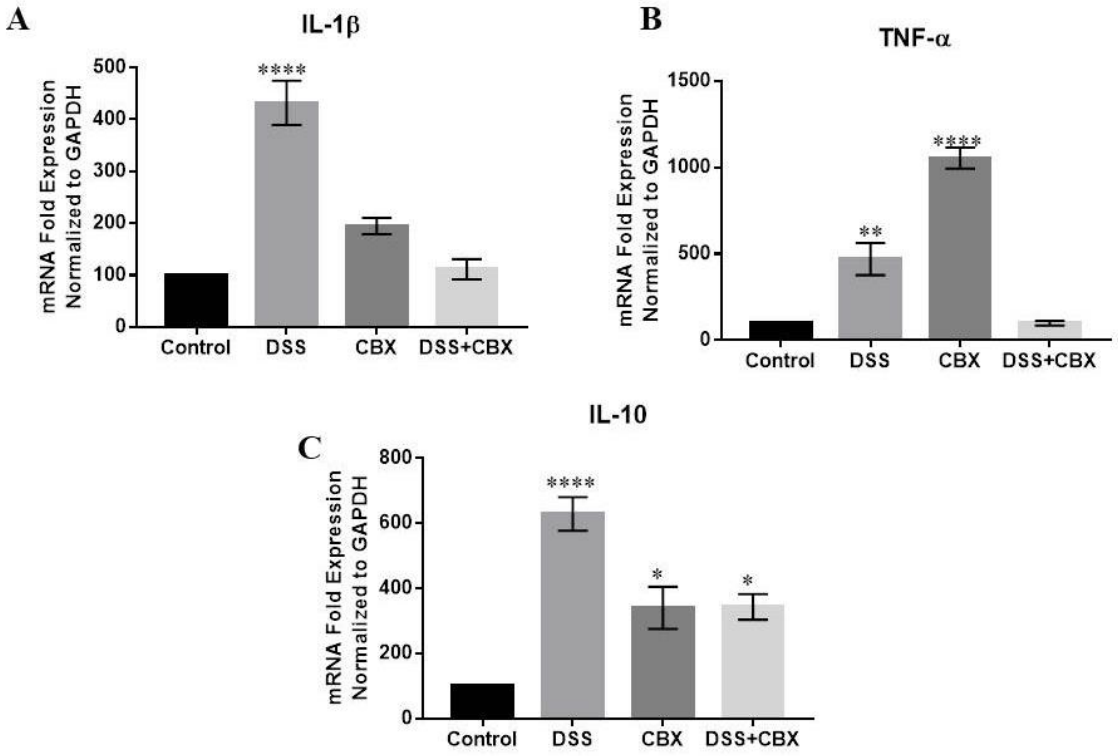

Figure 7. mRNA expression level of pro-inflammatory (IL-1β, TNF-α) and anti-inflammatory cytokines (IL-10) in the distal colon. A. Quantification of IL-1β, **B.** TNF-α, and C. IL-10 mRNA levels assessed by RT-qPCR in control, CBX, DSS, and CBX+DSS-treated groups in the distal colon normalized to GAPDH. Values are means ± SEM for n=4 mice for each group. , **P* <0.05; ***P* <0.005; *****P* < 0.0001.

B. Connexin 43 Expression

Inhibiting gap junctions reduces Cx43 intercellular localization after DSS-induced inflammation in the distal colon

Cx43 is a main component of gap junctions that are being associated to IBD pathogenesis. Therefore, we assessed the expression and the localization of Cx43, on paraffin-embedded distal colon tissue sections. **Figure 8** shows an increase in Cx43 expression in the DSS-treated colon when compared to control and it is localized between IECs and between them and the inflammatory cells infiltrated between the crypts. While in double-treated colon, it is almost between the epithelial cells where it acts mainly to facilitate cell-cell communication despite its disruption by CBX.

These results indicate that connexin 43 changes its location in the IECs according to the inflammatory condition.

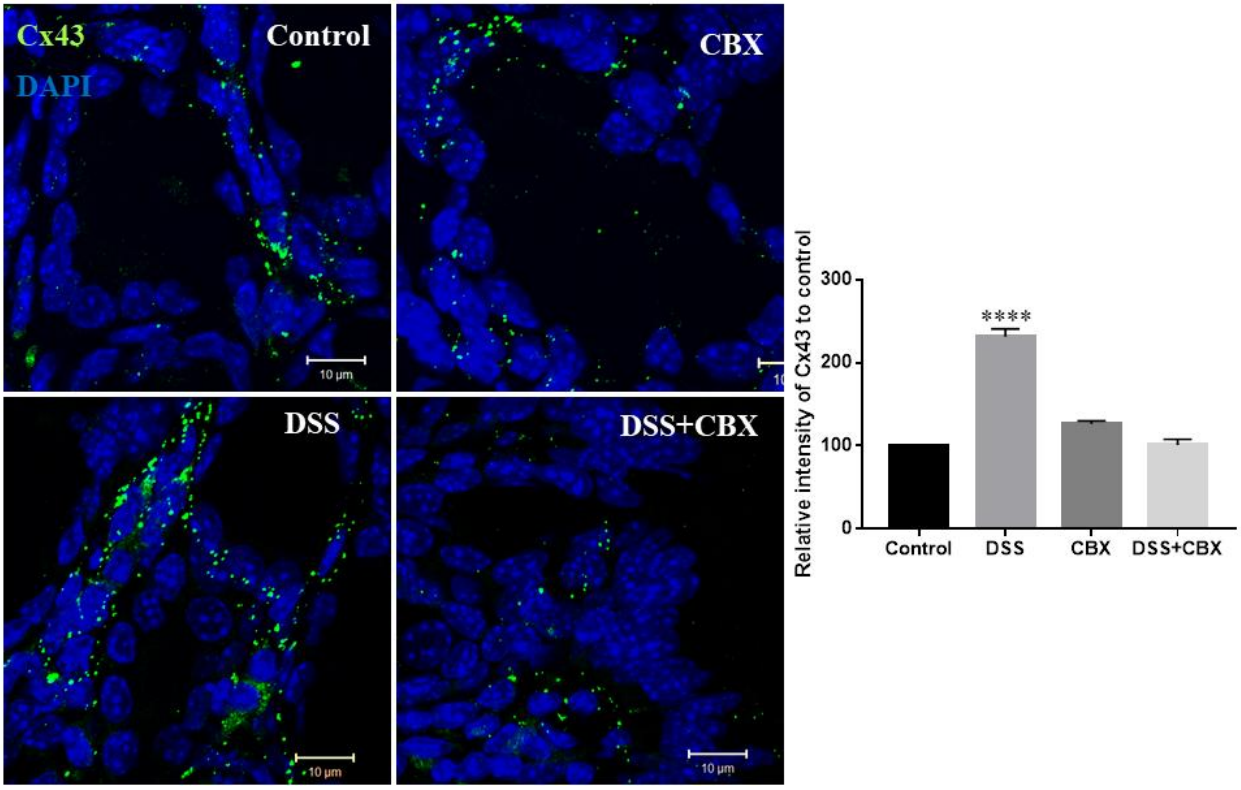

Figure 8. Cx43 expression and localization on distal colon tissue sections of control, CBX, DSS, and CBX+DSS-treated groups. Representative images of Cx43 staining

(green) of 3 independent experiments of n=3 each. Images captured at a 63x/1.46 Oil Plan-Apochromatic objective. Scale bar: 10 μm.

Inhibiting gap junctions reduces the increase of Cx43 mRNA expression and protein level after DSS-induced inflammation in the distal colon

To assess Cx43 expression level at a molecular level, RT-qPCR and western blot were performed. Consistent with the results shown before, Cx43 mRNA expression and protein level increase significantly in DSS-treated distal colon while it stays the same as control in CBX and double-treated colon (figure 9).

This indicates that inflammation increases the expression of gap junctions and specifically Cx43. But the blockage of Cx43 after inflammatory onset reduces its expression to normal average and changes its location in the tissue to the normal state although the blockage alone doesn't affect the expression of Cx43 in the colon.

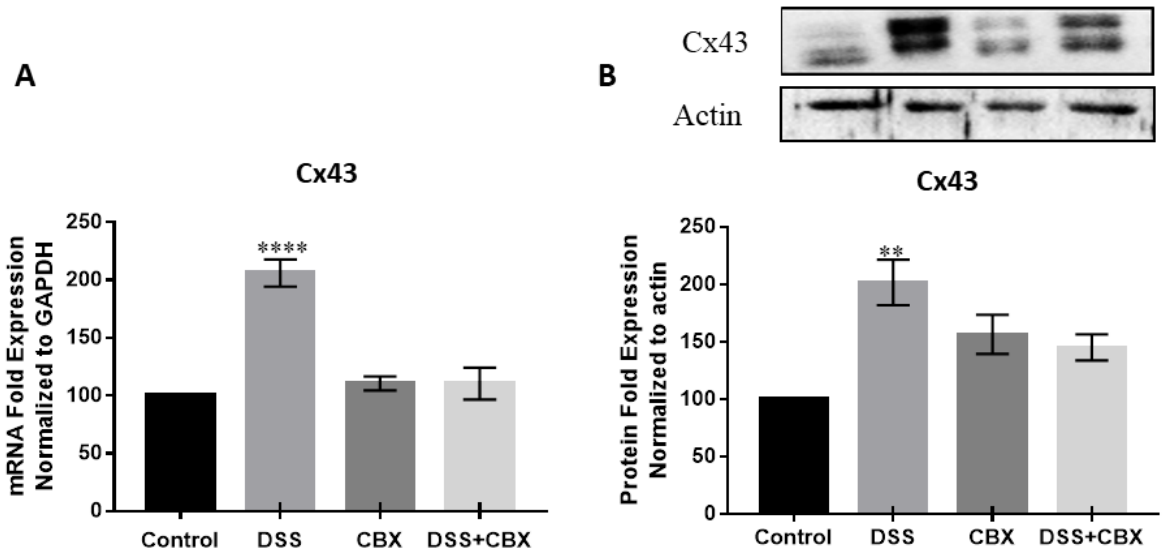

Figure 9. Cx43 mRNA and protein expression level in control, CBX, DSS, and CBX+DSS-treated mice. A. Quantification of Cx43 mRNA levels assessed by RT-qPCR in the four groups of mice normalized to GAPDH. **B.** Western blot and quantification of Cx43 expression levels in the groups, normalized to Actin. Values are means ± SEM for n=4 mice for each group. , **P* <0.05; ***P* <0.005; *****P* < 0.0001.

Part 2: TET2 expression in DSS-induced colitis in mice

A. TET2 expression

TET2 gains a nuclear localization in DSS-induced colitis mice

TET2 was tightly linked to leukemia and was shown to have antitumoral properties. To assess whether TET2 may have an anti-inflammatory potential and whether it is through gap junction regulation, we checked first TET2 expression in colon tissues of control, DSS-treated, CBX-treated, and DSS+CBX-treated mice. Figure 10 shows a nucleic localization of TET-2 upon induced inflammation in all treated mice while being cytosolic in the control untreated condition.

This data may indicate the anti-inflammatory role of TET2 is stimulated along with its nuclear localization and secretion to interact with anti-tumoral genes and regulate their expression.

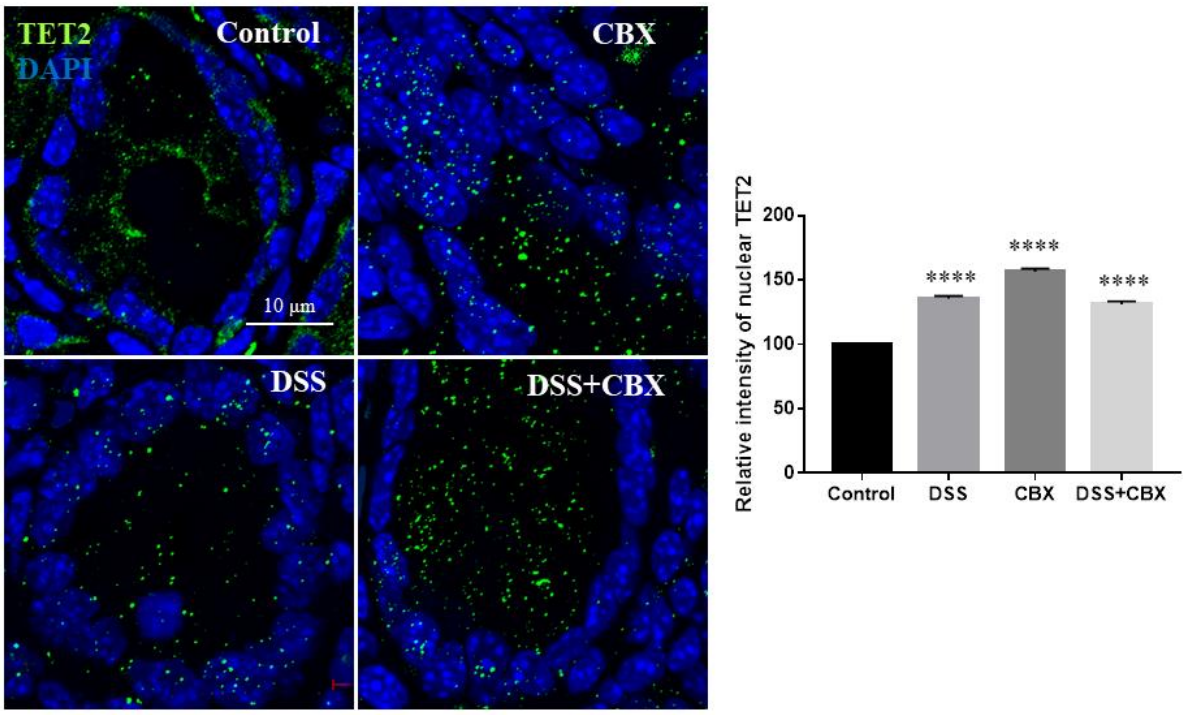

Figure 10. TET2 localization in distal colon tissues of control, CBX, DSS, and CBX+DSS-treated groups. Representative images of TET2 staining (green) of 3 independent experiments of n=3 each. Images captured at a 63x/1.46 Oil Plan-Apochromatic objective. Scale bar: 10 μm.

TET2 expression level is increased in DSS-induced colitis mouse

To assess TET2 expression level at a molecular level, RT-qPCR and western blot were performed. TET2 mRNA expression and protein level increase significantly in CBX-treated distal colon while it stays almost the same as control in DSS and double-treated colon.

This indicates that the disruption of gap junctions alters the expression of TET2 in the distal colon and thus a link between both regulators exists.

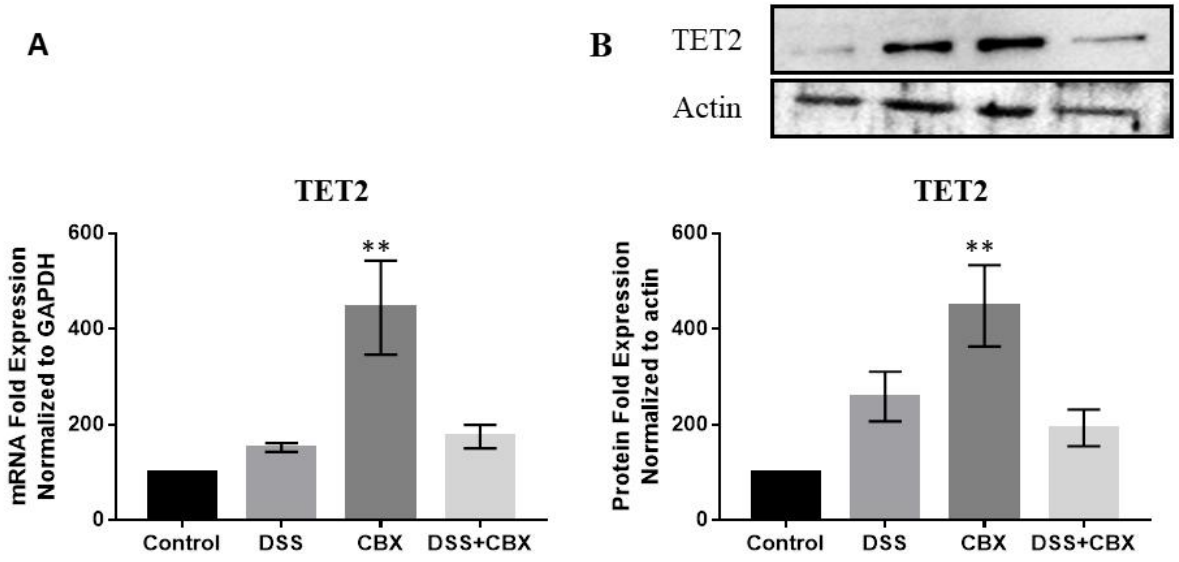

Figure 11. TET2 expression A. Quantification of TET2 mRNA levels assessed by RT-qPCR in the four groups of mice normalized to GAPDH. B. Western blot and quantification of TET-2 expression levels in the four groups, normalized to Actin. Values are means ± SEM for n=4 mice for each group. , **P* <0.05; ***P* <0.005; *****P* < 0.0001.

B. TET2 function

DSS treatment does not seem to affect TET-2 function on the level of 5-hmC

TET2 is a protein that catalyzes the conversion of the modified methylated DNA base methylcytosine (mC) to 5-hydroxymethylcytosine (5-hmC) as a first step. To assess the effect of DSS-induced inflammation on TET2 function, we assessed 5-hmC accumulation. The DNA dot blot (figure 13) and immuno-fluorescent (figure 12) assay show a change in 5-hmC expression upon inhibiting gap junctions. The nuclear localization of 5-hmC in IECs is clear in all conditions.

This data may indicate that TET2 function on the level of 5-hmC accumulation is presumably affected by gap junctions.

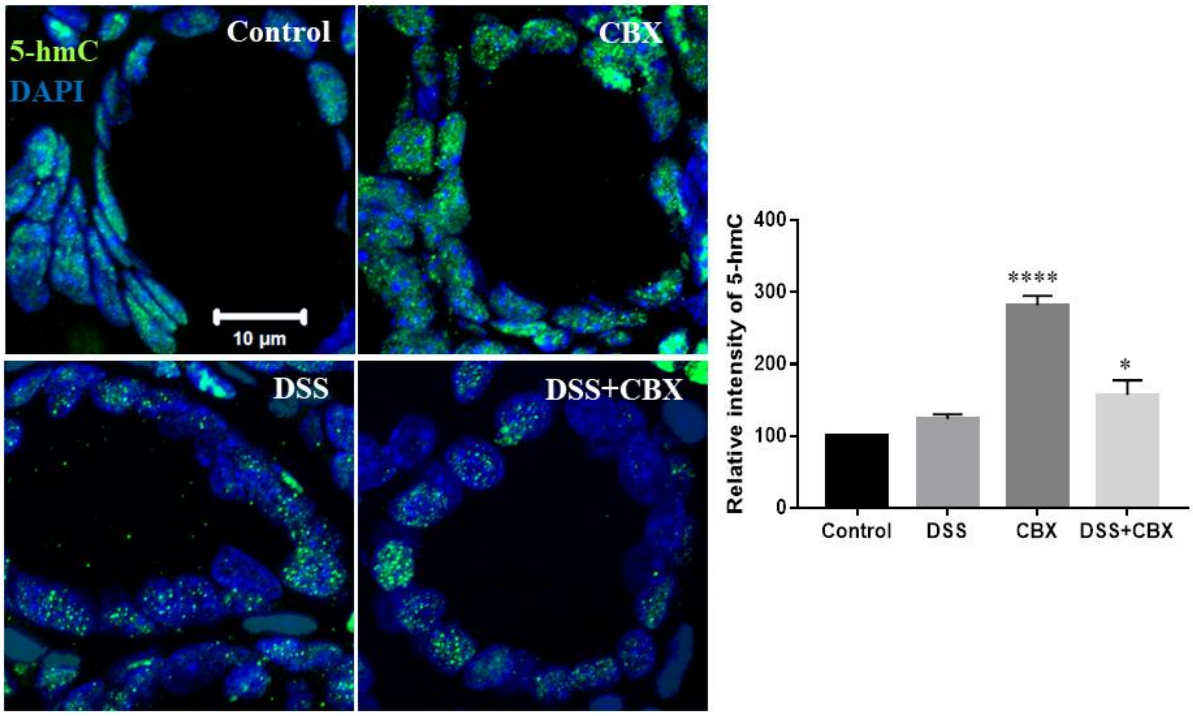

Figure 12. 5-hmc localization in distal colon tissue sections. Representative images of 5-hmc staining (green) in the IECs of 3 independent experiments of n=3 each. Images captured at a 63x/1.46 Oil Plan-Apochromatic objective. Scale bar: 10 µm.

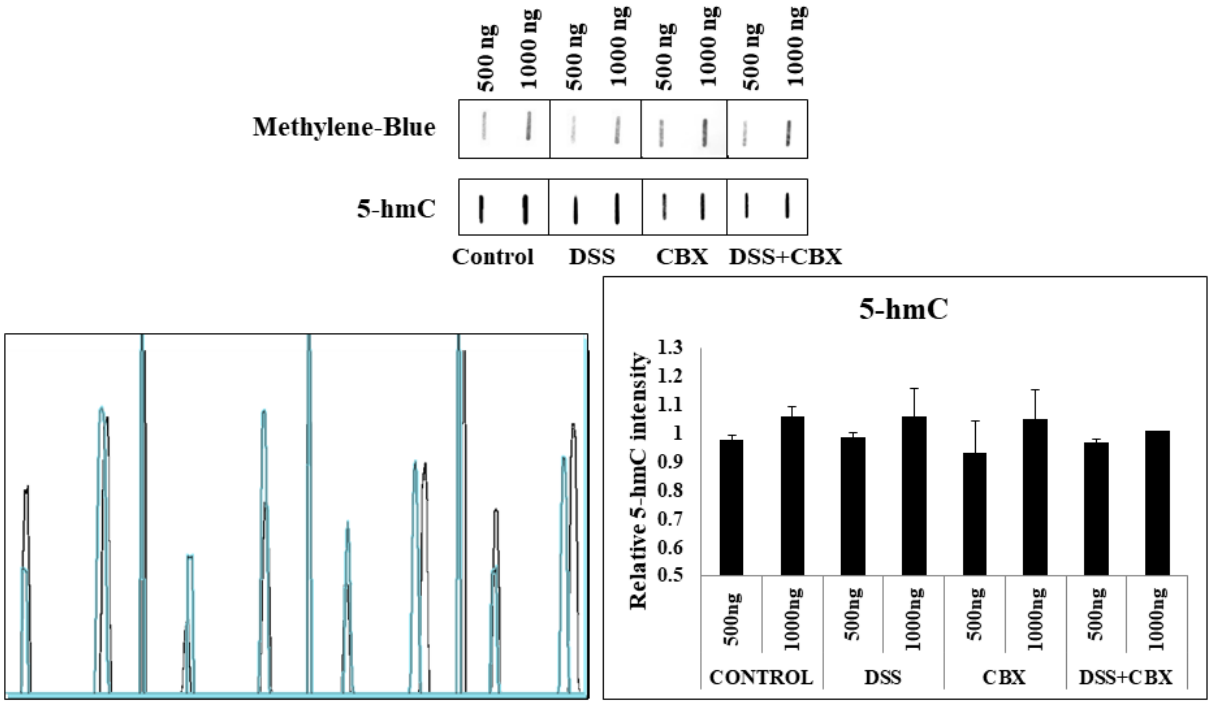

Figure 13. 5-hmC expression in the distal colon. Representative image of DNA dot blot of 5-hmC expression (upper panel), densitometry (lower panel), and relative intensity of 5-hmC blots normalized to methylene blue stain for different quantities (500 and 1000 ng) in the distal colon of control, DSS, CBX and DSS+CBX-treated mice of n=3 each.

CHAPTER V

DISCUSSION

In this study, we aimed to determine the role of both gap junctions and epigenetic modification particularly TET2 in the DSS-induced colitis model.

First, the DSS-induced colitis model was characterized through many elements of disease activity index including body weight, diarrhea, and rectal bleeding. Indeed, BALB/c DSS-treated mice had a significant weight loss in addition to diarrhea and archorrhageia (data not shown) consistent with the literature. Interestingly, mice treated with repetitive CBX showed continuous and irreversible weight loss, unlike the DSS group that regained weight after DSS removal. Moreover, colon length shortening is another indicator of DSS-induced colitis. Surprisingly, DSS treatment resulted in a significant increase of colon length compared to control when sacrificed 11 days after DSS removal. According to Yan *et al*., body weight increases and colon length recovers and goes back to normal range 9 days after DSS withdrawal [62]. Thus, this increase may be explained as a result of a compensation mechanism after inflammation onset.

The inflammatory onset induced in IBD is characterized by the disruption of the intestinal epithelial barrier and the infiltration of activated inflammatory cells mainly the macrophages leading consequently to the increase in inflammatory cytokines secretion. The infiltration of the inflammatory cells was observed in all the treated colons with a varied number of goblet cells relative to the untreated control colon. Moreover, the infiltration of activated macrophages towards the lumen is clearly detected by CD68

expression. Interestingly, CBX seems to exert a dual role in this context: when administered alone, it causes an increase in the inflammatory profile similar to that in DSS, while it seems to exert an anti-inflammatory effect when administered after DSS exposure, since it reduces the number of infiltrated macrophages and goblet cells density. This may be explained by the first use of CBX for gastric ulcers healing. It increases the amount of mucus secreted by goblet cells in the mucosa of patients with chronic gastric ulcerations as a protective mechanism [63].

IL-1β and TNF-α are from the main inflammatory cytokines secreted under inflammation. Upon intestinal inflammation, epithelial and immune cells are activated as a result of tissue damage to proceed with the healing mechanism triggered by the biochemical mediators [64]. As expected, pro-inflammatory cytokines- IL-1β and TNF-α- increased in the DSS-treated group confirming the DSS-induced colitis model while they are down-regulated after CBX administration showing a potential therapeutic role for the latter upon inflammation. Moreover, IL-10 is an anti-inflammatory cytokine capable of regenerating the intestinal epithelial barrier. Blocking gap junctions has shown to decrease IL-10 levels in the double-treated group but remained significantly higher than the control. This indicates that, IL-10 secretion can be regulated by the disruption of the communication between immune cells and epithelial cells under inflammation [65].

We further investigated the effect of inflammation and gap junctions' intercellular communication (GJIC) disruption on the expression of Cx43. We showed that DSS increases the expression of Cx43 with respect to the control group and they are formed between IECs and immune cells for hetero-cellular communication. Moreover, despite the

inhibiting role of CBX, Cx43 level increases in CBX-treated group suggesting a compensation mechanism of Cx43 after gap junctions' blockage. These findings are consistent with previous results showed in our lab and by others under the same conditions [15, 29].

Since TET2 mRNA level is significantly increased in IBD patients more than the level of TET1 and TET3 [66], in our study, we decided to assess the impact of inflammation and gap junctions' inhibition on its expression and localization. Our data showed that TET2 increases upon inflammation and it is more pronounced with the inhibition of gap junctions. Moreover, TET2 gains a nuclear localization in the treated conditions. This suggests a potential role of TET2 under inflammation by regulating the methylation/demethylation pattern of different genes involved in UC pathogenesis. This was shown in recent studies that altogether confirmed that hypermethylation of the promoters of different genes involved in UC is common in patients. For instance, the promoter of CDH1, the gene coding for E-cadherin, is hypermethylated in UC patients reducing E-cadherin expression leading consequently to a long-lasting inflammation [67]. So, to prevent the progression of inflammation into colorectal cancer, the demethylases such as TET2 could be increased to regulate the methylome status.

Finally, TET2 functionality was assessed under the same conditions. Data shows no significant changes observed on 5-hmC level in all conditions when using the whole tissues for analysis. While the nuclear localization of 5-hmC in the IECs in the CBX-treated group may indicate that the functional feature of TET2 in blocking gap junctions is

affected on the level of 5-hmC formation. Thus, TET2 expression is presumably related to gap junctions.

CHAPTER VI

PERSPECTIVES

More investigations are needed to get new insights on the relation between IBD pathogenesis, GJ and epigenetics.

Knowing that CBX is a broadly used gap junction inhibitor, whether other connexins family including Cx26 are impacted should be assessed, since Cx26 is an important partner of Cx43 in the gut. Moreover, GJ blockade can be improved since CBX is a non-selective inhibitor with reversible action [68]. To overcome these issues, flufenamic acid will be used as selective GJ inhibitor for Cx26, 30, 43, 46, and 50 and exerts an irreversible effect.

Furthermore, in the aim of assessing gap junctions' functionality, Fluorescence Recovery After Photo bleaching and dye tracking assays can be applied on cultured treated tissues.

Moreover, junctions are associated to many partners including ZO-1 and E-cadherin that were shown to be reduced in IBD after the hypermethylation of their promoters. Therefore it is interesting to assess the impact of inflammation and GJ inhibition on ZO-1 and E-cadherin expression levels.

Since the chronic inflammation in IBD increases the risk of colorectal cancer development, assessing the variation of TET2 expression and its nuclear translocation in a developed colorectal cancer *in vivo* model (DMH model) is a must.

Finally, as 5-hmC does not seem to be affected, the levels of 5-caC and 5-fC can be assessed in order to detect any changes correlated with TET2 expression variation.

mTarget (mGene)	Real-time mouse primers sequences	Ta (˚C)
mTNFα	F: 5'-AATGGCCTCCCTCTCATCAGT-3'	57
	R: 5'-CCACTTGGTGGTTTGCTACGA-3'	
mIL-10	F: 5'-CACAAAGCAGCCTTGCAGAA-3'	60
	R: 5'-AGAGCAGGCAGCATAGCAGTG-3'	
mGAPDH	F: 5'-CATGGCCTTCCGTGTTCCTA-3'	57
	R: 5'CCTGCTTCACCACCTTCTTGAT-3'	
mTET-2	F: 5'-CAAGGTGTGCTTGGCAATTTT-3'	60
	R: 5'-CATCACTGTGCGTCAATCAAGA-3'	
mConnexin 43	F: 5'-ACAGCGGTTGAGTCAGCTTG-3'	60
	R: 5'-GAGAGATGGGGAAGGACTTGT-3'	
mIL-1β	F: 5'-CTCCACCTCAATGGACAGAA-3'	57
	R: 5'-GCCGTCTTTCATTACACAGG-3'	

Table 1. RT-qPCR Primers.

Primary Rabbit Antibodies	Catalogue number	Dilution used
β-actin	Sigma	1:1000
Connexin 43	SAB-4501175	1:400
TET-2	ab-94580	1:1000

Table 2. Primary antibodies used in western blot.

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