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EFFECT OF EPSTEIN-BARR VIRUS DNA ON THE SEVERITY OF INTESTINAL INFLAMMATION IN A MOUSE MODEL OF ACUTE COLITIS

by

SIRINE IMAD ANDARI

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Experimental Pathology, Immunology and Microbiology
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
June 2020

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SIRINE IMAD ANDARI

Approved by:

Elias A. Rahal, PhD, Associate Professor — Advisor
Department of Experimental Pathology, Immunology and Microbiology

Ghassan Matar, PhD, Professor and Chairperson — Member of Committee
Department of Experimental Pathology, Immunology and Microbiology

Marwan El-Sabban, PhD, Professor — Member of Committee
Department of Anatomy, Cell Biology and Physiological Sciences

Margret Shirinian, PhD, Assistant Professor — Member of Committee
Department of Experimental Pathology, Immunology and Microbiology

Date of thesis: June 19, 2020

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ACKNOWLEDGMENTS

Foremost, I would like to express my deepest appreciation to my advisor, Dr. Elias Rahal, for the considerable support, encouragement, patience, thoughtful comments, and guidance throughout this research. His insight and knowledge of the subject matter steered me in the right direction and made this MSc study an inspiring experience. Secondly, I would like to extend my gratitude to my thesis committee members: Dr. Margret Shirinian, Dr. Ghassan Matar, and Dr. Marwan El-Sabban, for their constructive advice and motivation.

My sincere thanks go to my fellow lab members, particularly Hadi Hussein, Sukayna Fadlallah, and Dr. Marwa Chehab, for all their invaluable support, assistance, and feedback. This thesis work would not have been completed without their helpful contributions.

A very special thanks to my support system in life, my parents and brother, who never ceased to do everything possible for my sake. I am deeply honored and blessed to have you as my family; nothing would have been done without you.

Finally, my endless thanks go to my life partner and soulmate, Tarek. You were a source of unconditional support, inspiration, strength, and empowerment throughout my MSc study.

Thank God for everything.

AN ABSTRACT OF THE THESIS OF

Sirine Imad Andari for Master of Science
Major: Microbiology and Immunology

Title: Effect of Epstein-Barr Virus DNA on the Severity of Intestinal Inflammation in a Mouse Model of Acute Colitis

Background: Epstein-Barr virus (EBV) infection has been lately associated with inflammatory bowel disease (IBD), which is a collective term for chronic relapsing inflammatory disorders of the gastrointestinal tract with a poorly defined etiopathogenesis. In recent research, our group showed that feeding flies EBV DNA aggravates cellular and humoral innate inflammatory responses in a fruit fly model of gut inflammation. Whether such an exacerbatory role is exhibited by EBV DNA in a complex mammalian system, such as in mice, remains to be investigated. Therefore, the main objective of this study was to determine the effect of EBV DNA on the severity of intestinal inflammation in a murine model of acute colitis.

Methods: To assess the colitogenic potential of Dextran Sodium Sulfate (DSS)-induced colitis in C57BL/6J mice and standardize the optimal DSS concentration for examining the effect of EBV DNA on gut inflammation in subsequent aims, four groups of female C57BL/6J mice were administered 0.5%, 1%, 1.5%, or 2.5% DSS in drinking water for seven days to induce acute colitis. A fifth group served as control and was fed normal drinking water. On day 3, mice were given sterile water by rectal gavage (to control for the route of administration of EBV DNA in this study). All mice were clinically scored on a daily basis for changes in body weight, stool consistency, and fecal blood in assessment of the disease activity index (DAI). On day 7, mice were sacrificed and their colon lengths were measured for macroscopic evaluation of colonic inflammation. The 1.5% DSS concentration was employed in the investigation of the effect of EBV DNA on clinical manifestations and macroscopic inflammatory markers of colitis in the C57BL/6J mouse model of the disease. For this experiment, mouse groups received either 1.5% DSS-containing or normal drinking water for 7 days. Two DSS-treated groups were then rectally administered sterile water or 288 x 10^3 copies of EBV DNA in sterile water on day 3. Two other normal drinking water-fed groups served as controls and received sterile water or EBV DNA by rectal gavage on day 3. The severity of colitis was evaluated on the basis of the DAI and the colon length shortening that were determined in a similar manner as in the DSS concentration selection protocol.

Results: In the DSS dosage determination experiment, mice-treated with 1.5% DSS in drinking water presented a progressive aggravation of clinical colitis together with a moderately intense DAI and a prominent colon shortening showing statistically significant differences in comparison to the normal water-fed control group, which led us to investigate the effect of EBV DNA on colitis severity using the 1.5% DSS concentration in

the C57BL/6J mouse model of the disease. Mouse groups that received DSS alone or EBV DNA in addition to DSS exhibited markedly higher values of body weight loss, DAI, and colon length shortening compared to the normal water-fed control group. Moreover, the increase in the DAI score was significantly more elevated in mice treated with EBV DNA in addition to DSS than in mice treated with DSS alone. In line with the DAI results, mice receiving DSS plus EBV DNA showed a significantly more pronounced reduction in their colon lengths compared to mice receiving DSS alone.

Conclusion: The observed worsening of clinical symptoms and colonic inflammation upon administration of EBV DNA with DSS over treatment with DSS alone indicates that EBV DNA is associated with increased severity of colitis in a mouse model of the disease. These findings merit further investigation to understand the underlying mechanisms and eventually gain insight into potential therapeutic targets that may mitigate inflammatory responses attributed to EBV DNA.

CONTENTS

ILLUSTRATIONS

Figure Page

TABLES

CHAPTER I

INTRODUCTION

Inflammatory Bowel Disease (IBD) is a chronic autoimmune syndrome characterized by recurrent gastrointestinal inflammation affecting more than 10 million people around the world. IBD has an unclear multifactorial pathophysiology developing from the convergence of genetic predisposing factors, immune system dysregulations, environmental triggers, and intestinal microbiota alterations. In this regard, animal models provide a valuable approach to improve our understanding of the complex processes involved in the pathogenesis of IBD and to develop potential therapeutic strategies that contribute to controlling intestinal inflammation in patients. Of the several animal colitis models, dextran sodium sulfate (DSS)-induced colitis is largely used in murine models. For many years Epstein-Barr virus (EBV), a herpes virus that usually establishes latency in infected memory B lymphocytes, has been known to be associated with autoimmune and chronic inflammatory diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), and is more recently proposed to play a role in the pathogenesis of IBD. Knowing that EBV has a potential to reactivate after primary infection whereby EBV DNA is shed, a previous study by our group assessed the possibility of triggering pro-autoimmune processes through persistent EBV DNA. The study revealed that the levels of interleukin 17A (IL-17A), which is a proinflammatory cytokine associated with autoimmune processes, are elevated in response to intraperitoneal injection of EBV DNA in mice. In correlation to IBD, a recent study was carried out by our group to determine the effect of EBV DNA on intestinal damage induced

by DSS in *Drosophila melanogaster* as a model system. The results of the study showed that feeding flies EBV DNA aggravates the gut inflammation produced by DSS but does not initiate the inflammation by itself. While fruit flies possess innate immunity components, they do not have an adaptive immune system; hence, studying the effect of EBV DNA on more complex pathways requires establishing a higher model system. The overall objective of this study is to determine whether EBV DNA results in enhanced severity of intestinal inflammation in a murine model of acute colitis.

The specific aims of the study at hand, hence, were to:

1. Examine the functionality of the DSS-induced murine colitis model in C57BL/6J mice and determine the appropriate DSS concentration to be employed in subsequent investigations.
2. Assess the effect of EBV DNA on colitis activity and colonic morphology in a mouse model of the disease.

CHAPTER II

LITERATURE REVIEW

A. Epstein-Barr Virus (EBV)

Epstein-Barr virus (EBV), also designated as *human herpesvirus 4* (HHV-4), belongs to the *Herpesviridae* family of viruses whose members typically establish latency following primary infection (1, 2). According to the exercised viral classification by the International Committee on the Taxonomy of Viruses (ICTV), EBV is part of the *Gammaherpesvirinae* subfamily and falls under its *Lymphocryptovirus* genus (1,3, 4). This human pathogen was originally identified in 1964 by Epstein and Barr following its isolation from cultured Burkitt's lymphoma cells and now is recognized as one of the most prevalent viruses with the world population being seropositive in 90 to 95% of adults (5 - 10).

1. EBV Structure

EBV is a large enveloped double-stranded (ds) DNA virus that shares the common virion structure of herpesviruses (11, 12). In this sense, the genome of EBV is encapsulated by an icosahedral nucleocapsid constituted of 162 virally-encoded capsomeres (4, 12 - 14). Surrounding the capsid is an amorphous tegument or matrix composed of proteins involved in the viral replication (12, 15). Following the tegument is an outer lipid-containing envelope that is acquired from the host membrane during the release stage of the viral life cycle (12). Found on the envelope are virally-encoded glycoprotein (gp) spikes that interact

with host receptors (4). The most abundant of which is gp350/220 that binds to CD21 receptor on the surface of B cells (16, 17).

2. EBV Genomic Structure and Genes

Like other herpesviruses, the EBV genome is composed of a linear double-stranded DNA molecule (12). The genome is approximately 172 kilo base pairs (kb) and is arranged into internal repeat (IR) and terminal repeat (TR) sequences (4, 12, 18). The IRs are four in number and divide the genome into unique sequences of different sizes. The first IR (IR1) constitutes the major IR as it separates the genome into a short unique sequence (Us) and a long one that is further partitioned by the remaining IRs into four shorter sequences, termed U2, U3, U4, and U5 (4) (19, 20). As for the TRs, these are located at both ends of the viral genome and mediate its circularization into an episome in infected cells during latency (12, 21). EBV genome encodes for 85 genes that majorly participate in the EBV lytic cycle (18, 22). However, a limited set of these genes is only expressed in latently infected cells and referred to as latent viral genes (23). These latent genes consist of virally encoded proteins, non-coding EBER RNAs, and microRNAs. The virally encoded protein can be further grouped into six nuclear proteins (EBNAs 1,2,3A,3B,3C, and LP) and three latent membrane proteins (LMP-1, LMP-2A, and LMP-2B). EBV latent genes have multiple functions related to the maintenance of EBV infection, B cell transformation, and tumorigenesis (12).

a. EBNA1

The first identified EBV protein was the EBV nuclear antigen 1 (EBNA1) that mediates several EBV-related functions (24). Among these functions is its integral role in the maintenance of the viral episome in EBV-infected cells. This protein tethers EBV DNA to cellular chromosomal DNA and promotes its replication. It also regulates the transcription of several latent proteins, such as EBNA 1 and 2. Moreover, it escapes proteasomal degradation inhibiting its antigen presentation to $CD8^+$ cytotoxic T lymphocytes (CTLs) through the MHC I pathway (9, 12). Of the EBV latent proteins, EBNA1 is the only one that is expressed in all EBV-associated malignancies (25).

b. EBNA2

EBNA2 is among the first genes expressed in EBV-infected B cells (26). This nuclear protein acts as a transcriptional regulator for latent viral and cellular gene expression as well as an inducer of primary B cell growth transformation (12).

c. EBNA3

EBNA3 family of proteins encompasses three gene products, namely EBNA3A, EBNA3B, and EBNA3C, that contribute to EBV latency. Of these proteins, only EBNA3A and EBNA3C are considered essential for B cell transformation and are regarded as oncogenic, while all of them are thought to support the viability of EBV-infected lymphoblastoid cell lines (12).

d. EBNA-LP

EBNA leader protein (EBNA-LP) is the first latent protein produced by EBV-infected B cells and participates, in conjunction with EBNA2, in B cell growth transformation (12).

e. LMP1 and LMP2

Latent membrane proteins (LMPs) are integral membrane proteins required for the establishment of viral latency. LMP1 and LMP2A function as viral mimics of the co-stimulatory receptor CD40 and the B cell antigen receptor (BCR), respectively, allowing the activation of signaling pathways in a ligand-independent manner. These viral mediators also appear to play an important role in the EBV-mediated B cell transformation and in the induction of oncogenesis (9).

f. EBER-1 and EBER-2

The EBV-encoded small RNAs (EBERs) are the most abundant non-coding nuclear RNAs expressed in latently EBV-infected cells (12, 27). While the role of EBERs in the viral life cycle remains to be elucidated, findings have demonstrated that they participate in the antiviral innate immunity through binding to the RNA-activated protein kinase (PKR) and inhibiting its mediated apoptotic control mechanism (28). The involvement of EBERs in oncogenesis and in growth transformation of EBV-infected B cells has been reported (28).

g. MicroRNA

EBV DNA encodes several microRNAs (miRNAs) (9). Although their function is relatively unknown, emerging evidence has indicated that they play a role in immune

evasion through inhibiting the expression of viral antigens, in suppressing lytic replication, and in maintaining latency in infected cells (29). Moreover, studies have indicated that these genes support the development and progression of EBV-associated malignant tumors (30).

3. EBV Types

A widespread distribution of two types of EBV, classified as EBV-1 and EBV-2, exists in the human population (4). The most significant variation between these two types at the genomic level is in their EBNA2 gene that exhibits more efficient *in vitro* B cell transformation properties in EBV-1 than EBV-2 (4, 12). As for their geographic distribution, EBV-1 appears to be more prevalent in the US and Europe, whereas both seem to be equally distributed in Africa (8).

4. Epidemiology and Transmission

EBV is widely disseminated around the world, with humans serving as its only natural host (4). Studies show than more than 90% of the world's adults get infected with EBV at some point in their lives and develop antibodies that provide durable immunity (4, 8, 12). The age at which primary infection occurs differs greatly between world populations. In developing countries, children get infected with EBV early in life, while in developed countries, infection usually occurs in early adulthood. The early acquisition of primary EBV infection in developing countries is suggested to be related to low socioeconomic conditions and poor hygienic practices (4). The main mode of transmission of EBV is orally through the saliva during infection (31, 32). Evidence for the transmission of EBV

by sexual intercourse, blood transfusion, and organ transplantation have also been documented, yet these cases are rare (33).

5. EBV-related Diseases

EBV is the etiological agent of Infectious Mononucleosis (IM). Although most EBV infections are inapparent or mild, in some cases, they can lead to complications, and even more can trigger the development of different malignancies. EBV infections also constitute a risk factor for the development of autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS), and more recently inflammatory bowel disease (34 - 36).

During childhood, primary EBV infections are common and usually asymptomatic or subclinical. When symptoms do occur, they are manifested by ear, gastrointestinal, and upper respiratory tract indications (10, 37). However, when EBV infections are acquired during adulthood, they give rise to IM in 35 to 50% of cases (38). IM is the most common clinical manifestation of EBV that is characterized by tonsillar pharyngitis and cervical adenopathy. Following an incubation period of 30 to 50 days, patients infected with EBV present with malaise, headache, fever, sore throat, lymphadenopathy, anorexia, as well as with sporadic cases of hepatomegaly and jaundice (10, 33, 37). Moreover, IM patients can develop lymphocytosis, usually with atypical lymphocytes on peripheral blood smears (37). IM specific symptoms resolve within 1 to 2 weeks, but patients may continue to experience fatigue for months (10). In rare cases, complications related to EBV infection are established. Acute complications of IM include morbilliform rash, splenic rupture, airway

obstruction, hematologic abnormalities, and neurological disturbances such as Guillain-Barre syndrome and meningoencephalitis (10, 33, 37).

EBV infections are also associated with a number of delayed complications that can be categorized into epithelial diseases, hematologic conditions, and lymphoproliferative disorders. One delayed complication is oral hairy leukoplakia (OHL), which is a disease of the lingual epithelium with affected patients showing benign white lesions on the lateral portion of their tongue (37, 39). This disease is usually described in relation to Human Immunodeficiency virus (HIV) infection, but it can also occur in other cases of immunodeficiency (11, 37, 40). Examples of blood disorders that develop in association with EBV infection in immunocompromised subjects include hemophagocytic lymphohistiocytosis (HLH) and lymphomatoid granulomatosis (10, 37). Likewise, EBV-induced lymphoproliferative disorders are also observed in individuals with congenital or acquired immunodeficiencies and include the X-linked (XLP) and the post-transplant (PTLP) lymphoproliferative diseases (12, 37).

Being a DNA tumor virus, EBV has also been shown to play a pathogenic role in the development of a variety of human cancers (37). These include Burkitt lymphoma (BL), which is situated in the jaw and most prevalent among childhood tumors in Africa. Another B cell neoplasm associated with EBV infection is Hodgkin lymphoma (HL), where several studies have indicated the presence of the EBV genome in tissues obtained from HL patients (10, 33). As for people infected with HIV, high EBV loads promote the development of non-Hodgkin lymphoma (NHL) (10, 12). EBV is also associated with nasopharyngeal carcinoma, which is a rare epithelial cell cancer that typically affects populations in southern China (33). Finally, EBV-associated malignancies do not spare T

cells, and cases of T cell lymphoma have been observed in individuals with EBV infection (41).

The association of EBV with autoimmune diseases (AIDs) dates back to 1971 following the detection of elevated levels of EBV-specific antibodies (Abs) in SLE patients (42, 43). After encountering an antigen, specific lymphocytes may become activated and induce an immune response, or they may be inactivated or deleted, resulting in tolerance. The process of immunological self-tolerance is a fundamental property of the normal immune system. When this process fails, an immune response against the host is mounted, a state referred to as autoimmunity (44). A combination of different factors contributes to the development of autoimmunity. These include genetic susceptibility, flawed immune regulations, and environmental triggers such as infections and local tissue injury (34). EBV is an example of an environmental factor whose potential involvement in the pathogenesis of AIDs has been explained by a number of proposed mechanisms, including molecular mimicry, bystander activation of autoreactive T and B cells, and epitope spreading, among others (34). Molecular mimicry is a mechanism by which sequence similarities between foreign antigens of an infectious agent and self-antigens of the host result in cross-reactive immune responses (33, 44, 45).This can be exemplified by the cross-reactive response of antibodies directed against an EBNA-1 epitope in MS patients to the myelin basic protein (MBP) of the central nervous system (46). Other findings in MS patients suggest that cytotoxic T cell immune responses primarily directed against EBV could result in bystander damage to the CNS (47, 48). During this process, EBV infections trigger the activation of antigen presenting cells (APCs) that in turn might drive the activation of non-specific T cells such as pre-primed autoreactive T cells, apart from the EBV-specific T

cells, and thus promote the development of AIDs (34, 35). Nevertheless, despite all the evidence linking EBV to autoimmune diseases, the definitive role of EBV in these diseases remains unconfirmed and requires further study. Our group previously reported that the systemic administration of EBV DNA in mice enhances the secretion of interleukin 17A (IL-17A), an autoimmune-related proinflammatory cytokine (11). In follow-up studies, our group demonstrated that Toll-like receptors 3, 7, and 9 (TLR9) are involved in the increase in IL-17A production in response to EBV DNA (49). In support of our observation of an enhanced effect of EBV DNA on IL-17A levels in mice, our group further indicated that the EBV DNA load in RA patients correlates to a higher serum level of IL-17A (49). More recently, our group showed that EBV DNA contributes to IBD progression in a *Drosophila melanogaster* model of gut inflammation induced by a dextran sodium sulfate (DSS), a colitogenic chemical, whereby more enhanced cellular and humoral innate inflammatory responses were observed in flies treated with DSS then EBV DNA rather than in flies treated with DSS and EBV DNA at the same time (50). Several aspects of the correlation between EBV and AIDs remain to be elucidated.

6. EBV Life Cycle and Pathogenesis

EBV has a life cycle that is adapted to immune cell compartments where it evades eliminatory host immune responses, specifically cytotoxic T cell surveillance, and progresses to lifelong persistence in a transcriptionally quiescent state in resting memory B cells while exploiting the physiology of normal B cell differentiation (9, 12, 51). The life cycle of EBV is initiated following the spreading of the virus in the saliva and its arrival to the oropharynx, where it enters the epithelium surrounding the pharyngeal lymphoid ring

and undergoes lytic infection (4, 12, 31, 52). Penetration of EBV into epithelial cells is facilitated by the binding of the viral BMRF-2 proteins with the β1 integrins, then the binding of the viral gH/gL with αvβ6 or αvβ8 integrins that further promote the fusion of EBV envelope with the epithelial cell membrane (12, 53 – 55). These oropharyngeal epithelial cells are considered the primary cellular targets of EBV and act as sites for productive replication (4, 56 – 58). After invading the nasopharyngeal epithelial barrier, the virus migrates to the neighboring lymphoid tissues and infects naïve B cells resulting in a state of latent infection (4, 31, 33). Interaction between the virus and B cells occurs through a different set of mediators than those associated with epithelial cells. In this sense, B cell infection is initiated by the binding of the major EBV envelope glycoprotein gp350/220 with the B cell surface molecule CD21, which is also referred to as complement receptor type 2 (CR2) as it interacts with the C3d complement component (12, 17, 59, 60). The penetration of EBV into B cells is also mediated by the attachment of a second membrane glycoprotein, gp42, to the Major Histocompatibility Complex class II (MHCII) molecule on the cell surface (4, 12, 61). Following adsorption and endocytosis into membrane vesicles, the viral envelope fuses with cell membrane allowing the release of the nucleocapsid into the B cell cytoplasm in a process involving the gp85, gp25, and gp42 glycoproteins (4, 12, 62 – 64). When EBV infects these naïve B cells, it drives their transformation into activated lymphoblasts that remain latently infected and express the latent genes that define the third latency program in B cell (4, 33). In addition to epithelial and B cells, *in vitro* studies have demonstrated that EBV also infects other human cells like T cells and monocytes, yet through less understood mechanisms (4, 12, 65, 66).

During latency, four different transcription programs, termed as latency programs 0, I, II, and III, are noted in latently infected B cells based on the expression of different sets of EBV latent proteins (4, 9). Transition to the latent phase begins with the latency III program that activates the transformation of B cells into lymphoblasts (4, 67). In this program, all latent genes are expressed, but EBNA-1, two non-coding small ENA, and microRNAs are the only genes that continue to be expressed in all of the other forms of latency (4). Following the type III latency program, activated B cells migrate into the lymph node follicle and begin to proliferate, forming the germinal center (GC) (4). In the GC, B cells stimulate the type II latency program, which is also known as the default program where a limited number of latent genes are expressed (4). At this stage, B cells produce the EBNA1 and LMP1/2A proteins, differentiate into memory B cells, and acquire the ability to survive for long periods in response to survival signals provided by the default program (68, 69). These cells then exit the GC and recirculate in the peripheral blood (70). The long-lived memory B cells maintain a completely silenced viral gene expression and assume a form of latent infection termed latency 0 (4). The lifelong persistence of EBV is only confined to the resting memory B cells that bypass the immune system due to their downregulated gene expression (4, 33, 71). During the latent phase, the linear viral genome circularizes and remains in the cell in the form of extrachromosomal double-stranded plasmids, also known as episomes (12, 72). When the EBV-infected memory B cells divide, they express the EBNA1 protein that is responsible for maintaining the episome and enabling its replication during the S phase of the cell cycle (12, 72, 73). In these cells, the episome divides under the influence of the cellular DNA polymerase, unlike lytic phase cells where the EBV genome replication is carried out by the viral DNA polymerase (74 –

76). The EBNA1-only program is referred to as latency I. In the long run, latently-infected memory B cells can move back to the tonsils and resume their role as the reservoirs of EBV reactivation where EBV regains its potential for lytic replication and productive viral progeny synthesis (9, 33, 71).

Apart from the induction of the viral lytic cycle during primary infection, viruses can reenter the lytic replication phase after latency. This is initiated following the disruption of latency by cellular signals that result in the expression of the viral transcription factor BZLF1 that brings about this switch (9, 12). During lytic replication, latently infected memory B cells resume their differentiation into plasma cells, and the virus reproduces with associated infection of naïve B cells and epithelial cells (12, 20). Lytic cycle genes are grouped into different temporal phases, based on the time they are expressed in association with DNA replication, and include immediate early, early, and late proteins (13, 33). Immediate early genes represent the genes that are transcribed in newly infected cells prior to any new synthesis of viral proteins and encode for proteins that transactivate early gene promoters. Two main immediate-early proteins are the BZLF1 and BRLF1 transcriptional activators aforementioned as stimulators for switching from latent to lytic infection (12). The expression of early genes results in the production of protein products that function as enzymes during viral DNA replication (33, 77). Finally, late genes encode mostly for structural proteins of the viral particle such as the viral capsid antigen (VCA) against which antibody-mediated immune responses to EBV are directed (12, 20, 33).

B. Inflammatory Bowel Disease (IBD)

Inflammatory Bowel Disease (IBD) is a chronic idiopathic disorder characterized by uncontrolled inflammation in the gastrointestinal tract with repeated cycles of relapse and remission (78 – 80). Although the etiology of IBD is poorly defined, it is likely to be multifactorial and precipitated, as other autoimmune diseases, by a complex interaction of multiple pathogenic factors including susceptibility gene variants, environmental triggers, gut microbiota changes, and immune response dysregulations (80, 81).

1. Types of IBD

IBD encompasses mainly two major forms, Crohn's disease (CD) and Ulcerative Colitis (UC), that can be classified based on their distinguishing anatomical, histological and clinical features (78, 82 – 85). Concerning the affected location of the GI tract, Crohn's disease can engage any portion of the GI tract from the mouth to the perianal region. Still, in most cases, it affects the terminal ileum and the colon (79, 82 – 84, 86, 87). Areas of involvement in CD are characterized by the presence of skip lesions, which are normal segments between affected regions in the bowel resulting in a non-continuous patchy pattern of inflammation (79, 83, 84, 87). In contrast, inflammation in UC is confined to the colon; it arises in the rectum and spreads continuously to cover more sections of the colon (79, 83, 85 – 87). On the histological level, CD can be distinguished from UC in that inflammation in this subtype is typically transmural involving all layers of the intestinal wall, while in UC it is superficial and limited to the mucosa and submucosa (79, 82, 86, 87). Lymphocytic cell infiltration is observed in both diseases. These infiltrates are accompanied by dense accumulations of macrophages that contribute to the development of

granulomas in CD, while they involve luminal penetrations of polymorphonuclear cells that give rise to crypt abscess with loss of goblet cell in UC (79). Clinically, patients with Crohn's disease present with abdominal pain and prolonged diarrhea without a gross manifestation of blood. The transmural nature of the disease is associated with the narrowing of the gut lumen and the development of fibrotic strictures. CD may also give rise to sinus tracts that expand to form tunnels connecting epithelial lined organs known as fistulas (79, 83, 84). On the other hand, symptoms associated with UC include bloody diarrhea and gradual loss of the peristaltic function of the colon without the development of fibrosis and strictures. Unlike the patchy inflammation in CD that spares the rectum, inflammation in UC patients is initiated in the rectal region that may exhibit blood upon examination. In both forms of IBD, patients may also develop systemic symptoms such as fatigue, fever, and weight loss. In addition to the GI tract, CD and UC patients share several extra-intestinal manifestations that may involve the joints, skin, liver, eyes, lungs along with other organ systems (79, 82 -85).

2. Epidemiology

Despite the substantial progress in the understanding of the pathogenesis of IBD, the burden of this disease is continuously expanding on a global scale (88). Population-based studies describe a bimodal age distribution for IBD with a peak age of onset occurring between 15 to 30 years and another peak period extending from 50 to 70 years (78, 83). A second demographic factor that slightly differs among IBD groups is sex. It has been reported that the incidence of ulcerative colitis is higher in males, while Crohn's disease occurs more frequently in females; however, this gender-based difference is almost

negligible in both groups (78, 83). Moreover, epidemiological studies have shown that North America, northern Europe, and the United Kingdom represent regions of high prevalence for ulcerative colitis and Crohn's disease with an estimated average range of 100 to 200 cases per 10^5 individuals (82, 89). The disease incidence was described to follow a pattern of a north-to-south gradient with areas of South America, Southeast Asia, Africa, and the Middle East accounting for lower incidence rates (83, 89). This gap between the two latitudes has been narrowing with the rise in the incidence of IBD in developing countries of historically low rates (86, 89). The observed variation in the rates of IBD was attributed partially to environmental factors aside from the underlying diverse genetic backgrounds of the world population and interpreted as a possible result of the different extents of industrialization, migration to other geographical regions, and westernization of lifestyle such as changes in the diet and smoking (78, 83, 89). The link between diet and IBD is inconclusive, but some data indicated that the higher ingestion of food additives and fatty acids and the decreased intake of vitamin D appear to contribute to IBD (81, 78, 90). Given that vitamin D is involved in the regulation of the mucosal immune system, the increased risk of IBD in northern areas might be, to a certain degree, related to the less sunlight exposure of residents there (81, 83, 91). In the case of cigarette smoking, its effect has been consistently proven to be discordant between the two forms of IBD (78, 89). While smoking is associated with increased risk of developing CD, it seems to play a protective role against UC (78, 90). These observations support the unique pathophysiology of each type of IBD (78). Furthermore, an inverse relationship was postulated to exist between the incidence of IBD and the degree of sanitation (81). This correlation was given credence by the hygiene hypothesis which highlights that excessive sanitation can limit the

early exposure to diverse microbial agents and impair the fundamental education of the immune system predisposing subjects to immune-mediated diseases later in life (89). The markedly increased risk of both forms of IBD in higher socioeconomic groups was relatively linked to the generally favorable housing sanitary conditions, the better access to health care, and the repeated exposure to antibiotics in early life (78, 81). For example, children living in overcrowded poor families have a higher propensity for infection with different environmental agents and thus develop a more-balanced gut microbiota that reduces their risk of acquiring IBD in comparison to children raised in spacious conditions with minimal sharing of belongings (78, 81). Overall, comprehensive epidemiological findings could potentially provide a means for evaluating the relationship between environmental exposures and IBD.

3. Genetic Factors in IBD

Advances in IBD genetics have provided a substantial amount of evidence to a role for genetic factors in the susceptibility to IBD. The strongest evidence for such a contributory role is indicated by twin studies. These studies have shown that monozygotic twins tend to develop the same type of IBD more commonly than dizygotic twins do (78). They have also pointed out that the concordance rate for CD is substantially higher than that for UC, more explicitly with 30 to 35% in CD compared to 10 to 15% in UC, suggesting a greater contribution of genetic factor in CD than in UC (79, 87, 92). Moreover, family studies have shown that first degree relatives of IBD patients are around 3 to 20 times more prone to developing IBD than relatives of unaffected families (92 -94). Furthermore, it was reported that families with IBD affected members have concordance for the developed disease type

in 75 to 80% of the cases (78, 82, 95). In other words, all affected individuals in the aforementioned families solely develop either CD or UC, while in less common cases, both types of IBD are exhibited among different family members. Collectively, these observations indicate that a genetically complex non-Mendelian pattern of inheritance is associated with IBD risk (78, 79, 92).

To date, genome-wide association studies (GWAS) have allowed the identification of over 200 distinct susceptibility loci for IBD (92, 96). Comparative analysis of these genetic associations between the two types of IBD revealed that many of these genes are shared between CD and UC, while some are associated exclusively with one IBD type (96, 99). A number of observations have shown that genetic variants that alter innate immune responses are found to confer CD risk, while genes affecting the adaptive immune system are implicated in both CD and UC (82, 96).

a. Innate Immune Pathways in IBD Genetics

i. Defects in NOD2 Pathway

Nucleotide-binding oligomerization domain 2 (NOD2) mutations are key pathogenic variants that confer increased susceptibility to Crohn's disease (81, 82, 92, 96). NOD2 gene, also recognized as caspase activation and recruitment domain 15 (CARD15), was identified in the first IBD susceptibility (IBD1) locus of chromosome 16 (78, 79). This gene encodes for a cytosolic pattern recognition receptor, NOD2, that belongs to the NOD-like receptor (NLR) family (78, 79, 81, 82). The NOD2 protein is expressed in the cytosol of intestinal epithelial cells (IECs) and phagocytes, and allows response to pathogenic bacteria in the gastrointestinal tract (78, 79, 86, 92, 96). It is also expressed by Paneth cells

at which it stimulates secretion of antimicrobial defensins (78, 86). This protein responds to a bacterial peptidoglycan component, the muramyl dipeptide (MPD), which is found in both Gram-positive and Gram-negative bacteria. Then it gets stimulated to activate the nuclear factor kappa B (NF- κB) signaling pathway, which in turn facilitates the production of inflammatory mediators and the clearance of bacteria (78, 82, 87, 96, 100 -102). CD-associated NOD2 variants result in loss of its functional outcomes (96). Despite the decreased NOD2 associated activation of the NF- κB pathway that is assumed to cause reduced inflammation, several explanations have been raised in support of the involvement of NOD2 mutations in the pathogenesis of Chron's disease (78). One hypothesis suggests that the absence of NOD2 expression may result in persistent survival of intracellular bacteria and, subsequently, a chronic inflammatory response (78, 86). Its defective expression in IECs may also lead to a loss of barrier function due to decreased NOD2-related innate responses that allow bacterial proliferation (78, 79). Another theory suggests that the inflammatory responses observed in CD may arise from upregulated adaptive immune processes that come in compensation to the aberrant innate immunity (78). A third hypothesis proses that altered NOD2 functions impair the conditioning of antigen-presenting cells, which normally regulate T cell responses, resulting in a failed maintenance of intestinal homeostasis (78, 79).

ii. Defects in Autophagy

Autophagy is a cell-autonomous innate homeostatic process that contributes to the clearance of intracellular pathogens as protection against their growth in host cells, besides

its role in the recycling of cytoplasmic organelles (82, 87, 96, 103, 104). Loss-of-function genetic variants in the Autophagy Related 16-like 1 (ATG16L1) and the more recently described ones in the Immunity-Related GTPase M (IRGM) genes that are involved in autophagy have been associated with increased risk for Crohn's disease (81, 86, 87, 96, 105). ATG16L1 is ubiquitously expressed in various cells of the innate and adaptive immunity, involving epithelial, dendritic, T and B cells; yet, most impairments in its autophagic function have been focused only in intestinal tissues owing to the vast microbial weight in the gut (82, 86, 87). Defects associated with ATG16L1 mutations include reduced pathogen clearance and abnormal Paneth cells properties such as atypical granule size and distribution and restricted antimicrobial peptide production (87, 96). Evidence shows that NOD2 and ATG16L1 pathways are integrated and disease-associated (81, 87, 96). Under normal conditions, the activation of NOD2 activates autophagy in an ATG16L1-dependent manner, while in Crohn's diseases, genetic defects in both pathways promote impaired immune activities related to the pathogen clearance, cell-signaling regulation, and adaptive system communication (81, 87, 96).

b. Adaptive Immune Pathways in IBD Genetics

IBD gene variants regulating adaptive proinflammatory and immunoregulatory pathways have been identified in both CD and UC (82, 96). One of the significant genetic variant associations reported in both forms of IBD is in the gene encoding the IL-23 receptor (82, 92, 96, 106). A key component in this association is mediated through the involvement of IL-23 in the generation of Th17 cells that are characterized by their production of the proinflammatory IL-17 cytokine. In fact, genomic regions containing components of the

IL-23 signaling pathways, namely IL-12B which is a common subunit shared by IL-12 and IL-23, JAK2 signaling molecule, and STAT3 transcription factor, showed association with IBD, thereby highlighting a strong implication for this pathway in the pathogenesis of IBD (82, 96). At the same time, studies have also provided evidence for the presence of a protective IL-23R variant against the risk of IBD; carriers of this variant exhibit a decreased response to IL-23 and reduced levels of circulating Th17 cells (96, 107). In parallel to the upregulated expression of the proinflammatory Th17/IL-23 pathway-related genetic loci in IBD, loss of function mutations in the anti-inflammatory IL-10 pathway also represent a rare IBD-implicated locus involved in the very early onset of the disease (92, 96, 108, 109).

4. The Gastrointestinal Mucosal Immune System

The gastrointestinal epithelium is a tube-shaped structure lined by a monolayer of tightly connected columnar epithelial cells that form a mechanical barrier to the external environment. At the basolateral surface of the epithelium immediately lies the lamina propria, which is a highly vascular layer of loose connective tissue composed of a dense network of blood vessels, lymphatic capillaries, a range of immune cells, and secondary lymphoid tissues (110). The intestinal lumen harbors the largest microbial community in the body, approximately containing 10^{14} organisms (78, 81, 110). Many of these microbes are growing in the lumen as commensals referred to as the gut microbiota, while a very small number of microbes might be pathogenic (110, 111). Given the constant exposure of the mucosal immune system to a high density of luminal microflora, the host and the microbiota have coevolved mechanisms of mutual benefit with maintenance of intestinal homeostasis (96, 111). Thus, beyond their role in nutrition and energy metabolism,

commensal organisms are also involved in the development and the maturation of the intestinal immune system (81, 96, 112). This host-microbiome interaction allows for the proper conditioning of immune cells that display unique characteristics within intestinal tissues. A failure in the establishment of a symbiotic relationship of tolerance can lead to a chronic destructive immune response that may promote the development of IBD in the context of underlying genetic defects (78, 113).

Among the cells exhibiting a unique phenotype within the intestine are the resident macrophages. In a healthy gut mucosa, resident macrophages produce attenuated levels of proinflammatory cytokines but show enhanced phagocytic and bactericidal activities in response to potential microbial ligands compared to circulating macrophages (78, 81, 86, 87, 114). As a mechanism for controlling excessive inflammatory responses, less than 10% of resident macrophages in the intestinal lamina propria express the triggering receptor expressed on myeloid cells-1 (TREM-1), which is a cell surface molecule that potently stimulates neutrophil, monocyte, and macrophage-mediated inflammatory response by inducing proinflammatory cytokines secretion (78). In addition, resident macrophages also inhibit inflammation by secreting anti-inflammatory cytokines, such as IL-10 (96). These combined local properties promote pathogenic microbial clearance with a minimal tissue injury (96).

Another type of cell that plays a central role in maintaining the intestinal homeostasis is the intestinal dendritic cells (DCs). These cells function as important monitors of microenvironment as they express the full array of TLR and NLR which allows them to distinguish between commensals and pathogens, thereby inducing tolerance or inciting a proinflammatory response, respectively (81, 87, 89). Under normal

circumstances, DCs sample Ags, exhibit an immature phenotype and silence T cell responses to control tolerance towards commensals. During such response, DCs promote naïve T cells differentiation into regulatory $CD4^+$ T (T_{reg}) cells instead of effector Th1 or Th2 cells (81, 115). With the presence of potentially proinflammatory pathogens, DCs mature to develop an activated phenotype and initiate immunity. Mucosal DCs also contribute to the maintenance of intestinal homeostasis through modulating the interaction between innate and adaptive immunity (81, 116). For example, in a healthy state, the crosstalk between epithelial cells and DCs drives the differentiation of anti-inflammatory T_{reg} cells and promotes tolerance. Through this mechanism, intestinal epithelial cells release the vitamin A metabolite retinoic acid and the cytokines thymic stromal lymphopoietin (TSLP) and transforming growth factor-β (TGF-β) inducing DCs to foster T_{reg} cell differentiation (81, 87, 117, 118). A failure of such interaction could give rise to pathologic intestinal inflammation (87).

In this context, for health to be maintained, the gut depends on a functional epithelial barrier, an innate, and an adaptive immune system to defend the host against pathogens.

a. Intact Epithelial Barrier and Innate Immunity

The epithelial barrier comprises the first line of defense of the mucosal immune system (119). It provides three major ways to prevent microbial invasion into intestinal tissues (111). First, it is coated by a pre-epithelial mucus layer in which microbes get embedded to limit their ability to access the epithelium (110, 89). Second, the continuous single layer of

epithelial cells physically impedes the penetration of luminal microbes by the selectively permeable tight junctions and kills pathogens in the lumen by the secretion of protective factors into the mucus layer (96, 110, 111). Third, beneath the epithelial layer, plasma cells in the lamina propria secrete IgA that gets transported to the apical epithelial cell surface to neutralize pathogens in the lumen (89, 111). Together, these active functions of the epithelial barrier are essentials for the maintenance of a healthy intestinal environment (79).

i. Role of Epithelial Cells in Innate Immunity of a Healthy Gut

The intestinal epithelium is in constant contact with the microbiome in the lumen and the immune cells in the underlying connective tissue. Besides forming a physical barrier through attaching in tight junctions, IECs maintain intestinal homeostasis by regulating innate and adaptive immune responses (86, 87). Recent studies have shown that IECs express TLRs and NLRs and activate signaling cascades that trigger immune responses to pathogens while limiting inflammatory responses to commensal bacteria (79, 86, 111, 120). In addition to the columnar IECs, the epithelial barrier includes specialized cells that are interspersed along the crypt-villus axis and play different roles in immune defense. These highly specialized enterocyte populations are namely the goblet, Paneth, and microfold (M) cells. Goblet cells are responsible for the formation of a viscous mucus layer that provides protection through preventing microbes from contacting the epithelial cell lining (82). The epithelium also contains Paneth cells that contribute to the innate immune defense through the secretion of potent antimicrobial peptides known as defensins (89, 121). The defensins are peptides with amphipathic properties that allow them to insert into and lyse microbial membranes resulting in a lethal effect on microbes (89, 111). In the colon, defensins are

produced by absorptive epithelial cells. Moreover, neutrophils release defensins as part of their antimicrobial effector mechanisms. M cells are also important components of the epithelium. They overlie lymphoid tissues and function as a conduit sampling Ags (whole microbes and soluble Ags) to APCs found in the Peyer's patches of the small intestine and secondary lymphoid organs of the colon (89).

ii. Role of Innate Lymphoid Cells (ILCs) in a Healthy Gut

If luminal microorganisms were able to cross the epithelial barrier, they would get in direct contact with components of the mucosal innate immune system that is populated with cells poised to defend the gut against invading microbes. Dendritic cells, macrophages, neutrophils, natural killer cells, and innate lymphoid cells (ILCs) are crucial cellular components of the innate immune system that provide an initial response during infection or inflammation.

Innate lymphoid cells are a distinct population of lymphoid cells that are derived from the common lymphoid progenitor (CLP) that gives rise to lymphocytes and NK cells. These cells have lymphoid morphology but do not express antigen-specific T or B cell receptors (BCR or TCR) (80, 111). The ILCs are subdivided into three major subsets based on their lineage-specific expression of transcription factors and their production of distinct cytokine profiles. The subsets include ILC1, ILC2, and ILC3 that are respectively analogous to Th1, Th2, and Th17 cells producing an array of effector cytokines that are similar to those of the CD4 T helper cells (111). ILC1 require T-bet, a common transcription factor of Th1 cells, for their development and function, and they secrete IFN-γ

following activation for protection against intracellular microbial pathogens (122). In analogy to Th2, the second subset of cells shares the transcription factor GATA-3 and produces IL-5 and IL-13 for defense against helminths and the development of allergic inflammation (111). The third group of ILCs is subdivided into natural cytotoxicity receptor $(NCR)^+$ ILC3s that produce IL-22 and NCR^- ILC3s that secrete both IL-22 and IL-17 serving as the innate counterpart for Th17 cells (80). The third subset requires RORγt as a transcription factor. It is involved in the initiation of immune responses against extracellular bacteria and fungi as well as in the maintenance of intestinal homeostasis (80, 123, 124). All the innate lymphoid cells are always resident in the epithelial barrier tissues at the interface between the environment and the associated lymphoid tissues (122). This enables them to promptly react against invading pathogens, providing a first-line of immunological defense before having this role assumed by the more specific effector T cells (111). While ILCs have an important function in the regulation of intestinal homeostasis, constant stimulation of these cells can result in major inflammation and tissue damage (122).

b. Adaptive Immunity in the GI Tract

Adaptive immunity is primarily comprised of B and T lymphocytes, which mediate the specific humoral and cellular immunity, respectively. In the gut, adaptive immune responses are initiated in gut-associated lymphoid tissues (GALT) and the mesenteric lymph nodes (MLNs), which function as inductive sites for the mucosal responses (96, 111). M cells in the intestinal mucosal continuously monitor luminal content and directly

transport antigens and microbes without processing them to GALTs where they are picked up by APCs. At the same time, dendritic cells in the lamina propria trap antigens either by projecting long processes between epithelial cells into the lumen or by directly picking up invading microbes that cross the epithelial barrier (86, 89, 111, 125). These activated dendritic cells process protein Ags from microbes and migrate to the MLN via afferent lymphatic vessels. In the GALTs and the MLNs, DCs present the foreign Ags to naïve T cells and promote their differentiation into effector and regulatory T cells (81). Differentiation of naive B cells into IgA secreting plasma cells also occurs at these sites through their interaction with activated effector T follicular helper (Tfh) cells (111). After their differentiation, effector lymphocytes home back to the lamina propria that functions as an effector site of the adaptive immune system (86).

5. Defective Mucosal Immunity in IBD

A vast body of literature outline roles for both defective mucosal barrier and dysfunctional intestinal immune system to luminal antigens in the pathogenesis of IBD.

a. Defective Intestinal Barrier Function

A dysregulated epithelial barrier represents a key component in IBD pathogenesis (81). Defects in the mucosal barrier include increased intestinal permeability, altered intestinal mucus, and defective antimicrobial peptide production (96, 126).

i. Intestinal Permeability

IBD is associated with increased intestinal epithelial lining permeability whereby a leaky gut barrier could facilitate the access of luminal antigens to the underlying mucosal tissues resulting in constant activation of the mucosal immune system (78, 81, 127). Abnormal intestinal permeability has been described in patients with Crohn's disease and ulcerative colitis (81, 89, 128). This has also been found in some healthy first-degree relatives of patients with IBD suggesting that the increased barrier permeability could be the primary defect involved in the initiation of inflammation and the predisposition IBD (78, 81, 86, 89, 129 – 131). The effect of a leaky barrier was shown in animal studies with the observation of increased tendency for the development of severe inflammation in areas of the intestine that lie beneath the disrupted locations (78, 79).

ii. Mucus Production

Goblet cell secreted gel-forming mucins constitute the loose mucus layer overlying the surface of the intestinal epithelium. In the small intestine, muc2 forms the main constituent of the mucus layer that is populated by luminal commensal microbes. In both forms of IBD, defects in the mucus production have been reported (132). Studies on $MUC2^{-/-}$ mice have reported the development of colitis, which demonstrates the contribution of mucus in providing protection against microbial invasion and intestinal inflammation (81, 133).

iii. Antimicrobial Peptide Production

One of the main classes of antimicrobial peptides secreted from Paneth cells is defensins. These peptides can be classified into α and β defensins. While β defensins are ubiquitously

distributed on the luminal surface of both the small and the large intestine of the gastrointestinal tract, α defensins are only expressed in the small intestine (89). Some observations have shown a decreased expression of β defensins in colonic Crohn's disease patients (81, 134). In comparison, the expression of Paneth cell α defensins was reduced in patients with ileal Crohn's disease (81, 86, 135). These combined data among others suggest an association of Paneth cell deficiency with an increased risk of the development of Crohn's disease (86, 96).

b. Innate Immune Dysregulation in IBD

IBD is characterized by excessive infiltration and immunoregulatory defects in various cellular components of the mucosal innate immune responses that are mediated by classic immune cells and nonimmune cells.

i. Role of Epithelial Cells in IBD

A disruption in the intestinal epithelial cell innate immune mechanisms has been found to contribute to IBD pathogenesis. One reported defect in IBD patients is the altered expression of TLRs on IECs. In a healthy state, IECs tend to constitutively express TLR3 and TLR5 and very scarcely express TLR4 (89). In the case of active Crohn's disease, the expression of TLR3 was observed to be significantly reduced (89). TLR5 is known to sense flagellin from invading bacteria. Due to its basolateral expression on IECs, TLR5 signaling is normally inactivated (96). During intestinal tissue injury, bacterial flagellin gains access to TLR5, stimulating the receptor to release proinflammatory mediators (87). CBir1

flagellin is a colitogenic antigen of intestinal luminal bacterial flora (136). In relation to IBD, seroreactivity to CBir1 bacterial flagellin was detected in around 50% of Crohn's disease patients (87, 136). At the same time, an upregulated expression of TLR4 was documented in both forms of IBD. Another IECs defect that is relevant to IBD pathogenesis is their abnormal antigen-presenting function that contributes to T-cell immunoregulatory responses (81, 96). IECS normally induce anergy in $CD4^+$ T cells and stimulate nonspecific $CD8^+$ T suppressor cells in *in vitro* systems (81, 89, 137). Conversely, IBD derived IECs acquire an activated phenotype with expression of costimulatory molecules and show a defective ability to stimulate $CD8^+$ T suppressor cells (81, 89).

ii. Involvement of Neutrophils and Macrophages in IBD

Neutrophils are the first type of leukocytes to home from the blood into sites of infection or tissue injury. Accordingly, the infiltration of the gut mucosa with neutrophils that occurs in response to chemokines produced by tissue-resident macrophages accounts for one of the earliest signs of intestinal inflammation (81, 111). Neutrophils play a key role in inflammatory processes contributing to IBD pathogenesis through a variety of mechanisms (78, 81). In one way, they release antimicrobial peptides and reactive oxygen species that further promote tissue destruction. They also secrete chemokines and multiples proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF- α), IL-1, IL-6, and IL-8, to recruit and activate macrophages that are derived from circulating monocytes and thereby perpetuate inflammation (78, 81, 138, 139). The recruited macrophages, unlike

resident macrophages, express TREM1 and release a broad array of proinflammatory mediators that also aggravate inflammation (86). An increased number of these macrophages was seen in the intestinal mucosa of patients with active IBD (140). These cells amplify inflammatory innate immune responses through the secretion of TNF-α, IL-1, IL-6, and IL12 and IL-18 (81, 140).

iii. Role of Dendritic Cells

In people with IBD, dendritic cells exhibit a defective antigen-presenting activity. As a consequence of a markedly reduced release of TSLP cytokines by IECs, DCs become improperly conditioned in Crohn's disease patients (81). Animal and *in vitro* studies show that these cells stimulate naïve T cell differentiation into effector Th1 and Th17 cells in response to commensal microbiota, which indicates a loss of tolerance towards commensals (89). These studies correlate with the increased frequency of activated DCs observed in the inflamed gut of patients with active IBD (89). Moreover, DCs in Crohn's disease upregulate their expression of chemokine receptors that mediate their migration and retention in the inflamed mucosa (81).

iv. Role of ILCs in IBD

A role for innate lymphoid cells in the pathogenesis of IBD was recently identified (81). The pathogenic function of ILCs is orchestrated by their IBD-relevant cytokines (81). IL-17 and IFN-γ secreting ILCs have been mainly associated with IBD (96). Increased production of IL-23 induced IL-17 cytokines in ILC3s was first described in mouse models of colitis

before the identification of ILCs in the gut of patients with IBD (81, 82, 141). In a complementary manner, genes linked to the ILC3 function were detected in ILCs isolated from IBD patients (141). Similarly, an increased frequency of ILC1 cells in the intestine of Crohn's disease patients was determined by various studies (122, 142). Moreover, humanized mice treated with the Dextran sodium sulfate (DSS), colitis-inducing agent, showed increased levels of IFN-γ-producing ILC1 in inflamed lesions of their intestines (122). In evidence for the involvement of ILC2s in IBD exacerbation, ILC2s secreted IL13 cytokines were reported to contribute to intestinal fibrotic lesions in CD patients (81). Overall, the intestinal accumulation of ILCs and the combination of their secreted cytokines suggest a contributory role for these cells in IBD.

c. Adaptive Immune Dysregulation in IBD

Intestinal homeostasis is maintained through a balance between proinflammatory and anti-inflammatory signals. A failure to maintain this balance results in intestinal inflammation that can happen due to an insufficient Treg cell function in the face of an excessive effector cell response (79, 87). In IBD, this balance is disturbed with evidence supporting that Crohn's disease is characterized by an excessive Th1 and Th17 response, while Ulcerative colitis is associated with an excessive atypical Th2 phenotype (81, 86).

i. Effector T-cell Abnormalities in Crohn's Disease

Crohn's disease was previously designated as a Th1 condition due to the biased production of cytokines associated with Th1 cells (79, 81, 82). Following the identification of IL-17 secreting Th17 cells and their implication in intestinal inflammation, the disease was

redefined as a mix of Th1 and Th17 profiles (81, 82). The Th1 phenotype of CD is exemplified by several colitis mouse models and human IBD investigations. The administration of 2,4,6-Trinitrobenzenesulfonic acid (TNBS), a chemically induced experimental colitis model, into mice is associated with a Th1-mediated mucosal inflammation that is characterized by an inclination towards the increased production of IL-12 and IFN-γ. At the histopathological level, TNBS gives rise to a transmural inflammation in the bowel wall with marked inflammatory cell infiltration (79, 143). The manifestations observed in TBNS colitis closely resemble the histopathological features of human CD (79). Human specimen research provided further evidence that Th1 cells are important mediators of inflammation in CD. As one example, an *ex vivo* study showed that IL-12 was overproduced by macrophages isolated from the mucosa of CD patients, while these cytokines secreted in reduced amounts by macrophage from UC patients, compared with those of healthy subjects (79, 144). $CD4^+$ T cells isolated from lesions of patients with CD also exhibited increased amounts of other proinflammatory mediators such as IFN-γ cytokines, and activated STAT4 and T-bet transcription factors in other studies (78, 96, 145). Recent IBD research has focused on IL-17-secreting Th17 cells as key factors in the disease pathogenesis. These cells are characterized by their production of IL-17 under the effect of IL-23 among other inflammatory mediators. A number of studies found immense Th17 cells infiltration in the mucosa and increased IL-17 expression in the intestinal mucosa and the serum of IBD patients compared to healthy controls (80, 81, 146, 147). These findings were higher in CD patients than in UC patients (147). In addition to IL-17, other Th17-derived cytokines were also identified at elevated levels in the gut of patients

with both forms of IBD (80, 96, 148, 149). Providing profound support for the role of th17 cells in the progression of IBD are experiments that highlight the importance of IL-23 in the induction of colitis. This can be exemplified in studies carried out on mice with mutations in the IL-23 receptor that result in reduced risk of colitis, suggesting that IL-23 is a key driver for the development of intestinal inflammation in murine models of colitis (86, 96, 150).

ii. Effector T-cell Abnormalities in Ulcerative Colitis

Unlike the Th1 condition associated with CD, ulcerative colitis exhibits a Th2-mediated immune response marked by an elevated production of Th2 cytokine profile. Although there is no proof for an enhanced expression of IL-4, the typical Th2 cytokine, in UC, sizeable amount of data from mouse colitis and human IBD studies support that mucosal inflammation in UC is mediated by Th2 processes. The administration of oxazolone, a chemically induced colitis model, promotes a Th2 response accompanied by markedly elevated IL-13 and IL-5 production (79). It was recently shown that IL-13-producing natural-killer T (NKT) cells contribute to the intestinal inflammation observed in the oxazolone-induced colitis whereby the depletion of these cells or the blockade of IL-13 strikingly ameliorates the condition (79). Histopathologic examinations of oxazolone-induced colitis indicate that this Th2-cell-mediated model mimics human UC and induces a relatively superficial mucosal inflammation characterized by epithelial cell loss and colonic infiltration by neutrophils and lymphocytes (79). Similar to oxazolone-induced colitis cytokine patterns, lamina propria cells derived from UC patients showed increased secretions of IL-13 and IL-5 cytokines (96). Additional evidence for the presence of Th2

phenotype in UC is the finding of activated humoral immune responses manifested by the production of autoantibodies in this form of IBD. This indication is in accordance with the dependence of the B cell-mediated immune responses on their activation by Th2 cells to a greater extent than Th1 cells (96). Among the observed autoantibodies is the atypical perinuclear antineutrophil cytoplasmic antibody (pANCA) that is prevalent in the sera of UC patients at a range from 50 to 90% (79, 81, 96). These patients also exhibit an antibody response against a putative colonic autoantigen tentatively recognized as tropomyosin (88, 90, 105). Moreover, examinations of the produced immunoglobulin subclass in UC patients again demonstrate a bias toward the Th2 immune response with the expansive of IgG1 and IgG4, which are Th2 related (79).

iii. Defective T-cell-mediated Regulation in IBD

Given that Tregs perform a critical role in maintaining tolerance towards intestinal commensal organisms and controlling the development of severe inflammations, prompts the notion that an underlying defect in oral tolerance and in the suppressor function of Tregs can be involved in the pathogenesis of IBD. Such association was reflected in an *in vitro* assay that compared the difference between the response of T cells isolated from IBD patients and healthy controls when culture with their own mucosal microflora. In the study, only the T cells of IBD patients induced an immune response against their own mucosal antigens and showed an increased proliferation and cytokine production in culture, indicating a loss of tolerance towards commensals in the mucosa (79, 96, 151). *In vivo* studies also support this association as they show increased susceptibility to IBD in models of aberrant Treg cell function. For example, intestinal inflammation was observed in IL-10

knockout mice and in those with impaired Treg related TGF-β signaling (79, 80, 152, 153). Additionally, there is also evidence that Tregs can reverse the development of intestinal inflammation. This was shown in a study conducted on immunodeficient mice in which colitis induced by the administration of naïve T cells lacking Tregs was prevented after the introduction of $CD4^+CD25^+$ Tregs (78, 80, 154, 155).

6. Association of EBV with IBD

Over the past years, a wide range of pathogens, including bacteria, parasites, and viruses, have been implicated in the pathogenesis of IBD. The infection of genetically susceptible hosts with these agents is proposed to activate immunopathologic mechanisms that eventually result in chronic inflammation (34, 156). Given that EBV is ubiquitous in the general population and that its reactivation from latency can occur at any site where B cells reside, a number of studies have repeatedly considered the possible association of EBV infection with IBD; however, it remains unclear whether the virus contributes to the pathogenesis or the exacerbation of the disease (35, 36, 157 -161). As a first step in exploring the potential role of EBV in IBD, Wakefield et al. (157) examined the prevalence of EBV in colonic specimens of IBD patients using polymerase chain reaction (PCR). The study reported more frequent numbers of EBV-infected cells in UC and, to a lesser extent in CD samples, compared to control tissues obtained from patients with non-inflammatory diseases. These results were replicated by several subsequent studies that took further measures to elucidate the correlation between EBV infection and the development of IBD (36, 158 -160). In an attempt to provide evidence of EBV infection in precise locations of

the colonic mucosa, Yanai et al. (158) performed in situ hybridization for EBV-encoded RNA 1 (EBER-1) as a marker of latent infection. The authors documented a preferential localization of EBER-1-positive cells to areas of inflamed mucosa compared to areas of non-inflammatory activity in the colonic specimens of both forms of the disease reflecting a possible contribution of EBV infection to the local inflammation observed in IBD. Furthermore, this group also reported that the EBER-1 positivity was localized to non-epithelial cells in IBD tissues, a finding that aligned with other relevant studies (36, 159). In this context, based on in situ hybridization morphological data, Spieker and Herbst indicated that all EBER-positive cells were lymphocytes and specified a predominance of B cells (36). The work of these researchers brought further distinction between UC and CD in terms of EBV distribution and gene expression. They documented that the more frequent EBER-positive cells in ulcerative colitis accumulated in a transmural pattern in sub- and intraepithelial layers of the colonic specimens and suggested that this intra-mucosal expansion of EBV-infected cells might be associated with the locally impaired antiviral immunity that aggravated the inflammatory processes in IBD (36, 161). Moreover, Spieker and Herbst used EBER (1 and 2) and BamHI-Z leftward frame number 1 (BZLF1) markers to detect the presence of EBV antigens associated with latent and lytic infection, respectively. Expression of BZLF1 was detected only in cases of ulcerative colitis in that study along with EBER positivity, which signified that both active viral replication and latent EBV infection of lymphocytes contribute to the high viral levels in UC. Knowing that in immunocompetent hosts, EBV-infected cells escape detection by cytotoxic T cells due to the limited latent gene expression, the occurrence of active EBV replication might indicate an altered immune responsiveness to this virus in UC patients. In this regard, one

possible explanation has been attributed to the predominant Th2 lymphocyte profile in UC that produce cytokines known to stimulate the growth of EBV transformed B cell and to the EBV viral IL-10 homologue that inhibits the production of Th1 and NK cell cytokines (35, 36, 157, 159, 162). Overall, these findings raise the possibility for a role of EBV infection in the perpetuation of UC associated inflammation (35, 36, 159). Yet, no clinical correlates were addressed in these studies, and only a small number of references in the literature were found on the clinical relevance of EBV colonic infection in IBD (162 – 165). One cross-sectional study assessed the relation between EBV prevalence in colonic lesions of Chinese IBD patients and the clinical disease activity (162). Investigators determined the clinical activity of UC and CD through the Mayo Clinic score and Crohn's disease activity index, respectively. They showed that the EBV load increased as the clinical activity of the disease aggravated, indicating a directly proportional correction between the two variables. EBV infection was also shown to result in increased clinical complications in IBD patients, especially in those being treated with immunosuppressive drugs as described in few case reports (164, 165). Despite all of these observations, the definitive involvement of EBV in IBD cannot be inferred, and different features of this relation remain to be elucidated.

7. Treatment

Classical treatment of IBD involves the intensive administration of immunomodulators and biologics, individually or in combination, coupled with surgery that is often specified as a last resort. One example of current therapy involving a combination of the immunosuppressive drug azathioprine together with infliximab, which is an anti-tumor necrosis factor (TNF) agent, was shown to be effective in maintaining disease remission for

both forms of IBD (80). Progress in the understanding of the factors involved in IBD pathogenesis continues to provide a roadmap for the development of new targeted treatment approaches to this disease. Among the emerging therapies are those that target cytokines relevant to the inflammatory cascade of IBD. Given that Th1 and Th17 cell responses are important mediators of inflammation in Crohn's disease, inhibition of their respective cytokines was foreseen as a possible promising method. In this regard, neutralizing monoclonal antibodies against cytokines or their receptors were employed. It was documented that antibodies specific for TNF effectively treated Crohn's disease case secondary to their apoptotic action on Th1 effector cells (79). The use of antibodies against the proinflammatory IL-12 and IL-23 cytokines that promote the differentiation and maintenance of Th1 and Th17 cells was also proven to attenuate colitis severity in mouse models (80). However, the relevance of blocking IL-17 cytokine in the treatment of intestinal inflammation is still disputed as conflicting results were observed in different experimental models of colitis (80, 166). In the case of UC, it has been proposed that using antibodies against the overly produced Th2 cytokines, such as IL-13 specific antibodies, might possibly yield a positive therapeutic effect on this disease (79). Another potential strategy for IBD treatment involves blocking the cell-signaling pathways that are associated with the perpetuated activation of NF-κB in IBD patients (78). One suggested approach to achieve this is through the use of proteasome inhibitors that prevent the degradation of the inhibitory kappa B (IκB) protein and consequently block the activation of the NF-κB pathway (167). An additional target for therapeutic intervention is the α4β7 integrin that is expressed on the surface of lymphocytes. Antibodies targeting this integrin block the

homing of lymphocytes to the intestinal mucosa in an effort to reduce the excessive inflammation (79). Moreover, a promising option that is increasingly being explored in animal and human studies is restoring the immune tolerance by recomposing the commensal microflora in the gut (78, 168). This is performed by the introduction of nonpathogenic enteric or genetically engineered bacteria in the inflamed mucosa in order to reconstitute the gut microbiota and induce the activity of regulatory cells (79). Considering that predisposing genetic variants contribute to the pathogenesis of IBD, gene therapy might possibly present a curative approach whereby the defective genes are replaced. Recent preclinical studies for intestinal gene transfer demonstrate the feasibility of this approach (143, 169). With the constant drive to gain further insight into the pathogenesis of this multifactorial disease, new therapeutic opportunities for targeting the inflammatory processes in a tailored manner will come to light.

CHAPTER III

MATERIALS AND METHODS

A. Mice

Female C57BL/6J mice between six to eight weeks old were used in the study. These mice were obtained from the Animal Care Facility at the American University of Beirut (AUB), after approval of the experimental protocols by the Institutional Animal Care and Use Committee (IACUC) of the university, and treated in accordance with the institutional guidelines.

B. Induction of Acute DSS Colitis in C57BL/6J Mice

Various animal models have been instrumental in the understanding of the complex mechanisms underlying the development and pathogenesis of IBD. Among the array of chemical incitants of experimental colitis, dextran sodium sulfate (DSS) model is commonly adopted because it provides results immediately and controllably in a relatively simple and reproducible manner. This chemical is a sulfated polysaccharide employed in mice to permit the development of intestinal inflammation with features representative of human IBD, particularly UC, secondary to its toxic effect to colonic epithelium (170 -172). The approach to establishing acute colitis is based on feeding mice for an average of 7 successive days with DSS dissolved in drinking water. Prior to assessing whether EBV DNA is associated with increased severity of colitis in mice, it was essential to investigate the possible execution of DSS-induced colitis in C57BL/6J mice and to optimize the model

for application in future experiments. To this end, a total of twenty-five C57BL/6J mice were used. These mice were equally divided into five groups, each containing five mice. Four mouse groups were orally administered 0.5%, 1%, 1.5% or 2.5% DSS (molecular weight 40kDa; Chondrex, Redmond, WA) in drinking water for 7 days. The fifth group served as a control and received normal sterile water that was not supplemented with DSS. All groups were also given sterile water, the vehicle, by rectal gavage on day 3 to account for the route of DNA administration in the experiment assessing the effect of the nucleic acid as described in section C below. Per group, water bottles were filled with 100 ml of normal or DSS water, and these were replaced with freshly prepared solutions every 2 days (170), while rectal administrations were performed on sevoflurane-anesthetized mice using a 3.5 French catheter and consisted of a volume of 100μl. The clinical course of each mouse was monitored on a daily basis and data pertaining to their body weight, stool consistency, and fecal blood was collected and used to calculate the disease activity index (DAI) as per the scoring method previously established by Cooper et al. (173) and described in section D below. After the experimental period, mice were sacrificed by cardiac puncture following anesthesia with sevoflurane and their colons were collected for total length measurement. The scheme and timeline of the experiment are shown in Figure 1.

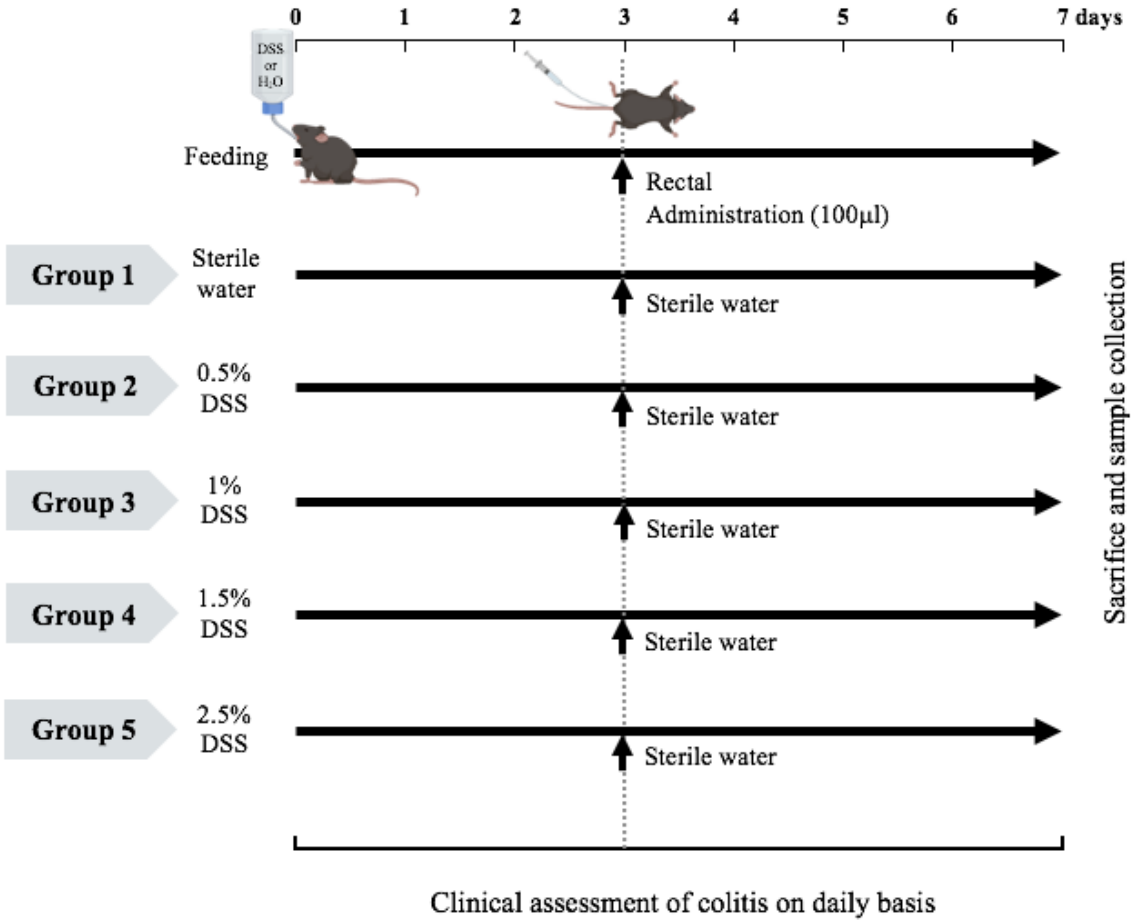

Figure 1: Experimental setup and timeline of dextran sodium sulfate (DSS) concentration selection for the C57BL/6J mouse acute colitis model. DSS was dissolved in sterile drinking water at a concentration of 0.5%, 1%, 1.5%, and 2.5% and given respectively to mice of groups 2, 3, 4, and 5 from day 0 to 7. On the other hand, mice of group 1 served as a control and received sterile drinking water instead of being challenged with DSS. On day 3, mice were rectally administered sterile water. All mice (n = 5 per group) were weighed and evaluated for clinical symptoms on a daily basis throughout the experimental period. Mice were sacrificed on day 7 for collection of biological samples.

C. Treatment of Colitic Mice with EBV DNA

To assess the effect of EBV DNA on the severity of colitis in mice, a total of forty-six mice were obtained and distributed into four groups. Group one included nine mice and served as

a negative control whereby mice received 100µl of sterile water (the DNA diluent) by rectal gavage on day 3 to control for any inflammatory response that might result from the method of DNA administration and had no DSS added to their sterilized drinking water throughout the experimental period. Acute colitis was induced by oral administration of 1.5% (w/v) DSS in autoclaved drinking water *ad libitum* from day 0 to 7 in the second group of fourteen mice that were also rectally given 100µl of sterile water on day 3. The optimal concentration of DSS was determined based on the evaluation of clinical observations in the DSS assessment experiment described in section B. In group three, the fourteen mice were treated with 1.5% DSS in the same manner as in group two and rectally received EDV DNA (Vircell, Granada, Spain) as a dose of 288 x 10^3 DNA copies in 100µl of sterile water on day 3. The EBV DNA copy number was chosen after considering the 144 x 10^3 copies of DNA that induced the highest levels of IL-17A following its systemic injection in mice in a previous study conducted by our group and then doubling the concentration in an optimization experiment to account for any DNA degradation by the vast proportion of microbial flora in the gut owing to rectal administered (11). Moreover, for comparison purposes, the fourth group that included nine mice received EBV DNA by rectal gavage, together with oral intake of drinking water from day 0 without being treated with DSS. The DSS feeding and rectal administration were performed in a similar manner as in the previous section. During the experimental period, all mice were evaluated on a daily basis for weight loss, stool consistency, and presence of blood in stool in assessment of the DAI. On day 7, mice were sacrificed by cardiac puncture after sedation with sevoflurane and their colons were excised. The full length of each mouse colon, expected to

become shorter with increased severity of colitis, was measured. Then, a fragment from the colon distal end was fixed in 10% formaldehyde for future histological injury assessment of hematoxylin and eosin stained sections. The remaining colon portions were placed in individually labeled cryovials containing 1ml of freezing media (90% fetal bovine serum + 10% dimethyl sulfoxide) and stored in liquid nitrogen for further analysis of immune cell composition using flow cytometry at a later time. The schematic diagram of the experimental design is shown in Figure 2.

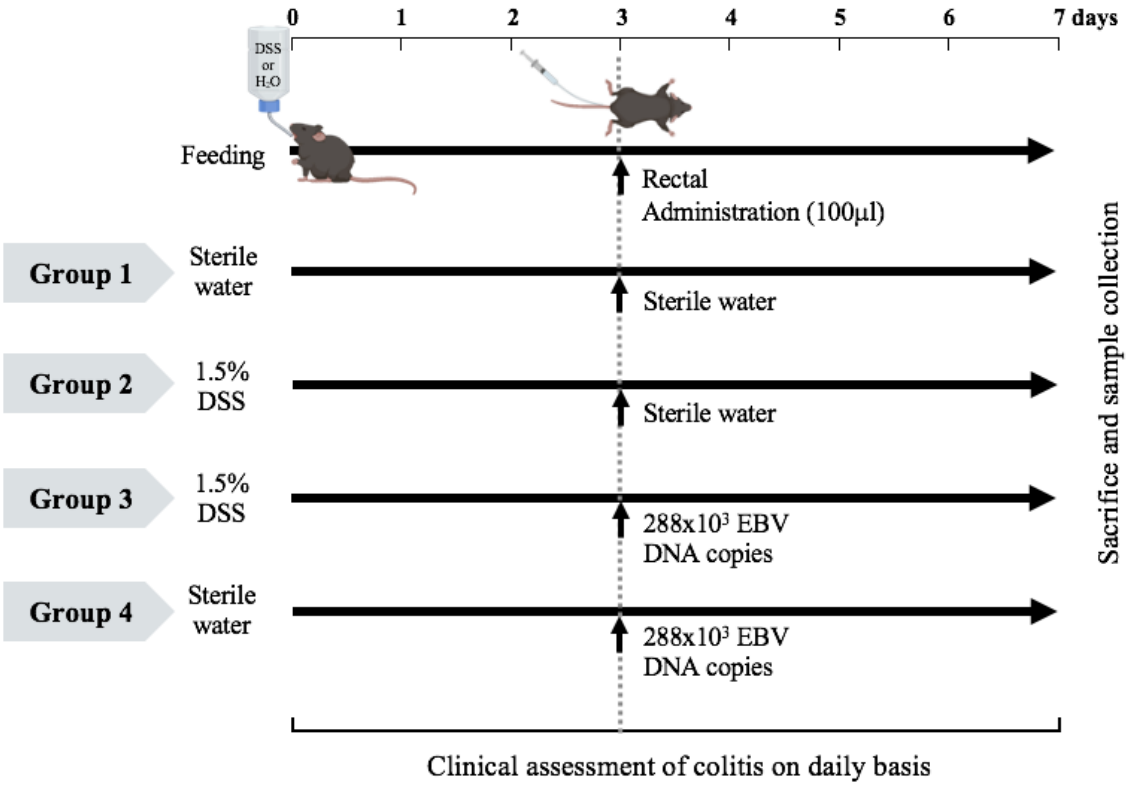

Figure 2: Experimental design used to assess the effect of Epstein-Barr virus (EBV) DNA on colitis severity in C57BL/6J mice. C57BL/6J mice were assigned to one of four groups. In groups 2 and 3 (n = 14), acute colitis was induced by administration of 1.5% dextran sodium sulfate (DSS) in drinking water for 7 days. On the third day of the experimental period, group 2 was rectally administered sterile water, while group 3 was rectally administered EBV DNA in sterile water. The other two groups (n = 9) were included as

controls. Both groups fed on DSS-free sterile water, but group 4 received EBV DNA in sterile water on day 3 by rectal gavage. All mouse groups were clinically assessed for colitis activity on daily basis and then sacrificed on day 7 for collection of biological samples.

D. Clinical Evaluation of Colitis

Clinical activity of colitis was utilized as a surrogate marker to assess the degree of disease severity. In essence, oral administration of DSS to mice via drinking water induces severe colitis that is characterized by weight loss, watery diarrhea, and fecal bleeding. These clinical criteria were summarized as the disease activity index (DAI) in accordance to Cooper's grading system of DSS-induced colitis model (173). In this manner, all mice were examined and scored for changes in body weight, stool consistency, and occult/gross fecal blood positivity from day 0 and continuing on daily basis to determine the DAI. Then, the DAI was calculated as the combined score of these three criteria which ranges from 0 to 12. The method of scoring is shown in table 1.

1. Weight Loss

The body weight of each mouse was measured from day 0 prior to the onset of the experiment till the sacrifice day and the weight loss was calculated as the percentage difference between the weight on any specific day (X) and the baseline weight on day 0 according to the following formula:% Weight change =

$$\left| \frac{(\text{weight on day 0} - \text{weight on day X})}{\text{weight of day 0}} \right| \text{x100.}$$

The resulting percentages were scored from 0 to 4 as follows: 0: ≥ 0% weight change, 1: 1-5% weight loss, 2: 6-10% weight loss, 3: 11-15% weight loss, 4: >15% weight loss. Generally, any weight loss of 20 to 25% relative to the initial weight was considered a humane endpoint in compliance with the IACUC guidelines.

2. Stool Consistency

Fresh stool samples were collected daily from all mice and evaluated for consistency. This was performed by handling each mouse properly by the scruff of the neck and turning it around on the ventral surface to collect feces in individual Eppendorf tubes as it defecates. Using a pair of forceps or a disposable pipette tip, obtained stool samples were then pressed down to determine their firmness. Well-formed solid pellets were assigned a score of 0, loose stools that readily become pasty and semi-formed upon pressing down were given a score of 2, and watery stool that gets stuck around the anus and also on the base of the tail in advanced stages received a score of 4. On the sacrifice day, stool samples were collected from the colon and assessed accordingly.

3. Blood in Stools

The presence of blood in the stools was assessed every day following stool collection using a benzidine guaiac method for fecal occult blood detection as previously described by Hughes with some modifications (174). This type of benzidine test allows for the identification of blood in stools that is not readily visible upon gross inspection through the oxidation of this chromogen in acid solution and the production of a blue color. The oxidation reaction that takes place is catalyzed by the iron (Fe) contained in hemoglobin

that acts as the peroxidase enzyme facilitating the transfer of an oxygen atom from hydrogen peroxide to benzidine. After recording benzidine test results, the status of fecal blood was then scored with 0 for negative occult blood results, 2 for positive hemoccult results, and 4 for stool with gross blood signs that also became visible on the mouse anal region in advanced stages of colitis.

a. Benzidine Fecal Occult Blood Test Procedure

A fresh solution of saturated benzidine in glacial acetic acid was prepared daily before each run of tests since its shelf-life is 8 hours (5g benzidine + 50 ml glacial acetic acid). Then, 1ml of this solution was transferred to 2 ml Eppendorf tubes each containing a pea-sized piece of the collected stools per mouse per data point and vigorously mixed using an applicator stick to obtain an emulsion. For the positive control, a drop of blood was placed in an Eppendorf tube instead of stools, while the negative control tube was left empty before the addition of saturated benzidine. Subsequently, one or two drops of each feces solution were slowly added to a glass test tube two-thirds filled with 3% hydrogen peroxide and observed for color change against a white background. As the drop starts to descend downwards in the test tube, it forms a cloud that remains white in the case of a normal negative sample but becomes slightly or completely blue-green with a positive sample.

Table 1: Disease activity index (DAI) scoring system used for evaluation of dextran sodium sulfate (DSS) colitis in C57BL/6J mice (173, 175).

	Clinical parameter		
Score	**Weight loss (%)**	**Stool consistency**	**Blood in feces**
0	None	Normal	None
1	1-5	-	-
2	6-10	Loose stools	Occult bleeding
3	11-15	-	-
4	>15	Diarrhea	Gross bleeding

E. Colon Macroscopic Assessment

Another indirect metric of the severity of colitis is the overall colonic length that tends to shorten with the growing inflammation (176, 177). At the end of the clinical monitoring period, euthanasia was performed on all mice by cardiac puncture after inhaled sevoflurane anesthesia and followed by a midline laparotomy incision for the entire collection of colon samples from the ileocecal junction to the distal end of the rectum. Each isolated colon was then straightened without stretching over a ruler and its full length was measured with the exclusion of the cecum. Subsequently, every colon tissue was then cleaned by repetitive flushing with 1x ice-cold phosphate-buffered saline (PBS) using a 25G needle affixed to a 10ml syringe until its fecal content was cleared. Eventually, the cleaned colons were cut into small fragments to be further processed for histological damage grading and immune cell profile analysis at a future stage.

F. Statistical Analysis

Statistical analysis was carried out using the GraphPad Prism software. The differences in the disease activity index scores were evaluated using the Mann-Whitney U test. The unpaired t-test was performed to assess the statistical significance of body weight changes and colon length measurements. P-values less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

A. Selection of Dextran Sodium Sulfate (DSS) Concentration for the C57BL/6J Mouse Acute Colitis Model

Prior to examining the potential role for EBV DNA in the exacerbation of colitis severity in mice, it was necessary to assess the functionality of a murine model of colitis induced by DSS in C57BL/6J strain and to standardize the optimal DSS dosage at which disease development occurs while leaving a margin to observe an additive effect of EBV DNA on its activity in subsequent aims. In this experiment, 0.5%, 1%, 1.5%, and 2.5% of DSS were administered in drinking water for induction of colitis in C57BL/6J mice and comparison between these concentrations was carried out on the basis of DAI and colon length assessments that served as indicators of colitis severity.

At this stage, the DAI, which is a composite measure of weight loss, stool consistency, and blood in stools, was determined daily to evaluate the clinical course of each group of C57BL/6J mice in response to different concentrations of DSS. In comparison to the control group that fed on normal drinking water, all DSS-treated groups presented increased DAI scores (Table 2, Figure 3). Mice subjected to 2.5% DSS showed a variability in increasing DAI scores during the first 3 days and then started to manifest significantly elevated scores compared to the control group from day 4 (average DAI of 2.6 in 2.5% DSS group vs 0.6 in control group, $p = 0.0219$) until the end of the experiment on day 7 (average DAI of 11 in 2.5% DSS group vs 1 in control group, $p = 0.0106$). In

contrast, the first significant rise in the average DAI score of mice treated with 1.5% DSS was observed on day 5 (DAI of 2.4 in 1.5% DSS group vs 0.6 in control group, $p = 0.0219$). Moreover in this group, the DAI score increased in a gradual and steady manner starting from day 2 and then continued to rise more dramatically from day 4, reaching an average DAI of 8.6 in 1.5% DSS group vs 1 in the control group ($p = 0.0117$) on day 7. In a similar way to the 2.5% treated mice, the average DAI score of the 1% DSS group fluctuated from day to day; however, no significant elevation in the score was observed before day 7 (4.2 in 1% DSS group vs 1 in control group, $p = 0.0306$). On the other hand, the time frame of the experiment did not allow for the development of diarrhea and any gross signs of blood in stools in the 0.5% DSS group which showed a slight and insignificant increase compared to the control group (p-value > 0.05). In fact, gross signs of rectal bleeding were only clearly manifested in the 1.5% and the 2.5% DSS treated groups, appearing one day earlier in the latter group than the 1.5% DSS group which presented rectal bleeding on day 7. Figure 4 is a representative of this gross rectal bleeding in the 1.5% DSS mouse group on day 7 of treatment. Collectively, these results indicate that the colitogenic potential of DSS and the clinical severity of the induced colitis, which is reflected by the DAI, are directly dependent on the administered concentration of DSS.

Another marker of disease severity is colon length shortening that commonly accompanies the development of DSS-induced acute colitis and serves as a macroscopic indicator of colonic inflammation (171). On day 7 of DSS administration, all mice were sacrificed and the length of their collected colons was measured. Macroscopic examination of the obtained colons revealed that the 1%, 1.5%, and 2.5% DSS-treated groups exhibited shorter colons than those of the normal water-fed control group; however, the decrease in

the colon length was only significant in the groups that received 1.5% or 2.5% DSS in drinking water (Figure 5). The average colon length was 6.6 cm in the control group. The 0.5% DSS group showed comparable measurements to the control group with an average colon length of 6.58 cm ($p = 0.9441$). The average colon length was 6.36 cm in the 1% DSS treated group ($p = 0.4111$), 5.46 cm in the 1.5% DSS treated group ($p = 0.0009$), and 5 cm in the 2.5% DSS treated group ($p = 0.0001$). Moreover, the average colon length in the 2.5% DSS was also markedly shorter than that of the 1.5% DSS treated group ($p = 0.0016$). These results indicate that a more prominent reduction in the colon length and thus further colonic inflammation is observed with increasing concentrations of DSS. These findings also indicate a positive correlation between the degree of colon length shortening and the DAI with reference to DSS-induced colitis in C57BL/J6 mice as previously reported (178 -180).

Overall, these results taken together show that C57BL/J6 mice can develop acute experimental colitis induced by DSS of a clinical severity and colonic inflammatory extent that correlate with the DSS concentration. In this regard, the progressive onset of colitis, together with the moderately intense severity of clinical symptoms, and the evident colonic shrinkage that were observed with 1.5% DSS treatment in this experiment, provided grounds for the selection of this concentration to investigate the additive effect of EBV DNA on the severity of colitis.

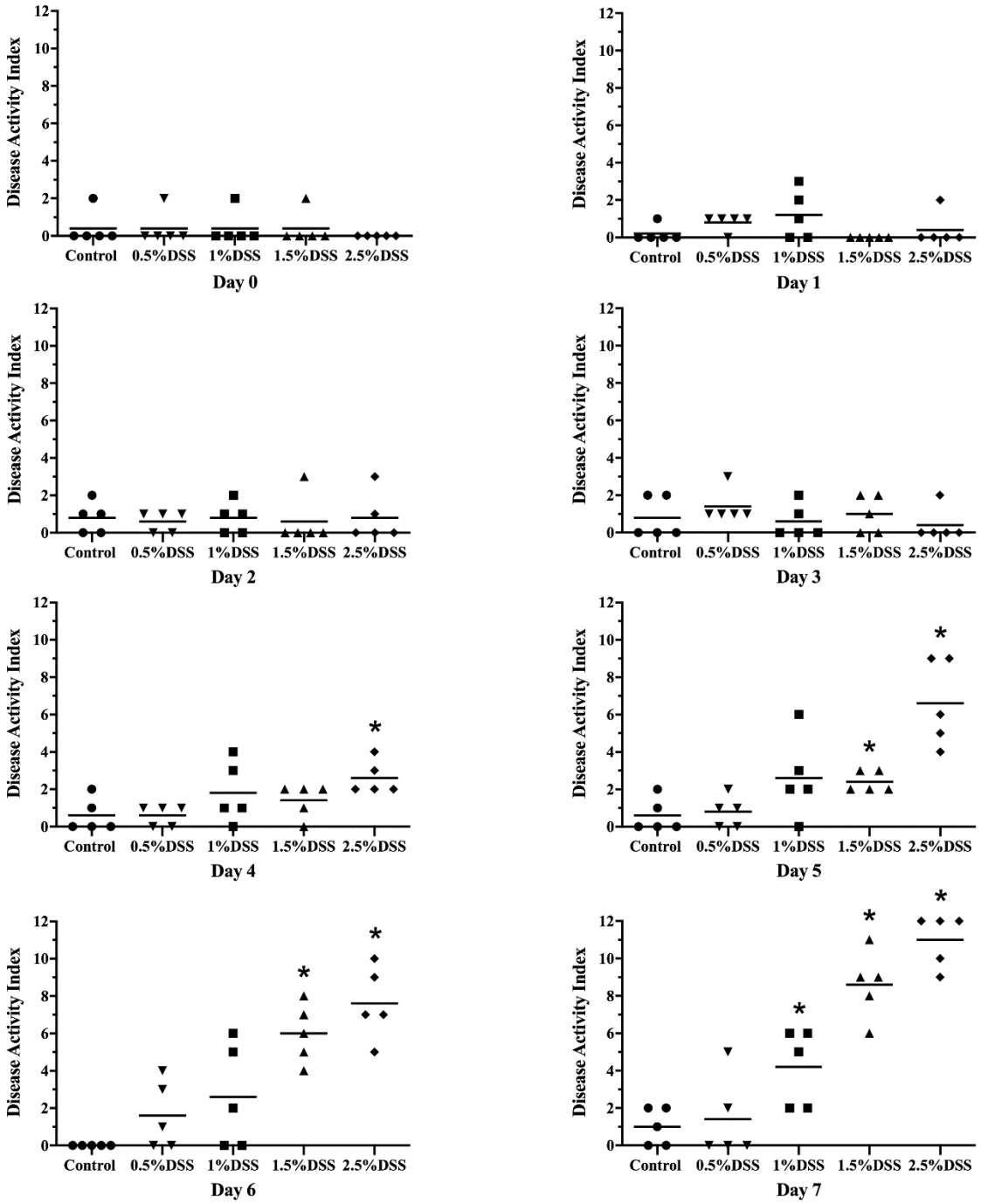

Figure 3: Disease activity index (DAI) scores in each group of C57BL/6J mice of the dextran sodium sulfate (DSS) concentration selection protocol. Mouse groups were orally administered 0.5%, 1%, 1.5%, or 2.5% DSS in drinking water from day 0 to 7 to induce acute colitis except for the control group which was fed on normal drinking water. On day 3, mice were rectally administered sterile water. The DAI was determined daily as a composite measure of the scores of body weight loss, stool consistency, and fecal blood. *indicates p-value <0.05 when compared to the control group on the same day.

Figure 4: Representative of gross rectal bleeding exhibited on day 7 by C57BL/6J mice treated with 1.5% dextran sodium sulfate (DSS) in drinking water.

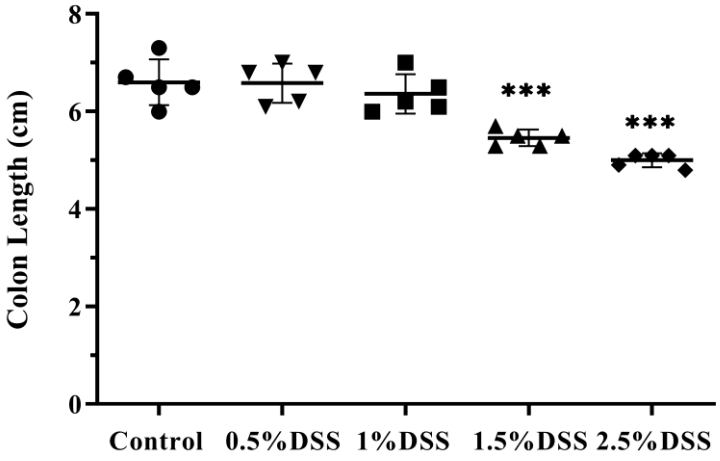

Figure 5: Colon length measurements in each group of C57BL/6J mice after treatment with different concentrations of dextran sodium sulfate (DSS) in drinking water for 7 days for DSS concentration selection. Mouse groups were orally administered 0.5%, 1%, 1.5% or 2.5% DSS in drinking water from day 0 to 7 to induce acute colitis except for the control group which was fed on normal drinking water (n= 5 mice per group). On day 3, mice were rectally administered sterile water. On day 7, mice were sacrificed and their colon lengths were measured. *** p-value <0.001, compared to the control group.

Table 2: Average disease activity index (DAI) in each group of C57BL/6J mice of the dextran sodium sulfate (DSS) concentration selection protocol.

Mouse group	Disease activity index							
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Control (n = 5)	0.4 ± 0.89	0.2 ± 0.45	0.8 ± 0.84	0.8 ± 1.10	0.6 ± 0.89	0.6 ± 0.89	0 ± 0	1 ± 1.00
0.5% DSS (n = 5)	0.4 ± 0.89	0.8 ± 0.45	0.6 ± 0.55	1.4 ± 0.89	0.6 ± 0.55	0.8 ± 0.84	1.6 ± 1.82	1.4 ± 2.19
1% DSS (n = 5)	0.4 ± 0.89	1.2 ± 1.30	0.8 ± 0.84	0.6 ± 0.89	1.8 ± 1.64	2.6 ± 2.19	2.6 ± 2.79	4.2 ± 2.05
1.5% DSS (n = 5)	0.4 ± 0.89	0 ± 0	0.6 ± 1.34	1 ± 1.00	1.4 ± 0.89	2.4 ± 0.55	6 ± 1.58	8.6 ± 1.82
2.5% DSS (n = 5)	0 ± 0	0.4 ± 0.89	0.8 ± 1.3	0.4 ± 0.89	2.6 ± 0.89	6.6 ± 2.3	7.6 ± 1.95	11 ± 1.41
Values are expressed as average DAI per group ± standard deviation.								

B. Effect of EBV DNA on Clinical Manifestations and Macroscopic Inflammatory Markers of Colitis in a Mouse Model of the Disease

After evaluating the colitogenic potential of the DSS-induced colitis in C57BL/6J mice and determining the optimal conditions that serve the objective of this study, the effect of EBV DNA on the severity of colitis was subsequently assessed according to clinical manifestations and macroscopic inflammatory markers. Herein, evaluation of body weight changes, DAI progression, and colon length shortening was carried out on the experimental and control groups of mice that were assigned for this aim.

The body weight of each mouse group was measured every day and expressed as a percent difference between its value on a particular day and that of day 0 to represent disease progression during the study period and to provide a measure for comparison that eliminates any significant initial weight difference between mouse groups (Figure 6).

Initially, all mouse groups showed weight gain until day 5. This pattern of weight gain continued till the end of the experiment in the normal and the EBV DNA control groups which exhibited an average weight gain of 2.6% and 4.9%, respectively. In contrast, DSS treatment resulted in a decrease in the body weight of affected mice starting from day 6 and reaching on day 7 an average of 2.8% weight loss in the group that fed on DSS alone and an average of 6.4% weight loss in the group that received EBV DNA in addition to DSS. Moreover, these groups manifested a significant weight loss relative to the control group after day six (DSS vs control $p = 0.011$, DSS plus EBV DNA vs control $p = 0.0005$); however, no significant difference in the weight loss between the two DSS-treated groups was observed throughout the experiment. Worth indicating is that the reduction in the body weight was noted with the initiation of fecal bleeding in the DSS-treated groups.

Similarly, the DAI of all mouse groups was scored on a daily basis to evaluate the severity of the clinical course of colitis disease. The DAI score was increased in the two DSS-administered groups compared to the normal water-fed group (Table 3, Figure 7). Initially, the DAI scores gradually increased in both groups after day 2; this increase became significantly higher than the normal control on day 5. At this time point, the average DAI score was 2.86 for the group that fed on DSS alone ($p = 0.0013$) and 3.50 for the group that received EBV DNA in addition to DSS ($p = 0.0004$), indicating that the latter group had a higher level of significance than the other DSS group with respect to the normal control. Following day 5, both groups exhibited a dramatically increased rise (p-value <0.0001) in their DAI scores which was associated with a significant worsening of the clinical signs of colitis disease. Toward the end of the experiment on day 7, a statistically significant difference ($p = 0.0044$) was obtained between these DSS-treated

groups with an average DAI score of 10.07 for the DSS and EBV DNA group but an average score of 8.50 for the DSS alone group. Moreover, the DAI scores of all but one mouse in the group that received EBV DNA in addition to DSS was 9 or higher, whereas half of the mice that received DSS alone had a DAI score of 9 or higher. As for the control groups, a low average DAI score of 0.78 and 1 was observed in the normal water and EBV DNA alone groups, respectively.

The severity of colitis in the IBD mouse model was also evaluated based on macroscopic examination of colon samples collected from each mouse group on day 7 for any colon length shortening, which is a marker of inflammation. Consistent with the DAI, the colon length was significantly shorter in both groups that received DSS compared to the normal water-fed control group; however, the greatest colon shortening was observed in the group that received EBV DNA in addition to DSS (average colon length in DSS alone group of 5.7 cm vs 6.6 cm in control group, $p = 0.0003$; DSS plus EBV DNA: 4.8 cm, $p = 0.0001$) (Figure 8). Moreover, there was also a significant reduction in the colon length comparing the DSS alone group and the DSS plus EBV DNA group ($p = 0.0002$). In particular, the majority of mice in the group that received EBV DNA in addition to DSS had a colon length of 5.3 cm or shorter, while most of the mice that received DSS alone had a colon length of 5.3 cm or longer.

Overall, the observation of a significantly higher DAI and more marked colon length shortening, together with an increased body weight loss in mice treated with EBV DNA in addition to DSS in comparison to mice treated with DSS alone indicates that EBV DNA exacerbates the clinical signs and colonic inflammation of colitis in the mouse model of the disease.

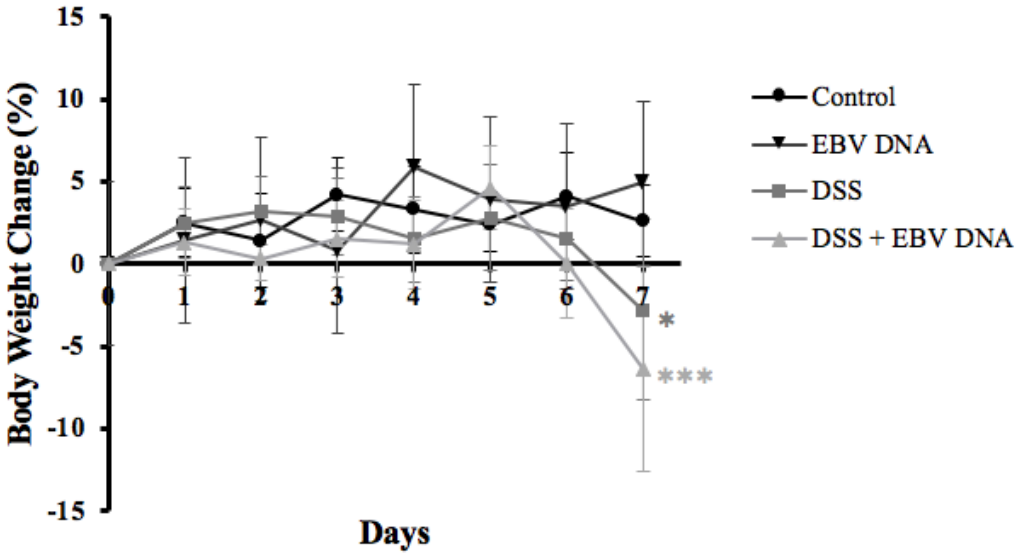

Figure 6: Average percent body weight change in control and experimental mouse groups used to assess the effect of Epstein-Barr virus (EBV) DNA on colitis severity in C57BL/6J mice. Mouse groups received either 1.5% DSS-containing or normal drinking water for 7 days. The two DSS-treated groups were then rectally administered sterile water or Epstein-Barr virus DNA in sterile water on day 3 (n = 14 mice per group). The two other normal drinking water-fed groups were included as controls and also received sterile water or EBV DNA by rectal gavage on day 3 (n = 9). Mouse body weight was evaluated daily and the percent body weight change was calculated per mouse per group compared to its initial weight on day 0. * p-value <0.05, *** p <0.001, compared to the control group on the respective day of measurement.

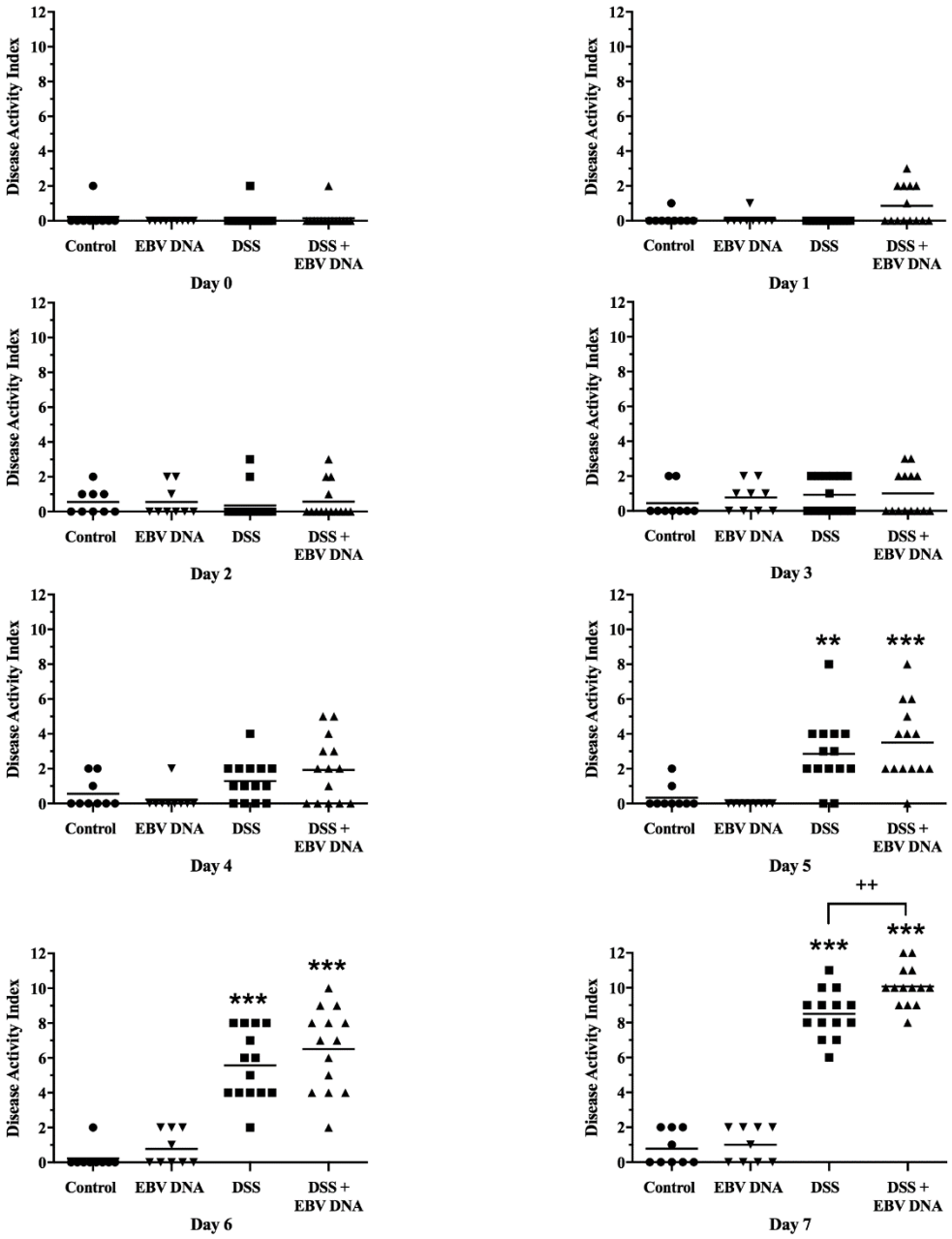

Figure 7: Disease activity index (DAI) scores in control and experimental mouse groups used to assess the effect of Epstein-Barr virus (EBV) DNA on colitis severity in C57BL/6J mice. Mouse groups received either 1.5% DSS-containing or normal drinking water for 7 days. The two DSS-treated groups were then rectally administered with sterile water or Epstein-Barr virus DNA in sterile water on day 3 (n = 14 mice per group). The two other normal drinking water-fed groups were included as controls and also received sterile water or EBV DNA by rectal gavage on day 3 (n = 9). The DAI was determined daily as a composite measure of the scores of body weight loss, stool consistency and fecal blood. **p-value <0.01, *** p <0.001, compared to the control group on the same day; ++ p <0.01, compared to the DSS group on the same day.

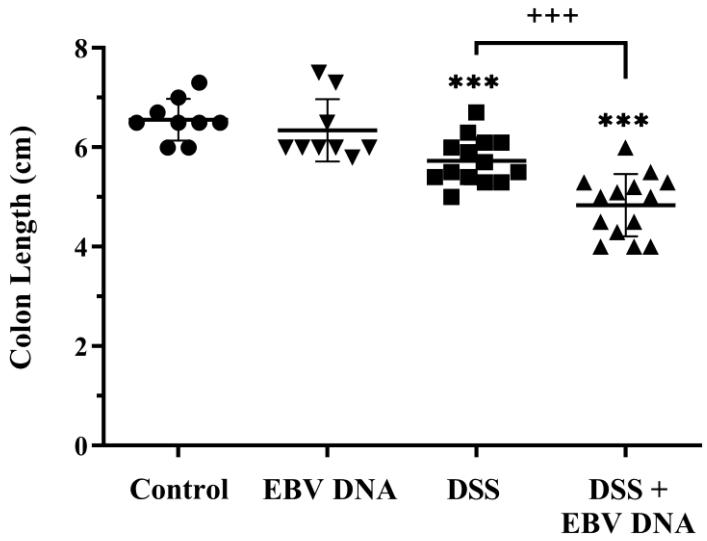

Figure 8: Colon length measurements in control and experimental mouse groups used to assess the effect of Epstein-Barr virus (EBV) DNA on colitis severity in C57BL/6J mice. Mouse groups received either 1.5% DSS-containing or normal drinking water for 7 days. The two DSS-treated groups were then rectally administered with sterile water or Epstein-Barr virus DNA in sterile water on day 3 (n = 14 mice per group). The two other normal drinking water-fed groups were included as controls and also received sterile water or EBV DNA by rectal gavage on day 3 (n = 9). After 7 days, mice were sacrificed and their colon lengths were measured. *** $p < 0.001$, compared to the control group; +++ $p < 0.001$, compared to the DSS group.

Table 3: Average disease activity index (DAI) in C57BL/6J mice used to assess the effect of Epstein-Barr virus (EBV) DNA on colitis severity.

Mouse group	**Disease activity index**							
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Control (n = 9)	0.22 ± 0.67	0.11 ± 0.33	0.56 ± 0.73	0.44 ± 0.88	0.56 ± 0.88	0.33 ± 0.71	0.22 ± 0.67	0.78 ± 0.97
EBV DNA (n = 9)	0 ± 0	0.11 ± 0.33	0.56 ± 0.88	0.78 ± 0.83	0.22 ± 0.67	0 ± 0	0.78 ± 0.97	1 ± 1
DSS (n = 14)	0.14 ± 0.53	0 ± 0	0.36 ± 0.93	0.93 ± 1	1.29 ± 1.14	2.86 ± 1.99	5.57 ± 1.99	8.5 ± 1.34
DSS + EBV DNA (n = 14)	0.14 ± 0.53	0.86 ± 1.1	0.57 ± 1.02	1 ± 1.24	1.93 ± 1.86	3.5 ± 2.18	6.5 ± 2.38	10.07 ± 1.14
Values are expressed as average DAI per group ± standard deviation.								

CHAPTER V
DISCUSSION

Epidemiological studies show that more than 90% of the world population is seropositive for EBV (8). Primary infection with this viral agent is usually characterized by asymptomatic or flu-like symptoms in children but may be associated with infectious mononucleosis among adults (181). In addition to causing IM, EBV constitutes a risk factor for the development of several malignancies, lymphoproliferative disorders, and most relevant to the present study, autoimmune diseases. Following the initial infection, the virus establishes latency in resting memory B cells with a potential of causing recurrent infections whereby viral DNA can be shed upon reactivation (182, 183). It has been established that unmethylated CpG DNA motifs, which are abundant in the EBV genome, induce immune-stimulatory pathways through Toll-like receptor 9 (TLR9) (184, 185). Moreover, studies have shown that the DNA of *Herpes simplex virus 1* (HSV-1), which belongs to the same family as EBV, promotes T helper type 1 (Th1) cell immune responses (185). Such observations previously led our group to assess whether the persistent EBV DNA is possibly capable of triggering pro-autoimmune responses in mice (11). The study revealed that intraperitoneal injection of mice with EBV DNA increases the production of interleukin 17A (IL-17A), which is a proinflammatory cytokine consistently linked with autoimmune pathways. Having observed these findings in mice that did not exhibit an autoimmune disease, our group next aimed to establish animal models of different types of inflammatory diseases and evaluate the association between EBV DNA and these diseases. In the study at hand, a murine model of acute colitis was employed.

Growing evidence confirms that reactivation of EBV from latency can occur at any mucosal site where B cells reside. Aside from the long-known associations of EBV with systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis, the latest research continues to demonstrate a possible implication of EBV in the pathogenesis of inflammatory bowel disease (34, 35, 36, 157-161). Several studies reported the detection of increased numbers of EBV-infected cells in colonic specimens from patients with IBD compared to samples obtained from healthy controls (35, 157, 158, 160). Evidence from other relevant studies also indicated a possible perpetuation of inflammation in UC patients as a result of active EBV replication in colonic lymphoid cells (36, 159). However, the definitive role of EBV in IBD remains unclear and warrants further investigation. In light of the above data, a recent study conducted by our group established a *Drosophila melanogaster* model system to discern the possible role that EBV DNA plays in IBD by examining markers of the cellular and humoral innate immune response in the fly gut (50). In this study, feeding flies DSS was the approach to induce localized inflammation in the gut secondary to the toxic effect of this chemical on intestinal epithelial cells; DSS compromises the epithelial barrier integrity resulting in the dissemination of intestinal content and subsequent activation of inflammatory responses (169-170). Upon assessment of results, enhanced levels of hemocytes, which resemble mammalian macrophages and promote inflammatory responses, were observed in the hindguts of flies receiving DSS then EBV DNA compared to the group feeding on DSS and EBV DNA at the same time and the group that fed on DSS alone, indicating that establishment of inflammation paves the way for EBV DNA to further augment the inflammatory cellular response. Moreover, at the humoral level, administration of EBV DNA after DSS resulted in increased expression of

the diptericin gene, a marker of activation of the Immune Deficiency (IMD) pathway in flies; this pathway is comparable to Tumor Necrosis Factor-α (TNFα) receptor signaling in mammalian systems (186). Our observations in flies suggest that EBV DNA participates in the exacerbation rather than the initiation of inflammatory processes of bowel diseases with autoimmune backgrounds. Having observed that EBV DNA could play such an exacerbatory role in the simple fly model, which are believed to lack adaptive immune responses, we intended to establish a similar model but in a higher system in which both innate and adaptive immune systems are involved. Hence, the aim of the present study was to determine the effect of EBV DNA on the severity of colitis in a murine model of the disease. The overall objective is to be able to use such a system to explore further questions pertaining to the role played by EBV DNA in inflammatory diseases.

Prior to investigating the possible involvement of EBV DNA in the exacerbated presentation of colitis, it was necessary to assess the functionality of DSS-induced colitis in C57BL/6J mice and to optimize the model in an effort to provide a margin for observing an additive effective of EBV DNA, should this occur. In rodents, oral administration of DSS via drinking water is a widely employed approach to induce a form of colonic mucosal inflammation that closely recapitulates several characteristics relevant to human UC, such as weight loss, diarrhea, blood in stool, colon shortening, and mucosal ulceration (175, 187). However, the effectiveness of DSS in inducing colitis and the severity of the clinical and histological course are influenced by numerous factors (171, 172, 188). These include the molecular weight, concentration, duration, frequency, and manufacturer of the administered DSS, as well as genetic factors such as the age, gender, and animal strain. Against this background and in search of a potential enhancement effect of EBV DNA on

colitis severity in the subsequent aim, the response of C57BL/6J mice, which are well-documented to develop less severe colitis than C3H mice, to a range of low DSS concentrations was clinically and macroscopically examined for colon changes, while taking into account the other factors that affect DSS-induced pathology (189, 190). According to clinical manifestations, disease severity was evaluated daily in all mouse groups based on the disease activity index score, a combined score of body weight loss, stool consistency, and fecal blood parameters. Treatment with 2.5% DSS in drinking water resulted in the earliest and the highest increase in the DAI score among all other mouse groups. Moreover, mice fed on 2.5%, 1.5%, and 1% DSS showed statistically significant differences in their DAI scores in comparison to the normal water-fed group starting on days 4, 5, and 7, respectively. However, a slow and steady onset of colitis was only observed in the 1.5% DSS mouse group. A previously published protocol by Chassaing et al. (170) indicated that the attainment of a gradual onset of colitis by DSS in early experiments allows for reproducibility of results in future studies. The obtained results indicate that the colitogenic potential of DSS and the severity of the clinical symptoms in C57BL/6J mice with induced colitis are directly dependent on the concentration of the DSS treatment. These results agree with a previous study conducted by Nunes et al. (175) who administered different concentrations of DSS in C57BL/6J mice and showed that the clinical disease severity correlated with the increase in DSS concentration. Additional information on the severity of DSS-induced colitis is provided by the degree of colon shortening which is indicative of colonic inflammation. The obtained macroscopic findings from extracted colons of each mouse group indicate that more pronounced colon length reductions and thus further colonic inflammation occur with increasing DSS concentrations

in C57BL/6J mice, whereby mice treated with 2.5% DSS manifested the highest extent of colon shortening among the DSS-receiving groups. Moreover, treating C57BL/6J mice with DSS for a period of 7 days was only associated with significantly reduced colons in the groups receiving a concentration of 1.5% or 2.5% DSS in drinking water compared to the control group. In a study by Egger et al. (179), findings also indicated that the severity of colonic mucosal injury is dependent on the increased DSS concentrations. Likewise, the positive association between the elicited clinical disease activity and the macroscopic colon changes of DSS-induced colitis that we observed was also previously described by other studies (178 -180). Overall, the obtained results as a whole suggest the DSS treatment can trigger the development of acute colonic mucosal injury in C57BL/6J mice with a concentration-dependent clinical severity and colonic inflammatory outcome. Accordingly, the gradually progressing fashion of colitis development and the moderately intense clinical course, as well as the reduced colon length that was elicited in C57BL/6J mice treated with 1.5% DSS, provide evidence for selecting this concentration for assessing the involvement of EBV DNA in IBD exacerbation.

Having determined the optimal DSS concentration for the C57BL/6J mouse acute colitis model, we next aimed to assess the effect of EBV DNA on the severity of colitis, particularly in terms of clinical disease activity and macroscopic colon morphology. Therefore, clinical evaluation of body weight loss and disease activity index and macroscopic assessment of colon shortening were carried out on experimental and control groups of mice assigned for the addressed aim. Reduced body weight was only observed in mice treated with DSS. These groups exhibited a significant percentage of weight loss relative to the normal water-fed group, indicating a successful induction of colitis with

DSS. However, there was no significant difference in the weight loss between the DSS and the DSS plus EBV DNA mouse groups despite the more apparent decrease in the latter group. However, when scores of other factors were added to the calculations so as to determine the DAI, a more comprehensive representation of the clinical course severity was obtained. In line with the body weight change observations, the DAI score was only increased in the two DSS-treated groups. Moreover, mice receiving EBV DNA in addition to DSS exhibited a higher increase in their DAI score than mice receiving DSS alone starting from day 4, and this difference became statistically significant on the last day of the experiment, indicating that EBV DNA aggravates the clinical symptoms of colitis in the mouse model of the disease. Further support to the potential involvement of EBV DNA in the severity of colitis was provided by the obtained colon shortening results, whereby the most pronounced reduction in colon length was observed in the group receiving EBV DNA in addition to DSS. More importantly, this group showed significantly shorter colons than those of the group receiving DSS alone, indicating that EBV DNA potentially increases the severity of colonic inflammation in the mouse model of acute colitis.

Our results taken together indicate that EBV DNA plays a role in the exacerbation of colitis pathology by increasing the severity of the disease in a mouse model. This association requires further investigation to understand the underlying mechanisms by which EBV DNA contributes to disease progression. The disruption of the epithelial barrier and the uncontrolled immune dysregulation that characterize DSS colitis induction in mice shed light on possible predisposing conditions by which underlying damage provides a favorable environment for EBV DNA to instigate or aggravate IBD. Following primary infection in immunocompetent individuals, EBV persists for life in resting memory cells

and escapes cytotoxic T cell surveillance by limiting viral gene expression. Possibly, the absence of immune surveillance that is associated with impaired mucosal immunity and immunosuppressive therapy in IBD cases predisposes patients to reactivation of EBV infection resulting in increased mucosal inflammation and further disease perpetuation (35, 36, 164). Alternatively, minor mucosal damage in subjects with active EBV replication in their gastrointestinal tract may result in IBD due to the enhanced inflammation induced by the viral DNA. Assessment of the role played by EBV DNA in subjects with IBD is being investigated by our group.

In addition to the clinical data described in our model above, the effect of EBV DNA on colitis severity will also be assessed based on histological and immunological parameters. These investigations were halted due to the SARS-CoV-2 pandemic that began in 2019 and which necessitated a shutdown of non-essential laboratory activities during 2020. Colons collected on the sacrifice day from each mouse group were cut into small fragments and appropriately stored in preparation for histological injury assessment and immune cell profile analysis. The histological grading will be performed on H&E-stained colon sections and scored based on a system previously described by Mu et al. (187) and Xiao et al. (191) that accounts for the severity and depth of inflammation along with the damage in crypt architecture. Subsequently, as a means to identify the effect of EBV DNA on the immune status of the colitis mouse model, colonic lamina propria cells will be isolated from the stored colons and processed for flow cytometric determination of the immune cell composition according to a combination of surface markers, cytokines, and transcription factors.

In conclusion, our study suggests an association between EBV DNA and increased severity of colitis in a mouse model of IBD. Further research efforts are required to examine the mechanism by which EBV DNA aggravates the inflammatory process. A better understanding of the link between recurrent EBV infections and IBD exacerbation may have notable implications for preventive strategies and therapeutic interventions, such as screening for EBV infection before initiation of drug therapy in patients. In a previous study by our group, findings indicated that TLR3, 7, and 9 were involved in the increase of IL-17A in response to EBV DNA, whereby treatment of mice or mouse PBMCs with TLR inhibitors resulted in a significant reduction in IL-17A levels triggered by EBV DNA (49). Thus, TLR inhibitors may be useful as therapeutic agents in IBD patients. Moreover, adopting the DSS-induced colitis model in C57BL/6 mice for future studies will help investigate the different factors that affect the pathogenesis of colitis. One possible consideration would be to examine whether EBV DNA is associated with altered colonic microbiota composition in the colitis mouse model, given the long-studied role of gut microbiota changes in the pathogenesis of IBD. Furthermore, since EBV DNA triggered the IMD pathway in the fly model of gut inflammation and this pathway is comparable to the TNFα receptor signaling in mammals (50, 186), the present colitis mouse model would allow us to determine whether EBV DNA results in a similar response in mice. In the long run, it would be of great interest to examine whether our findings are applicable to humans who have IBD.

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