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THE EFFECT OF EPSTEIN-BARR VIRUS DNA ON T-HELPER 17 AND REGULATORY T-CELL SELECTED MARKER EXPRESSION IN MICE

by
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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
April 2017

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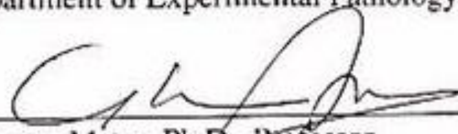

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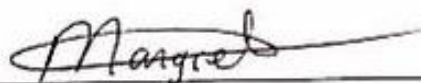

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ACKNOWLEDGMENTS

Foremost, I would like to express my sincere gratitude to my advisor Dr. Elias Rahal for his continuous support, patience, motivation, knowledge, and guidance throughout this research.

Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Alexander Abdelnoor, Dr. Ghassan Matar, and Dr. Margret Shirinian, for their encouragement and insightful comments.

My sincere thanks also go to Ms. Noor Salloum and Ms. Marwa Chehab for their invaluable efforts, passionate encouragement and continuous assistance.

A very special thanks to my lab mate and fiancée, Nour Sherri, who was beside me every step of the way for the last couple of years, with whom I shared all the fun and all the exhaustion that accompanied this thesis and without whom it would not have been possible to achieve this work.

Lastly, I would like to thank my parents and all my family for supporting me in every way possible.

AN ABSTRACT OF THE THESIS OF

Hadi Malek Hussein for Master of Science
Major: Microbiology and Immunology

Title: The effect of Epstein-Barr virus DNA on T-helper 17 and regulatory T-cell selected marker expression in mice.

Introduction: Epstein-Barr Virus (EBV) establishes latency following a primary infection mainly in B-lymphocytes and is capable of reactivating at any time potentially shedding its viral DNA. A previous study at the Department of Experimental Pathology, Immunology and Microbiology indicated that EBV DNA increased the levels of the autoimmune-related proinflammatory cytokine interleukin-17 (IL-17) in mice suggesting that EBV DNA may trigger autoimmune pathways. IL-17 is produced mainly by the $CD4^+$ T helper 17 (Th17) lymphocytes and is associated with various autoimmune diseases. On the other hand, regulatory T cells (Treg), possess suppressor and anti-inflammatory properties and play a role in the maintenance of immune homeostasis in contrast with the function of Th17 cells. The main objective of this study was to investigate whether regulatory T cell activities are affected by EBV DNA.

Methods: To assess the effect of EBV DNA on the activity of Th17 and regulatory T cell pathways, 27 BALB/c mice divided into three groups, each containing 9 mice, were used. Mouse groups were intraperitoneally injected with sterile distilled water as a negative control, EBV DNA (144×10^3 copies) or *Staphylococcus epidermidis* DNA (28.3 pg) as a non-viral control DNA. Three mice were sacrificed per group on days 3, 6, and 9 post-injection, then RNA was extracted from mouse spleens per group per time point to assess the transcriptional levels of Th17 markers (IL-17A, IL-21 and RORγT) in addition to regulatory T-cell markers (FOXP3 and CTLA4) by real-time reverse transcriptase PCR.

Results: Normalized to transcriptional levels in mice that received a sterile water injection and assessed on day 3 post injection, the transcriptional levels of RORγT, IL-17 and IL-21 increased with the highest levels being 524 ($p=0.0094$), 512 ($p=0.0006$) and 1462 folds ($p=0.0077$) respectively on day 6 post-injection with EBV DNA. These results indicate that EBV DNA induces Th17 lymphocyte activity which subsequently leads to the production of proinflammatory cytokines. On the other hand, the transcriptional levels of FOXP3 were increased by EBV DNA to a lesser extent than *S. epidermidis* DNA injection with a relatively low level of 12 folds coinciding with the highest levels of Th17 markers on day 6 post-injection. Additionally, CTLA4 expression in the EBV DNA-injected group was not only lower than the *S. epidermidis* DNA-injected groups at all time points, but also significantly lower than the water injected group by about 4 folds ($p=0.0447$) on day 6 and by 6 folds ($p=0.0408$) on day 9 post-injection.

Conclusions: Results indicate that EBV DNA favors Th17 cell activity at the expense of regulatory T cell pathways. The exact mechanisms by which EBV DNA induces a Th17/IL-17 mediated inflammatory response while repressing regulatory T cell activities, remain to be fully investigated.

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CHAPTER I

INTRODUCTION

Epstein-Barr Virus (EBV) is a DNA herpesvirus associated with infectious mononucleosis. EBV has been shown to be involved in various autoimmune diseases including rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus. Following the primary infection, EBV establishes latency in the host, and is able to cause recurrent infections during which the viral DNA can be shed. EBV DNA has been seen to trigger the production of the proinflammatory cytokine interleukin-17 (IL-17) in mice. IL-17 is mainly secreted by T helper 17 (Th17) cells, and is associated with the development of multiple autoimmune diseases. Th17 cells also produce IL-21 and IL-22. These cytokines stimulate different types of cells to secrete pro-inflammatory molecules such as IL-1, IL-6, TNF-α among others. Another T cell subset, regulatory T cells (Treg), have suppressor and anti-inflammatory properties and play a role in the maintenance of immune tolerance in contrast with the action of Th17 lymphocytes. The overall objective of this study is to examine whether regulatory T cells activities are affected in response to EBV DNA in mice concurrent with changes in the Th17 response.

This study aimed to:

1. Assess the effect of injecting BALB/c mice with Epstein-Barr Virus DNA on the expression of the Th17 markers IL-17, IL-21 and RORγT in mouse splenic tissues.
2. Assess the effect of injecting BALB/c mice with Epstein-Barr Virus DNA on the expression of regulatory T cell markers FOXP3 and CTLA4 in mouse splenic tissues.

CHAPTER II

LITERATURE REVIEW

A. Epstein-Barr virus (EBV)

The Epstein-Barr virus (EBV), was originally discovered by Epstein and Barr when isolated from a Burkitt lymphoma cell line; it was the first isolated human tumor virus (1). EBV belongs to the Lymphocryptovirus which is part of the gamma subfamily of the *Herpesviridae* family, it is formally known as Human Herpes Virus 4 (HHV-4) (2).

1. Structure

The size of EBV ranges between 122 and 180 nanometers and it is made-up of three major components: the first is an icosahedral nucleocapsid containing 162 capsomeres and housing the EBV genome (3), the second is a lipid bilayer envelope obtained from the host cell following the budding of the freshly formed virion through the membrane of the infected cell. This envelope contains spikes which are glycoproteins encoded by the viral genome, such as glycoprotein B (gB), glycoprotein (gH), glycoprotein (gL) and glycoprotein 350 (gp350) which recognizes CD21 on B lymphocyte surfaces and binds it (4, 5). The third component is the tegument which is formed by around 17 amorphous proteins localized between the nucleocapsid and the envelope. Some host proteins like actin and heat-shock protein 70 (Hsp 70) can be detected among these proteins (3).

2. *Structure of the viral genome*

The viral genome consists of a linear double-stranded DNA (100×10^6 Daltons). It encodes nearly 85 genes and is around 172 kilo base pairs (kb) long (6). Furthermore, it has a greater fraction of cytidine (58%) and guanidine (42%) than the human genome (7). The major internal repeat, IR1, contains between 5 and 10 copies of a sequence around 3 kbp in length. IR1 separates between the short unique sequence (U_S), which is 12 kbp in length, and the 134 kbp long unique sequence (U_L). IR1 contains the EBV nuclear antigens (EBNAs) promoter (Wp) (7). The long unique sequence (U_L) is divided into four shorter sequences (U2, U3, U4, and U5) by repetitive elements contained in other shorter internal repeats (IR2, IR3, and IR4) (8). The genetic material of the virus is maintained as an extrachromosomal episome in the host. A series of 0.5 kbp terminal repetitive sequences (TR) located on both extremities of the linear molecule intermediate the construction of this episome (9).

The bulk of the viral genes play a role in the EBV lytic (productive) cycle; whereas 12 genes and two types of noncoding RNAs, Epstein-Bar virus encoded small RNAs (EBERs) and micro RNAs (miRNAs) are exclusively associated with the latent (nonproductive) cycle of the virus (3).

3. Types of EBV

In humans, two major types of EBV exist: EBV-1 and EBV-2 differing in the sequence of the genes encoding EBV nuclear antigens (EBNA-2, EBNA-3A/3, EBNA-3B/4, and EBNA-3C/6) (2). EBV-1 immortalizes B cells with greater effectiveness than EBV-2 *in vitro*, and the viability of lymphoblastoid cell lines infected with EBV-1 is higher than EBV-2-infected lines (10).

4. Epidemiology and transmission

It has been shown that the percentage of adults throughout the world not infected with EBV is less than 10%; this demonstration was chiefly based on the detection of anti-VCA antibodies (antibodies specific to the viral capsid) and complement-fixing soluble (CF/S) antigens of EBV (11). Different studies have also demonstrated the great ubiquity of EBV around the world (12, 13). The age at primary infection varies markedly around the world, and vulnerability to an EBV infection is probably due to socioeconomic conditions (14) such as overpopulated areas having subnormal hygienic standards (11).

Despite being asymptomatic or exhibiting symptoms resembling other respiratory diseases in early childhood, primary EBV infections acquired at an older age, which is a trait usually associated with developed countries (15), can lead to infectious mononucleosis. EBV is mainly transmitted orally; however, it has been shown that transfusion can also be a way of transmission. Infection in the stage of early childhood is common in developing

countries, due to practices like pre-chewing food for infants, while infection is often delayed to adolescence in the developed world (16).

5. *Diseases*

Generally, between 25 and 75% of EBV-infected persons are diagnosed with infectious mononucleosis (IM) (14, 17) while around 1 out of 4 young adults who acquired a primary EBV infection are diagnosed with IM (18). The symptoms of IM include malaise, fever, adenopathy, pharyngitis, atypical lymphocytosis, hepatomegaly, splenomegaly and jaundice (19).

On the other hand, EBV has also been associated with Burkitt lymphoma. This disease is divided into three subtypes: 1) endemic Burkitt lymphoma, 2) sporadic Burkitt lymphoma, and 3) immunodeficiency-associated Burkitt lymphoma. The first subtype mostly targets New Guinean and equatorial African children, the second is acquired by children and young adults worldwide, and the third is mainly linked to HIV infections (20). It has been documented that EBV is detected in the tumor tissue of nearly all of the cases of endemic Burkitt lymphoma, but less in instances of the two other subtypes (20).

EBV is also commonly correlated with with oral hairy leukoplakia, nasopharyngeal carcinoma (21) as well as the mixed-cellular subtypes of Hodgkin lymphoma and rarely with the non-classical nodular lymphocyte-predominant subtype (22).

6. *Replication, latency and pathogenesis*

After being transmitted as a free virion, via infected cells in saliva, or both, EBV will invade the circulating B lymphocytes (23). Like all herpesviruses, EBV persists in the host

following the primary infection and can reactivate at any time (24). The mucosal epithelial cells are also targets for EBV infection; once these cells are invaded, viral productive replication occurs (25).

In immunocompetent carriers, the true repository for the latent virus seems to consist of B lymphocytes, particularly the resting memory B cells. Viral gene expression is extensively diminished in these cells (26); which may explain the mechanism by which these infected cells evade cytotoxic T lymphocyte surveillance (27).

It has been reported that EBV can permanently persist in more than 90% of human adults without leading to the manifestation of any disease. EBV proceeds from the primary infection to the lifelong persistence within the B lymphocyte compartment of the immunocompetent infected host using various combinations of latency gene expression (28).

EBV is transmitted in the saliva and then reaches the epithelium of the pharyngeal lymphoid ring which is located in the oropharynx. The virus initiates a lytic infection there resulting in amplification of viral numbers. After being infected by EBV, B lymphocytes present in the neighboring lymphoid tissues become activated lymphoblasts following the activation of the viral Latency III growth transcription program. Negative autoregulation of this program is then mediated by three EBV proteins: EBNA-3A, EBNA-3B, and EBNA-3C; subsequently, the cell moves into the follicle to commence a germinal center reaction, and to trigger the Latency II transcription program. The cell exits the germinal center as a memory B cell. Then, Latency 0 is initiated in the quiescent memory B cells; in this program, viral protein expression is absent. When these cells infrequently divide, they

trigger the EBNA- 1-only program referred to as Latency I. Ultimately, the memory B lymphocytes migrate to the tonsils, where they sporadically differentiate into plasma cells, which consequently elicits viral replication. The newly produced virus may then be delivered into saliva to infect other hosts or may spread to other B lymphocytes (28).

A potent cellular immune reaction is triggered by the primary EBV infection leading to the restraint of this infection, and it has been suggested that the T-cell response, which is antigen-specific, is effective in disposing of the newly infected host cells. Lifelong persistence in the infected organism can happen exclusively in dormant memory B lymphocytes where proteins of the virus are not expressed, and the virus is therefore protected from the immune system (28, 29).

EBV spreads to B lymphocytes by interacting through gp350/220, which is a glycoprotein found on the envelope of the virus, with CD21 (also referred to as CR2), which is the cellular receptor for the C3d complement component (30-32). EBV also uses gp42 that binds to major histocompatibility complex (MHC) class II as a cofactor to invade B-cells (33). The virion then merges its envelope with the membrane of the host-cell via three glycoproteins: gp85, gp25, and gp42 (34). Despite being known as a B-lymphotropic virus, EBV can also infect epithelial cells by binding through its glycoprotein BMRF2 with β1 integrins, then binding αvβ5, αvβ6, or αvβ8 integrins through gH (35). It has also been reported that EBV is capable of infecting T lymphocytes through less understood mechanisms as the virus can be detected in $CD4^+$ T cells, $CD8^+$ T cells, and γδ T cells in

various disease instances including EBV-associated hemophagocytic syndrome, NK/T-cell lymphomas and chronic active EBV infection (36, 37).

B. Autoimmune diseases

Autoimmunity refers to the failure of the immune system to distinguish between self and foreign bodies. Immune cells would then react to antigens expressed by the organism's own cells resulting in humoral and cellular immunologic abnormalities. This usually leads to organ-specific or systemic autoimmune diseases.

Factors that contribute to the rise of autoimmunity include genetic and environmental factors, encompassing microbial infections (31). Various research efforts have emphasized the pathogenic function of T-helper 17 (Th17) lymphocytes and its signature cytokine, Interleukin 17 (IL-17), in autoimmune diseases (38-40).

EBV is associated with a number of autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis (27). A previous study conducted at the Department of Experimental Pathology, Immunology and Microbiology at the Faculty of Medicine at the American University of Beirut reported that EBV DNA increased the secretion of the pro-inflammatory cytokine IL- 17 following injection of EBV DNA into mice (41).

Another study showed that high EBV viral loads were observed in the blood of patients with SLE, which indicated that EBV is highly linked to this disease. Furthermore, it has

been suggested that EBV has a crucial role in the pathogenesis of rheumatoid arthritis. The same study revealed an increase in the viral load of EBV in RA affected subjects (42). In addition, MS is highly linked with EBV infection since many studies and sero-epidemiological surveys revealed that there is an increased risk to have MS in EBV-infected subjects (43, 44).

C. Interleukin-17

IL-17A is a pro-inflammatory cytokine produced by T helper 17 cells (44, 45). IL-17 has six family members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F. Although IL-17A and IL-17F share the highest amino acid sequence homology (50%), they perform distinct functions; IL-17A plays a major role in the defense against extracellular bacterial and fungal infections but it is also involved in the development of autoimmune disorders, inflammation, and malignancies, whereas IL-17F is mainly involved in mucosal host defense mechanisms. IL-17E is an amplifier of Th2 immune responses. IL-17B, IL-17C, and IL-17D have similar ability to induce inflammatory mediators as IL-17A and IL-17F, however, their specific roles in the immune system remain unknown (46)**.** Both IL-17B and IL-17C induce Tumor Necrosis Factor (TNF) and IL-1β expression from a monocytic cell line and cause neutrophil infiltration (47, 48) while IL-17D, which is most homologous to IL-17B, induces the expression of IL-6, IL-8, and GM-CSF in endothelial cells (49). Other immune cells have also been reported to produce IL-17 including monocytes, neutrophils, NK-T cells and lymphoid tissue inducer (LTi)-like cells under specific circumstances (50).

The gene coding for IL-17A cytokine in humans is present on chromosome 6 (51, 52) while that for mouse IL-17A is on chromosome 1. Murine IL-17A is a 21 kDa glycoprotein containing 147 amino acid residues (46) while the human IL-17A consists of a 20 to 30 kDa protein composed of 155 amino acids (53, 54). Human IL-17A shows 63% similarity with that of mice (55). Furthermore, IL-17A stimulates neutrophils to secrete myeloperoxidase(56) and promotes the expression of MHC class II on dendritic cells (57). Also, keratinocytes secrete IL-6 and IL-8 in response to IL-17 stimulation (58, 59) which then stimulates epithelial cells to express these two cytokines (58, 60). In addition, IL-17A plays an important role in the maturation, chemotaxis and proliferation of neutrophils (40).

Several functions of IL-17A show its role in boosting tissue inflammation including the stimulation of different types of cells to produce factors involved in inflammation such as the granulocyte-macrophage colony-stimulating factor (GM-CSF), which is produced by some immune cells like macrophages and some non-immune cells like endothelial cells and fibroblasts, tumor necrosis factor-α (TNF- α), interleukin-6 (IL-6) and interleukin-8 (IL-8) produced mainly by macrophages, in addition to chemokines like the monocyte chemoattractant protein-1 (MCP-1) and the C-X-C motif ligand-2 (CXCL2) secreted primarily by macrophages and monocytes (58, 61). β-defensin 2, which is an antimicrobial peptide mainly produced by various types of epithelial cells, is also expressed following IL-17 stimulation (62).

Despite having a beneficial role in defending against bacterial and fungal infections (61), it is believed that IL-17A plays a substantial role in the pathogenesis of several autoimmune disorders such as inflammatory bowel disease (IBD), SLE and RA (46, 63) as

shown by increased levels of IL-17 in tissues and sera of patients with these diseases (61). Mice deficient in IL-17A or IL-17A receptor are less vulnerable to experimental autoimmune encephalomyelitis (EAE); they still acquire the disease but with less serious symptoms(45, 63) meaning delayed onset, diminished severity, better histological changes and early recovery (39). Furthermore, previous research efforts have reported that anti-IL17 or anti-IL-17R antibodies restrict the progress of arthritis (38, 63, 64).

D. T helper 17 cells

Various studies have shown that in the presence of IL-6 with or without other proinflammatory cytokines like IL-1β and IL-21, TGF-β was sufficient to promote the differentiation of naive T lymphocytes into Th17 lymphocytes (65-67)**.** IL-23 produced by activated dendritic cells, on the other hand, appears to play a role in the proliferation of IL-17-secreting $CD4^+$ T lymphocytes (68, 69).

Induction of IL-17A and IL-23R expression from naïve T lymphocytes is dependent on the presence of RORγT encoded by the RORC gene (70). Moreover, Rorc-/- mice do not react to IL-23 stimulation, have decreased counts of Th17 lymphocytes, and are protected from autoimmune diseases (71).

RORγT is expressed in lymphoid tissues and a number of immune cell types (72) including innate lymphoid cells, NK cells and NKT cells (73). Also, RORγT is induced in naïve CD4+T cells upon their activation by an antigen-presenting cell, in presence of a number of cytokines (IL-6, IL-21, IL-23, IL-1β, TGF-β) (74).

It has been shown that the expression of RORγT is required for the differentiation of lymphoid tissue inducer (LTi) cells, since RORγT-deficient mice lack Peyer's patches, cryptopatches and isolated lymphoid follicles in the intestine as well as peripheral lymph nodes. RORγT also plays a critical role in the generation of thymocytes; in the absence of RORγT, mice exhibit severe thymic atrophy. Furthermore, the expression of RORγT is induced at the transition from the double negative (DN) to the double positive (DP) stage of thymic T cell development. The absence of RORγT results in a dramatic decrease in the number of $CD4^{+}CD8^{+}$ DP and mature single positive (SP) $CD4^{-}CD8^{+}$ and $CD4^{+}CD8^{-}$ thymocytes (72).

RORγT plays an essential role in establishing and maintaining adaptive immune responses by regulating thymic T cell development and Th17 cell differentiation which makes it a potential target for the treatment of autoimmune diseases like psoriasis (72).

The discovery of pathogens that favor Th17 lymphocyte responses and that are capable of inducing autoimmune diseases orchestrated by Th17 is very suggestive of an association between infection and autoimmunity (74). For instance, a recent study suggested that Coxsackievirus B3 was capable of directly inducing Th17 cell differentiation from naive $CD4^{+}$ T cells by promoting acetyl-Stat3-mediated RORγT synthesis (75).

Rheumatoid arthritis is one of the first examples of a correlation between IL-17 and disease in humans. Synovial cells were shown to secret TNFα, IL-8, and IL-6 in response to an IL-17 signal, which led to the proposal that IL-17 could act on stromal cells to enhance inflammation (53, 76). Moreover, the presumptive formation of osteoclasts was early

observed as a result of treating cocultures of mouse hemopoeitic cells and primary osteoblasts with recombinant human IL-17 (76). Later, it was shown that IL-17 produced by Th17 cells regulates osteoclastogenesis in RA patients through the up-regulation of RANKL expression in fibroblast-like synoviocytes (FLS) and the direct induction of osteoclast differentiation chiefly through the NF-κB and AP-1 pathways (77).

On the other hand, MS patients have shown large numbers of Th17 cells, high levels of IL-17 mRNA in addition to $CD4^{+}IL\text{-}17^{+}$ and $CD8^{+}IL\text{-}17^{+}$ T lymphocytes in their brain lesions. Furthermore, astrocytes, and oligodendrocytes expressed IL-17 in active areas of MS lesions and the level of expression of IL-17 was consistently higher within these cells as well as in T cells within active areas of MS lesions than in control tissue (78). Moreover, Th17 lymphocytes have been isolated from the gut mucosa of Crohn's disease patients (79).

E. Regulatory T cells

The functions of regulatory T cells (Treg) are considered principal among several complex regulatory mechanisms that sustain immune homeostasis, prevent autoimmune diseases and control inflammation. Moreover, they are now considered as primary mediators for maintaining peripheral tolerance (80, 81). Tregs also prevent the development of autoimmune diseases (80, 81), and restrict chronic inflammatory diseases (81, 82).

Forkhead box P3 (FOXP3) is a key transcription factor that is needed for the development of the Tregs as well as their maintenance and function (83, 84). It was demonstrated that the lack of FOXP3 in both mice and humans results in the development

of autoimmune-like lymphoproliferative diseases which graphically highlights the important role that Treg cells play in maintaining peripheral tolerance (85-87). FOXP3 has been suggested to be the major mediator of Treg cells responsible for regulating the transcription of genes that orchestrate their regulatory activity (88, 89).

Natural CD4+CD25+ FOXP3+, regulatory T-cell (nTreg) development occurs in the thymus. These cells present a distinct T-cell receptor (TCR) repertoire that recognizes self antigens (90, 91). On the other hand, Tregs may also be induced from effector T lymphocytes throughout inflammation in peripheral tissues, or experimentally developed for therapeutic purposes (92-94). FOXP3 promotes the expression of CTLA4 on the Treg cell surface. CTLA-4 is an inhibitory molecule related to the T cell costimulatory molecule CD28. CTLA-4 and CD28 bind to shared ligands (CD80, CD86) on antigen presenting cells. While CD28 signaling promotes T cell activation, CTLA-4 serves an immunoregulatory function, suppressing the T cell response. The first evidence of the immunoregulatory function of CTLA4 was indicated when mice deficient in CTLA4 suffered a fatal lymphoproliferative syndrome. CTLA-4 is induced in non-Treg T lymphocyte populations following activation; however, it is constitutively expressed in FOXP3+ Tregs. Various studies have shown that CTLA4 significantly contributes to Treg immunoregulatory functions (95).

Other Treg subsets have also been identified, including induced Tregs (iTregs) such as Th3 and Tr1 cells. Th3 cells are peripheral $CD4^{+}CD25^{-}$ cells that are induced to express Foxp3 in response to foreign antigens and that are capable of producing TGF-β and IL-10;

these cells resemble nTregs in their suppressive functions. On the other hand, Tr1 cells are $CD4^+$ $CD25^+$ that do not express FOXP3, but also have suppressor functions mainly by secreting the anti-inflammatory cytokine IL-10. These cells are mostly found in the lungs and draining lymph nodes (96). Additionally, a subset of FOXP3+CD8+ Tregs have also been detected in tonsils and rarely in peripheral blood. These cells can be induced *in vitro* in naive CD8+ T cells by polyclonal stimulation and produce high levels of TNF-α, IFN-γ, and granzyme B (96).

The different suggested suppression mechanisms utilized by Tregs include the use of inhibitory cytokines, cytolysis, metabolic disruption and modulation of dendritic cells. It was demonstrated that Treg-cell induced suppression is mediated by the action of inhibitory cytokines such as IL-10 and TGF-β (81). For instance, in allergy and asthma models, evidence suggests that both nTregs and iTregs control disease in an IL-10-dependent manner (93). IL-10 can act on various immune cells including dendritic cells, and effector/memory T cells which is an important function in the prevention of T cell-mediated colitis. It has also been shown that IL-10R signaling is needed in Tregs as well as in Th17 cells in order to suppress colonic Th17 responses (97).

Cytolysis, caused by the production of granzymes is a major function of CD8+ cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (98). nTregs have been also been shown to mediate cytolytic activity via the adhesion of granzyme A and perforin to CD18 (99). Although CD4+ T cells present in mouse lack cytolytic activity, early gene

expression arrays in mouse Tregs cells demonstrated that granzyme B expression is upregulated (99, 100).

It has been suggested that the increased level of CD25 expression promotes the consumption of IL-2 by Tregs and subsequently restricts it from reaching the expanding effector T lymphocytes that need this cytokine to survive (101, 102). Despite earlier research proposing that this was not a genuine Treg mechanism (103, 104), multiple studies have shown that Tregs promote apoptosis induced by IL-2 restriction (105).

Tregs can also regulate the maturation and function of dendritic cells (106). Studies using intravital microscopy have revealed direct interactions between Treg cells and dendritic cells *in vivo,* these interactions were suggested to decrease effector T-lymphocyte activation by dendritic cells (107, 108) in a CTLA4-dependent mechanism. (109, 110). It was also shown that Treg cells could condition dendritic cells to express indoleamine 2,3-dioxygenase (IDO), a potent regulatory enzyme which is known to induce the production of pro-apoptotic metabolites from the catabolism of tryptophan, resulting in the suppression of effector T cells (111).

F. Th17/Treg balance

The relation between Th17 and Treg cells represents the equilibrium between inflammation and suppression which may affect the end result of autoimmune diseases, cancer and other disorders. Diverse mechanisms result in opposite effects in these two populations of cells,

inducing Tregs and inhibiting Th17 lymphocytes at the same time or the inverse. This has for example been shown in the human gastrointestinal tract. The differentiation of Foxp3+ Treg cells was promoted and that of Th17 cells was inhibited following the production of retinoic acid by dendritic cells located in the gut, in the presence of TGF-β (112). Retinoic acid was also capable of redirecting the differentiation of naive T lymphocytes from Th17 to Treg cells even in the presence of IL-6 and TGF-β in the gut of mice (113).

IL-2 was similarly seen to activate the transcription factor STAT5 favoring the differentiation of naïve T lymphocytes into Tregs while blocking the differentiation of Th17 cells in mice (114). On the other hand, Tregs may have a direct influence on the expansion of Th17 lymphocytes and their cytokine production. For example, Tregs obtained from joints of RA patients appear to hinder the production of IL-17 from effector T lymphocytes (115).

On the other hand, human nTregs can transform *ex vivo* into Th17 cells in the presence of proinflammatory cytokines such as IL-1β, IL-23 and IL-21 (116). RORγT and FOXP3 were found to be co-expressed in naïve $CD4^+$ T cells exposed to TGF-β and in a subset of T cells in the small intestinal lamina propria (LP) of mice (117). *In vitro*, FOXP3 inhibited RORγT function in part through biochemical interaction detected via co-immunoprecipitation experiments. Hence LP T cells that co-express both transcription factors were shown to produce less IL-17 than those that express RORγT alone However, IL-6, IL-21 and IL-23 relieve FOXP3-mediated inhibition of RORγT, thereby promoting Th17 cell differentiation (65, 117).

CHAPTER III

MATERIALS AND METHODS

A. Mice

To study the effect of Epstein-Barr virus (EBV) DNA on the expression of the immunological markers RORγT, IL-17A, IL-21, FOXP3 and CTLA4, 3 groups of 4-6-week-old female BALB/c mice were used. Each group included 9 mice; hence, a total of 27 mice was used.

Mice were obtained from the Animal Care Facility at the American University of Beirut (AUB) and treated according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at AUB.

B. Injections

EBV DNA was obtained from Advanced Biotechnologies (Columbia, MD). Mouse group 1 received a 100μl sterile water injection only, thus this group served as a negative control. Group 2 received $144 x 10^3$copies of EBV DNA in sterile water. Group 3 received 28.3 pg of *Staphylococcus epidermidis* DNA which is equivalent to the weight of EBV DNA used (Department of Experimental Pathology, Immunology and Microbiology collected isolates). This group served as a non-viral DNA control. All injections were administered intraperitoneally and each consisted of 100μl.

C. **Specimen procurement**

After sedation with Forane, three mice from each group were sacrificed per day by cardiac puncture on days 3, 6 and 9 post-injection and their spleens were collected.

D. **RNA Extraction**

RNA extraction was performed using the QIAzol® lysis reagent (Qiagen, Germany) according to the manufacturer's specifications.

1. Reagents

- QIAzol® lysis reagent
- Chloroform
- Isopropyl alcohol
- RNase-free water
- Ethanol (75%)

2. Protocol

Per 100 mg of spleen tissue collected 1 ml of lysis reagent was added. The spleens were completely homogenized using a tissue homogenizer to form a viscous solution. The tube containing the homogenate was then left at room temperature for 5 minutes. Then, 0.2 ml of chloroform was added to the homogenate and the tube was vigorously shaken. The tube was again left at room temperature for 3 minutes and then centrifuged at 12,000g for 15 minutes at 4°C. The upper aqueous phase was transferred to a new tube and 0.5ml of isopropanol was added to it and mixed thoroughly by vortexing. The tube was subsequently placed on the bench top for another 10 minutes and then centrifuged at 12,000g for 10

minutes at 4°C. The supernatant was aspirated and discarded. One ml of 75% ethanol was added to the RNA pellet before centrifuging at 7500g for 5 minutes at 4°C. The supernatant was completely removed, and the RNA pellet was air-dried. The RNA was redissolved in 70 µl of RNase-free water.

The concentration and purity of RNA was then assessed using a nanodrop spectrophotometer (ds11 Denovix Tc 312, Wilmington, DE).

E. Reverse Transcriptase Real Time Polymerase Chain Reaction

1. cDNA synthesis

cDNA synthesis was performed using the QuantiTect® Reverse Transcription Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

a. Reagents

- gDNA Wipeout Buffer (provided by the kit)
- Quantiscript Reverse Transcriptase: also contains RNase inhibitor (provided by the kit)
- Quantiscript RT Buffer (provided by the kit)
- RT Primer Mix: contains oligo-dT and random primers dissolved in water (provided by the kit)
- RNase-Free Water (provided by the kit)

b. <u>Protocol</u>

Before starting, all the reagents were thawed and then kept on ice throughout the preparation and procedure. The QuantiTect Reverse Transcription procedure comprises two main steps: elimination of genomic DNA (gDNA) and reverse transcription.

For elimination of the gDNA, 1µg of RNA in a volume of 2µl were placed per sample in a sterile, nuclease-free, thin-walled PCR tube. Then 2µl of the gDNA Wipeout Buffer were added. Samples were then incubated for two minutes at 42ºC on a heat block, and then placed immediately on ice.

For the reverse-transcription reaction, the following components were added to each sample tube: 1 µl of Quantiscript Reverse Transcriptase, 4 µl of Quantiscript RT Buffer, and 1 µl of RT Primer Mix. These were then gently mixed and then incubated for 15 minutes at 42ºC. The Quantiscript Reverse Transcriptase was then inactivated by incubating the samples at 95ºC for 3 minutes. These incubations were performed using a thermal cycler (PCR SPRINT, Thermo Electron Corporation, Waltham, MA) with a heated lid.

After the allotted time, the sample tubes containing the cDNA were immediately placed on ice and then stored at -20ºC for later use.

2. *Real-time polymerase chain reaction*

Real-time PCR was performed to detect the relative gene expression of: RORγT, IL-17A and IL-21 which are markers of T-helper 17 cell activity, in addition to FOXP3 and CTLA4 which are regulatory T-cell activity markers.

a. Primers

The primers used for assessing the expression of the RORγT, IL-17A, IL-21, FOXP3 and CTLA4 genes, in addition to those for β-actin used as a reference gene, were obtained from Thermo Scientific (Ulm, Germany). Primers used to assess the expression of RORγT and CTLA4 were designed using the NCBI primer designing tool whereas previously published primers were used to assess the expression of IL-17A (118), IL-21 (119), FOXP3 (120) and β-actin (121). Primers were blasted using the NCBI primer BLAST tool to ensure specificity.

The forward and reverse primers for RORγT had the following respective sequences:5'-GACTTTCCCTCTGGCACACA-3' and 5'- ATCCGGTCCTCTGCTTCTCT-3'; the expected product length was 135 base pairs (bp). *The forward and reverse primers for* IL-17A had the following respective sequences: 5'-TTAAGGTTCTCTCCTCTGAA-3' and 5'-TAGGGAGCTAAATTATCCAA-3'; the expected product length was 104 bp. *The forward and reverse primers for* IL-21 had the following respective sequences: 5′-CTCCAGCCTCAGTCTCCTCT-3′ and 5′-ACCTCTGGTCTCTTGGCTCA-3′; the expected product length was 177 bp. *The forward and reverse primers for* FOXP3 had the following respective sequences: 5'-TGGGTGTCAGGAGCCCACCAG-3' and 5'-AGGGCCACAGCATGGGTCTGT-3'; the expected product length was 91 bp. *The forward and reverse primers for* CTLA4 had the following respective sequences: 5′-GCCAGTGGTTCCAAAGGTTG-3′ and 5′- CACTGTGGGACGACACTGAT-3'; the expected product length was 133 bp. The forward and reverse primers for β-actin had the

following respective sequences: 5’-GGC ATT GTT ACC AAC TGGGAC GAC-3’ and 5’-CCA GAG GCA TAC AGG GAC AGCACA G-3’; the expected product length was 218 bp (Table.1).

b. Protocol

For real time PCR assessment of the RORγT, IL-21, FOXP3 and CTLA4 genes, 25 µl reactions were prepared, each reaction containing 12.5 µl of SYBR green, 5 picomoles of the forward primer, 5 picomoles of the reverse primer and 100 ng of cDNA. For the assessment of IL-17A gene, 10 µl reactions were prepared, each reaction containing 5 µl of SYBR green, 150 picomoles of the forward primer, 150 picomoles of the reverse primer and 150 ng of cDNA. Each sample was run in triplicates. Real time detection was then performed in a BioRad CFX96 Real Time System using a C1000 Thermal Cycler (Hercules, CA). The cycling conditions were as follows: PCR initial activation step took place at 95ºC for 5 minutes and was followed by 40 cycles of 95ºC for 15 seconds followed by the annealing temperature for 30 seconds. The annealing temperatures for RORγT, IL-17A, IL-21, FOXP3, CTLA4 and β-actin were 56°C, 56°C, 60.8°C, 60.3°C, 60.9°C and 58.6°C respectively (Table.1).

c. Normalized Relative Gene Expression

Upon obtaining the threshold cycle (C_T) for each target gene per sample in addition to that of β- actin used as a reference gene, the normalized relative expression was calculated. First the ΔC_T was calculated using the following formulas:

→ ΔC_T (test) = C_T (target, test) – C_T (ref, test)

→ ΔC_T (calibrator) = C_T (target, calibrator) – C_T (ref, calibrator)

Where 'test' indicates an experimental mouse group, 'calibrator' indicates the water injected control mouse group used for expression normalization, 'target' indicates the gene of interest and 'ref' indicates the reference gene, β-actin.

Then, the $\Delta\Delta C_T$ was calculated as follows:

→ $\Delta\Delta C_T = \Delta C_T$ (test) – ΔC_T (calibrator)

Finally, relative expression (RE) per sample normalized to that of the water-injected calibrator group was calculated, employing the $\Delta\Delta C_T$ determined above, using the following formula (122):

→ $RE = 2^{-\Delta\Delta CT}$

F. Statistical analysis

To assess statistical significance, unpaired t-tests were performed using the Graphpad software. The p-values for experimental groups were calculated in comparison to the water-injected mouse group on day 3; p-values less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

A. Relative gene expression of T helper 17 selected markers:

Intraperitoneal injection of 144 x 10^3 copies of EBV DNA into BALB/c mice led to an increase in the transcriptional levels of IL-17A by 357 folds on day 3 (p=0.0002), 512 folds on day 6 (p=0.0006) and 183 folds on day 9 (p=0.0232) post-injection normalized to its expression on day 3 post-injection in the mouse group treated with sterile distilled water (Table.2, Figure.1). Although injection with *S. epidermidis* DNA resulted in some increase in IL-17 transcription, the highest fold increase was by 78 folds on day 9. Similarly, IL-21 gene expression increased in response to EBV DNA injection by 165 folds on day 3 (p=0.0128), 1462 folds on day 6 (p=0.0077) and 1236 folds on day 9 (p=0.0017) post injection (Table.3, Figure.2). The highest level of IL-21 transcript fold increase induced by treatment with *S. epidermidis* DNA was by 141 folds on day 9. A statistically significant increase was also noted in RORγT gene expression by 76 folds on day 3 post-injection (p=0.001), by 524 folds on day 6 post-injection (p=0.0094) and by 28 folds on day 9 post-injection (p=0.0045) (Table.4, Figure.3). The highest level of RORγT transcript detection upon treatment with *S. epidermidis* DNA was on day 6; this increase was by 74 folds.

Worth indicating is that transcriptional level increases induced by *S. epidermidis* DNA in the expression of IL-17A, IL-21 and RORγT were notably less than those triggered by EBV DNA at all time points.

B. Relative gene expression of regulatory T cell selected markers:

Normalized to its expression in the mouse group treated with sterile distilled water on day 3 post-injection, the level of FOXP3 expression upon intraperitoneal injection of 144 x 10^3 copies of EBV DNA into BALB/c mice was increased by 11 folds on day 3 (p=0.0172), 12 folds on day 6 (p=0.0995) and by 53 folds on day 9 (p=<0.0001) post-injection. On the other hand, in contrast with the results observed for Th17 activity markers, the injection of *S. epidermidis* DNA also led to an increase in FOXP3 gene expression by 41 folds on day 3 (p=0.0777), by 15 folds on day 6 (p=0.0431) and by 146 folds on day 9 (p=0.0282) post-injection; these levels were higher than the increases observed upon injection with EBV DNA (Table.5, Figure.4).

In the case of CTLA4, the injection of 144 x 10^3 copies of EBV DNA did not lead to a statistically significant increase in the level of gene expression. On the other hand, the injection of *S. epidermidis* DNA resulted in a statistically significant increase in the level of CTLA4 gene expression by 40 folds on day 3 (p=0.0003), by 15 folds on day 6 (p=0.0007) and by 83 folds on day 9 (p=0.0374) post-injection normalized to its expression on day 3 post-injection with sterile distilled water (Table.6, Figure.5).

Table 1: Primer sequences, annealing temperatures and expected product lengths.

Gene	Primers	Annealing temperature	Product length (bp)
RORγT	F: 5'-GACTTTCCCTCTGGCACACA-3' R: 5'-ATCCGGTCCTCTGCTTCTCT-3'	56°C	135
IL-17A	F: 5'-TTAAGGTTCTCTCCTCTGAA-3' R: 5'-TAGGGAGCTAAATTATCCAA-3'	56°C	104
IL-21	F: 5'-CTCCAGCCTCAGTCTCCTCT-3' R: 5'-ACCTCTGGTCTCTTGGCTCA-3'	60.8°C	177
FOXP3	F: 5'-TGGGTGTCAGGAGCCCACCAG-3' R: 5'-AGGGCCACAGCATGGGTCTGT-3'	60.3°C	91
CTLA4	F: 5'-GCCAGTGGTTCCAAAGGTTG-3' R: 5'-CACTGTGGGACGACACTGAT-3'	60.9°C	133
β -actin	F: 5'-GGCATTGTTACCAACTGGGACGAC-3’ R: 5'-CCAGAGGCATACAGGGACAGCACAG-3’	58.6°C	218

Table 2: IL-17A relative gene expression in BALB/c mice.

	Treatment	IL-17A relative gene expression	SD	P-value
Day 3	Water	1	0.33	
	EBV DNA (144x10^3 copies)	357.72	7.89	0.0002
	S. epidermidis DNA (28.3pg)	30.60	1.06	0.0007
Day 6	Water	2.23	0.44	0.0893
	EBV DNA (144×10^3 copies)	512.83	17.28	0.0006
	S. epidermidis DNA (28.3pg)	53.04	34.63	0.1675
Day 9	Water	0	0	0.0532
	EBV DNA (144×10^3 copies)	183.56	39.98	0.0232
	S. epidermidis DNA (28.3pg)	77.98	53.66	0.1796

Table 3: IL-21 relative gene expression in BALB/c mice.

	Treatment	IL-21 relative gene expression	SD	P-value
Day 3	Water	1	0.37	
	EBV DNA (144x10^3 copies)	165.53	41.31	0.0128
	S. epidermidis DNA (28.3pg)	19.96	1.15	0.002
Day 6	Water	1.91	0.23	0.099
	EBV DNA (144×10^3 copies)	1462.95	181.88	0.0077
	S. epidermidis DNA (28.3pg)	34.14	48.28	0.4341
Day 9	Water	0	0	0.0631
	EBV DNA (144×10^3 copies)	1235.69	153.91	0.0017
	S. epidermidis DNA (28.3pg)	141.32	153.86	0.3262

Table 4: RORγT relative gene expression in BALB/c mice.

	Treatment	**RORγT relative gene expression**	**SD**	**P-value**
Day 3	Water	1	0.39	
	EBV DNA (144x10^3 copies)	76.78	3.32	0.001
	S. epidermidis DNA (28.3pg)	4.26	1.27	0.074
Day 6	Water	12.23	3.19	0.0387
	EBV DNA (144×10^3 copies)	524.38	72.13	0.0094
	S. epidermidis DNA (28.3pg)	74.11	5.78	0.0031
Day 9	Water	22.35	3.88	0.0163
	EBV DNA (144×10^3 copies)	28.12	2.56	0.0045
	S. epidermidis DNA (28.3pg)	20.18	6.39	0.0515

Table 2: FOXP3 relative gene expression in BALB/c mice.

	Treatment	**FOXP3 relative gene expression**	**SD**	**P-value**
Day 3	Water	1	0.10	
	EBV DNA (144x10^3 copies)	10.94	1.86	0.0172
	S. epidermidis DNA (28.3pg)	41.18	16.83	0.0777
Day 6	Water	0	0	0.005
	EBV DNA (144×10^3 copies)	11.79	5.21	0.0995
	S. epidermidis DNA (28.3pg)	15.16	4.29	0.0431
Day 9	Water	0	0	0.005
	EBV DNA (144×10^3 copies)	53.26	0.07	< 0.0001
	S. epidermidis DNA (28.3pg)	146.25	35.25	0.0282

Table 3: CTLA4 relative gene expression in BALB/c mice.

	Treatment	CTLA4 relative gene expression	SD	P-value
Day 3	Water	1	0.14	
	EBV DNA (144x10^3 copies)	5.23	3.60	0.2384
	S. epidermidis DNA (28.3pg)	40.91	11.58	0.0003
Day 6	Water	8.22	6.65	0.0129
	EBV DNA (144×10^3 copies)	1.23	0.81	0.7275
	S. epidermidis DNA (28.3pg)	14.99	0.50	0.0007
Day 9	Water	11.43	2.64	0.0308
	EBV DNA (144×10^3 copies)	1.95	0.89	0.2761
	S. epidermidis DNA (28.3pg)	83.16	23.12	0.0374

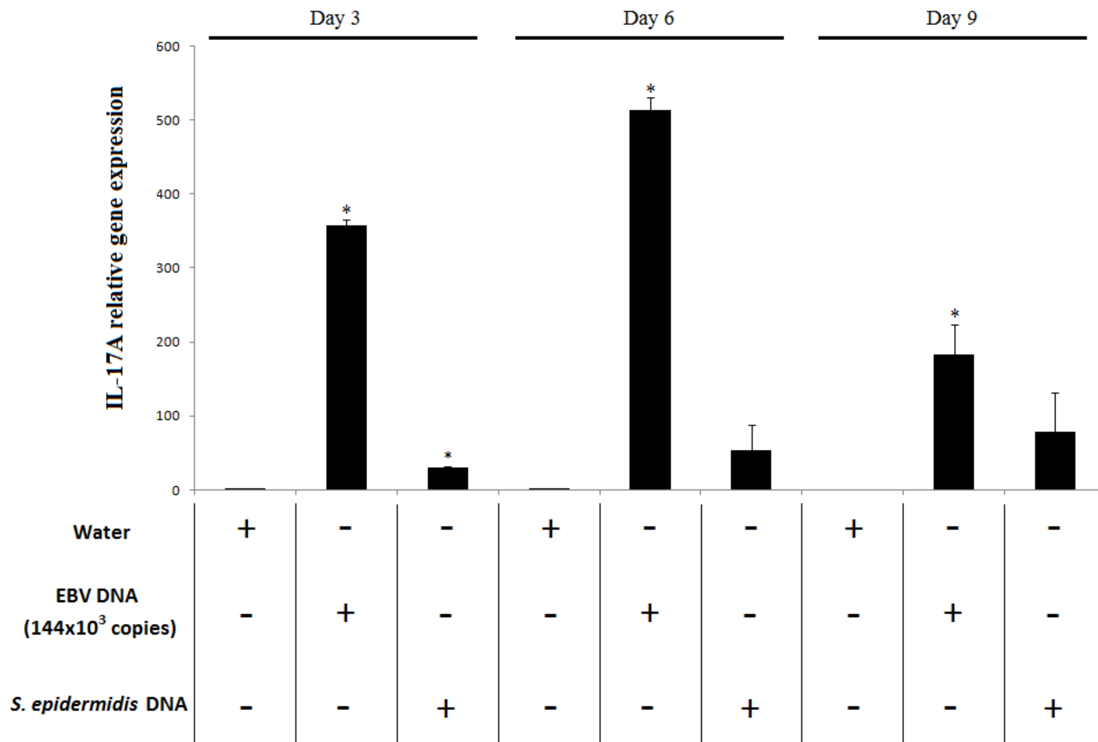
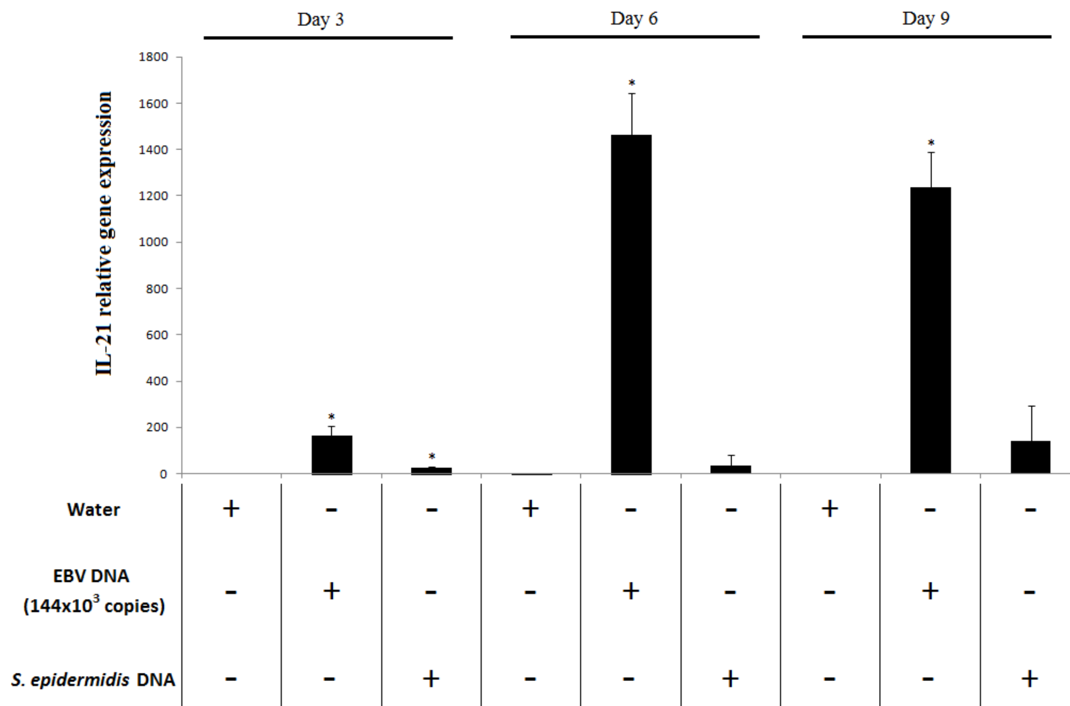


Figure 1: IL-17A relative gene expression in spleens of BALB/c mice treated with sterile distilled water, EBV DNA (144x10^3 copies), and *S. epidermidis* (28.3pg) DNA on days 3, 6 and 9 post-injection. Levels are normalized to those on day 3 post-injection with sterile distilled water. * indicates $p<0.05$



Water	+	-	-	+	-	-	+	-	-
EBV DNA ($144x10^3$ copies)	-	+	-	-	+	-	-	+	-
S. epidermidis DNA	-	-	+	-	-	+	-	-	+

Figure 2: IL-21 relative gene expression in spleens of BALB/c mice treated with sterile distilled water, EBV DNA ($144x10^3$ copies), and *S. epidermidis* (28.3pg) DNA on days 3, 6 and 9 post-injection. Levels are normalized to those on day 3 post-injection with sterile distilled water. * indicates $p<0.05$

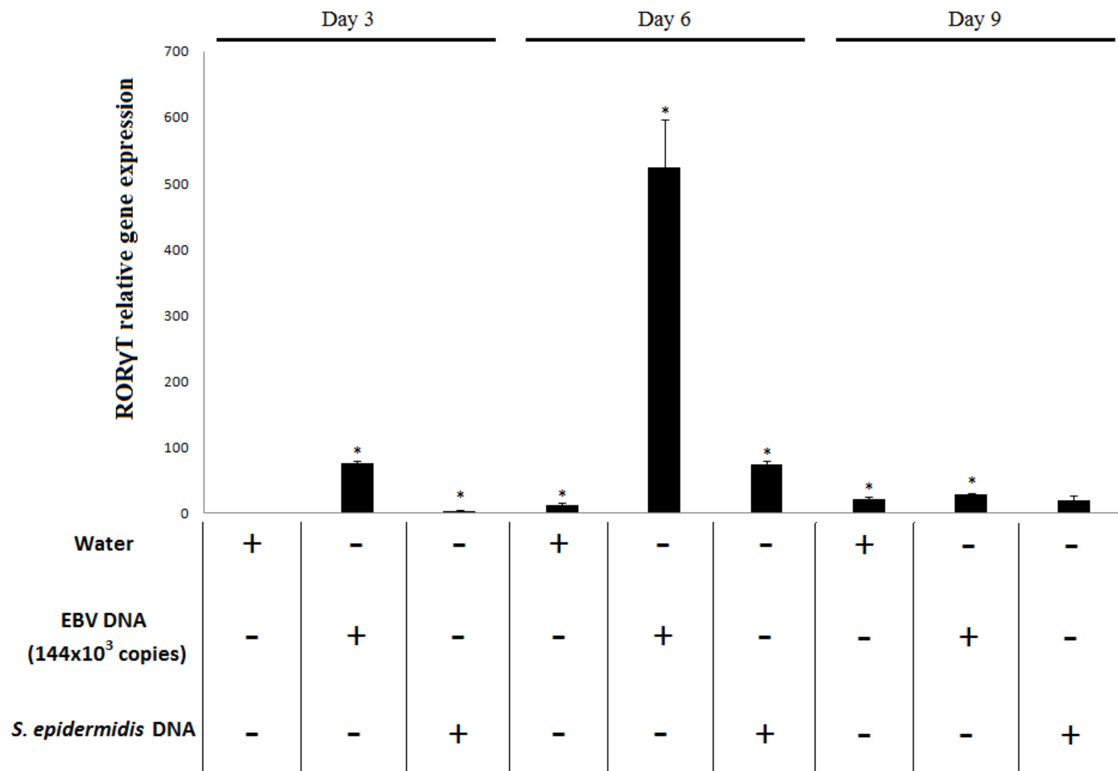


Figure 3: RORγT relative gene expression in spleens of BALB/c mice treated with sterile distilled water, EBV DNA (144x10^3 copies), and *S. epidermidis* (28.3pg) DNA on days 3, 6 and 9 post-injection. Levels are normalized to those on day 3 post-injection with sterile distilled water.* indicates $p<0.05$

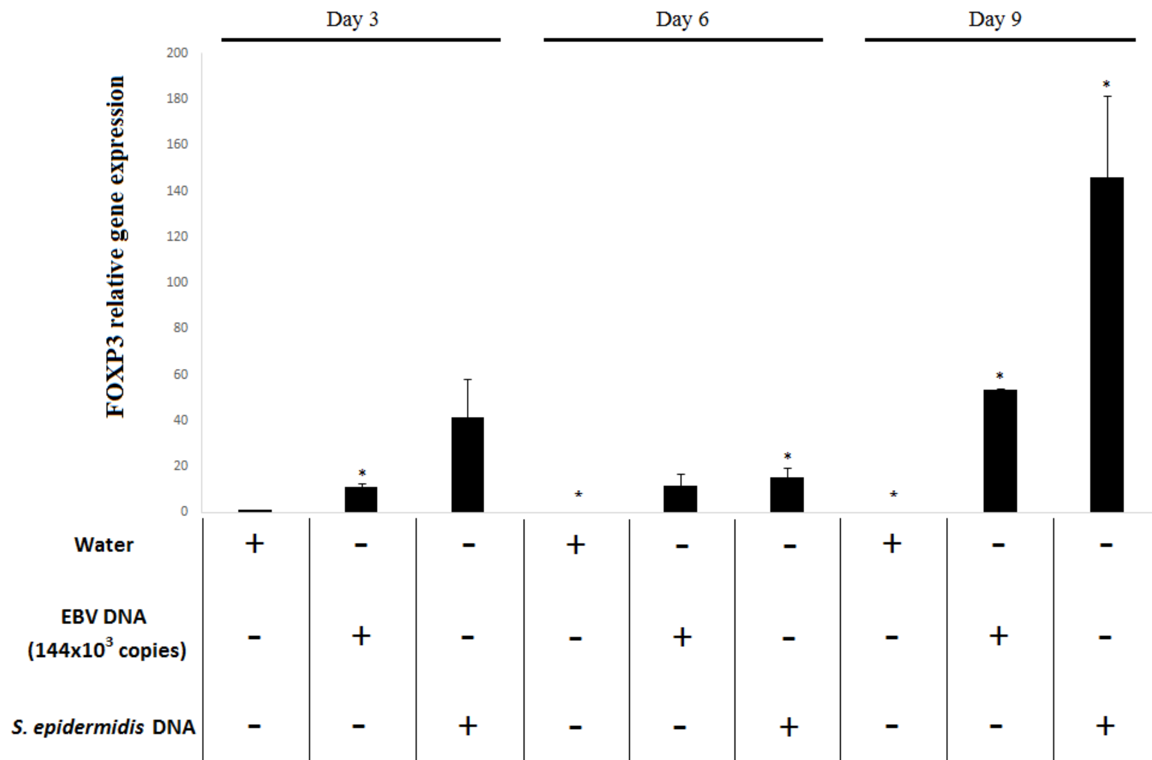


Figure 1: FOXP3 relative gene expression in spleens of BALB/c mice treated with sterile distilled water, EBV DNA (144x10^3 copies), and *S. epidermidis* (28.3pg) DNA on days 3, 6 and 9 post-injection. Levels are normalized to those on day 3 post-injection with sterile distilled water. * indicates $p<0.05$

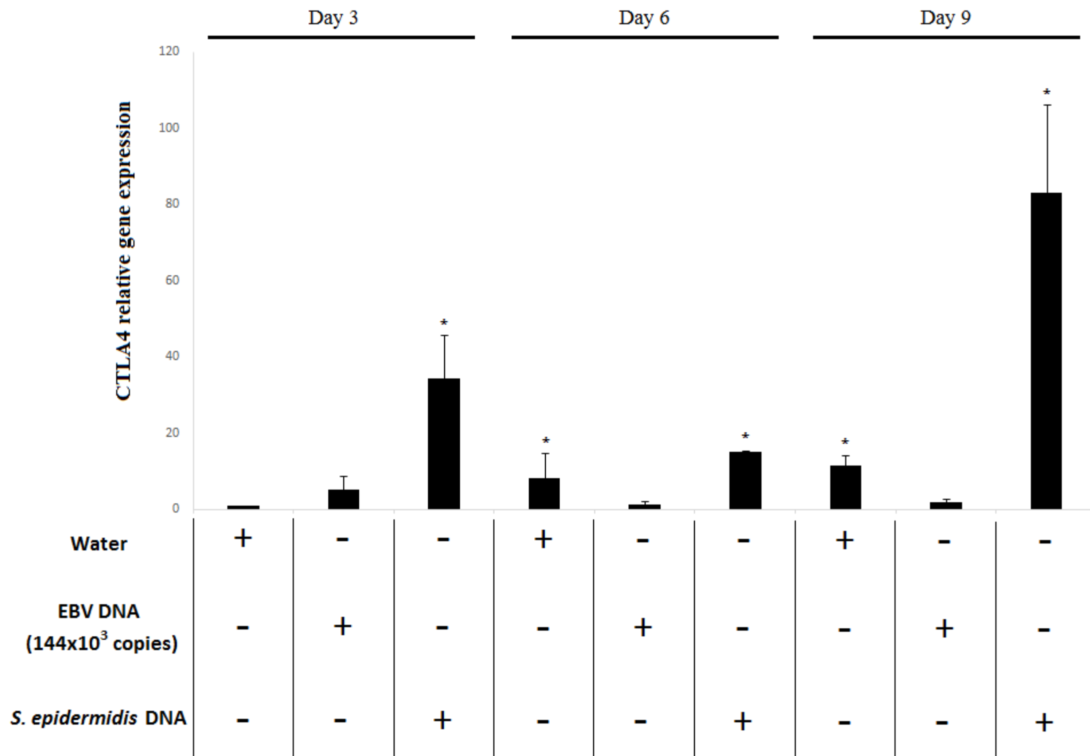


Figure 2: CTLA4 relative gene expression in spleens of BALB/c mice treated with sterile distilled water, EBV DNA (144x10^3 copies), and *S. epidermidis* (28.3pg) DNA on days 3, 6 and 9 post-injection. Levels are normalized to those on day 3 post-injection with sterile distilled water.* indicates $p<0.05$

CHAPTER V

DISCUSSION

Epstein-Barr virus has long been associated with autoimmunity (27, 42, 43), similarly, the association between the proinflammatory cytokine IL-17 and autoimmune diseases has been the subject of many studies (39, 58, 61). Being a herpesvirus, EBV enters a phase of latency in the host following the primary infection, and is capable of reactivating at any time potentially shedding its viral DNA (24). Moreover, a previous study has shown that EBV DNA triggers the production of IL-17 in BALB/c mice (41). Furthermore, being the major source of IL-17, the induction of Th17 mediated immune responses and the activation of these cells has been consequently associated with the development of inflammation and autoimmunity (71). On the other hand, regulatory T cell functions have been considered principal among several complex regulatory mechanisms that sustain immune homeostasis, prevent autoimmune diseases and control inflammation (80, 81). Therefore, this study aimed to examine whether Th17 and regulatory T cell activities are affected by EBV DNA *in vivo* in BALB/c mice.

The transcriptional levels of RORγT, a transcription factor essential for the Th17 program, increased in mice splenic tissue with the highest normalized level being 524 (p=0.0094), on day 6 after injection with EBV DNA. Furthermore, the normalized transcriptional levels of IL-17 and IL-21, two cytokines produced by activated Th17 cells, also increased with their highest levels being 512 (p=0.0006) and 1462 (p=0.0077), respectively, also on day 6. These results indicate that EBV DNA induces Th17

lymphocytes which subsequently leads to the production of proinflammatory cytokines. This is concurrent with our previous observations indicating that IL-17 increases at the protein level in mice injected with EBV DNA (41). On the other hand, transcriptional levels of the assessed Th17 markers were lower in *S. epidermidis* DNA-injected mice than in those injected with EBV DNA on all examined days. This indicates that EBV DNA has a higher potency of activating Th17 responses.

In contrast to Th17 markers, those of regulatory T cells were higher in *S. epidermidis* DNA-injected mice than in EBV DNA-injected ones on all days tested. Transcriptional levels of FOXP3, a transcription factor essential for the Treg program, were induced by EBV DNA however to a lesser extent than *S.epidermidis* DNA injection. A low level of FOXP3 transcriptional increase in the EBV DNA injected mice, that of 12 folds, coincided with the highest levels of Th17 markers; this indicates that pro-Th17 responses may adversely affect regulatory T cell responses. This notion is further supported by the detected transcriptional levels of CTLA4 which also were at their lowest on day 6 post injection with EBV DNA. CTLA4 is a T cell regulatory activity marker and is constitutively expressed on Tregs. Not only were the levels of CTLA4 expression lower in the EBV DNA-injected group than the *S. epidermidis* DNA-injected group on all days tested, they were also significantly lower than the water-injected group on days 6 and 9 post injection; the normalized level of CTLA4 was 4.39 in the water-injected group while it was 1.23 in the EBV DNA-injected group ($p=0.0447$) on day 6. On the other hand, its normalized level was 11.43 in the water-injected while it was 1.95 in the EBV DNA injected group ($p=0.0408$) on day 9. This may indicate that CTLA4 expression is rather suppressed by EBV DNA.

These results indicate that EBV DNA favors Th17 cell activity at the expense of regulatory T cells. This may be explained by the fact that critical factors required for Th17 lymphocytes, including the transcription factors STAT3 and RORγT, in addition to proinflammatory cytokines such as IL-6, IL-1β and IL-21 in the presence of TGF-β (75), simultaneously restrict the expression of factors needed for Treg programming such as STAT5 and FOXP3, which are usually induced by TGF-β alone (112, 113).

In conclusion, the potential involvement of EBV DNA in inflammatory reactions may be mediated by the modification of two major cell compartments with opposing functions; on one hand, inducing Th17 cells which contribute to the development of inflammation and autoimmunity by producing IL-17, and on the other hand, repressing regulatory T cell activities which are responsible for suppressing inflammation and restricting the development of autoimmunity. The exact mechanisms by which EBV DNA induces a Th17/IL-17 mediated inflammatory response while repressing regulatory T cell activities, remain to be fully investigated. Assessing these mechanisms could eventually provide therapeutic targets to control the development of inflammation and autoimmunity in an EBV infection. Further research efforts are also required to specifically examine the rate of proliferation, differentiation and activation of the studied cell compartments in different mammalian tissues.

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