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EPSTEIN-BARR VIRUS GENOMIC DNA AND EPSTEIN-BARR NUCLEAR ANTIGEN 3A (EBNA-3A) AS TRIGGERS OF PRO-INFLAMMATORY MECHANISMS

by
DIALA MHD ALI ALHAKIM

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AN ABSTRACT OF THE THESIS OF

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Title: Epstein-Barr Virus Genomic DNA and Epstein-Barr Nuclear Antigen 3A (EBNA-3A) as Triggers of Pro-Inflammatory Mechanisms

Epstein-Barr virus (EBV), a member of the *Herpesviridea* family, is a DNA virus that establishes latent infections that often later reactivate. Potential associations between EBV and several autoimmune diseases such as rheumatoid arthritis (RA) have been reported. Previous studies have also demonstrated a role for EBV nuclear antigens, such as EBNA-3A, in B lymphocyte transformation; moreover, cytotoxic T-lymphocytes specific to EBNA-3 protein family members are the most prevalent CD8+T cells during a latent EBV infection. On the other hand, studies conducted at our department have shown that EBV DNA may enhance the production of the pro-inflammatory cytokine Interleukin 17 (IL-17), which has been shown to contribute to a large number of autoimmune diseases. Therefore, we intended to study the effect of EBNA-3A and EBV DNA on autoimmune pathways. We examined whether these viral components are capable of triggering IL-17 production in female BALB/c mice and in human peripheral blood mononuclear cells (PBMCs) from patients with RA in culture. Different mouse groups, each containing 9 female BALB/c mice, were intraperitoneally injected with an increasing amount of EBNA-3A with or without EBV DNA. One group was injected only with sterile water (the DNA and EBNA-3A solvent) and another was left uninjected. After 3, 6, and 9 days of injection, IL-17 and IL-10, as a pro-inflammatory and an anti-inflammatory cytokine, respectively, were measured in mice sera using an enzyme-linked immunosorbent assay (ELISA). On the other hand, PBMCs from 7 EBV positive RA patients and 7 non-RA control subjects were cultured in the presence or absence of EBNA-3A and/or EBV DNA for 24 hrs. Culture supernatants were then collected and examined for IL-17 and IL-10 levels by ELISA. Mouse groups injected with EBV DNA showed increased levels of IL-17 starting from day 6 post-injection. Similarly, when received alone, 5 and 10 μg of EBNA-3A increased IL-17 levels in mice after 9 days of injection. These levels were also increased in all mouse groups receiving different concentrations of EBNA-3A with EBV DNA by day 9 post-injection. In contrast, a reduction in IL-17 levels was recorded in supernatants of both non-RA control and RA PBMCs incubated with EBNA-3A and EBV DNA compared to respective untreated cells of the same type. EBV DNA alone did not result in a significant change in IL-17 levels in non-RA control or RA PBMC supernatants compared to respective untreated cells of the same type. On the other hand, the IL-10 levels detected in supernatants from PBMCs treated with EBV components were variable among cells from different subjects and did not display any notable changes upon treatment with viral components. In conclusion, EBV DNA and EBNA-3A are possible triggers of pro-inflammatory mediators *in vivo* as demonstrated in mice; however, similar effects were not observed *ex vivo* in PBMCs from RA subjects. Whether these components serve as triggers of pro-inflammatory pathways in a human subject is therefore yet to be determined.

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To The Loving
Memory of My Father
(Love & Miss you)

CHAPTER I

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most prevalent autoimmune diseases globally. Triggering RA is believed to require the association of a genetic predisposition with the presence of an environmental factor. Previous studies have implicated the Epstein-Barr virus (EBV) in triggering RA in genetically predisposed individuals. EBV is a herpes virus that infects and perpetuates in B lymphocytes where the viral genome remains in a latent form for life. During latency, some viral proteins, including the EBV nuclear antigen 3A (EBNA-3A), continue to be expressed. It is thought that an EBV infection may impair the tolerance of self-antigens hence resulting in an autoimmune disease in genetically susceptible subjects. A previous study at the Department of Experimental Pathology, Immunology and Microbiology indicated an increase in interleukin 17 (IL-17) production in mice injected with EBV DNA. A similar observation was seen in peripheral blood mononuclear cells (PBMCs) from multiple sclerosis subjects previously infected with HHV-6 when these PBMCs were treated with HHV-6 or EBV DNA. Based on these findings, the overall objective of this study was to assess whether EBNA-3A in conjunction with EBV DNA is capable of triggering IL-17 as a pro-inflammatory mediator and to determine the effect of such a treatment on IL-10, the potential anti-inflammatory cytokine. The aims of this study were to:

1. Assess the effect of EBNA-3A and EBV DNA on IL-17, as a pro-inflammatory marker of autoimmunity, and IL-10, as a potential anti-inflammatory marker, *in vivo* in BALB/c mice.

2. Examine the effect of EBNA-3A and EBV DNA on IL-17 and IL-10 production in PBMCs from RA patients and non-RA control subjects *ex vivo*.

CHAPTER II

LITERATURE REVIEW

A. Epstein-Barr Virus (EBV)

1. *Discovery and Classification*

In 1964, M. A. Epstein and Y. M. Barr discovered the fourth virus in the human herpesvirus family in a Burkitt's lymphoma cell line and named it after themselves, the Epstein-Barr virus (EBV) (Epstein & Achong, 1964) [1] EBV is the *Human herpesvirus 4* (HHV-4) species of the *Lymphocryptovirus* genus that belongs to *Gammaherpesvirinae* subfamily of the *Herpesviridae* family [2]. Eight herpesviruses are known to be capable of infecting humans [3]. Once an infection occurs, viruses of this family remain in the host for life in a latent form with possible recurrent reactivation [4]. Herpesviruses are distinct in their cell type tropism, content of their genome, and the immune response they trigger [5]. They all encode, however, a conserved set of proteins, encoded by 40 conserved genes [6].

2. *Structure*

The Epstein-Barr virus structure, as all herpesviruses, consists of four major components: 1) The envelope which is a lipid bilayer acquired from the infected cell while the newly formed virion buds through the host cell membrane ; it also contains virally encoded glycoproteins (spikes) [7]. The major envelope glycoprotein is gp350 which binds CD21, the viral receptor, on B lymphocyte surfaces. 2) The tegument or viral matrix is a layer between the nucleocapsid and the envelope and formed of about 17 amorphous proteins. Among the proteins constituting the viral tegument, host

proteins can be found like heat-shock protein 70 (Hsp 70) and actin. 3) An icosadeltahedral nucleocapsid that measures 100 nm in diameter and has 162 capsomeres [7]. 4) Finally, the core contains the viral genetic material and consists of double-stranded linear DNA that is about 172 kilo base pairs (kbp) and encodes for 100 genes [4].

3. *Genomic Properties*

Studying the genomic constitution of the virus revealed that both ends of the linear double-stranded DNA (dsDNA) contain an inconstant number of 538 base pair repeats called the direct terminal repeats (TRs). The major internal repeat (IR1) contains 5-10 copies of a sequence consisting of about 3 kilo base pairs (kbp) that separates between the short (12 kbp) and the long (134 kbp) unique sequences (U_s and U_L, respectively). IR1 contains the promoter, Wp, for the genes encoding EBV nuclear antigens (EBNAs). There are other shorter internal repeats (IR2, IR3, and IR4) that contain repetitive elements and divide the long unique sequence (U_L) into four shorter sequences (U2, U3, U4, and U5) [8]. Open reading frames (ORFs) of the EBV genome that can be translated into proteins were identified resulting in an estimate of 80-85 proteins that can potentially be expressed [4]. During lytic infection, which can happen in both epithelial cells and B lymphocytes, the virus productively replicates its genome, expresses most of its proteins, produces new progeny (virions), and is released by budding through the host cell membrane. Whereas, in the latent infection, mostly in B lymphocytes, only some proteins are expressed by the viral DNA which becomes circular and persists in B cells as an episome (integrated to the host cell genome) for life [4]. Proteins expressed in the lytic phase, identified so far, include the immediate early

viral proteins (BRLF1 and BZLF1) that initiate the expression of the early antigens including the virally encoded DNA polymerase [9], in addition to the late antigens such as the viral capsid antigen (VCA) and the membrane antigen (MA). The human interleukin 10 homologue (vIL-10) is also produced in the lytic infection [10]. On the other hand, twelve genes have been reported to be expressed during the latent (nonproductive) phase. Of these genes, nine proteins and three noncoding RNAs are expressed. Latently expressed proteins are classified into two classes, the EBV-encoded nuclear antigens (EBNAs) that include EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-LP, and the latent membrane proteins (LMPs) include LMP-1, LMP-2A, and LMP-2B. The noncoding RNAs expressed during latency are the EBV-encoded small RNAs (EBER 1 and 2) and the BamH1-A rightward transcripts (BARTs) [11]. A cellular polymerase is responsible for the viral episome maintenance; on the other hand, the production of new linear DNA for the new virions, upon reactivation is accomplished by a viral polymerase [12]. Transcription of EBNA-1, LMP-1, 2A, 2B, and EBERs continues during EBV reactivation [10].

a. Immunostimulatory Properties of EBV DNA

A study conducted at the Department of Experimental Pathology, Immunology and Microbiology at the American university of Beirut has demonstrated that EBV DNA is capable of inducing the production of IL-17 from mice *in vivo* and from human peripheral blood mononuclear cells (PBMCs) obtained from multiple sclerosis patients *ex vivo* [13]. Moreover, EBV DNA has been shown to induce the release of IL-8 from primary monocytes and IFN-α from plasmacytoid dendritic cells via Toll-like receptor 9 (TLR9) signaling [14]. TLR9 is associated with the recognition of unmethylated

cytosine-p-guanosine (CpG) motifs which are present in the EBV genome [15]. CpG motifs are believed to contribute to triggering autoimmune diseases in predisposed individuals [16]. Experimental evidence supports this notion. For example, autoreactive B cells from mice with lupus-like disease treated with CpG-containing immune complexes produced large amounts of autoantibodies [16]. Moreover, healthy mice injected with bacterial CpG intra-articularly develop aseptic arthritis [17]. Previous studies have also shown that the *Human herpes virus 1* (HSV-1) purified genome can induce a type 1 helper T cells (Th1) immune response via its unmethylated CpG motifs when introduced to mice or when mouse splenocytes are treated with HSV-1 DNA *ex vivo* [18]. Viral nucleic acids may also be recognized by intracellular receptors other than TLR9. These include the nucleotide oligomerization domain (NOD)-like receptors (e.g. NOD1 and NOD2), the cytosolic DNA sensors (e.g. DAI and AIM2) and the retinoic acid inducible gene 1 (RIG-1)-like receptors (RLRs) [19].

4. *EBV Types and Genomic Variability*

Although most of the EBV genome is conserved [20], some genomic variability exists. EBV strains are currently classified as either Type 1 or Type 2 based on the sequence of the EBNA-2A gene; these are also referred to as Type A and Type B respectively. While Type 1 is the more commonly encountered variant worldwide, Type 2 is more common in particular regions of Africa [21].

The EBNA-2 proteins obtained from the B95-8 strain, the prototype of EBV type 1, and the AG876 strain, the prototype of EBV type 2, are 55% homologous [22]; whereas EBNA-3 proteins (A, B, and C) show 84, 80, and 72% homology respectively between the two strains[23]. This difference results in EBV type 2 being less efficient in

inducing B cell transformation *in vitro* than EBV type 1. This further highlights the importance of EBNA-2 and EBNA-3 gene products in the induction of B cell immortalization [24].

Some ORFs may differ between EBV isolates within the same strain, for example the LMP-1 ORF has a 30 base pairs (bp) deletion in some isolates that results in the production of an LMP-1 protein lacking the C-terminal domain [25].

5. *Infection with EBV*

The majority of EBV infections are transmitted orally via saliva and most likely during childhood [26]. The virus is also transmitted sexually [27] and through organ transplantation which may lead to post transplantation lymphoproliferative disease (PTLD) [28]. Low concentrations of the virus have been detected in breast milk; transmission through breast feeding is potential, however, is not confirmed [29].

The virus first enters the differentiated oropharyngeal cells in oral transmission cases [26]; it replicates in these cells, then spreads into salivary glands, oropharyngeal lymphoid tissues, and B lymphocytes of the tonsils where the virus can produce new virions (lytic cycle) or stays inside the memory B cells without progeny production (latent cycle) [10]. The latently infected memory B cells are attacked by cytotoxic T cells (CTLs); few of them, however, survive and remain in circulation for life [30]. Intermittent viral reactivation occurs in latently infected lymphocytes and the new virions produced infect the oropharyngeal epithelial cells where lytic cycle of the virus is predominant giving rise to new infectious progeny [31]. The reactivation of the virus from a latently infected memory B cell is essential for two reasons, maintenance of a

persistent EBV infection in memory B cell, and transmission of the virus to new naïve human hosts [31].

While some studies indicate that latency can occur in both epithelial cells and B lymphocytes , it is evident that transmission from person to person is always happening through infected B lymphocytes [31]. T cells may also be infected by EBV and the virus has been associated with a subset of T-cell lymphomas [32]. Interestingly, virions generated from B cells are oriented to infect epithelial cells; whereas those generated from epithelial cells are inclined to infect B cells. The virion tropism is dictated by the ratio of glycoptotein complexes on its envelope [31].

6. ***Diseases Associated with EBV***

Due to the diversity of host immune responses that are likely controlled by genetic factors, diseases associated with EBV are variable [33]. They can be divided into infectious mononucleosis, epithelial diseases, and other associated diseases.

a. Infectious Mononucleosis (IM)

If primary infection with EBV occurs during childhood, it is usually asymptomatic. On the other hand, 30-50% of immunocompetent adults exhibit IM upon acute infection. IM is a self-limiting lymphoproliferative disease that is normally resolved within several weeks without therapeutic intervention [34]. However, treatment might be necessary in some cases as described in the treatment section below [section A.11]. Common symptoms of IM include malaise, sore throat (pharyngitis), swollen lymph nodes of the head and neck (cervical lymphadenopathy), enlarged liver and spleen (hepatosplenomegaly), and a high lymphocyte count (10,000-20,000 cells per cm^3) with

increased ratio of the atypical (active) cells. Young children exhibit more exudative pharyngitis and hepatosplenomegaly with occasional exanthema than adults do. Other symptoms that may associate a primary infection with EBV include cough, vomiting, diarrhea and fever that can be prolonged and intermittent [34]. Serious complications may include lung infection (pneumonia), chronic active liver infection (hepatitis), internal bleeding (gastrointestinal hemorrhage), and bone marrow failure [33]. In extremely rare cases, chronic active EBV-infection (CAEBV) may occur, in which some of the IM symptoms may persist for up to 6 months. Persistent symptoms in CAEBV include intermittent fever, aplastic anemia, hepatitis, uveitis, and splenomegaly associated with high serum titers of lytic phase EBV-antibodies and antigens in addition to high viral load [35]. CAEBV can be severe with a high rate of mortality [33]. This type of infection is characterized by the presence of an EBV-infected CD4+T and natural killer (NK) cells. In patients with X-linked lymphoproliferative syndrome IM is fatal due to liver necrosis and failure [35]. In this case T cells that control the proliferation of EBV-infected B cells are impaired due to a genetic disorder that leads to the production of a defected signaling adaptor SLAM-associated protein (SAP) involved in the lymphocytic activation molecule (SLAM or CD150) signaling [36]. This results in impaired perforin-dependent cytotoxicity, which diminishes the capability of CD+8 T cells of eliminating EBV infected B cells [37].

i. Burkitt's Lymphoma

EBV infection triggers burkitt's lymphoma in genetically susceptible individuals. These patients have a chromosomal translocation that places the c-myc proto-oncogene adjacent to the immunoglobulin regulatory elements resulting in the activation of c-

myc. EBV is believed to induce Burkitt's lymphoma by acting as a cofactor to c-myc, and enhancing the proliferation of B lymphocytes that harbor this translocation and consequently leading to the selection of such clones [38].

ii. EBV Immunoblastic Lymphomas

In severely immunosuppressed hosts, T cells are incapable of controlling the proliferation of EBV-infected B cells [39]. Whether the infection is primary or latent, EBV-infected B cells will expand continuously leading to immunoblastic lymphomas in these subjects. A direct example is PTLD which may be diffused or localized to bone marrow by infiltration. PTLD begins as a polyclonal B lymphocyte proliferation that is reversible and responsive to therapy, then biclonal, and ends with monoclonal B cell proliferation that is irreversible, refractory to therapy, and rapidly fatal [28].

iii. Hodgkin's Lymphoma (HL)

EBV contributes directly to the oncogenesis of HL by affording the infected B lymphocyte the means to survive apoptosis through the viral anti-apoptotic protein LMP-2A [40].

iv. Other Lymphomas

EBV can rarely infect T and NK cells causing T-cell and NK-cell lymphomas. These are fatal diseases that usually progress aggressively [32].

b. Epithelial Diseases

The lytic cycle of the virus is predominant in epithelial cells [31]. EBV-associated diseases that affect epithelial cells include:

i. Hairy Leukoplakia (HLP)

HLP is a benign white lesion on the tongue in its lateral borders, contains massive numbers of EBV in its replicative (lytic) form [41]; however, latent genes are also expressed in this form of lesions making the replication of the virus in the infected epithelial cells not completely cytolytic [42]. It usually happens when the host is significantly immunosuppressed as in the case of patients infected with the human immunodeficiency virus (HIV) infection or on an immunosuppressive regimen. HLP is resolved upon antiviral therapy [41].

ii. Nasopharyngeal Carcinoma (NPC)

It has been hypothesized that NPC is the result of reactivation of latent virus giving rise to new virions that will induce dimer IgA synthesis. This latter will facilitate virion entry to new epithelial cells in the nasopharynx. The newly infected nasopharyngeal epithelial cells may have an altered program of gene expression due to years of exposure to environmental carcinogens. Dietary co-factors are also implicated in NPC. This may render the infected cells more susceptible to EBV leading to latent infection and transformation of these epithelial cells and later triggering their clonal proliferation resulting in NPC [43].

iii. Gastric and Breast Cancers

EBV has also been detected in 10% and 30% of gastric and breast cancers, respectively. Although EBV is not confirmed as an etiologic agent of these cancers, it is widely believed that it causes epigenetic changes in the infected epithelial cell [44].

c. Other Diseases

i. EBV-Associated Hemophagocytic Lymphohistiocytosis (EBV-HLH)

In EBV-HLH the infected cells are T lymphocyte (CD8+T) and NK cells rather than the usual B lymphocytes in IM. The presence of phagocytosed histiocytes in bone marrow and secondary lymphoid organs distinguishes HLH from IM. EBV-HLH is associated with high morbidity and mortality [45].

ii. Leiomyosarcoma

Leiomyosarcoma is a soft tissue cancer that may develop anywhere in the body (prostate, urinary bladder, uterus…etc.). The malignant neoplasms show variant degrees of smooth muscle differentiation [46]. These neoplasms express CD 21, also referred to as complement receptor 2 (CR2), that plays the role of an EBV receptor [46]. In acquired immune deficiency syndrome (AIDS) and immunocompromised patients, leiomyosarcomas are latently infected with EBV [47].

7. *Epidemiology*

About 90% of the population are seropositive for EBV infection, they have the latent form of the virus that can be reactivated asymptomatically [48].

The geographic distribution of EBV and its strains is directly correlated with the socioeconomic status. In under-developed countries and rural areas where hygienic customs are inadequate, infection is usually acquired early in life and during childhood starting from 2 years of age, most of the cases are asymptomatic. On the other hand, EBV infection in developed countries is delayed until adolescence or even adulthood, and patients usually manifest infectious mononucleosis upon primary infection. Both EBV types, EBV-1 and EBV-2, are widespread in tropical areas in Africa and New Guinea, whereas in the Western communities, the EBV-2 type is predominant [49].

The natural host of EBV is humans, but multiple types of animals can be infected particularly in an experimental setting [50].

8. *The Epstein-Barr Virus Nuclear Antigen-3 (EBNA-3) Family*

Three members of the EBNA-3 family have been identified, EBNA-3A, B and C (also known as EBNA-3, 4, and 6, respectively). All seem to result from single gene progenitor by duplication; however, the three genes now exhibit little sequence homology although they still share similar motifs. EBNA-3A and C have transactivation domains on their carboxylic termini and they can bind to many host cell transcriptional factors [51]. The EBNA-3 proteins can bind to the G2/M checkpoint kinase (chk2/cds1), disrupting the involved checkpoint which drives infected cells to skip arrest when treated with genotoxic agents [52]. This is believed to contribute to the EBV effect in inducing cell growth and proliferation during a particular phase of latency, namely the Latency III program, during which EBNA-3 proteins are expressed [10]. Furthermore, EBNA-3A and C can convert the chromatin from a transcriptionally active to a silent condition by modulating the histone acetylation state [53].

EBNA3-A and B suppress the expression of the pro-apoptotic tumor suppressor Bam [54]; additionally EBNA-3A and C were shown to be essential for the transformation of B cells [55, 56]. Subklewe et al. [57] have also reported that dendritic cell viability was reduced upon EBNA-3A expression in these cells. Noteworthy is that during an EBV latent infection, CTLs specific to EBNA-3 proteins (A, B, and C) are the most prevalent form of CD8+T cells [58]. (see section A.9) .

9. *The Immune Response against EBV Infection*

The immune response against an EBV infection includes aspects of both humoral and cellular immunity, and it becomes discernable after the incubation period of the virus which lasts 4-6 weeks [58].

The humoral response targets primarily the newly formed virions, thus antibodies appear rapidly upon an EBV-primary infection and are directed against replicative antigens like the viral capsid antigen (VCA), early antigens (EAs), membrane antigens (MA) that include gp350/220, and, later, the EBV nuclear antigens (EBNAs). Anti-MA antibodies are capable of neutralizing the virus and enhancing the antibody-dependent cellular cytotoxicity (ADCC). Generally, IgM antibodies against EA and VCA start to appear 1-2 weeks after an EBV-infection. Anti-EA are detected even before anti-VCA. Antibodies of several isotypes (IgG, IgM, and IgA) against a variety of viral proteins and protein complexes are formed [58]. Asymptomatic infections are usually accompanied with stable titers of anti-VCA, MA, and EBNA antibodies [59]. Infectious mononucleosis (IM), however, is characterized by an exaggerated humoral response due to polyclonal activation of EBV infected B cells, which even leads to the formation of autoantibodies like rheumatoid factor [60].

The cell-mediated response, on the other hand, targets the infected cells thus controlling unlimited B cell proliferation. This type of response involves NK and T cell cytotoxicity [61]. Primary infection with EBV causes a robust cellular response characterized by the over expansion of EBV-specific cytotoxic CD8+ T lymphocytes and observed as lymphocytosis in IM. These CD8+ T cells are reactive against both lytic and latent viral antigens and provide immunosurveillance over infected B cells in carrier individuals. The numbers of these cells are reduced when acute infection is resolved. CD8+ T cells with epitope specificity against EBNA proteins start to increase during convalescence of IM, and they predominate in carrier individuals as cytotoxic T cell precursors (CTLp) specific against EBV antigens and constitute about 1% of the total circulating CD8+ memory T cells [62]. The epitope specificity of these CTLp cells is narrow, mainly against EBNA-3 proteins (EBNA-3A, EBNA-3B, and EBNA-3C) and less predominantly against LMP-2, and they contribute to the immunosurveillance that prevents virus reactivation in infected B cells [63].

CD4+ T cells also contribute to the cellular response through IFN-γ releasing and cytolytic activities [64]. These helper T cells recognize HLA-DR bound to BHRF1 (an EBV early antigen) and induce elimination of the infected B cells [65]. This response is significant in primary infection; however, CD4+ T cells specific to EBV are hardly detected in healthy carriers suggesting that these cells die after the primary infection, lose their functional capacity, or that only a small number of CD4+ T cells is needed to accomplish the immune surveillance required [64]. Thus, an immunosuppressive therapy or an HIV infection that impairs T cell response allows infected B cells to proliferate indefinitely [66].

Dendritic cells (DCs), on the other hand, play an important role in adaptive and innate immune responses against EBV [67]. DCs are not directly infected, but rather process particles of the virus and of dying infected B and epithelial cells. These particles are presented on the DC surface bound to MHC class I and II molecules thus stimulating CD8+ and CD4+T cells, respectively. The primed T lymphocytes then control EBV infection and infected B cell proliferation. EBV-stimulated DCs produce IFN type 1 (IFNα/β) that protects B cells from transformation by the virus for the first 24 hours [68]. Natural Killer (NK) cells activated by the EBV-stimulated DCs produce IFN type II (IFNγ) at a later stage which then expands B cell protection against transformation about 3-4 days [69]. DCs are ten-fold more efficient in stimulating CD8+T cells against the viral proteins EBNA-3A and LMP-2 than virally transformed B cells. This is thought to result from the presence of more costimulatory and adhesion molecules on DCs than on B cells [70].

10. ***Diagnosis***

Infectious mononucleosis is diagnosed via its clinical manifestations, a complete blood count (CBC) test showing increased white blood cells and atypical lymphocytes, and an increased titer of anti-EA/VCA IgG and IgM [71]. The immunofluorescent antibody (IFA) assay is the gold standard; enzyme-linked immunosorbent assay (ELISA) is used as well. The presence of anti-EBNA antibodies is indicative of latent state [72, 73]. In case of EBV-related PTLD, the immunosuppressive therapy decreases the humoral response, which makes viral load (DNA or messenger RNA) measuring in peripheral blood lymphocytes (PBL), serum, plasma, ascites, transudates and effusions of any source more efficient as a diagnostic tool [71]. Polymerase chain reaction techniques

(PCR and real time PCR) amplifying EBERs gene are the most used in detection of the EBV latent form [72].

11. *Treatment*

A primary infection with EBV is usually self-limiting and does not require treatment. However, if the infection is severe and involves multiple organs, intravenous immunoglobulin (IVIG) are indicated to neutralize the virus and enhance ADCC [74]. Addition of corticosteroids and anti-viral drugs (Acyclovir, Gancyclovir) to the therapy is also recommended. Therapy is considered as well when infection is not resolved in the expected 1-2 weeks period [75]. In case of PTLD, the immunosuppressive therapy should be decreased as the first therapeutic intervention. This is usually a sufficient treatment if the proliferating cells are polyclonal in nature or have a low copy number of the virus. However, if this is not the case, changing the immunosuppressive therapy type and administration of an antiviral drug are considered. If monoclonal cells are present, this indicates the development of cancer; cytotoxic therapy is then required. Recently, cytotoxic T cells are addressed in therapy to enhance their control over the proliferating B cells. This is achieved by deriving T cell clones that can react against the patient's B cell lines *in vitro*, or by treating the patient with rituximab, a monoclonal anti-CD20 antibody [76]. In malignancies caused by EBV, early detection is essential for a successful therapy [77]. Prevention of EBV infection is now under investigation exploiting the neutralizing capacity of anti-viral membrane (anti-MA) antibodies in developing vaccines against EBV infection [78].

B. Immunological Tolerance

1. *T Cell Tolerance*

During embryonic life (8-9 weeks of gestation), newly formed T cells migrate from bone marrow to the thymus where central tolerance is induced [79]. Via positive selection, only T cells with low affinity to self-MHC peptide complexes survive; while those that show no affinity at all die. This process occurs in thymic cortex where cortical thymic epithelial cells (cTEC) present a wide range of self-antigen bound to MHC class I or II molecules [80]. In the currently predominant model, negative selection takes place in the thymic medulla where medullary thymic epithelial cells "mTEC" and thymic dendritic cells "tDC" present self-peptides bound to MHC class I or II. Here, the strength of interaction, between CD+8 or CD+4 cells and the self-peptides complexed to MHC I or II, respectively, dictates the fate of the T cell. If the interaction is too strong, apoptosis is triggered and the T cell dies; cells exhibiting weak interactions survive and thus are able to reach the periphery as mature naïve lymphocytes, either as helper T cells (CD+4) or cytotoxic T cells (CD+8) [80]. However, some clones presenting a moderate avidity T cell receptors (TCR) on their surfaces survive negative selection; yet they are functionally disabled (anergic). Such cells have a regulatory role contributing to the peripheral tolerance hence referred to as regulatory T cells (Treg) and they are capable of controlling autoreactive T cells that escaped clonal selection in the thymus [80].

The need for two signals to activate T lymphocytes is one mechanism of peripheral tolerance. In order for T cells to be fully activated and engage in the immune response, binding of their TCR with antigen-MHC complex expressed on the surface of an antigen presenting cell (APC) is not enough [81]. A second co-stimulatory signal is

required and provided by binding of TCR (CD28) on T lymphocytes to one of its ligands (CD80 or CD86) on an APC [82].

2. *B Cell Tolerance*

Induction of central tolerance in B lymphocytes takes place in the bone marrow, where cells that recognize self-antigens are subject to negative selection and undergo apoptosis [79]. However, some autoreactive B cells with low avidity to self-peptides can escape the central tolerance and reach the periphery [83].

In the periphery, recognition of a self-antigen is not sufficient to fully activate an autoreactive B cell [84, 85]. They require cytokines like type I interferon (IFN) and B cell activating factor (BAFF) which is essential for the proliferation and survival of B cells and is produced by dendritic cells (DC) under the effect of IFN type I. Self-reactive B cells, however, have limited responsiveness to BAFF due to the decreased amount of BAFF receptors compared to those expressed on the surface of the outnumbering non-self reactive B cells [86]. Hence, BAFF levels are rather limiting consequently resulting in the death of the autoreactive cells [87].

3. *Helper T 17 (Th17) Cells*

Th17 cells are believed to differentiate *in vivo* from naïve helper T cell (Th_0) potentially under the effect of IL-23 [88]. However, the polarization of Th_0 toward Th17 *in vitro* is independent on IL-23; instead, it requires the presence of the transforming growth factor beta TGF-β and IL-6 in combination with the stimulation of T cell receptor [89]. In mice, TGF-β alone induces Foxp3 and the retinoic acid-related orphan receptor γt (RORγt). Foxp3, which favors the conversion of Th_0 toward Treg, is capable of

inhibiting the translation of mRNA for IL-17A (a member of IL-17 family), thus Treg programming will prevail. On the other hand, the presence of IL-6 in the milieu with TGF-β favors Th17 differentiation through two means, inhibiting the expression of Foxp3[90], and the activation of the signaling pathway IL-6/gp130/STAT3 (signal transducers and activators of transcription 3) that activates RORγt expression which steers the conversion of Th_0 toward Th17 cell [91].

The resulting Th17 cells secrete IL-17 which mobilizes neutrophils in bacterial and fungal infections to benefit from their capacity in phagocytosis [92]. IL-17 receptors are also present on epithelial cells, endothelial cells, fibroblasts, adipocytes, and osteoblasts. Binding of IL-17 to its receptors activates signaling via NF-κB, which in turn induces target cells to secrete various cytokines [93]. The family of IL-17 comprises 6 members, IL-17A, B, C, D, E (also referred to as IL-25), and F, among which IL-17A and F are believed to mediate pro-inflammatory interactions [94]. The Th17 response also triggers the production of IL-1, IL-6, IL-8, IL-22, and TNF-α that recruits other immune cells such as monocytes, neutrophils, and macrophages. Previous studies implicated Th17 cells in autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA) [92, 95].

4. *Interleukin 10 (IL-10)*

IL-10 is an anti-inflammatory cytokine released by various kinds of cells including DCs, monocytes and macrophages, neutrophils, eosinophils, mast cells, CD4+T cells, CD8+T cells, B cells, NK cells in addition to keratinocytes, endothelial cells and other tissue resident cells. According to the cell type, variable factors trigger IL-10 production [96]. These factors include TLR or FcR activation for macrophages, transforming

growth factor beta (TGB-β) and IL-6 or IL-27 for all types of T helper cells, IL-12 for type 1 helper T (Th1) cells specifically, IL-2 and/or IL-12 for NK cells [96, 97]. IL-10 impairs the maturation of antigen presenting cells (APC), like DCs, monocytes and macrophages, decreases the ability of these cells to produce pro-inflammatory cytokines (tumor necrosis factor (TNF), IL-1β, IL-6, IL-8, and IL-12) [98, 99], impairs the co-stimulation of T lymphocytes through CD80/CD86, and reduces MHC class II expression [100]. Interestingly, IL-10 activates receptors involved in immune-complex recognition and phagocytosis (CD14, CD16, CD32, and CD64) on monocytes [101]. Furthermore, IL-10 activates and drives the proliferation of CD8+T, NK, and B cells [99]. However, the anti-inflammatory effects of IL-10 are believed to be predominant; it therefore may have an immunosuppressive role that allows the regulation and alleviation of the immune response leading to a decrease in the resultant tissue damage [102]. It may also hinder pathogenic inflammation [103]. EBV exploits IL-10 immunosuppressive properties by producing a viral homologue to human IL-10 to evade the elimination of the infected B cell [10]. IL-10 dysregulation may play a role in autoimmune diseases. Rheumatoid arthritis is associated with low levels of IL-10 [104].

C. Infection and Autoimmunity

Several mechanisms have been proposed to explain the association between some specific pathogens and certain autoimmune diseases.

1. *Molecular Mimicry*

By definition, molecular mimicry is the structural similarity between two distinct molecules coming from different origins (e.g. between two different viruses or between viral and human proteins) [105].

In order for a foreign protein to result in a human autoimmune disease through molecular mimicry, 3 criteria should be fulfilled: 1) structural resemblance to a human antigen 2) adequate difference from self-epitopes to allow discrimination by immune cells as foreign triggering an immune response, and 3) the localization of the resembling self-peptide on a biologically important tissue where an immunogenic injury will result in a discernable functional disturbance leading to a disease. Examples of autoimmune diseases triggered by infections through molecular mimicry include, but not limited to, dementia associated with HIV, dengue hemorrhagic shock syndrome [106], and rheumatic fever following infection with *Streptococcus Pyogenes* [107]. Molecular mimicry is also one of the mechanisms involved in triggering autoimmune diseases by an EBV infection due to the structural resemblance between some viral components with human antigens [108]. Examples include EBNA-1 protein harboring epitopes that share similarities with myelin basic protein (MBP) in multiple sclerosis [109, 110], La antigen that may trigger sjögren's syndrome, and Sm (the small nuclear ribonucleoprotein) in systemic lupus erythematosis (SLE) [108].

2. *Bystander Activation*

During an inflammation course, injured tissue and cells, bearing self-antigens, will be phagocytosed by APCs at the site of inflammation. The self-antigens might be processed, bound to MHC molecules, and presented to autoreactive T cells which are

bystander elements in this case [111]. The consistent inflammatory signals would help in perpetuating this autoreactive response. For example continuous inflammatory signals in infectious mononucleosis caused by EBV have been associated with the activation of autoreactive lymphocytes leading to SLE [112].

3. *Epitope Spreading*

Epitope spreading occurs when tissue damage caused by an infection leads to the release of self-antigens that can be taken up by APCs and presented to autoreactive cells which become subsequently activated. Hence, the inflammatory reaction is mediated against self-epitopes different from the microbial ones that initially provoked the immune response. Intramolecular spreading, on the other hand, occurs when inflammatory signals lead to enhanced antigen processing by an APC; as a result, cryptic epitopes within a protein become exposed to immune cells when they usually are not [113]. For example, anti-EBNA-1 antibodies cross-react with the Sm and Ro self-antigens [114]. This results in damage that exposes further cryptic self-antigens to the immune system. As a consequence, this leads to epitope spreading and antibodies against other nuclear antigens, including dsDNA, are engendered. This leads eventually to the appearance of SLE clinical symptoms [100].

4. *Perpetuation of Autoreactive B Cells*

This is believed to be the result of a B cell expressing an aberrant receptor. If this cells is also autoreactive, the binding of an auto-antigen may trigger an altered immune response eventually leads to a self-perpetuating autoreactive B cell [115]. For example the EBV latent membrane proteins (LMP-2A and B) localize on the infected B cell

surface and act as a constitutively active B cell receptor [116]. Some infected B cells may be autoreactive and may thus produce autoantibodies indefinitely. Alternatively, the infected cell may be a non-self reactive B cell that can present a self-antigen to an autoreactive T cell resulting in bystander activation [111].

5. ***Cross-Reaction of Idiotypes***

Idiotypes are antigenic determinants found on the variable region of antibodies and the production of anti-idiotypes is a normal part of the immune response. Anti-idiotypes hence bare similarity to the original triggering antigen. These anti-idiotypes are thought to provide regulation to the immune response by binding to the original antibody or to act as antigenic reminders to memory immune cells due to their resemblance to the original antigen [117]. Antibodies against particular infectious agent may bear idiotypes that share antigenic similarity with self-antigens [118] hence anti-idiotypes against these antibodies may cross react with self-antigens. This mechanism may be involved in SLE pathogenesis. Anti-DNA antibodies from SLE patients have idiotypes that antigenically resemble a cellular glycolipid in *Mycobacteria* [119].

6. ***Sequestered Antigen Release***

Viral replication inside a host cell may lead to the necrosis of the latter releasing endogenous cellular constituents (self-antigens) that were otherwise hidden from immune cells. The newly exposed components are recognized by immune cells as foreign epitopes. APCs uptake these self-antigens and present them to CD4+T cells which activate B cells to produce autoantibodies. In systemic lupus erythematosus,

antibodies against the nucleosomes are formed suggesting that these nucleosomes were somehow exposed to immune cells potentially after an infection [120].

No one specific exclusive mechanism is believed to be responsible for triggering an autoimmune disease after exposure to a specific infection. An interplay between several mechanisms probably takes place [113].

D. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease characterized by synovitis leading to joint destruction. It also may affect extra-articular organs like lungs, heart, and kidneys among other organs. Four or more bilateral and symmetric sites are affected, starting from small peripheral joints, then advances gradually involving major ones [121]. The prevalence of RA is about 0.5 to 1% of the global population [122], with women being three times more affected than men [123].

1. *Etiology*

The etiology of rheumatoid arthritis (RA) is multifactorial comprising genetic and environmental elements [124]. Antibodies against the Fc portion of the immunoglobulin IgG are formed [125]. The risk of having the disease within the same family of an RA patient is two to ten times higher than the normal population. Some HLA-DRB1 locus alleles (HLA-DRB1*401/0404) as well as HLA-B27 are more susceptible to have RA [126, 127]. Other loci also have strong associations with the disease, these include the protein tyrosine phosphatase 22 (PTPNN22) [128], the tumor necrosis factor alpha-induced protein 3 (TNFAIP3) [129], the tumor necrosis factor associated factor-1 and the C5 complement (TRAF1-C5) [130], and the signal transducer and activator of

transcription-4 (STAT-4) loci [131]. Environmental contributions to RA include smoking and inflammation [132]. The joint destruction process results from the invasion of a proliferating pannus mediated by several cytokines, namely IL-1, IL-2, IL-6, IL-17, TNF-α, TGF-α/β, and IFN-γ. These cytokines activate T and B lymphocytes, in addition to neutrophils, mast cells, fibroblasts, and chondrocytes. They also promote matrix metalloproteinase production that mediates joint injury which is typified by the hypertrophy of the synovium lining layer accompanied by cell infiltration and the formation of new blood vessels [133]. The case can be reversible if treated at this stage. If untreated, however, the inflammation can cause erosions to the juxta-articular bone leading to an irreversible joint damage [134].

2. *Infections Associated with Rheumatoid Arthritis (RA)*

Some infectious agents are strongly associated with RA in genetically susceptible patients [135]. These agents are believed to develop the disease in a Th1 mediated process that leads to the release of proteases resulting in the destruction of proteoglycans in the articular cartilage. It is believed that EBV provide the trigger of this process [136]. Other pathogens associated with RA include bacteria such as *Borrelia burgdorferi* [137], *Tropheryma whippeli* [138], mycobacteria [139], as well as viruses like parvovirus, rubella, hepatitis B and C viruses, and HIV [140], in addition to fungi like *Cryptococcus* [141]. Studies concerned in defining the etiology of RA revealed a correlation between the disease and EBV infection [142]. An increased anti-EBV antibodies (anti-EA IgG) were found in RA patients (21.7%) compared with healthy subjects (7.1%) [143]. Furthermore, the viral DNA load in peripheral blood lymphocytes was about 10 fold increased in RA patients compared with EBV-

seropositive normal subjects [124]. This association between EBV and RA is thought to be due to impaired T cell control of infected B cells, which is also explained by decreased MHC class I on EBV-infected B cells [144].

3. *Diagnosis*

According to the American College of Rheumatology (ACR) [145], an RA patient must have synovitis in at least one joint in addition to achieving a total score of 6 or more in four domains:

1. Number and site of affected joints (Score of 0 to 5)
2. Serological irregularities (Score of 0 to 3) including the rheumatoid factor test and anti-citrullinated protein antibody
3. Increased acute-phase reactants (Score of 0 or1)
4. Duration of symptoms (Score of 0 or 1).

4. *Treatment*

Central to the treatment of RA is the use of glucocorticoids which reduce inflammatory episodes [146]. T cell inhibitors (Cyclosporine A), as well as a folate inhibitor (Methotrexate), are also useful since they decrease immune cell proliferation [147]. Recently anti-TNF-α antibodies were introduced to RA therapy with promising outcomes [148].

CHAPTER III

MATERIALS AND METHODS

A. Effect of EBNA-3A and EBV DNA on IL-10 and IL-17 in BALB/c mice.

1. Mice

To study the effect of Epstein-Barr virus nuclear antigen 3A (EBNA-3A) and EBV DNA on interleukin 10 (IL-10) and 17 (IL-17), 81 female BALB/c mice, 4-6 weeks old, grouped into 9 groups (9 mice per group) were used. Mice were obtained from the Animal Care Facility at the American University of Beirut (AUB). The study was approved by the Institutional Animal Care and Use Committee (IACUC) at AUB and the animals were handled according to the committee guidelines.

2. EBV DNA and EBNA-3A Preparation

EBV DNA and EBNA-3A were obtained from Advanced Biotechnologies (Columbia, MD) and JPT (Peptide Technologies Berlin, Germany), respectively.

The EBV DNA stock solution vial contains 1.2 X 10^4 DNA copies/µl according to the manufacturer. Mouse EBV DNA injections were prepared by diluting the stock solution with water to a concentration of 360 copies/µl and administering 100 µl to each mouse. Hence, mice injected with EBV DNA were each given 36 x 10^3 copies of DNA. This copy number was selected since it was the least copy number to result in a significant increase in mouse serum IL-17 levels in a previous study conducted in the Department of Experimental Pathology, Immunology and Microbiology at the American University of Beirut [13].

EBNA-3A was reconstituted, as recommended by the manufacturer, by adding 975 µl autoclaved distilled water to the stock vial to obtain a concentration of 6000 µg/ml. Three dilutions were prepared using distilled water to obtain concentrations of 10, 20 and 40 µg/ml. Depending on the mouse group, mice were injected with 250 µl of one of these dilutions to administer either 2.5, 5 or 10 µg of EBNA-3A as described in section III.A.3 below.

3. Mouse Group Injections

Mice in group 1 were left without injection, while group 2 was injected with 100 µl of autoclaved distilled water. These two groups served as negative controls. Groups 3, 4, and 5 were injected with 2.5, 5, or 10 µg of EBNA-3A, respectively. Group 6 was injected with 36 X 10^3 EBV DNA copies in 100 µl of autoclaved distilled water. Groups 7, 8, and 9 were injected with 36 X 10^3 EBV DNA copies each, along with 2.5, 5, or 10 µg of EBNA-3A, respectively. All injections were performed intraperitoneally (Table 1).

4. Specimen Procurement

Three mice were sacrificed per group on days 3, 6, and 9 post-injection. Blood was collected by cardiac puncture into sterile tubes and centrifuged at 3500 round per minute (RPM) for 15 min. Serum was then separated, consolidated per time point per group and used for measuring IL-10 and IL-17 levels as described below.

5. Enzyme-Linked Immunosorbent Assay (ELISA) for Mouse IL-17 and IL-10

IL-10 and IL-17 levels were assayed in mice sera using mouse ELISA kits (Abcam, Cambridge, UK). Following the manufacturer instructions, samples were diluted 5 times, analyte standard dilutions were prepared, and reagents were reconstituted. All experiment steps were conducted at room temperature. First, 100 µl of standards and samples were added to the 96 well plate, covered, and incubated for 2.5 hours. Then, the plate was washed 4 times by adding 300 µl washing buffer per well and discarding it each time. One hundred microliters of Biotinylated IL-17 or IL-10 Detection Antibody (according to the type of interleukin assayed) were added to each well; the plate was covered and incubated again for 1 hour. The plate was then washed as described above; 100 µl horse radish peroxidase (HRP)-Streptavidin solution was then added to each well. The plate was covered and incubated one more time for 45 min. Wells were washed again and 100 µl of Tetramethylbenzidine (TMB) One-Step Substrate was added to each well, followed by a 30 min incubation period in the dark. Finally, 50 µl of Stop Solution were added to each well. Absorbance was measured at a 450 nm wavelength using an ELISA reader (BIO-TEK, Winooski, VT). Samples and standards were assayed in duplicates. The mean absorbance was calculated and a standard curve was plotted using Microsoft Excel. Sample concentrations were then calculated using the standard curve formula.

B. Effect of EBNA-3A and EBV DNA on IL-10 and IL-17 Production from Human PBMCs

1. Sample Collection

Approval of the Institutional Research Board (IRB) was obtained to collect blood samples from seven patients diagnosed with rheumatoid arthritis and respective age and sex-matched non-RA controls. Patient age range was 21-72 including 3 males and 4

females. Controls who reported having no autoimmune disease themselves or in their immediate family were included in the study. All participants were older than 18 years and signed a consent form. Patients with rheumatoid arthritis were recruited by Dr. Imad Uthman, rheumatologist physician at the American University of Beirut Medical Centre (AUBMC) and diagnosed according to the American College of Rheumatology (ACR) criteria [145]. Three milliliter blood samples were collected from each subject by the vacuum tube method using 21G needles (Greiner Bio-One, kremsmünster, Austria) and sterile EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ).

2. Serology for EBV

Blood samples were centrifuged at 3500 rpm for 15 min at room temperature using a Biofuge Stratos, Heraeus centrifuge (Kendro, Osterode, Germany). Plasma was then transferred aseptically to another plastic tube with no additives using sterile transfer pipettes in a class II biological safety cabinet (Nuaire, Plymouth, MN). Plasma was assayed by the AUBMC Pathology and Laboratory Medicine Department for EBV viral capsid antibodies (EBV-VCA IgM and IgG) using Vidas (BioMérieux, Craponne, France).

3. Human PBMC Separation by Ficoll-Isopaque

All steps of PBMC separation were performed in a class II biological safety cabinet. The plasma volume previously transferred from blood samples (section B.2.) was substituted by phosphate buffered saline (PBS) (SIGMA-ALDRICH, St. Louis, MO). Blood-PBS mixture was further diluted 2 fold with PBS. Then the blood-PBS final

mixture was layered onto 3 ml Ficoll-Isopaque (Lonza, Walkersville, MD) in a sterile 15 ml tube (Corning CentriStar, NY) and centrifuged at 900 g for 30 min at 20º C. Four layers were obtained; these are, from top to bottom: 1) PBS 2) off-white layer containing mononuclear cells 3) Ficoll-Isopaque layer 4) erythrocytes and granulocytes. The off-white layer was transferred to a glass rounded bottom tube (Pyrex, Tewksbury, MA) using a sterile dropper; cells were washed with double their volume of PBS and another centrifugation was performed at 400 g for 15 min at 20º C. The supernatant was discarded and the cell pellet was suspended in the remaining few drops of PBS, then 5 ml PBS were added again for another wash step and resuspended in PBS; cells were counted using a Neubauer chamber (Neubauer, Eberstadt, Germany) under a microscope (Olympus CX21) using 400X magnification, then cells were centrifuged at 400 g for 15 min at 20º C. Finally, the supernatant was discarded and cells were resuspended in a cryopreservative medium [50% Fetal Bovine Serum (FBS) (Lonza, BioWhittaker,Verviers, Belgium), 40% sterile Roswell Park Memorial Institute (RPMI) with L-Glutamine medium (Lonza), and 10% Dimethyl Sulfoxide (DMSO) (FisherBiotech, Fair Lawn, NJ). Cells were resuspended at a density of 10^6 cell/ml and then aliquoted in cryotubes (SARSTEDT Aktiengeselischaft & Co, Nümbrecht, Germany). Then, cryotubes were placed in an isopropanol box at -80ºC Freezer (New Brunswick, Enfield, CT) for 24 hours before they were transferred to a liquid nitrogen container (Thermo Scientific, Dubuque, Iowa) for storage.

4. Cell Culture

Plates of 96 wells (Sarstedt, Newton, NC) were used for PBMC culturing. A culture medium was prepared of 89% RPMI 1640 without L-Glutamine and phenol red, 10%

heat inactivated FBS and 1% penicillin-streptomycin (Pen-Strep) (Lonza). An amount of 25 X 10^4 cell was added to each well. Five wells were seeded per cell sample. EBV DNA (6.164 X 10^3 copies) was added to the first well, EBNA-3A (1.71 µg) to the second, both EBV DNA (6.164 X 10^3 copies) and EBNA-3A (1.71 µg) to the third; *Staphylococcus epidermidis* (1.162 pg) DNA to the fourth, and culture medium to the fifth (Table 2). Each well contained a total volume of 250 µl. Cell and test substance preparation is described below.

PBMCs were cultured for 24 hours at 37° C in the presence of 5% CO_2. The contents of each well were then transferred into Eppendorf tubes and centrifuged at 14000 rpm for 5 min. Supernatants were then collected, aliquoted in pairs, stored at -20° C, and then assessed, within a period not exceeding one month, for IL-10 and IL-17 levels using ELISA as described below in section III.A.5.

a. Preparing Human PBMCs for Culture

PBMCs were thawed in a 37°C water bath for 1.5-2 minutes, then poured directly into 3 ml culture medium in 15 ml conical tubes and brought to 37°C. PBMCs were then centrifuged at 100g for 5 min. The supernatant was discarded and PBMCs in the pellet were resuspended in 1 ml culture medium. Live cells were counted with Trypan blue under a microscope. Finally, culture medium was added to PBMCs to obtain a concentration of 25 X 10^4 cells/150 µl.

b. Preparing EBV DNA for Incubation with Human PBMCs

The amount of EBV DNA (6.164 X 10^3 copies) used in this study was deduced using the following formula: EBV DNA copies per well = [(EBV DNA copies per mouse) X (PBMCs per well)] / (Total number of PBMCs per mouse)

The EBV DNA copy number injected to mice in this formula is $36X10^3$ copies. PBMC count in BALB/c mice was considered to be 1000 cells/microliter [149], mouse blood volume was estimated to be about 1.46 ml and the number of PBMCs added to each cell culture well is $25X10^4$.

C. Preparing EBNA-3A for Incubation with Human PBMCs

Similarly, the EBNA-3A amount added per well (1.71µg) and incubated with the human PBMCs was extrapolated from the amount associated with a significant increase ($P<0.05$) in IL-17 levels upon injection into mice (10 µg). The formula used for deducing the amount of EBNA-3A to be implemented is: EBNA-3A µg per well = [(EBNA-3A µg per mouse) x (PBMCs per well)] / (Total number of PBMCs per mouse)

d. *S. epidermidis* DNA

An *S. epidermidis* isolate stored at -80 C at the Department of Experimental Pathology, Immunology and Microbiology was cultured on blood agar and incubated at 37° C for 18 hours. Seven McFarland of fresh colonies in normal saline were boiled in an eppendorf for one hour at 95° C using a thermal cycler (Thermo, Electron Corporation, MA). The eppendorf was then centrifuged at 14000 rpm for 15 min and the supernatant containing bacterial DNA was collected. Thirty microliters (1/10 of the supernatant's volume) of sodium acetate (3M, PH 5.2) (Riedel-deHaën,Seelze, Germany) and 1 ml (3

times the supernatant's volume) of ethanol (100%, -80° C) (Sigma-Aldrich) were added. This mixture was stored at -80° C overnight. Then, the eppendorf was centrifuged at 13000 rpm for 15 min. The supernatant was discarded. The DNA pellet was then washed 3 times by adding 1 ml room temperature ethanol (70%), centrifuging at 13000 rpm for 15 min, and then discarding the supernatant each time. After the last wash, the eppendorf was moved to a biosafety cabinet where the supernatant was discarded and the pellet was exposed to open air for 1-2 minutes to allow for ethanol evaporation. The pellet was then resuspended in 20 µl of autoclaved distilled water and incubated at 37° C for 15 min to ensure complete DNA dissolution. About 2 µl of the solution was then taken aseptically for DNA concentration measurement using a Jenway Genova Nano Micro-volume spectrophotometer (Staffordshire, UK).

An amount of *S. epidermidis* DNA equal to EBV DNA designated for incubation with human PBMCs was calculated as follows:

EBV DNA amount (Da) = EBV DNA copy number X Number of nucleotide base pairs in EBV DNA X Average base pair molecular weight.

Where the EBV DNA copy number is 6.164×10^3, the number of nucleotide base pairs in EBV DNA is 172×10^3 [150], and the average weight of a base pair is 660 Dalton (Da). Therefore, the calculated amount of *S. epidermidis* DNA, 6.99×10^{11} Da, which is equivalent to 1.162 pg was used.

5. ELISA for Human IL-17 and IL-10

Levels of IL-17 and IL-10 were assayed in culture supernatants using human cytokine specific ELISA kits (Abcam). Samples were tested in duplicates. The procedures used were similar to those described above in section III. A.5.

C. Statistical Analysis

Statistical analysis was performed using the Graphpad software. Two-sample t-tests were conducted and p-values less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

A. Effect of EBNA-3A on IL-10 and IL-17 in BALB/c mice:

1. Mouse Serum (IL-10) Levels

Intraperitoneal injection of 2.5 µg EBNA-3A into BALB/c mice had no significant effect on serum IL-10 levels compared to the water-injected group at all time points. Injection of 5 µg EBNA-3A, however, led to a decrease in IL-10 on day 3, followed by an increase on day 6, then a return to similar levels seen in the water-injected group on day 9 post-injection. While IL-10 levels in the group receiving 10 µg EBNA-3A remained close to those of the water-injected group on day 3, these levels dropped on day 6 reaching the lowest detected IL-10 values among all groups at all time points; IL-10 levels recovered on day 9 to levels higher than those seen in water receiving group (Table 3, Figure 1).

Both mouse groups receiving 36 X 10^3 copies of EBV DNA alone or with 2.5 µg EBNA-3A showed a similar pattern of IL-10 levels, which started with decreased amounts on day 3 compared to the water-injected group, then increased on day 6 and continued increasing ending up higher than the latter group by day 9. In mice receiving EBV DNA with 5 µg EBNA-3A, IL-10 amounts were close to those of the water-injected group on day 3, then increased steadily on days 6 and 9. It is noteworthy that IL-10 levels in this mouse group were the highest among all other groups at all time points; the highest level was observed on day 9 post-injection. On the other hand, the group receiving EBV DNA with 10 µg EBNA-3A showed decreased levels of IL-10 on day 3 post-injection, while no discernable change was seen in the cytokine's

concentrations on days 6 and 9 compared to the group receiving water injection; the lowest level of IL-10 was observed on day 6 in the group receiving 10 µg of EBNA-3A alone. Injection of EBV DNA alone was accompanied by a continued increase in IL-10 levels with time (Table 3, Figure 2).

2. *Mouse Serum (IL-17) Levels*

The mouse group injected with 2.5 µg EBNA-3A did not show a notable change in serum IL-17 on days 3 and 9 post-injection; however, on day 6 this group showed an 8% decrease compared to the water-injected group ($p=0.019$). The group injected with 5 µg EBNA-3A exhibited decreased levels of IL-17 on day 3, while no significant change was seen on day 6 and an increased concentration was detected on day 9 post-injection compared to the water injected group. When 10 µg of the viral protein were administered to mice, IL-17 levels were significantly increased by 27.9% on day 3 compared to the water injected group ($p=0.0055$), dropped on day 6, but recovered again to show a marked increase of 26.2% compared to the water injected group by day 9 ($p=0.01$). The level of IL-17 detected on days 3 and 9 in this group was the highest recorded among all groups receiving EBNA-3A alone (Table 4, Figure 3).

As seen with IL-10, changes in IL-17 levels in mouse groups injected with EBV DNA alone and in association with 2.5 µg EBNA-3A were similar in pattern. In both of these groups, IL-17 steadily increased starting from a level below that of the water-injected group on day 3 but significantly increasing on day 6 and then on day 9 ($p<0.05$). Both mouse groups injected with EBV DNA with either 5 or 10 µg EBNA-3A showed a significant increase in IL-17 on day 9 post-injection ($p<0.05$), while no marked change was observed in its levels on both days 3 and 6 (Table 4).

Worth noting is that except for the group that received 2.5 µg of EBNA-3A, IL-17 levels in all mouse groups that received EBV DNA, EBNA-3A, or both, were significantly higher by day 9 compared to the water-injected mouse group ($p<0.05$). The highest level of IL-17 among all mice groups was seen in that receiving EBV DNA with 5 µg EBNA-3A (Figure 4).

Since injection of 10 µg EBNA-3A was associated with the highest increase in mouse serum IL-17 levels among groups receiving the viral protein alone, this was used to infer the amount of viral protein employed in assays using human PBMCs described in section B below. This calculation is further elaborated upon in section III.B.4.c of the Materials and Methods chapter.

B. Effect of EBNA-3A on IL-10 and IL-17 Levels in Human PBMCs Culture Supernatants:

1. IL-10 Levels in Cell Culture Supernatants

Incubation of PBMCs from EBV positive non-RA control subjects and RA patients with EBV DNA, EBNA-3A or both, yielded similar average levels of IL-10 in the supernatants. Nevertheless, although not statistically significant, the highest IL-10 average level was detected in supernatants from RA PBMCs treated with EBV DNA (Table 5, Figure 5). A notable variability in individual responses from different patients and controls to treatment with viral components was detected (Figure 7).

2. IL-17 Levels in Cell Culture Supernatants

Except for cells treated with both viral components, average IL -17 levels in culture supernatants from all RA PBMCs were significantly higher than those of respective control cells ($P<0.05$). The average IL-17 level in culture supernatants of cells treated

with both EBV DNA and EBNA-3A together was the lowest within both cell types. Culture supernatants of RA PBMCs incubated with EBNA-3A alone exhibited decreased average levels of IL-17 compared with untreated (P=0.0034), *S. epidermidis* (P=0.0163) and EBV DNA-treated cells (P=0.0031) of the same cell type. Within the control group, however, only PBMCs treated with both viral components showed a significant decrease in average IL-17 levels compared with the untreated cells (P=0.0092) and those treated with the viral DNA alone (P=0.0194) (Table 6, Figure 6).

Worth noting is that individual cells from non-RA control subjects had a higher level of variability in their IL-17 responses whereas cells from RA subjects displayed a smaller margin of variability. This was observed in all treatments except when cells were cultured in the presence of both EBV DNA and EBNA-3A (Figure 8).

Table 1: Protocol employed for injecting BALB/c mice with EBV components

Group (n=9) / Injection	1	2	3	4	5	6	7	8	9
Sterile Water	-	+	-	-	-	-	-	-	-
EBV DNA (36 X 10^3 copies)	-	-	-	-	-	+	+	+	+
EBNA-3A (µg)	-	-	2.5	5	10	-	2.5	5	10

Table 2: Treatment protocol for PBMCs from RA and non-RA control subjects

Treatment	**RA patient PBMCs**	**Non-RA control PBMCs**
1	Culture medium	Culture medium
2	*S. epidermidis* DNA (1.162 µg)	*S. epidermidis* DNA (1.162 µg)
3	EBV DNA ($36x10^3$ copies)	EBV DNA ($36x10^3$ copies)
4	EBNA-3A (1.71 µg)	EBNA-3A (1.71 µg)
5	EBV DNA ($36x10^3$ copies) and EBNA-3A (1.71 µg)	EBV DNA ($36x10^3$ copies) and EBNA-3A (1.71 µg)

Table 3: Serum IL-10 levels (pg/ml) in BALB/c mice on days 3, 6 and 9 post-injection with EBV components

Group (n=9)	Injection	Day 3 post-injection	Day 6 post-injection	Day 9 post-injection
G1	**-**	45100 ±919.2	69450 ±141.4	76250 ±4101.2
G2	**Sterile water**	97550 ±1979.9	71150 ±2404.2	60300 ±1060.7
G3	**EBNA-3A (2.5µg)**	88500 ±8697.4	70900 ±1060.7	59500 ±4879
G4	**EBNA-3A (5µg)**	62550 ±7071.1	84100 ±2192	55700 ±2333.5
G5	**EBNA-3A (10µg)**	105000 ±2616.3	31400 ±2899.1	91450 ±1555.6
G6	**EBV DNA (36 X 10^3 copies)**	65600 ±2192	75950 ±1555.6	84150 ±6222.5
G7	**EBV DNA (36 X 10^3 copies) + EBNA-3A (2.5µg)**	51800 ±70.7	67400 ±1484.9	87750 ±4666.9
G8	**EBV DNA (36 X 10^3 copies) + EBNA-3A (5µg)**	101800 ±4454.8	108200 ±70.7	140950 ±3677
G9	**EBV DNA (36 X 10^3 copies) + EBNA-3A (10µg)**	58950 ±2969.8	71300 ±5727.6	67200 ±4454.8

Table 4: Serum IL-17 levels (pg/ml) in BALB/c mice on days 3, 6 and 9 post-injection with EBV components

Group (n=9)	Injection	Day 3 post-injection	Day 6 post-injection	Day 9 post-injection
G1	-	1826.6 ±16.433	1570.1 ±1.0607	1896.2 ±38.906
G2	**Sterile water**	1822.3 ±28.73	1749.4 ±21.997	1432.1 ±33.342
G3	**EBNA-3A (2.5µg)**	1892.9 ±111.15	1606 ±17.97	1471.2 ±33.342
G4	**EBNA-3A (5µg)**	1487.5 ±50.251	1622.3 ±44.102	1629.9 ±34.295
G5	**EBNA-3A (10µg)**	2526.6 ±68.697	1535.3 ±50.251	2392.9 ±132.67
G6	**EBV DNA (36 X 10^3 copies)**	1476.6 ±78.873	2128.8 ±25.656	2210.3 ±77.336
G7	**EBV DNA (36 X 10^3 copies) + EBNA-3A (2.5µg)**	1413.6 ±26.609	2127.7 ±91.755	2151.6 ±34.295
G8	**EBV DNA (36 X 10^3 copies) + EBNA-3A (5µg)**	2042.9 ±98.857	1740.8 ±12.774	3391.8 ±41.028
G9	**EBV DNA (36 X 10^3 copies) + EBNA-3A (10µg)**	1719 ±92.708	2127.7 ±91.755	1752.7 ±48.129

Table 5: IL-10 levels (pg/ml) in culture supernatants from human PBMCs treated with EBV components

Treatment / Cell Type	Culture Medium	*S. epidermidis* DNA (1.162 pg)	EBV DNA (6.164 X 10^3 copies)	EBNA-3A (1.71µg)	EBV DNA (6.164 X 10^3 copies) + EBNA-3A (1.71µg)
Non-RA control PBMCs	356.7 ±218.7	457 ±271.9	412 ±199.2	503.8 ±269.2	410.4 ±281.7
RA Patients' PBMCs	579.7 ±677.4	582.3 ±588	749.2 ±609.7	625.9 ±619.1	509.1 ±617.8

Table 6: IL-17 levels (pg/ml) in culture supernatants from human PBMCs treated with EBV components

Treatment / Cell Type	Culture Medium	*S. epidermidis* DNA (1.162 pg)	EBV DNA (6.164 X 10^3 copies)	EBNA-3A (1.71µg)	EBV DNA (6.164 X 10^3 copies) + EBNA-3A (1.71µg)
Non-RA control PBMCs	10.8 ±5	10.3 ±4.8	9 ±3.9	6.7 ±3	4.1 ±2.8
RA Patients' PBMCs	18.3 ±3	16.4 ±2.1	18.1 ±2.8	13.5 ±1.8	5.7 ±3.5

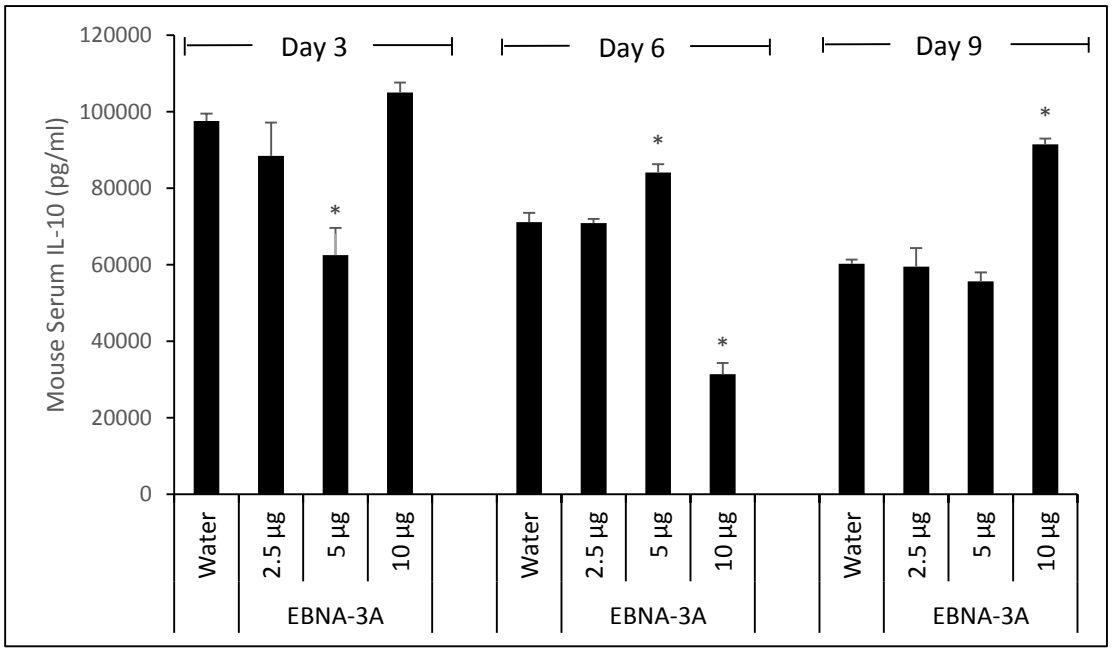

Figure 1: Average IL-10 levels in mouse sera after injection with 2.5, 5 or 10 µg of EBNA-3A on days 3, 6, and 9 post-injection. * indicates $p<0.05$ in comparison to mouse group treated with water asessed on same post-injection day.

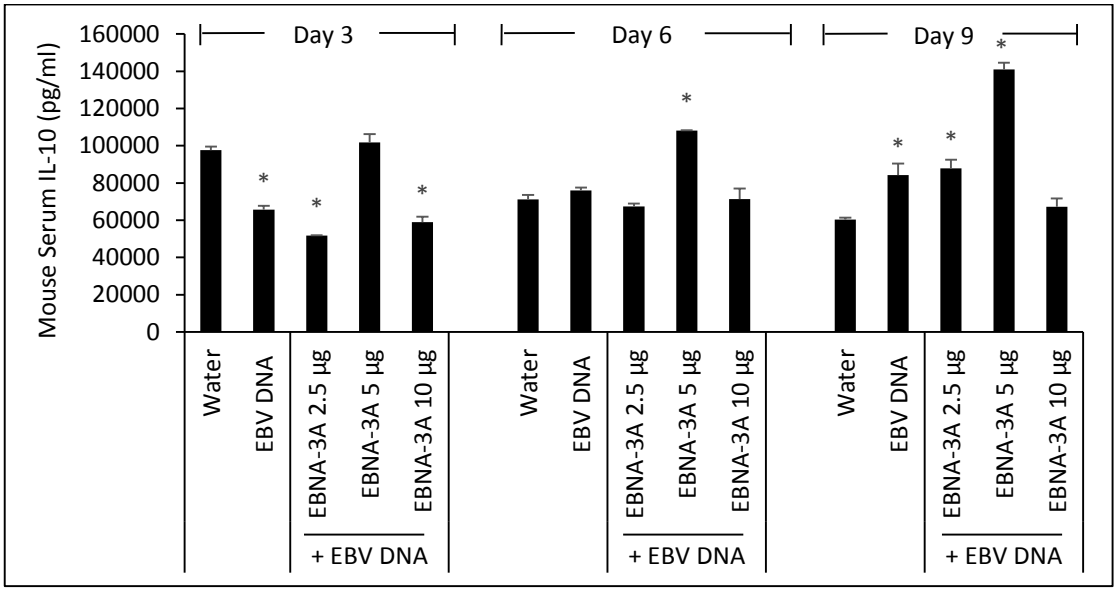

Figure 2: Average IL-10 levels in mouse sera after injection with 36 X 10^3 copies of EBV DNA alone or along with 2.5, 5, or 10 µg of EBNA-3A at different time points on days 3, 6, and 9 post-injection. Mice injected with water were used as controls. * indicates $p<0.05$ in comparison to mouse group treated with water asessed on same post-injection day

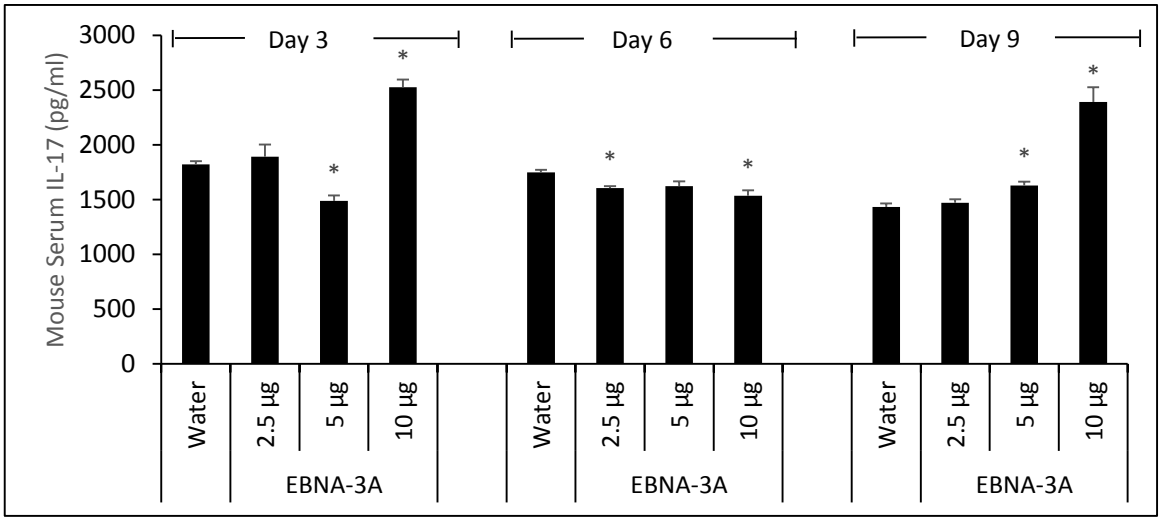

Figure 3: Average IL-17 levels in mouse sera after injection with 2.5, 5 or 10 µg of EBNA-3A on days 3, 6, and 9 post-injection. * indicates $p<0.05$ in comparison to mouse group treated with water asessed on same post-injection day.

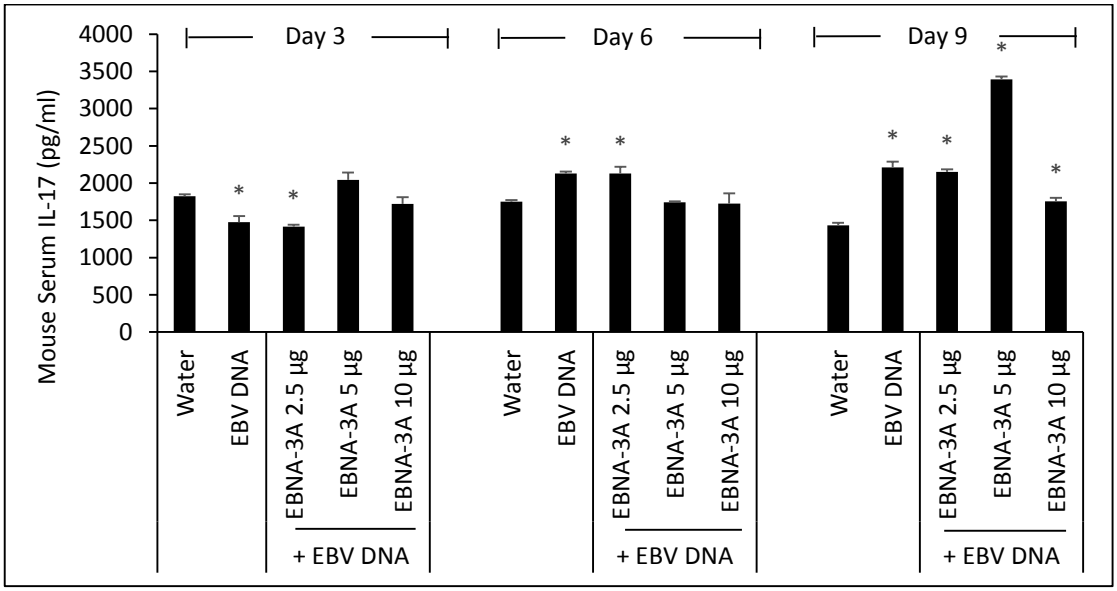

Figure 4: Average IL-17 levels in mouse sera after injection with 36 X 10^3 copies of EBV DNA alone or along with 2.5, 5, or 10 µg of EBNA-3A at different time points on days 3, 6, and 9 post-injection. Mice injected with water were used as controls. * indicates $p<0.05$ in comparison to mouse group treated with water asessed on same post-injection day

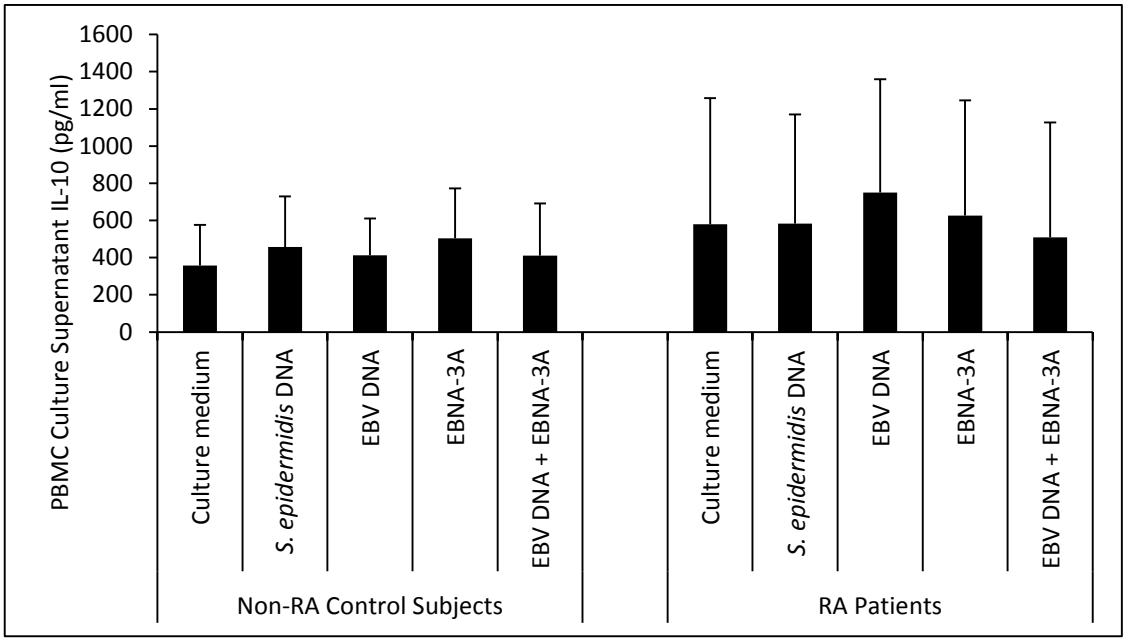

Figure 5: Average IL-10 levels in culture supernatants of PBMCs from seven RA patients and respective non-RA control subjects after 24 hours of incubation with EBV-DNA (6.164 X 10^3 copies), EBNA-3A (1.71 µg) or both. Cells cultured with *S. epidermidis* DNA (1.162 µg) were assessed as a control.

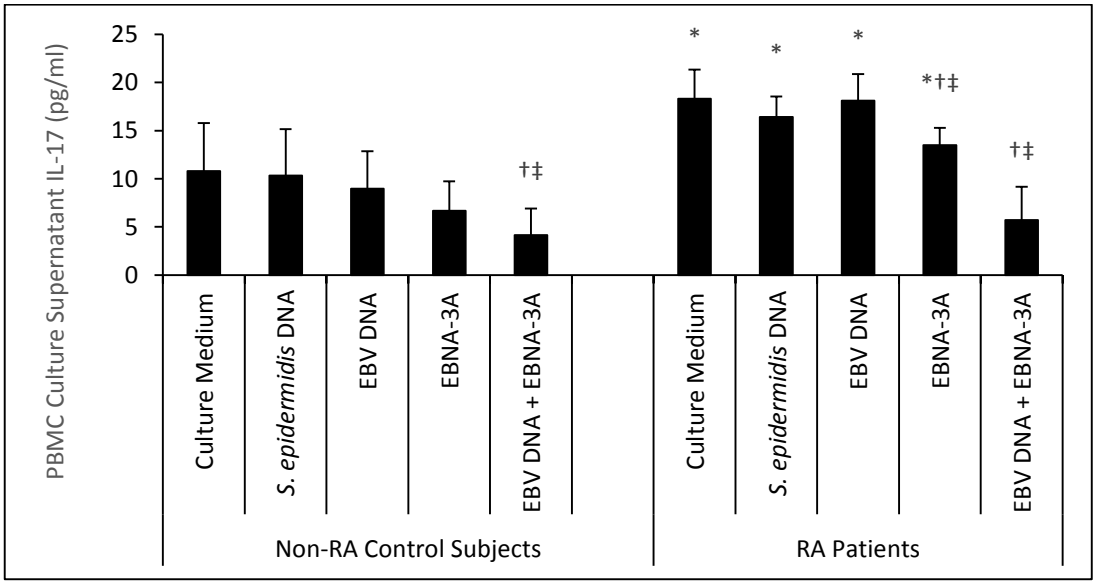

Figure 6: Average IL-17 levels in culture supernatants of PBMCs from seven RA patients and respective non-RA control subjects after 24 hours of incubation with EBV-DNA (6.164 X 10^3 copies), EBNA-3A (1.71 µg) or both. Cells cultured with *S. epidermidis* DNA (1.162 µg) were assessed as a control. * indicates $p<0.05$ when compared to respectively treated non-RA control subject PBMCs. † indicates $p<0.05$ when compared to respective cells cultured in the absence of infectious agent components. ‡ indicates $p<0.05$ when compared to respective cells cultured in the presence of EBV DNA.

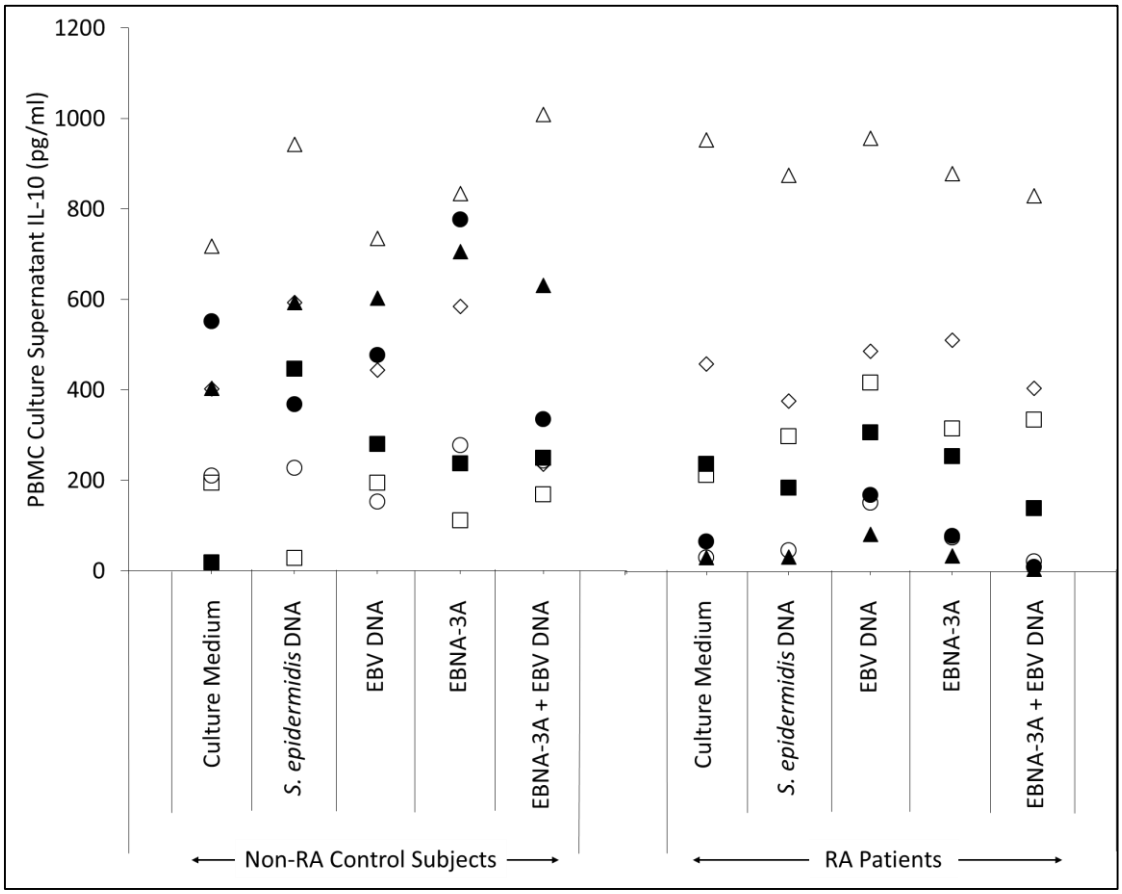

Figure 7: Individual IL-10 levels in culture supernatants of PBMCs from seven RA patients and respective non-RA control subjects after 24 hours of incubation with the culture medium, *S. epidermidis* DNA (1.162 pg), EBV-DNA (6.164 X 10^3 copies), EBNA-3A (1.71 µg) or both EBV components.

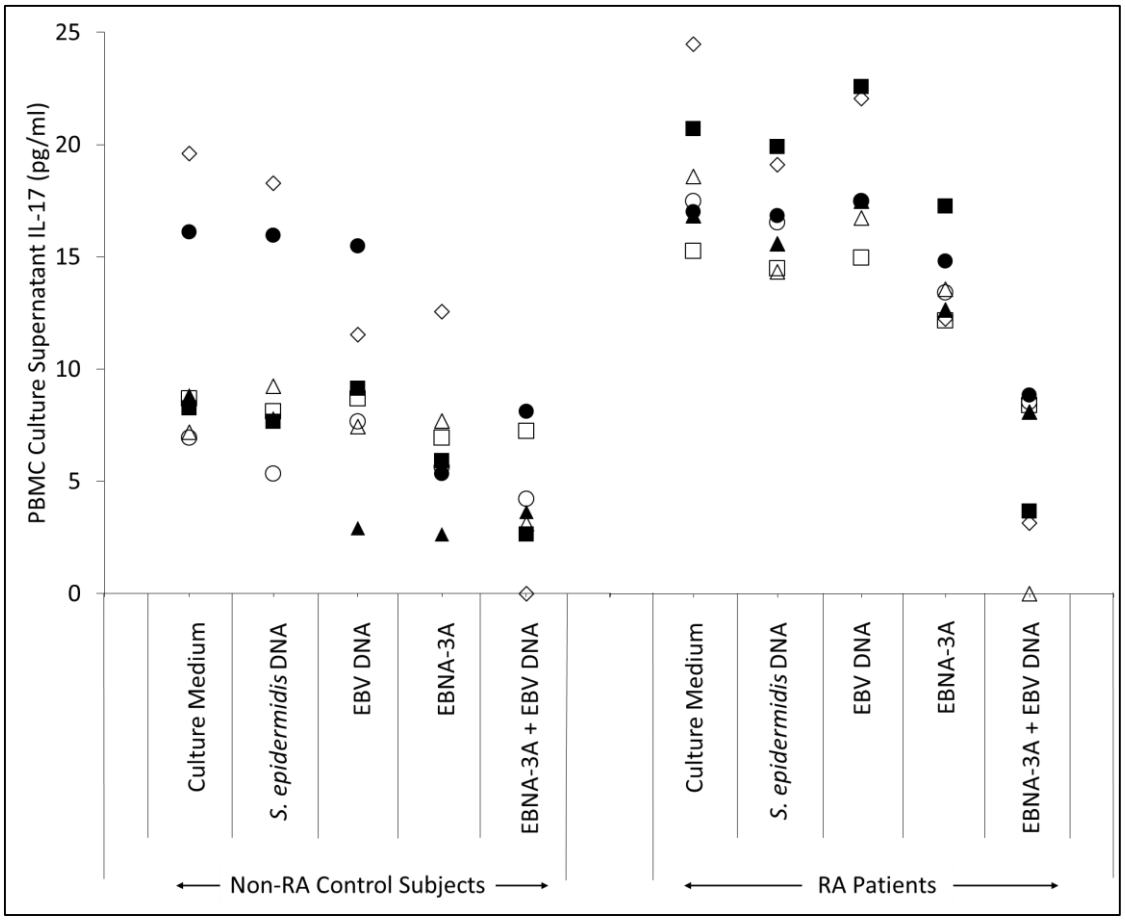

Figure 8: Individual IL-17 levels in culture supernatants of PBMCs from seven RA patients and respective non-RA control subjects after 24 hours of incubation with the culture medium, *S. epidermidis* DNA (1.162 pg), EBV-DNA (6.164 X 10^3 copies), EBNA-3A (1.71 µg) or both EBV components.

CHAPTER V

DISCUSSION

The increase in serum IL-17 levels on days 6 and 9 post-injection in the mouse group administered EBV DNA is concurrent with data obtained from a previous study conducted at the Department of Experimental Pathology, Microbiology and Immunology [13]. This is potentially due to the binding of EBV DNA to NOD-like receptors, RIG1-like receptors, DAI, AIM2, or some TLR family members among others [15, 19]; these are cellular receptors that have been shown to possibly interact with foreign nucleic acids. Such receptors may then trigger pro-inflammatory pathways [19].

Injection of 2.5 µg of the viral protein EBNA-3A appeared to have no effect on IL-17 levels in mice sera; therefore, the increase in IL-17 levels seen when 2.5 µg EBNA-3A was injected with EBV DNA is likely due to the influence of the latter. An EBNA-3A-associated increase in IL-17 levels when higher amounts of the viral protein were used (5 and 10 µg) on day 9 post-injection, with and without EBV DNA, is probably due to mechanisms activated by EBNA-3A itself. One such potential mechanism is the activation of the Notch signaling pathway. The Notch receptor is a transmembrane protein that normally binds to one of its ligands (Delta 1, 3 or Jagged 1, 2). Notch receptor-ligand binding activates the downstream effector CSL (CBF1/Su(H)/ Lag-1), also known as RBP-Jk or CBF-1, which is a transcriptional factor involved in cell proliferation and differentiation [151]. EBNA-3A can bind through its conserved domain in the amino terminus to CSL [152]. The Notch signaling pathway was shown to be required for Th-17 differentiation in both mice and humans; both IL-17 and

RORγt (which directs the differentiation of a Th_0 cell into a Th17 cell [91]) genes were shown to be direct transcriptional targets of Notch signaling in Th-17 cells in mice and humans [151].

Alternatively, EBNA-3A could bind the heat shock protein-70 (Hsp70) [153]. Hsp70 has been linked to the promotion of NF-kB activation which may in turn result in pro-inflammatory cytokine production [154, 155]. Moreover, Hsp70 was shown to induce IL-6 production [156, 157] which may then favor Th17-associated pathways [91]. Nevertheless, whether EBNA-3A binding to Hsp70 triggers its downstream effects remains to be determined.

IL-17 levels were increased in mouse sera 9 days after injecting EBV DNA along with 5 or 10 μg of EBNA-3A compared to the water-injected group, similar to what occurred when either component was administered alone. However IL-17 levels were higher in the group injected with EBV DNA along with 5 μg of EBNA-3A compared to the group administered EBV DNA along with 10 μg of EBNA-3A. This may indicate an interaction between the two viral components or a byproduct of their metabolism that could decrease their effects when 10 μg of EBNA-3A are used. Alternatively, these viral components may trigger competing pathways or inherent protective mechanisms by day 9 when 10 μg of EBNA-3A are administered.

IL-10 levels in mouse groups receiving 5 μg and 10 μg of EBNA-3A alone fluctuated throughout the different time points thus lacking a clear pattern. This may be due to the vast number of factors that influence the production of this cytokine *in vivo*. Various cells, including DCs, monocytes, macrophages, neutrophils, eosinophils, mast cells, CD4+T cells, CD8+T cells, B cells, NK cells, endothelial and other tissue resident cells, are capable of secreting IL-10 [96]. This variation in cell types releasing IL-10

and factors triggering this release according to the cell type might be the reason behind the fluctuating pattern. Factors triggering IL-10 production by various cells include IL-6, IL-12, and IL-27 [96]. Whether these cytokines are also released *in vivo* under the effect of the viral components is yet to be elucidated. Worth noting is that mouse groups receiving 10 μg EBNA-3A showed a similar pattern for both IL-10 and IL-17 at the three time points. This may indicate that administering 10 μg of the viral protein triggers a specific common mechanism that enhances or reduces the production of both cytokines. On the other hand, the introduction of EBV DNA resulted in a steady IL-10 increase with time. This effect was enhanced by the addition of 5 μg EBNA-3A; however, 10 μg of the viral protein abrogated the effect of EBV DNA and kept IL-10 levels below or near the water receiving mouse group levels. This possibly indicates that IL-10 stimulation or inhibition by EBNA-3A is concentration dependent. Whether mechanisms triggered by EBNA-3A are different from those triggered by EBV DNA remains to be determined.

To assess whether EBV components had similar effects in human cells, PBMCs from 7 RA patients and 7 non-RA controls were tested. Since the majority of the population has been infected with EBV [158], we ensured that all subjects included in the study were EBV seropositive. Untreated PBMCs from RA patients produced a higher baseline level of IL-17 compared to untreated PBMCs from non-RA controls; this concurs with various studies showing that RA patients exhibit high IL-17 levels in their sera [159, 160]. However, none of the treatments used increased IL-17 levels over baseline levels as opposed to what was observed in mice. This may indicate that higher concentrations of the components used are required to induce such a stimulation or that other cell types in addition to PBMCs play a role *in vivo*. Worth noting is that DNA

copy numbers and EBNA-3A amounts used were extrapolated from our mouse *in vivo* experiments; however, further concentrations should be tested. On the other hand, incubation time limitations may have curbed the effects of these components. While mice were assessed after 3, 6 and 9 days of injection, human PBMCs were only allowed a 24 hours incubation period with the viral components owing to the limited livelihood of such primary cells in culture.

A decrease in IL-17 levels was seen in human PBMCs incubated with EBNA-3A or EBNA-3A along with EBV DNA. This may indicate the presence of a mechanism through which cells curb their production of pro-inflammatory cytokines, such as IL-17, when exposed to triggering agents perhaps as a mean to prevent tissue damage caused by the excessive release of these cytokines. This is supported by the moderate but statistically significant decrease in IL-17 levels detected in the mouse group receiving EBV DNA along with 2.5 µg of EBNA-3A compared to the water-injected group 3 days post injection. This hindering mechanism may wane due to the persistence of the triggering agent with time. This is shown by the subsequent increase in the cytokine levels in the same mouse groups on days 6 and 9 post injection. Such a decrease in mice on day 3 was not observed with the groups injected with EBV DNA along with 5 µg or 10 µg of EBNA-3A; this may indicate that such a mechanism is overcome by higher concentrations of the triggering agents.

Worth noting is that the lowest IL-17 levels were produced by PBMCs incubated with both EBV DNA and EBNA-3A. Several mechanisms may have resulted in this reduction. The activation of Tregs, for example, might be further enhanced in the presence of both EBV DNA and EBNA-3A than with either component at a time. On the other hand, EBNA-3A may have associated with EBV DNA resulting in the

activation of a particular inhibitory mechanism. As noted above, when 10 µg of EBNA-3A were injected into mice along with EBV DNA a lesser extent of IL-17 elevation was observed on day 9 compared to injection of 5 µg of EBNA-3A along with the viral DNA. This supports a potential effect of EBNA-3A to DNA ratio that may have also played a role *in vitro* and should be further tested. If a direct EBNA-3A interaction with EBV DNA is behind triggering such an inhibitory effect, then this association may have been further enhanced by adding both components to the confined space of a culture well.

The IL-10 levels detected in culture medium from PBMCs treated with EBV components were variable among cells from different subjects and did not show any statistically significant differences. This variability in IL-10 and variabilities seen in IL-17 levels between cells obtained from subjects within the same type (RA patients and non-RA controls) may be due to non-assessed genetic determinants in these subjects; alternatively, this may be due to potential previous infections, including ones caused by other *Herpesviridae*, that were not assessed in this study.

In conclusion, EBV DNA appears to have the capability to enhance IL-17 production, this was particularly evident in mice. EBNA-3A may also have similar effects; however, this appears to be concentration-dependent.

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