AMERICAN UNIVERSITY OF BEIRUT

ROLE OF TOLL-LIKE RECEPTOR 9 IN EPSTEIN-BARR VIRUS DNA-TRIGGERED IL-17 PRODUCTION IN BALB/C MICE

by

RANA FADLLALA JAMMAZ

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Title: Role of Toll-Like Receptor 9 in Epstein-Barr Virus DNA-Triggered IL-17 Production in BALB/c Mice

Background: The Epstein-Barr virus (EBV) is a DNA virus that establishes latent infections that often reactivats. Previous studies demonstrated a relation between EBV and autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus. A previous study conducted at the Department of Experimental Pathology, Immunology and Microbiology demonstrated a relation between injecting EBV DNA and increased production of IL-17 in mice. IL-17 is a proinflammatory cytokine associated with various autoimmune diseases. On the other hand, reports investigating interaction of EBV with Toll-like receptors (TLRs) showed that EBV DNA is possibly recognized by TLR9.Therefore, the study at hand focused on the role of TLR9 on the enhancement of IL-17 production caused by EBV DNA in BALB/c mouse peripheral blood mononuclear cells (PBMCs) in culture as well as in BALB/c mice *in vivo*.

Methods (*Ex vivo*): Blood was collected from 11 BALB/c mice and PBMCs were separated using Ficoll-isopaque. Mouse PBMCs were then cultured for 24 hours with EBV DNA, EBV DNA and the TLR9 inhibitor ODN 2088, TLR9 inhibitor alone or with *Staphyloccocus epidermidis* DNA used as a non-viral DNA control. Untreated cells were included as well. Subsequently, the level of IL-17 was assessed in culture supernatants with an enzyme-linked immunosorbent assay (ELISA).

Methods (*In vivo*): To assess the role of TLR9 in the enhancement of IL-17 caused by EBV DNA *in vivo*, 45 female BALB/c mice were used. Mice were divided into 5 groups each containing 9 mice. Mouse groups were injected with EBV DNA, EBV DNA and the TLR9 inhibitor, TLR9 inhibitor alone or with *S. epidermidis* DNA. A group that was injected with sterile distilled water was assessed as well. Three mice were sacrificed per group on days 3, 6, and 9 post-injection. Blood was collected by cardiac puncture and pooled per group per time point. Serum was then collected to assess IL-17 levels by ELISA.

Results: Mouse PBMCs treated with EBV DNA displayed increased levels of IL-17; culturing cells with EBV DNA and TLR9 inhibitor at a concentration of 1.4 μM led to a significant decrease in IL-17 production by 73.41% (p=0.0088) compared to cells incubated with EBV DNA alone. Mice injected with EBV DNA also had higher levels of IL-17 on all days tested; however, mice injected with EBV DNA and 5.6 nmole of the TLR9 inhibitor demonstrated a decrease in IL-17 production on days 3, 6 and 9 post-injection compared to mice injected with EBV alone; the highest level of decrease, by 19.28 %, was observed on day 6 post injection (p=0.0139). In conclusion, TLR9 may play a role in triggering IL-17 production in response to EBV DNA both *ex vivo* in mouse PBMCs as well as *in vivo* in BALB/c mice. Whether similar observations

can be made in human cells or subjects remain to be seen.

Conclusion: In conclusion, TLR9 may play a role in triggering IL-17 production in response to EBV DNA both *ex vivo* in mouse PBMCs as well as *in vivo* in BALB/c mice.

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CHAPTER I

INTRODUCTION

The Epstein-Barr virus (EBV) is a DNA virus associated with glandular fever also referred to as infectious mononucleosis. EBV has been associated with various autoimmune diseases including rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus. EBV infects B cells resulting in latency and the ability to cause recurrent infections during which it can produce viral antigens like viral DNA. These antigens may then be recognized by specific receptors known as the pattern recognition receptors that include the Toll-like receptor (TLR) family. TLR9 has unmethylated CpG DNA as its agonist. Unmethylated CpG DNA is rarely found in vertebrate genomes and is present in newly produced EBV virions. A previous study conducted at the Department of Experimental Pathology, Immunology and Microbiology demonstrated a relation between injecting EBV DNA and increased production of IL-17 in mice. IL-17 is a proinflammatory cytokine associated with various autoimmune diseases. Moreover, IL-17 plays a role in combating various fungal and bacterial infectious agents. The overall aim of this study was to examine whether TLR9 plays a role in triggering IL-17 production in response to EBV DNA in mice.

The aims of this study were to:

A. Assess the role of TLR9 in the enhancement of IL-17 caused by EBV DNA upon treatment of mouse PBMCs with EBV DNA and the TLR 9 inhibitor, ODN 2088.

B.Assess the role of TLR9 in the enhancement of IL-17 caused by EBV DNA *in vivo* in BALB/c mice.

CHAPTER II

LITERATURE REVIEW

A. Epstein-Bar Virus

The Epstein-Barr virus (EBV), also referred *to Human herpes virus 4*, was first isolated from Burkitt lymphoma and was discovered by Epstein and Barr [1]. EBV belongs to the Lymphocryptovirus genus of the gamma subfamily of the *Herpesviridae* family [2].More than 100 known herpes viruses have been discovered and all of these viruses are able to establish latency in certain tissues. Eight of them can infect humans: the herpes simplex virus types 1 and 2 (HSV-1 and 2), varicella-zoster virus (VZV), EBV, cytomegalovirus (CMV), *Human herpes virus 6* (HHV-6), *Human herpes virus 7* (HHV-7), and Kaposi's sarcoma virus or *Human herpes virus 8* (HHV-8). All herpes viruses can establish latent infections [3]. These herpes viruses are divided into three groups. The first group is known as the alpha herpes viruses such as HSV-1, HSV-2 and VZV. These viruses have a short replicative cycle and wide range of hosts. The second group is the beta herpes viruses such as CMV, HHV-6 and HHV-7. These viruses have a relatively longer replicative cycle and a very narrow host range. The third group is the gamma herpes viruses; this group includes EBV and HHV-8; they have variable life cycle lengths and a very restricted host range [3].

1. EBV Structure

EBV is a 122-180 nm virus made-up of three major components: 1) an icosahedral nucleocapsid containing162 capsomeres and housing the EBV genome [4],

2) a lipid bilayer envelope acquired from the infected-cell as the newly formed virion buds through the cellular membrane. The envelope contains virally encoded glycoproteins (spikes) such as glycoprotein B (gB), glycoprotein (gH), glycoprotein (gL) and glycoprotein 350 (gp350) that binds CD21 on B lymphocyte surfaces [1, 2] 3) Between the nucleocapsid and the envelope, about 17 amorphous proteins constitute the tegument or matrix. Among those proteins constituting the viral tegument, host proteins can be found like heat-shock protein 70 (Hsp 70) and actin [4].

2. EBV Genomic Properties

The EBV genome consists of linear double-stranded DNA (100×10^6 Daltons). The viral genome encodes about 85 genes and is approximately 172 kilo base pairs (kb) in length [5]. The EBV genome has a higher percentage of cytidine and guanidine (C+G) nucleotides than the human genome (58% and 42%, respectively) [**6**].

The major internal repeat, IR1, contains 5-10 copies of a sequence about 3 kilo base pair (kbp) in length. IR1 separates between the short (12 kbp) and the long (134 kbp) unique sequences (U_L and U_S, respectively). EBV nuclear antigens (EBNAs) promoter (Wp) are contained within IR1 [6]. There are other shorter internal repeats (IR2, IR3, and IR4) that contain repetitive elements and that divide the long unique sequence (U_L) into four shorter sequences (U2, U3, U4, and U5) [7]. The EBV viral genome is preserved as an extrachromosomal episome in the infected cells, where this episome formation is intermediated by a set of 0.5 kbp terminal repetitive (TR) sequences that are situated at either sides of the linear molecule [8].

The majority of EBV genes are involved in the lytic (productive) cycle of the virus; while only 12 genes and two classes of noncoding RNAs, Epstein-Bar virus

encoded small RNAs (EBERs) and micro RNA (miRNA) are expressed during the latent (nonproductive) cycle [4].

3. Types of EBV

EBV is divided into two types: EBV type one and EBV type two, based on genomic variability [1]. These two types differ in their ORF (open reading frame) for the EBNA-2 gene, whereby these two types only have 64% identical nucleotide sequences in this gene. This leads to notable variations in reactivation and transformation capabilities and also results in producing antigens with different immunogenicity. The most prevalent type world -widely is type 1 and the two types of EBV have equal prevalence in Africa[9].

4. Epidemiology of EBV Infection

EBV is highly prevalent throughout the world whereby according to the International Agency for Research on Cancer (IARC) more than 90 percent of adults are infected by this virus [10]. Infection with EBV mostly occurs in children between one and six and in adolescents and young adults between fourteen and twenty [10].

5. Diseases Caused by EBV

In healthy individuals the immune system controls the different effects caused by EBV, whereas in immunocompromised individuals, EBV is highly linked to several types of malignancies [11].

Primary infection with EBV at early life stages is usually asymptomatic, unlike during adolescence. This subclinical infection is believed to be due to the maternal antibodies that play a role in containing the infection [12] and this was demonstrated by the detection of VCA viral capsid antigen (VCA) -IgG seroconversion in infants who were infected by EBV in the first two years of life. Primary infection in early childhood could have symptoms similar to several respiratory illnesses. If the acquisition of EBV infection is delayed until adolescence, and this usually happens in developed countries [13], it usually causes infectious mononucleosis (IM).

a. Infectious Mononucleosis:

EBV causes IM in 25–75% of EBV-infected persons [10] withthe highest incidence in children and adolescents 15-24 years old with no seasonal peak[14]. The incubation period is 33–49 days. The symptoms of IM include malaise, fever, adenopathy, pharyngitis, atypical lymphocytosis, hepatomegaly, splenomegaly and jaundice [14].

b. EBV-Related Carcinogenesis

The International Agency for Research on Cancer classified EBV as the most carcinogenic virus in due to evidence indicating that EBV’s pathogenesis is highly associated with a wide range of malignancies [8] such as Burkitt's lymphoma (jaw tumor mostly seen in equatorial Africa), Hodgkin’s lymphoma, T/NK cell lymphoma, gastric carcinoma, lymphoma in HIV positive infected patients, post-transplant lymphoproliferative disorders, nasopharyngeal carcinoma(more prevalent insouthern Asian populations). This oncogenic potential for EBV resides in the ability of this virus to immortalize B cells [10, 15].

6. *Transmission of EBV*

EBV infection occurs through person-to-person transmission; no animal reservoir has been detected [16]. The majority of EBV infections are transmitted orally via saliva, and most likely during childhood [1,4]. EBV could also may be transmitted sexually since several examinations revealed that the replication of EBV was detected in the uterine cervix *in vivo* [17]. It could also be transmitted through organ transplantation which usually leads to post transplantation lymphoproliferative disease (PTLD) [18]. This virus has also been detected in breast milk but transmission through this route is not confirmed [18].

7. *Epstein-Bar Virus Replication Phases*

a. Lytic Replication:

The lytic replication of EBV is also called the productive infection phase and it is essential for the formation of new progeny and for viral transmission from one cell to the other, as well as for its transmission between different hosts [19].

The lytic replication takes place in B cells and in epithelial cells. In B cells the lytic replication usually takes place after the reactivation from latency as the circular viral DNA genome must linearize to undergo this reactivation, while in epithelial cells this replication takes place directly after viral entry [9]. The linear viral genome is copied by the viral DNA polymerase during lytic replication, while the circular viral DNA genome is copied using the host DNA polymerase during the latency phase [9].

During this stage of replication, the expression of gene products takes place in three sequential stages: immediate-early, early, and the late stage[9]**.** Immediate-early lytic gene products are expressed independent of any de-novo synthesized viral proteins.

This is followed by expression of early genes, and then by late genes [4]. These immediate early genes such as BZLF1 (BamHIZ leftward reading Frame 1) and BRLF1 (BamHI R fragment leftward open reading frame 1) enhance the expression of later lytic genes [9]. Early lytic gene products, such as BNLF2, are responsible for different functions such as metabolism, blockage of antigen processing and regulation of replication [9]. Genes expressed during the late stage include those that encode proteins used in assembly and egression of new virions out of the cell such as VCA. Genes expressed during this stage also include, BCRF1 which plays a role in in immune evasion [4].

Replication of the viral genome during the lytic cycle initiates from the two origins of DNA replication (known as oriLyt) located in the long unique sequence (U_L), and is dependent on viral machinery of replication. The immediate early viral protein Zta binds to DNA specific motifs present on several promoters of viral early-genes and initiates the cascade for expression of lytic-cycle genes.Activated by Zta, the transactivator Rta interacts with its response elements in the two oriLyt domains participating with Zta in viral DNA replication [4]. The assembly of the viral core occurs in the infected cell nucleus, then the budding of the nucleocapsid while passing through the nuclear membrane will take place, and this step is essential for acquiring the primary viral envelope that will soon lose it when it fuses with the external nuclear membrane [20]. As this nucleocapsid buds through the golgi apparatus, the protein tegument and secondary envelope will be acquired [20] and finally the newly formed progeny virions will be released by exocytosis.

a. Latency

The viral latency program starts after twelve to sixteen hours following EBV entry into B cells *in vitro* [4]. During latency no production of virions take place[9],where the viral genome circularizes in the cell nucleus as an episome [21]. Then several copies (10-100) of the episomal DNA are formed and conserved in the infected B cell nucleus [4]. During latency a small portion of EBV genes and latent membrane proteins are expressed [21]. Four latency programs of gene expression exist: Latency III, Latency II, Latency I, and Latency 0. During Latency III EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBER, EBNA-LP,LMP-1,LMP-2A,LMP-2B are expressed [4],while during Latency II, EBNA-1 ,EBNA-LP, LMP-1(latent membrane protein 1), LMP2A, LMP-2B and EBER are expressed. EBNA-1 and EBER are expressed during Latency I and finally there is no expression of any viral proteins during Latency 0 [22].

8. EBV Pathogenesis

EBV mainly infects resting B cells **[**15], where first it binds to CR 2 on B cell surfaces via the viral gp350. Binding then initiates signaling events and then endocytosis will be triggered (**9**). After that, CR2 will bind to gp220 and this will help the virus to gain nearer approach to the cellular membrane where gp42 will interact with MHC class II [23]. Theinteraction between gp42 with the MHC class II triggers the interaction between the core fusion machinery, gHgL and gB with the endosomal membrane and this may also lead to the initiation of further signaling events**.** After these sets of events occur, the fusion between the endosomal membrane and the virus will take place and this will allow the entry of the viral capsid into the cytosol [24].

EBV is a B-lymphotropic virus, but it can still infect many other types of cells such as NK cells, T lymphocytes, monocytes in addition to epithelial cells of the oropharynx or the cervix but at lower efficiencies.When EBV first infects a host it typically initially enters the differentiated oropharyngeal epithelial cells [17] where lytic infection takes place and this leads to amplification of viral numbers. Then, the virus will spread into the salivary glands, oropharyngeal lymphoid tissues, and B lymphocytes of the tonsils where it can produce new virions (lytic cycle) or stay inside the memory B cells without progeny production (latent cycle) [2]. EBV expresses Latency III genes in naïve B cells whereby three proteins in this latency phase (EBNA-3A, EBNA-3B, and EBNA-3C) will downregulate the growth program and this will permit the migration of these activated lymphoblasts to the germinal follicles where Latency II will be established. Then, with specific survival signals (chemokine mediated signals) these cells will leave the germinal follicles as memory B cells and Latency 0 is established where there is no expression of any genes. Upon division, memory B cells will express the EBNA-1 which allows viral genomic replication as well. These memory B cells could return back to the tonsils where they undergo plasma cell differentiation and replication to infect other B cells and other hosts through salivary secretions [4, 25]. The infected memory B cells that are present in the circulation are attacked by cytotoxic T cells and very few of them stay in the circulation [17]. However, the resting memory B cells where Latency 0 is established allow life time persistence of the virus [4, 25].

9. EBV and Autoimmune Diseases

EBV is associated with several auto immune diseases such as SLE (systemic lupus erythematosus, RA (rheumatoid arthritis) and MS (multiple sclerosis) [26].A previous study conducted at the Department of Experimental Pathology, Immunology and Microbiology indicated that EBV DNA was capable of enhancing the production of the pro-inflammatory cytokine IL- 17 when EBV DNA was injected to mice *in vivo*[27].

According to a previous study, EBV is highly linked to SLE since high levels of EBV DNA as well as several types of antibodies to (EBNA1 and VCA) were detected in the serum of these patients. EBV is also thought to have a very important role in the pathogenesis of RA. The same study revealed an increase in the viral load with high titers of EBV-directed antibodies to EBNA1, VCA and decreased cell-mediated control of EBV in RA patients[28]. In addition, MS is highly correlated with EBV infection as many prospective studies and seroepidemiological surveys revealed that there is an increased risk to have MS in EBV infected subjects [29].

B. Autoimmunity

Autoimmunity is the loss of immunological tolerance; hence, it is the loss of the ability of the individual to discriminate between self and non-self-antigens. Immune cells would then recognize and respond to self-antigens resulting in humoral and cellular immunologic abnormalities. This usually leads to organ-specific diseases or systemic autoimmune diseases. Factors that contribute to the rise of autoimmunity include genetic as well as environmental ones encompassing microbial infections [**30**]. Various studies have demonstrated the critical pathogenic role of Thelper 17 (Th17) cells and its hallmark, IL-17, in autoimmune diseases [31, 32, 33].

C. IL-17

IL-17 is mainly produced by Th17 cells [34]. Several other immune cell types can also produce IL-17 such as T-cells, NK cells, natural killer T (NKT) cells, dendritic cells (DCs), activated monocytes, mast cells, neutrophils and lymph tissue inducer (LTi) cells [35,36]. IL-17 is a family of 6 closely related cytokines that includes (IL-17A/IL-17/IL-17B/IL-17-C/IL-17-D/IL-17-E and IL-17 F). IL-17A and IL-17 F share a very high degree of similarity.

IL-17 is a pro-inflammatory cytokine which mobilizes neutrophils in bacterial and fungal infections [37]. IL-17A is the most well investigated member of the IL- 17 family and it enhances the production of various cytokines from different types of cells (such as TNFα and IL-6), chemokines (such as CXCL2 and MCP-1), mucins, acute phase proteins and matrix metalloproteinases [32, 38]. IL-17 is believed to play a role in several auto immune diseases including psoriasis, inflammatory bowel disease, and rheumatoid arthritis. The IL-17A receptor (IL-17R) is expressed in many cell types including endothelial cells, epithelial cells, fibroblasts and myeloid cells [39]. Th17 cells differentiate from Th_0 under the effect of differentiation factors (TGF-β plus IL-6 or IL-21), the growth and stabilization factor IL-23 and the transcription factors (STAT3, RORγt, and RORα) [40, 41]. Th17 cells produce IL-17A, IL-17F (**2**) and IL-22. Th17 cells also produce IL-1, IL-6, IL-8, IL-22, TNF-α and IL-21 [40, 41].

1. *IL-17 and Autoimmune Diseases*

Multiple studies have demonstrated high levels of IL-17 expression in various inflammatory autoimmune diseases in both human patients and in animals

[42].Evidence also showed that IL-17 mediates adverse effects in many autoimmune diseases including RA, MS and SLE [43].

IL-17 stimulates the production of several other inflammatory cytokines such as IL- β and TNFα from macrophages in addition to IL-6 and GM-CSF from fibroblasts. IL-17 is believed to play a relevant role in the pathogenesis of RA through several mechanisms. IL-17 enhances cartilage destruction by inducing the production of metalloproteinases and inhibiting the synthesis of proteoglycan. Moreover, IL-17 acts on osteoclasts stimulating COX-2 (cyclooxgenase-2) –dependent PGE_2 (prostaglandin E 2) synthesis and ODF (osteoclast differentiation factor) gene expression and this will induce the differentiation of osteoclast progenitors into mature osteoclasts. Hence, IL-17 is a crucial cytokine for osteoclastic bone resorption in RA patients [44].

D. Toll-Like Receptors (TLRs)

TLRs are pattern recognition receptors (PRR) that are mostly expressed in macrophages, dendritic cells and other phagocytes. TLRs recognize conserved microbial patterns known as pathogen associated molecular patterns (PAMPs) [45]. Twelve TLR genes have been identified to be expressed in mice, while only 10 are expressed in humans. Mice express TLR1 through 13 except for TLR10 whose gene appears to be defective in mice. Humans express TLR 1 through 10; TLR11, 12 and TLR13 are not found in humans. Some TLRs are located on the cell surface such as TLR1, TLR 2, TLR4, TLR5, TLR6 and TLR11 while TLR3, TLR7, TLR8, TLR9, and TLR13 are located on the endosomes or lysosomes.; in mice, TLR11 and TLR12 recognize profilin from *toxoplasma gondii* while TLR13 recognizes bacterial ribosomal RNA. In humans, TLR10 is still an orphan receptor. TLR1 and 2 heterodimerize to

recognize tri acyl lipopeptides, whereas TLR2 and 6 heterodimerize to recognize diacyl lipopeptides. TLR2 is a receptor for bacterial components particularly for Gram positive bacterial lipoteichoic acid, while TLR4 recognizes lipopolysaccharide. TLR5 is a receptor for flagellin while microbial unmethylated CpG DNA is recognized by TLR9. TLR7 and TLR8 recognize single stranded microbial RNA and finally, TLR3 is implicated in the recognition of viral dsRNA. TLRs mediate the initiation of innate immune responses and they are involved in several processes such as inflammation, cellular proliferation and regulation as they are part of the primary defense against a wide range of pathogens [46].

1. TLRs Signalling Pathways

TLRs follow two signalling cascade patterns. The first one is the MyD88-dependent signaling pathway (**1**) and the second is the MyD88-independent pathway [48].

The MyD88-dependent pathway is used by all types of TLRs except TLR3. The cytoplasmic domain known as the TIR domain is shared by all types of TLRs. Upon TLR activation the adaptor protein TIRAP (also known as MAL) results in the recruitment of MyD88 to the TIR domain of TLR 1, 2, 6 and 4. TLR5, 7, 8 and 9 do not require TIRAP to recruit MyD88. Recruitment of the MyD88 adaptor protein leads to the recruitment of IRAK 1, IRAK2 and IRAK 4. IRAK4 will phosphorylate IRAK 1 and IRAK 2 leading to their interaction with TRAF6, an E3 ubiquitin ligase. Subsequently, TRAF6 ubiquitylates TAK1 leading to its activation [49]. TAK1 then interacts with the IKK complex resulting in the phosphorylation of IκB and the nuclear localization of NF-κB. The production of different types of pro inflammatory cytokines

such as TNF-α, IL-1 and IL-12 will then be enhanced due to the expression of NF-κB. In addition, upon activation of TLR 7 and 9, MyD88 recruits TRAF3 which consequently activates TBK1 and IKKε; these will then phosphorylate the IRF7 transcription factor which then translocates to the nucleus resulting in expression of IFN-α.

On the other hand, the MyD88-independent pathway is utilized by TLR3 and by TLR4 as well. In this pathway, TRIF is recruited which in turn recruits TRAF3 resulting in activation of TBK1, and IKKε. This culminates in the phosphorylation of the IRF3 transcription factor, its translocation to the nucleus and IFN-β expression. Recruitment of TRIF to the TLR4 TIR domain requires the TRAM adaptor protein which is not required for TRIF's recruitment to TLR3. In addition to recruiting TRAF3, TRIF also recruits TRAF6 which in turn also culminates in expression of NF-κB.

2. TLR9 and CpG DNA

TLR9 recognizes unmethylated CpG DNA which has been detected in both viral and bacterial genomes. Vertebrate CpG DNA, on the other hand, is methylated [50]. The role of TLR9 as a pattern recognition receptor has been mostly studied in plasmacytoid dendritic cells (pDCs) and B cells [51]. TLR9 is also expressed by other cell types such as monocytes [52], neutrophils [53] and CD4 T cells [54]. Non-immune cells including pulmonary epithelial cells, keratinocytes and intestinal epithelium may also express TLR9.

The activation of monocytes by TLR9 agonists has been reported to result in the secretion of TGFβ, IL-6, IL-1, IL-23 and IL-21 [51]. These may then induce IL-17 production.

3. TLR 9 and EBV

EBV DNA is delivered as unmethylated DNA when it enters the newly infected B cells. After that, CpG motifs within the EBV genome of latently infected cells progressively become more methylated. As a result, the BZLF1 protein binds to methylated CpG motifs in the EBV genome enhancing the expression of several essential lytic genes; this will activate the initiation of the lytic phase. The lytically induced cells will then release viral progeny which contain viral DNA in its naïve state free of histones and with unmethylated CpGs [55, 56, 57]. EBV unmethylated CpG motifs can then induce TLR9 signaling and the production of cytokines released by activated antigen presenting cells (APCs) as a function of TLR9 agonist recognition [50, 56, 58].

CHAPTER III

MATERIALS AND METHODS

A. Role of TLR9 in the enhancement of EBV DNA-triggered IL-17 from mouse peripheral blood mononuclear cells in culture

1. Mice and Blood Collection

Eleven female BLAB/c mice, 4-6 weeks of age, 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. Blood was collected by cardiac puncture after sedation with Forane. After collection, the blood was pooled and the peripheral blood mononuclear cells (PBMCs) were separated using the Ficoll-isopaque method.

2. Mouse Peripheral Blood Mononuclear CellsSeparation by Ficoll-Isopaque

All steps of PBMC separation were performed in a class II biological safety cabinet. Blood was diluted 2 fold with phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO). Then diluted blood was layered onto 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 for 24 hours before they were transferred to a liquid nitrogen container (Thermo Scientific, Dubuque, Iowa) for storage.

3. EBV DNA

EBV DNA was obtained from Advanced Biotechnologies (Columbia, MD). Viral EBV genome copy numbers were specified by the manufacturer. The EBV DNA stock solution vial contains 2.4 X 10^4 DNA copies/µl, according to the manufacturer.

4. TLR9 Inhibitor

The TLR 9 inhibitor, ODN 2088, was obtained fromIntegrated DNA Technologies (Leuven, Belgium). The TLR 9 inhibitor was reconstituted, as recommended by the manufacturer, by adding 7.272 ml of autoclaved distilled water to the stock vial to obtain a concentration of 100µM.

5. ***Treatment of Mouse P***eripheral Blood Mononuclear Cells

PBMCs were cultured in a 96-well plate. Each well contained 0.25 x 10^6 cells in a total volume of 250 µl of culture medium (Sigma-Aldrich Chemie GmbH, Munich, Germany). Seven different treatments were assessed: cells were cultured with either EBV DNA (9 x 10^3 copies) alone, EBV DNA (9 x 10^3 copies) and TLR9 inhibitor (0.7µM), TLR9 inhibitor (0.7µM) alone, EBV DNA (9 x 10^3 copies) and TLR9 inhibitor (1.4µM) , or TLR9 inhibitor (1.4µM) alone. Cells cultured with *Staphylococcus epidermidis* DNA (1.7pg, equivalent to weight of 9000 copies of EBV DNA) as a non-viral DNA control in addition to untreated cells grown in culture medium were examined as well. Duplicates of each treatment were performed. The plate was incubated for 24 hours at 37° C with 5% CO_2. The contents of each well were then transferred into tubes and centrifuged at 13000 rpm for 10 min at 4 °C. Supernatants were then collected and assessed by enzyme-linked immunosorbent assay (ELISA) as described below.

We assessed the TLR9 inhibitor at concentrations of 0.7µM and 1.4 µM since these were extrapolated from previous literature demonstrating inhibitory effects of TLR9 on immune-associated functions [26]. We used 9000 copies of EBV DNA per well since this number demonstrated maximal production of IL-17 from mouse PBMCs in preliminary testing.

6. ***Enzyme-Linked Immunosorbent Assay for Mouse IL-17***

IL-17 levels were assayed in culture media using ELISA kits for mouse IL-17 (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 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 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. Role of TLR9 in the enhancement of EBV DNA-triggered IL-17 in BALB/c mice.

1. Mice

To assess the role of TLR 9 in the enhancement of IL-17 caused by EBV DNA, 45 female BALB/c mice, 4-6 weeks old, grouped into 5 groups (9 mice per group) were used. Mice were obtained from the ACF at AUB and treated as indicated in Section A. 1. above

2. EBV DNA

EBV DNA injections were prepared by diluting the stock solution (2.4×10^4 DNA copies/µl) with water to a concentration of 1440 copies/µl and administering 100 µl to each mouse. Hence, mice injected with EBV DNA were each given 144×10^3 copies. This copy number resulted 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 [25].

3. Mouse Group Injections

Mice in group 1 were injected with distilled water (this group served as a negative control), while those in group 2 were injected with 144×10^3 copies of DNA. Group 3 was injected with EBV DNA and 5.6 nmoles of the TLR9 inhibitor, Group 4 was injected with 5.6 nmole of TLR9 inhibitor only and finally, group 5 was injected with 100 µl of *S. epidermidis* DNA (27.2pg, equivalent to the weight of 144×10^3 copies of EBV DNA) which served as non-viral DNA control. We used 5.6 nmoles of the TLR9 inhibitor which was extrapolated from the concentration that inhibited EBV DNA-triggered IL-17 production in mouse PBMC *ex vivo* using the following formula: TLR9 *in vivo* dose = (TLR9 *ex vivo* dose x mouse total PBMCs)/(number of PBMCs used *ex vivo*).

All injections had a volume of 100 µl and were performed intraperitoneally.

4. Specimen Procurement

Three mice were sacrificed per group on days 3, 6, and 9 post-injection. Blood was collected by cardiac puncture into EDTA tubes and centrifuged at 3500 rpm for 15

min. Serum was then separated, consolidated per time point per group and used for measuring IL-17 levels as described below.

5. Enzyme-Linked Immunosorbent Assay for Mouse IL-17

Determination of IL-17 levels in mouse sera was performed as described in Section A. 6. above

C. Statistical analysis

Two-sample t-tests were performed using the Graphpad software; p-values less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

A. IL-17 Levels in Cell Culture Supernatants

Incubation of PBMCs with EBV DNA resulted in a 2.3-fold increase in the level of IL-17 production. On the other hand, culturing these cells with EBV DNA and the TLR9 inhibitor at a concentration of 1.4 μM led to a statistically significant decrease by 73.41% (p=0.0088) in IL-17 production compared to cells incubated with EBV DNA alone. Incubation with the TLR9 inhibitor at a concentration of 0.7 μM led to a decrease that was not statistically significant. Culturing cells with the TLR 9 inhibitor alone did not result in statistically significant changes. PBMCs cultured with *S. epidermidis* DNA, used as a non-viral DNA control, resulted in levels of IL-17 that were similar to those detected in untreated cells (Figure 1, Table 1).

B. Mouse serum IL-17 levels:

Intraperitoneal injection of EBV DNA into BALB/c mice led to a statistically significant increase in serum IL-17 levels in the group receiving 144 x 10^3 EBV genome copies on days 3(p=0.0019), 6 (p=0.0271) and 9 (p=0.0044) post-injection (Table 2, Figure 2) compared to a group that received sterile water. Mice receiving injection with EBV DNA and TLR9 inhibitor (5.6 nmol) had a decrease in IL-17 production on days 3, 6 and 9 post- injection compared to mice injected with EBV alone; the highest level of decrease, by 19.28 %, was observed on day 6 post injection (p=0.0139). Worth noting is that a group that received TLR9 inhibitor (5.6 nmole) showed a statistically significant increase in the level of IL-17 on days 3, 6 and 9 post injection compared to

the group that received injections with distilled water (Figure 2). The group of mice injected with the DNA o*f S. epidermidis* did not result in notable IL-17 changes compared to mice injected with sterile distilled water.

Table 1: IL-17 levels from mouse PBMCs

	IL-17 (pg/ml)	SD	P-value	Percent variation
Negative control (untreated cells)	4.064	1.81		
EBV DNA (9000 copies)	13.48	0	0.018	+231.69†
EBV DNA +TLR9 inhibitor (0.7µM)	7.58	2.71	0.0917	-39.29††
EBV DNA +TLR9 inhibitor(1.4µM)	2.88	1.41	0.0088	-73.41††
TLR9 inhibitor(0.7µM)	4.38	0	0.8259	+7.87†
TLR9 inhibitor(1.4µM)	6.46	1.58	0.2937	+59.05†
S. epidermidis DNA (1.7 pg)	4.54	0.67	0.759	+11.81†

† compared to untreated cells
†† compared to cells cultured with EBV DNA

Table 2: Serum IL-17 levels in BALB/c mice

		Serum IL-17 (pg/ml)	SD	P-value	Percent variation
Day 3	Water	8.90	0.11		
	EBV DNA (144×10^3 copies)	11.25	0.09	0.0019	+26.46†
	EBV DNA + TLR9 inhibitor (5.6 nmol)	10.29	0.06	0.0068	-8.50††
	TLR9 inhibitor (5.6 nmol)	10.62	0.44	0.0326	+16.90†
	S. epidermidis DNA (27.2pg)	8.75	0.09	0.2946	-1.59†
Day 6	Water	9.53	0.14		
	EBV DNA (144×10^3 copies)	12.81	0.40	0.0271	+34.47†
	EBV DNA + TLR9 inhibitor (5.6 nmol)	10.34	0.13	0.0139	-19.29††
	TLR9 inhibitor (5.6 nmol)	10.26	0.02	0.0173	+6.50†
	S. epidermidis DNA (27.2pg)	8.87	0.04	0.0226	-6.89†
Day 9	Water	8.77	0.04		
	EBV DNA (144×10^3 copies)	11.60	0.26	0.0044	+32.32†
	EBV DNA + TLR9 inhibitor (5.6 nmol)	10.83	0.13	0.0664	-6.62††
	TLR9 inhibitor (5.6 nmol)	10.26	0.15	0.0051	+17.93†
	S. epidermidis DNA (27.2pg)	8.69	0.32	0.7794	-0.84†

† compared to mice injected with water

†† compared to mice injected with EBV DNA

* indicates p<0.05 when compared to respective group of mice that are injected water (serving as negative control).

** indicates p<0.05 when compared to respective group of mice injected with EBV DNA

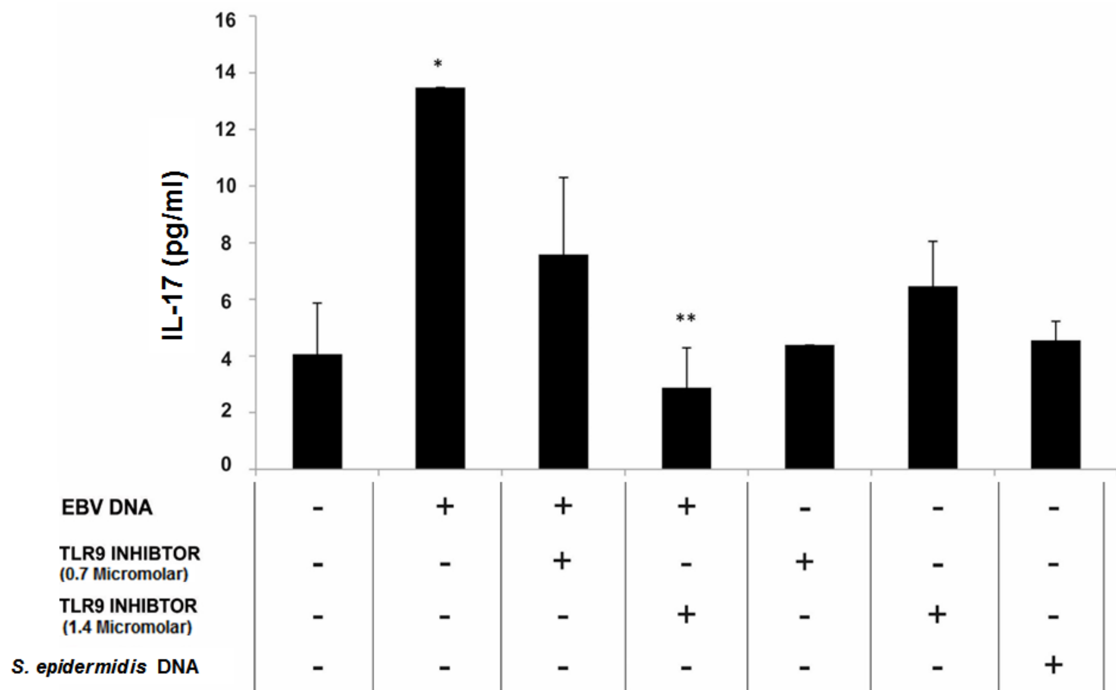

Figure 1: IL-17 levels from BALB/c mouse PBMCs treated with EBV DNA (9000 copies), EBV DNA and TLR9 inhibitor (0.7µM), EBV DNA and TLR9 inhibitor (1.4µM), TLR9 inhibitor alone or with Staphyloccocus epidermidis DNA after a 24-hour culture period. Untreated cells

* indicates $p<0.05$ when compared to untreated cells

** indicates $p<0.05$ when compared to cells cultured with EBV DNA

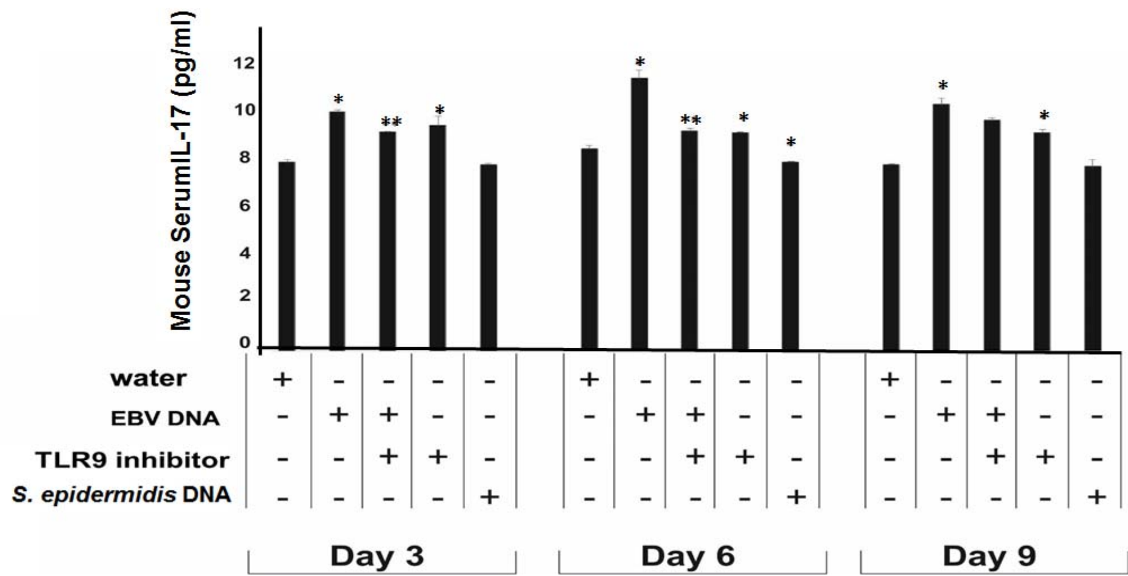

Figure 2: Serum IL-17 levels in BALB/c mice injected with EBV DNA (144x103 copies), EBV DNA +TLR9 inhibitor (5.6 nmol), TLR9 inhibitor alone or with Staphyloccocus epidermidis DNA (27.2pg) on days 3, 6 and 9 post-injection. Mice injected with water were included

CHAPTER V

DISCUSSION

Several studies have associated EBV infection with autoimmune diseases [15]. Furthermore, a previous study conducted at the Department of Experimental Pathology, Immunology and Microbiology indicated that EBV DNA induces the production of IL-17, the pro-inflammatory cytokine in BALB/c mice [27]. In addition, TLR 9 was previously demonstrated as a receptor that may be activated by EBV DNA [51]. Moreover, many other studies focused on using TLR inhibitors and its effect on the suppression of the innate immune response both (*in vivo*) and (*ex vivo*)[59,60]. Therefore, the study at hand focused on the role of TLR9 in the enhancement of IL-17 production caused by EBV DNA in BALB/c mouse PBMCs as well as *in vivo* in mice.

EBV DNA contains unmethylated CpG considered to be the ligand and the activator of TLR 9 [56].Here we assessed whether this receptor plays a role in EBV DNA-triggered IL-17 production. Mouse PBMCs cultured with TLR9 at 1.4 μM in presence of EBV DNA yielded a higher inhibition of IL-17 production by 30 % than the group of cells cultured with TLR9 inhibitor at 0.7 μM in the presence of EBV DNA indicating a proportionate dose effect. These observations indicate a possible role for TLR9 in this pathway. It has been previously reported that activation of monocytes by TLR9 agonists results in the secretion of TGFβ, IL-6, IL-1, IL-23 and IL-21. These factors may then promote IL-17 production [51].

Upon injection of BALB/c mice with 144 x 10^3 copies of EBV DNA, IL-17 levels were increased by 26.46-32.32% depending on the time point tested. This may be

due to the recognition of EBV DNA by TLR 9 leading to its activation and finally to the production of IL-17. Injection of EBV DNA along with the TLR9 inhibitor resulted in decreased levels of IL-17 *in vivo* corroborating our observations in cultured mouse PBMCs. The inhibition, although significant was not complete which may indicate that higher doses of the TLR9 inhibitor are required or that other receptors may play a role in this pathway. These receptors may include other members of the TLR family as well as other cellular receptors that are known to recognize nucleic acids [52].

Worth noting is that the group of mice injected with the TLR9 inhibitor alone showed a statistically significant increase in IL-17 production in comparison to the group injected with water. This may indicate that the TLR9 inhibitor may activate other receptors that trigger IL-17 production in the absence of EBV DNA.

In conclusion, TLR9 may play a role in triggering IL-17 production in response to EBV DNA both *ex vivo* in mouse PBMCs as well as *in vivo* in BALB/c mice. Whether similar observations can be made in human cells or subjects remain to be seen. Our current data may indicate a possible utility for TLR9 inhibitors as therapeutic agents in subjects with an autoimmune disease associated with an EBV infection. Our observation that the particular TLR9 inhibitor we employed in this study enhances IL-17 production in the absence of EBV DNA indicates that any potential therapeutic implementation would only be useful in subjects who are EBV DNA positive. Alternatively, other TLR9 inhibitors that do not result in activation of IL-17 production should be assessed.

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To my loving Family