Exploring the Role of Bacteria in Viral Reactivation and Pathogenesis

Ro Shauna S.Rothwell

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Approved by:

Jennifer Webster Cyriaque

Nancy Raab-Traub

Roland Arnold

Joseph Pagano

Blossom Damania

Abstract

Ro Shauna S.Rothwell. **Exploring the Role of Bacteria in Viral Reactivation and Pathogenesis** (Under the direction of Jennifer Webster-Cyriaque.)

Herpesviral sequences are frequently copresent with bacterial infection at multiple sites. Discerning mechanisms of bacterially induced viral reactivation would explain the molecular basis of polymicrobial infections. We hypothesized that bacterial end-products from oral and sexually transmitted pathogens and bacterial components initiate viral reactivation from latency and augment viral pathogenesis. Latently infected cell lines such as BCBL-1 (Kaposi's Sarcoma Associated Herpesvirus (KSHV)) and B958 (Epstein Barr Virus (EBV)) were incubated in vitro with crude spent media from oral bacteria. Cells were then assayed for promoter activation or state of infection by viral gene expression and Gardella gel analysis. To investigate mechanisms of reactivation, Histone Deacetylase (HDAC) inhibition potential and Protein Kinase C (PKC) activity were measured. Following incubation with crude spent media from bacteria, viral immediate early promoters were activated such as ORF –50 (KSHV), ICP0 (Herpes Simplex Virus (HSV), and BRLF1 (EBV). The KSHV early gene, Pan promoter, was upregulated and linear genomes were detected. HDAC inhibition activity as well as kinase activity increased significantly following pathogen spent media treatment from *Porphyromas gingivalis, Fusobacterium nucleatum*, and *Staphylococcus aureus*. Interestingly, distinct

oral bacterial pathogens such as *F.nucleatum, P.gingivalis*, *Prevotella intermedius*, and *S.aureus* differentially reactivated EBV, KSHV and HSV. Using crude spent media from STD pathogens, *Trichomonas vaginalis* and *Neisseria gonorrhea,* we demonstrated that they decreased HDAC activity and activated immediate early viral promoters such as ORF 50 (KSHV), and ICP0 (HSV). The ability of both oral and STD bacteria to decrease HDAC activity leads to decreases in innate immune responses. Using gene expression studies and promoter based assays it was demonstrated that induction of gene transcription of Interferon Regulatory factor 3 (IRF 3) and interferon alpha mediated induction of interferon stimulated gene transcription decreased subsequent to treatment with bacterial spent media from *P.gingivalis, F.nucleatum*, and *S.aureus*. Bacterial spent media dose dependently decreased activation of interferon beta promoter. Chromatin immunoprecipitation (ChIP) analysis detected decreased binding of RNA polymerase II on the IFNβ promoter following bacterial spent media treatment. Following viral challenge, there was decreased secretion of IFNβ from fibrosarcoma cell line, 2fTGH, in the presence of bacterial spent media. Interestingly, the presence of bacterial LPS in the spent media did not affect the ability of metabolites to decrease innate antiviral immune responses. Spent media treatment upregulated the expression of NLRX1, an antagonist of the innate mitochondrial antiviral signaling (MAVS) response. Collectively, these data demonstrate that bacteria in a polymicrobial environment may potentiate viral reactivation and enhance viral pathogenesis.

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Chapter I

Introduction

General Information

Herpesviruses are very complex. Most infections are thought to be ubiquitous since more than 90% of the world's population is infected with different types of human herpesviruses. The human herpesviruses share four significant biologic properties. First, all =encode specific enzymes involved in the biosynthesis of viral nucleic acids. These enzymes are genetically distinct from the host enzymes and provide unique therapeutic targets for inhibition by antiviral agents (26). Secondly, the synthesis of viral DNA is initiated in the nucleus. Assembly of the capsid is also initiated in the nucleus. Third, release of progeny virus from the infected cell is accompanied by cell death. Finally, all herpesviruses establish latent infection within tissues that are distinct for each virus, which demonstrates a tissue tropism for each member of this family. These virus' ability to establish lifelong latent infection of their host is denoted by the word herpes, which is derived from the Greek word *herpein*, which translates as "to creep" indicative of the virus's persistent and lurking infectious nature. Herpesviruses share a distinct icosahedral virion morphology that includes a double stranded DNA core, a capsid core that is 100-110 nm in diameterconsisting of 162 capsomers surrounded by a lipid bilayer envelope derived from the host cell membrane known as the tegument layer. Herpesvirus DNA varies in size from approximately 120 to 250 kilobase pairs, depending on the virus. The herpesvirus envelope contains polyamines, lipids, and glycoproteins (33). The glycoproteins of herpesviruses bestow distinguishing properties on each virus and provide distinct antigens to which the host is capable of responding (21, 57). To date

there are 8 human herpesviruses and these different viruses can be subdivided into 3 main groups, alpha, beta, and gamma based on sequence homology, pathogenic properties, and the type of cells they infect.

Alpha Herpesviruses

The alpha group includes Herpes Simplex 1 and 2 (HSV 1 and 2), and Varicella Zoster Virus (VZV), which are known to be neurotrophic. Following primary infection of the mucosal tissue these viruses typically establish a latent lifelong infection in sensory ganglia. The virus remains in this quiescent state unless triggered by reactivation stimuli. Reactivation stimuli of herpesviruses to date include radiation therapy, stress, immune suppression and environmental stressors (3, 8, 37, 38, 72, 96). Following reactivation stimuli of alpha herpesviruses, virus particles travel through the neuron in an anterograde motion and presentation of the virus usually occurs in the skin or mucosa. . Excellent examples of herpesvirus manifestation of disease occurring at the initial site of infection are cold sores and Herpes Whitlow. Herpes Whitlow is an occupational hazard of health care professionals, which usually occurs following an accidental puncture of the fingers or hands with a HSV infected syringe. HSV 1 and 2 infections occur in different compartments of the body and are horizontally transmitted via different routes. HSV-1 is transmitted orally whereas HSV-2 is frequently sexually transmitted . Varicella Zoster Virus (VSV) primary infection occurs most commonly in children, and the childhood illness, chicken pox, is characterized by viremia, fever, and scattered vesicular lesions of the skin. Reactivation of latent VSV in adults and the elderly is known as Herpes Zoster and commonly referred to as “Shingles”.

Beta Herpesviruses

The beta group members include Human Cytomegalovirus (HCMV), and Human Herpesvirus 6 and 7 (HHV6 and 7). Viruses in this group typically have a limited host range and grow slowly in culture. Infection with viruses from the beta group results in large, multinucleated cells (cytomegalia). Beta herpesviruses establish latent infection in salivary glands and kidneys. As a result, large quantities of virus can be shed in saliva and urine. Perinatal HCMV infection is a frequent occurrence in the United States presently with approximately one per cent of all live births. . Some children who excrete the virus at birth, but have no other symptoms, may later have impaired hearing. HCMV is also associated with a mononucleosis syndrome. This occurs in approximately 10 per cent of primary cytomegalovirus infections in older children and adults; the remaining 90 percent have asymptomatic primary infection (33). HCMV mononucleosis can be distinguished from Epstein-Barr virus mononucleosis by the lack of distinct antibodies to either nuclear or viral capsid antigens of Epstein-Barr virus. Immune compromised hosts such as organ transplant recipients, especially bone marrow transplant recipients, and individuals with human immunodeficiency virus are the cohort that often experience life-threatening disease from either primary or reactivated HCMV infection. In these patients, infection can involve the lungs, gastrointestinal tract, liver, retina, and central nervous system. Patients with human immunodeficiency virus infection and bone marrow transplant recipients seem particularly at risk for the development of CMV pneumonia. HHV6 and 7 primary infections are asymptomatic and there are no significant disease

burdens associated with these viruses. Recently HHV6 and to a lesser extent HHV7 infection has been associated with and probably cause exanthem subitum, or roseola. This illness is characterized by three to five days of fever, followed by the appearance of a maculopapular rash.

Gamma Herpesviruses

The gamma group is made up of Epstein Barr Virus (EBV) and Kaposi's Sarcoma associated herpesvirus (KSHV). KSHV has similarities genetically to herpesvirus saimiri and EBV. Since its discovery in 1994 by Chang *et al*, KSHV has become accepted as the etiological agent of Body Cavity Based Lymphoma, Multicentric Castleman's disease, as well as AIDS related and classical Kaposi's Sarcoma (KS) (13, 15, 35, 69, 94). KS is a debilitating multicellular, mesenchymal neoplasm characterized by the presence of spindle shaped tumor cells, angiogenesis, extravasated erythrocytes, edema, and a mononuclear inflammatory cell infiltrate (35). AIDS-KS is more aggressive, disseminated and resistant to treatment than all other forms of KS including those affiliated with immune suppression like post transplant KS. As the AIDS epidemic spread, KS became the most common neoplasm in patients infected with HIV and was recognized as a critical AIDS defining illness. Unlike the other human herpesviruses, KSHV infection seems not to be ubiquitous in the population and little is known about the precise way in which KSHV is transmitted (90). Significant data has shown that KSHV is a sexually transmitted pathogen among homosexual men with AIDS (32, 41, 50, 61, 63, 90, 93). However, Pauk et al's longitudinal studies of viral shedding showed that anal

intercourse among homosexual men was not the culprit of transmission of the virus since KSHV was not shed in appreciable amounts in seminal fluid, however it is found in oral cavities of severely immune compromised patients (12, 76). Subsequent research identified the possible culprits of KSHV transmission in the homosexual population were oral sex as well as deep kissing with an HIV positive partner (101).

Epstein Barr virus (EBV) is the prototypic gamma human herpesvirus (EBV) that causes African Burkitt's Lymhoma, Nasopharygeal carcinoma, and Oral Hairy Leukoplakia. Burkitt's Lymphoma (BL) can be stratified into 3 forms based on differences in EBV association and geographic distribution: endemic (eBL), sporadic (sBL) and HIV associated BL. The characterizing feature of BL is the translocation of the cMYC gene adjacent to the immunoglobin heavy or light chain loci. (6, 17, 42). EBV is unique in that it establishes several types of latent infections in different host, which are termed Latency I, II, and III. The EBNA 1 is expressed in all three forms of latency. Latency I is characterized by all Burkit's Lymphoma cells, and only expresses EBNA 1 (42). Latency II infections are characterized by all EBV positive Hodgkin Disease and Nasopharygeal Carcinoma infections and express EBNA 1 as well as latent membrane proteins 1 and 2. Latency III infections are observed in all B cell lymphomas of immune compromised patients, and all known latency associated proteins are expressed.Primary infection with EBV early in childhood is usually subclinical however primary infection during adolescence and older often results in Infectious Mononucleosis (IM). IM is characterized by malaise, sore throat, and an increase in production of EBV specific antibodies. Except for IM, and

Nasopharyngeal carcinoma (NPC) most of the diseases associated with EBV occur as a result of immune suppression. Add more EBV stuff

Herpesvirus State of Infection

Herpesvirus infection is characterized by two distinct states, latency (nonproductive) and lytic (productive) replication (47). During latency very few viral proteins are expressed which allows the virus to remain undetected by the host immune response (6, 17, 42) Herpesvirus reactivation occurs in a temporally ordered transcriptional cascade which is triggered following a reactivation stimulus leading to the expression of the immediate early genes, the viral transcriptional transactivators. Immediate early gene expression initiates the expression of the early genes, which encode enzymes for replicating viral DNA. The late genes encode the structural proteins of the virus (43). During latency the morphology of the genome is episomal whereas lytic replication of the virus is denoted by a linear genome. Certain genes are expressed during latency and lytic infection and the assignment of viral genes to the latent or lytic phase has benefited immensely from the ease of culturing cell lines that are latently infected with virus such as BCBL-1 cells, HH-B2, and BC-3 for KSHV and Raji, B958, and AGS cells for EBV (9, 13, 14, 40, 69, 79).

Chemical Inducers of Herpesvirus Reactivation

In latently infected cell lines, lytic reactivation can be induced by treatment of the cells with a variety of chemical compounds, most notably phorbol esters such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and Histone Deacetylase (HDAC) inhibitors such as sodium butyrate. TPA promotes the expression of immediate early genes through

activation of the cellular transcription factor AP1, an end point of the protein kinase C signaling pathway (36). TPA induced reactivation from latency is mediated by the Protein Kinase C and MEK/ERK mitogen-activated protein kinase (MAPK) pathways (27). Xie et al recently reported that besides the MEK/ERK pathway, the JNK and p38 MAPK pathways also mediates TPA induced KSHV reactivation from latency (103). TPA treatment also leads to the demethylation of RTA promoter and KSHV reactivation from latency (18). Histone deacetylase inhibitors have been shown to alter repressive chromatin structures on immediate early gene promoters by increasing acetyl groups and decreasing methylation of immediate early promoters thus promoting a favorable conformation for transcription factors to bind and initiate transcription of immediate early genes (1, 2, 51, 58). Chromatin modifications and nucleosome arrangement are important in the maintenance of stable gene expression patterns, especially in the repression of transcription (65). During latency, immediate early viral promoters are heavily methylated and histone deacetylases (HDAC) present at immediate early viral promoters prevent acetyl groups from binding to methylated promoters (18). HDAC inhibitors such as trichostatin A (TSA), sodium butyrate, and 5'AzaDC are HDAC inhibitors that have been demonstrated to reactivate latent herpesviruses by preventing HDACs from deterring the binding of acetyl groups to methylated promoters and facilitate acetylation of hstone tails via acetyl transferases (7). The increased presence of acetyl groups on histone tails relaxes DNA protein interactions allowing transcription factors to bind and activate transcription of immediate early genes. Following chemical induction of immediate early gene expression, viral gene expression switches from the latent dormant

program to an ordered cascade of lytic gene expression, leading to viral replication, virion production, cell lysis, and viral release. Little is known about reactivation of herpesvirus in vivo except that there is more presence of the virus following stressful stimuli, UV radiation and immune suppression (3, 8, 37, 38, 62, 72, 96, 97). Generally herpesvirus reactivation likely occurs spontaneously.

Increased Herpesvirus Presence in Periodontitis

Many categorial forms of periodontal disease are known. These include gingivitis, acute necrotizing ulcerative gingivitis, adult periodontitis, and localized juvenile periodontitis. Although periodontal diseases are thought to be widespread, serious cases of periodontitis are not common. Gingivitis is an inflammatory disease that is limited to the gingiva and there is no clinical evidence of loss of periodontal ligament or alveolar bone. The disease can be corrected subsequent to implementing proper oral hygiene. Periodontitis is more severe and is characterized by inflammatory destruction of the periodontal ligament and supporting bone. The tissues that are involved in periodontal diseases are the gums, which include the gingiva, periodontal ligament, cementum, and alveolar bone. The main area affected due to periodontal disease is the gingival sulcus, a pocket between the teeth and the gums. Gingivitis sites show pocket depths typically ranging from 2 to 4 mm. Periodontitis pockets exceede 4-mm depth on average and this disease plagues about 30% of the U.S. population (74). Periodontal pockets of depths of 7 mm or more are present in less than 5% of the U.S. adult population (74). Periodontitis is characterized as a polymicrobial infection since it contains human herpesviruses such as HCMV, EBV, HSV-1 and 2, and KSHV along with periodontopathic bacteria. Herpesviruses are

considered to be an important etiological factor of periodontal disease since herpesvirus sequences such as EBV, HCMV and KSHV gene products are consistently detected in periodontal lesions along with periodontopathic bacteria such as *porphyromonas gingivalis, Fusobacterium nucleautum, and Aggregatibacter actinomycetemcomitans,* (22, 23, 92, 99, 105). KSHV coinfection of gingival tissue has been described in AIDS associated perio disease (92). HCMV is detected in 65%, EBV1 in 71% and HCMV-EBV coinfection in 47% of the patients with severe periodontal disease. Herpesvirus and bacterial coinfection demonstrated a more rapid progression to periodontal disease (22). In addition HSV-1 gene products were detected in 4 out of 14 gingival biopsies from periodontal patients (30). Taken together these data suggest that there is a higher rate of herpesvirus presence in the gingival tissue sites than in healthy sites. Since the clinical circumstances of periodontal disease occur in the subgingival plaque. Saygun et al demonstrated that HCMV, EBV, and HSV-1 presence is associated with the presence of putative pathogenic bacteria (*P. gingivalis, P.revotella intermedia, Tannerallaa forsythia*, *Campylobacter rectus, A. actinomycetemcomitans*) in aggressive periodontitis lesions by conducting nested PCR of paper point samples from chronic periodontitis patients for HCMV, EBV, and HSV-1 and compared these results to randomly selected healthy subjects and determined that the prevalence of the virus/bacterial relationship in the subgingival region of chronic periodontitis patients was higher than healthy controls (85). A similar study was conducted by Yapar *et al* in which subgingival prevalence was examined in aggressive periodontitis patients compared to healthy controls and it was found that the rate of HCMV was 64.7% in aggressive periodontitis compared to 44.3%

of HCMV in chronic periodontitis and 70.6% of EBV presence in aggressive periodontitis compared to 16.7% of chronic periodontitis patients (105). The percentage of herpesvirus detection in subgingival plaque in healthy controls was hardly detected. A case report conducted by Prato *et al* demonstrated that HSV-1 presence facilitated rapid gingival recession and worsened periodontitis pronosis. Following surgery and treatment with acyclovir, periodontal prognosis improved with a decrease in gingival recession (77). In another report, 20 participant's periodontal status was assayed by gingival inflammation, bleeding on probing, probing depth, and clinical attachment loss. HCMV, EBV, and HSV-1 potentiated disease, with HSV-1 linked to more severe attactment loss (56). These data suggest that herpesvirus presence is heightened in the worst cases of periodontal disease.

Herpesvirus Modulation of Cell Mediated Adaptive Immunity

The increased presence as well as shedding of herpesviruses at sites of bacterial infection in certain disease states indicates that there could be a benefical polymicrobial relationship between herpesviruses and bacteria in the oral cavity as well as the genital tract, which facilitates viral reactivation and possibly aids in bacterial colonization. Microorganisms rarely grow in isolation in nature but rather persist in communities or in niches with other microorganisms as part of a polymicrobial infection. (11). Polymicrobial diseases themselves are acute and chronic that result from various combinations of viruses, bacteria, fungi and parasites. A key advantage resulting from the presence of herpesviruses is that they manipulate cellular immunity in order to persistently infect the host. The ability to modulate the cellular environment is an

attribute for other microbes in a polymicrobical infection of the oral cavity and the genital tract, since it could aid in their infection and pathogenesis. Immune cytokines can be divided into two groups, T helper 1 (Th1) and Th2. Th1 and Th2 cytokines have opposing functions in cell mediated immunity. Th1 cytokines (e.g., gamma interferon [IFN-γ], tumor necrosis factor alpha [TNF-α], and interleukin-2 [IL-2]) induce major histocompatibility complex molecules and activate T cells. Alternatively, Th2 cytokines (e.g., IL-4, IL-5, IL-6, and IL-10) activate B cells and stimulate antibody development. The Th1 cytokine IL-2 has been shown to suppress Th2 cytokine development, whereas IL-10, a Th2 cytokine, has been shown to suppress Th1 development. The polarization to a Th1 or Th2 response is mediated by chemokines, which are a large group of structurally related cytokines that stimulate directed migration of leukocytes through transmembrane G coupled receptors. Chemokines are classified into four subfamilies, CC, CXC, C, and CX3C, based on arrangement of conserved cysteine residues in the N-terminal region. Ultimately a Th1 environment favors recognition and clearance of virus-infected cells by cytotoxic T cells (CTLs). Cytotoxic T cell presence does not prevent herpesvirus latency or reactivation, which demonstrates how proficient herpesviruses are at controlling cell-mediated immunity. KSHV polarizes the cellular millieu to a Th2 environment by secreting virus encoded cytokines vCCL1, vCCL2, and vCCL3, which also secrete antagonistic signals for CC and CXC receptors of Th1 and NK lymphocytes. KSHV cytokines vCCL1 and vCCL3 activate CCR8 while vCCL2 activates CCR3 to cause a chemotaxis of Th2 mediators. KSHV encodes two zinc finger membrane proteins, K3 and K5, that function in the removal of MHC class 1 molecules from the cell surface. It

has been demonstrated that K5 significantly downregulates HLA-A and -B whereas HLA-C expression was minimally decreased. KSHV K5 did not decrease HLA-E expression. This selective decrease in expression of HLA allotypes by K5 is due to differences in amino acid sequences in their transmembrane regions. Unlike K5, K3 has been shown to decrease the expression of all four HLA allotypes (45). KSHV also employs the function of K5 to deter natural killer cells from eliminating cells that have decreased expression of MHC class 1 by decreasing the important ligands for NK cell mediated cytotoxicity, ICAM-1 and B7-2 (44). Impaired CTL function is a phenomenon shared by KSHV and EBV. KSHV infected cell lines such as BCBL-1, BCP-1, BC-1, and BC-2 have reduced expression of HLA class I on the cell surface because of decreased TAP-1 expression (10). EBV infection of B cells promotes a Th2 cellular milieu through its Latency Membrane Protein 1 (LMP1) by secreting CCL17 and CCL22 cytokines (68). Also sequence variation in LMP1 has been shown to affect antigen-presenting cell (APC) function of infected B cells and enhance immune escape by EBV-specific T cells (75). Using tumor biopsy specimens obtained from 36 Japanese nasopharyngeal carcinoma (NPC) patients, Takeshi et al demonstrated that LMP2, TAP1, tapasin and HLA class I antigens were downregulated in more than 65% of the lesions tested, while FasL, Fas and IL-10 were expressed in at least 60% of the lesions, which suggest that NPC cells may use immunoescape mechanisms (73). Host protein shut off is mediated by EBV protein BGLF5 during a productive infection(83). Baumforth et al recently demonstrated that EBV infection increased the expression of the chemokine

CCL20 in primary Hodgkin and Reed-Sternberg cells and Hodgkin and Reed-Sternberg cell-derived cell lines. The elevated levels of CCL20 in the supernatants of EBV-infected Hodgkin Lymphoma cell lines enhanced the migration of regulatory T cells (Tregs), which are specialized CD4(+) T cells that inhibit effector CD4(+) and CD8(+) T cells, which causes the loss of EBV specific immunity(5). HSV infection of T cells caused deregulation of the T cell receptor (TCR) which caused interleukin-10 production compared to mock infected T cells which secreted proinflammatory cytokines like tumor necrosis factor alpha, interferon gamma, and interleukin-2 (91). HSV-1 has been shown to up-regulate HLA-G expression in human neurons, which promotes tolerance(54). In neonatatal mice it has been demonstrated that there is a lack in expansion of CD8+ T cells due to reduced surface expression of CD25, IL-2 receptor α chain (31). To achieve selective interleukin-10 synthesis, HSV differentially affected TCR signaling pathways. HSV inhibited TCR-stimulated formation of the linker for activation of the T-cell signaling complex, and HSV inhibited TCR-stimulated NF-kappaB activation.HSV-1 protein ,USI, deters antigen presentation function of infected cells by inhibiting the ability of these cells to induce subsequent CD4(+) T cell activation (4). In addition HSV 1 and express an immediate-early protein, ICP47, that effectively inhibits the human transporter associated with antigen presentation (TAP), blocking major histocompatibility complex (MHC) class I antigen presentation to CD8+ T cells (78, 100)..

Kaposi's Sarcoma Associated Herpesvirus Modulation of Innate Immunity

Herpesviruses not only regulate adaptive immune responses but they also control innate immune responses as well. KSHV modulates innate immune responses by

expressing cellular homologs (66). KSHV encodes four viral interferon regulatory factors (IRFs), named vIRF-1, vIRF-2, vIRF-3 and vIRF-4, which function in controlling type 1 interferon gene expression. They are located in the 83- to 95-kb region of the KSHV genome between open reading frames (ORFs) 57 and 58 (25). These viral genes are expressed by ORF K9-encoded viral (v) IRF1, ORF K11.1-encoded vIRF2, and ORF K10.5-encoded viral interferon regulatory factor 3 (vIRF3)/latency-associated nuclear antigen2 (LANA2). KSHV vIRF-4 (ORFK10/K10.1) has been detected by gene array analysis, Northern blotting, and reverse transcription-PCR, but the protein is the least described of the viral IRFs. KSHV vIRF-1 is the most described of the viral IRFs and it deregulates the expression of type I inferferons by preventing phosphorlyated IRF-3 recruitment of CBP/p300 factors to the interferon beta gene (25, 46). In so doing, interferon beta expression is deterred and cell is unable to establish an antiviral state (55). The vIRF-3 protein has been named latency-associated nuclear antigen 2 (LANA-2), since it is expressed during latency and expressed in the nuclei of B cells and to distinguish it from ORF73-encoded LANA (80). Rivas et al demonstrated that LANA-2 is primarily expressed in subjects with primary effusion lymphoma and multicentric Castleman's disease; it was not expressed in KS (80). In addition this report demonstrated that LANA-2 inhibited p53-induced transcription and apoptosis. Other functions of KSHV vIRF it may either decrease the transcription of the IFN-α/ß genes by targeting IRF-3 and IRF-7 or transactivate them (59, 60). Other KSHV proteins that deter the innate antiviral interferon (IFN) response, include ORF45, viral IL-6 (vIL6) and the transactivator of the lytic cycle, RTA (16, 106, 107). Moreover KSHV controls cell

mediated immunity by encoding viral modulators of immune response (vMIRs), which function as E3 ubiquitin ligases and down-regulate MHC-I (24, 45). KSHV vMIR2 also decreases the expression of ICAM-1 and CD86 by increasing endocytosis, lysosomal targeting, and proteasome-mediated degradation and increases endocytosis of CD1d, leading to the evasion of virus infected cells from Natural Killer T cells (84). The majority of immune evasion strategies employed by KSHV are used during the lytic viral cycle, when most of these proteins are expressed and a rigorous host response occurs to deter viral dissemination.

Herpesvirus Facilitate Bacterial Pathogenesis in Periodontal disease

Herpesvirus modulation of the cellular milieu is an advantage to perio pathogens in the polymicrobial setting of a periodontal lesion since the induction of cytokines is advantageous for periodontal bacteria to orchestrate tissue destruction and bone loss associated with periodontal disease. For example, HCMV infection increases interleukin-1β and tumor necrosis factor alpha gene expression of monocytes and macrophages (22). The production of interleukin-1β and tumor necrosis factor alpha may increase the presence of matrix metalloproteinase, decrease tissue inhibitors of metalloproteinase, and facilitate periodontal bone destruction (22). In periodontal lesions, herpesvirus infection and cytopathic effect in fibroblasts, keratinocytes, endothelial cells, and inflammatory cells such as polymorphonuclear leukocytes, lymphocytes, and macrophages deters tissue turnover and repair (22).

Reports have shown that gingival herpesvirus infection may promote subgingival attachment and colonization of periodontopathic bacteria similar to the enhanced bacterial adherence to virus-infected cells observed in other infections. For example, HCMV infection enhances *A. actinomycetemcomitans* adherence to cells thus facilitating its pathogenesis (98). EBV can promote the environment of a periodontal lesion since it has the ability to induce proliferation and differentiation of B cells, which is a key feature of periodontal disease (22). Moreover, lytic EBV infection can also cause the production of anti-neutrophil antibodies and neutropenia, which augment bacterial pathogenesis and lead to the over-growth of perio-pathogens. EBV and HCMV infection of monocytes, macrophages, and lymphocytes deregulates their functions and this impairment may predispose to overgrowth by periodontal pathogens (22).

Bacterial Metabolites aid in Herpesvirus Reactivation

Since perio pathogens are allowed to persist more efficiently the amount of metabolite production increases. Oral bacteria, *P.gingivalis* and *F.nucleatum* , secrete proteases, lipopolysaccharide (LPS), and short chain fatty acids (SCFA), such as valproic acid and butyric acid, as a result of their metabolism. LPS is secreted by P.gingivalis and F.nucleatum in vesicles that are secreted from the cell membrane. *P.intermedia* is a gram-negative oral anaerobe that has an LPS but releases markedly reduced levels of SCFA compared to *P.gingivalis* and *F.nucleatum* (53). *P. gingivalis* is an asaccharolytic organism, dependent on nitrogenous substrates for energy. Although sugars such as glucose can be utilized by the organism, these compounds are not converted to metabolic end products but, rather, are used for the biosynthesis of intracellular macromolecules

(86, 87). F.nucleatum derive energy from amino acid and sugar fermentation (81, 82). In contrast gram-positive bacteria such as *S Mutans*, *P.streptococcus* and *S.Aureus* do not share the same metabolites as P.gingivalis or F.nucleatum because these organisms are saccharolytic and produce lactic acid and have lipotechoic acid (LTA). The salt form of butyric acid, sodium butyrate, is used in vitro to stimulate herpesvirus reactivation via histone deacetylase (HDAC) inhibition of immediate early viral promoters. Morris et al demonstrated that HDAC inhibition by metabolites from gram-negative oral bacteria such *P.gingivalis* and *F.nucleatum* facilitated KSHV reactivation by activating the P38 pathway, which is downstream of HDAC inhibition by co-treating BCBL-1 cells with P38 inhibitor, SB 202190 (4-(4 Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole)(67). Oral bacteria secrete LPS along with metabolites, which could activate virus in a similar manner as TPA since LPS signaling converges on the PKC pathway. LPS and LTA stimulation of toll like receptor 4 and 2 respectively causes the activation by receptor-mediated phosphorylation or dephosphorylation of proteins such as protein tyrosine kinases (PTKs)/Ras/IκB kinase/Raf-1/MEKK/MEK/MAPK, which leads to the translocation of AP-1 and NF-κB to the nucleus and subsequent modulation of gene expression (27, 28, 88, 95, 102). AP-1 binding to immediate early promoter is central to integral reactivation (64). LPS stimulation of herpesviruses as not been studied yet.

Innate Immune Response

Modulation of the cellular environment is integral for successful reactivation and propogation of human herpesviruses. An important component of host immunity is the innate immune response since it is the first line of defense against invading pathogens. Immediately following viral infection there is a production of type one interferons (39). Human interferons (IFNs) are cellular proteins important in innate immune defense that exhibit antiviral, immunoregulatory, and growth inhibitory functions. IFNs are classified according to cellular origin and receptor affinity. Type 1 interferons include interferon alpha and beta whereas IFN γ is a type 2 response. Leukocytes produce IFN α, fibroblasts produce IFN β, and activated T-cells and natural killer cells produce IFN γ. Type 1 interferons are encoded by many IFN α subtypes, a single IFN β gene and others like IFN ω, IFN ε, and IFN κ. The most recently described family of IFNs is IFN-λ, which includes IFN-λ1, -λ2, and -λ3 (52), alternatively named interleukin-28A (IL-28A), IL-28B, and IL-29, respectively (89). These cytokines share signaling similarities with IFN-α/β and activate ISRE-containing promoters (29). Secreted interferons have affinity for and activate the type 1 interferon receptor (a heterodimer of IFNAR1 and IFNAR2) in an autocrine and paracrine manner. The binding of secreted interferon to the receptor leads to the translocation of ISGF3, a trimer of signal transducer and activator of transcription 1(STAT1), STAT2, and IFN regulatory factor 9(IRF 9) forming the ISGF3 complex. The ISGF3 complex enters the nucleus, binds to conserved IFN-stimulated response element (ISRE) sequences on the promoters of IFN-α/β-stimulated genes (ISG) and increases their transcription rates. ISGs are essentially responsible for causing the

antiviral state initiated by secretion of interferons. Activation of ISGs such as 2'5' OAS causes activation of cellular RNase L, an endoribonuclease, which cleaves cellular and viral RNAs, which inhibits protein synthesis and viral propogation. PKR is an ISG, which leads to the phosphorylation of the α subunit of the eIF-2α translation initiation factor, causing complete inhibition of protein synthesis and viral replication. ISG54 (IFIT1) and ISG56 (IFIT2) are essential viral stress genes that are induced by the presence of dsRNA, interferon, and infection of by many different viruses. These related genes are both located on chromosome 10 and work to block initiation of translation by inhibiting eIF3.

Viral induction of type 1 interferons occurs following the identification of foreign antigens by toll like receptors (TLRs). The regulation of IFN-α and -β genes involves the Interferon regulatory factor (IRF) family of transcription factors, which in humans contains at least nine members. TLRs recognize conserved molecular patterns of microorganisms, which are not generated by the host and are integral for the microorganism survival. Antiviral signaling of both RNA and DNA viruses is mediated by Toll like receptors 3 and 7. Following viral infections, treatment with dsRNA and polynucleotides like Poly I.C., interferon regulatory factor 3 (IRF-3) is post translationally phosphorylated by the "Virus Activated Kinase (VAK), which includes Tank Binding Kinase 1 (TBK1), IκB kinase homologues, and IκB kinase-epsilon (IKKε). These factors allow for IRF-3 to be in its phosphorylated active form and translocate to the nucleus and initiate the transcription of the interferon beta gene by binding to positive regulatory domains (PRD) I and II in the promoter. Studies have shown that the

cytoplasmic arrangement of IRF-3 monomer is caused by prolonged export caused by a nuclear export signal (NES) in the molecule. IRF-3 is present in all cell types at appreciable levels. IRF-7 is closely related to IRF-3 in how both proteins' inactive form is found in the cytoplasm and their activation occurs as a result of phosphorylation of residues in the C terminus of the protein. Unlike IRF-3, IRF-7 is expressed at very low levels and its expression is greatly enhanced following IFN treatment.

Importance of HDACs in Type 1 Gene Expression

The expression of type 1 interferons relies on epigenetic modifications by HDACs. Nuizson et al demonstrated that virus infection stimulation of interferon beta gene expression as well as interferon alpha mediated transcription of interferon stimulated genes is dependant on histone deacetylase inhibitors (HDACs) by using HDAC inhibitor, Trichostatin A (TSA). The formation of the enhancesome at the interferon beta promoter decreased as well as secretion of interferon beta from 2fTGH cells such that cells were not able to protect themselves subsequent Sendai virus challenge at low concentrations of virus (71). HDAC inhibitor, TSA, did not decrease Stat 1/Stat 2/ IRF-9 trimer, ISGF3, translocation to the nucleus. TSA did decrease HDAC 1 at the ISGF3 promoter of ISGs thus decreasing interferon alpha mediated expression of ISGs (70).

Bacterial Infections Incite Innate Immune Responses

The type 1 interferon response is not only anti-viral but also has been shown to be anti-bacterial since lipopolysaccharide (LPS) stimulation of toll like receptor 4 has been

shown to cause the expression of IFNb through MyD88 dependant and independent mechanisms (49). The MyD88 independent signaling after LPS timulation of TLR 4 occurs through TRIF related adaptor molecule (TRAM) and TIR domain containing adaptor inducing IFNb (TRIF)(34, 104). The MyD88 dependant pathway is triggered by LPS and liptoteichoic acid (LTA) and signaling via the adaptor molecule TIRAP also known as Mal (34, 104). Both MyD88 dependant and indedendant pathways signal transductions originate from the intracellular Toll/interleukin-1 receptor TIR). TIR associates with MyD88 or other adaptor molecules such as TRIF/TRAM or TIRAP to ultimately cause the phosphorylation and translocation of activated IRF-3 to nucleaus to cause the expression of IFNβ. LPS and LTA stimulation also leads to the expression of ISGs through the IFNα/β positive feedback loop.

. As already mentioned human herpesviruses encode a multitude of genes that modulate cellular immunity, however prior to reactivation gene products responsible for dampening the immune response are not expressed. In the context of periodontitis and genital infections

Herpesvirus and Bacterial Interaction in STDs

Herpesvirus and bacterial coinfection in the genital urinary tract has been described for Pelvic Inflammatory Disease (PID), and Bacterial Vaginosis (BV). PID is an inflammatory disorder of the female upper genital tract. A consequence of long term PID includes infertility, ectopic pregnancy, and chronic pelvic pain. Cherpes et al demonstrated that women coinfected with HSV-2 and a lower genital tract pathogen such as N. gonorrhea had increased odds of acute endometrititis diagnosis than the presence of

HSV-2 or N. gonorrhea alone (20). HSV-2 is the most prevalent sexually transmitted disease as well as an important cofactor in global HIV pandemic. In a study conducted in Kenyan sex workers it has been noted that HSV-2 acquisition among women increases with Bacterial vaginosis (BV), which is a venereal disease that affects the lower genital tract and is characterized by an alteration of vaginal flora where there is a decrease in lactobacilli. Study by Kaul et al demonstated that the increase of HSV-2 aquistion by altered vaginal bacterial flora such as N. gonorrhea, T. vaginalis, and syphillis increased susceptibility of other sexually transmitted infections (STIs)(48). Further studies by Cherpes el al demonstrated that HSV-2 shedding was more prevalent in women along with the presence of bacterial vaginosis and a high density of Group B Streptococcus colonization (19). In the case of bacterial vaginosis HSV-2 reactivation has been demonstrated to upregulate STD pathogen N.gonorrhea and oral bacteria share butyric acid as a metabolic end product, which could activate herpesviruses in the genital urinary tract (Starling). Other STD bacterial infection such as bacterial vaginosis have been described to have a viral activation factor for HIV but the activation of other virus like herpesviruses is unknown. proinflammatory cytokines and chemokines in the female genital tract, as well as increase Nf-kB. Nf-KB enhances Neisseria gonorrhoeae in vitro.

Purpose- Herpesviral sequences are frequently associated with bacterial infection at multiple sites including the mouth, gut and genito-urinary tract. Recently, these viral infections have been detected in the oral cavity, particularly in severe cases of periodontitis. Many epidemiological reports have demonstrated that worsened disease

prognosis results from the combination of viruses and bacteria in the genital-urinary tract. Discerning mechanisms of bacterial induced viral reactivation underpins the molecular basis of periodontal disease and STD pathogenesis. We postulated that in a polymicrobial infection, bacterial metabolites that contain SCFA and bacterial components facilitate viral reactivation and aid viral pathogenesis by dampening type 1 interferon expression. Data presented in this report highlight the importance of analyzing the interactions between pathogens in a polymicrobial infection and provide a unique *in vivo* mechanism by which viruses are activated and modulate innate antiviral immunity.

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Chapter II

Bacteria Induction of Epstein Barr Virus, Herpes simplex virus and Kaposi's Sarcoma associated herpesvirus

Abstract

Herpesviral sequences are frequently associated with bacterial infection at multiple sites including the mouth, gut and genito-urinary tract. Recently, these viral infections have been detected in the oral cavity, particularly in severe cases of periodontitis. The detection of replicating virus in these tissues has incited investigation into the relationship between bacterial infection and herpesviral reactivation. We hypothesized that bacterial end-products including short chain fatty acids (SCFA), lipopolysaccharide (LPS) and lipoteichoic acid (LTA) secreted by oral bacteria initiate viral reactivation from latency. Latently infected herpesvirus cell lines were incubated with crude spent media containing secreted SCFA, and components of bacterial pathogens. Cells were then assayed for viral promoter activation, promoter-protein interactions and state of infection. Following incubation with crude spent media, viral immediate early promoters were activated, the viral early genes were upregulated and linear genomes were detected. HDAC inhibition activity as well as protein kinase C activity increased significantly following treatment with bacterial spent media. Interestingly EBV was preferentially reactivated following toll like receptor stimulation while KSHV and HSV-1 reactivation occurred following HDAC inhibition. This is the first published report describing selective bacterial mediated reactivation of herpesviruses.

Introduction

Herpesviruses cause a broad spectrum of disease (52). To date there are eight human herpesviruses that are divided into three subfamilies (α, β, γ). The alpha herpesviruses (Herpes Simplex HSV-1, HSV-2, and Varicella Zoster Virus VZV) have tropism for neurons, replicate rapidly in multiple cell types. The beta herpesviruses (Cytomegalovirus (CMV), (HHV-6, HHV-7) replicate slowly in a restricted range of cells, while the gamma herpesviruses (Epstein Barr virus (EBV) and Kaposi's Sarcoma associated Herpesvirus (KSHV)) replicate in endothelial and epithelial cells, and lymphocytes. EBV, HSV-1, HCMV, HHV6 and 7, and VZV infection have been shown to be ubiquitous in the population, and are transmissible in saliva. Likewise, it has been demonstrated that KSHV is also transmitted vertically in saliva in various endemic regions, shed horizontally in high levels in men who have sex with men (MSM), and oral KSHV has been shown to be transmissible in vitro (17, 33-35, 42). HCMV, EBV and HSV-1 have been detected in bacteria laden periodontal lesions (44, 49, 55). KSHV infection of gingival tissue has also been described in AIDS associated periodontal disease (44). HCMV is detected in 65%, EBV-1 in 71% and HCMV-EBV coinfection in 47% of the patients with severe periodontal disease. These viruses are thought to potentiate disease as periodontal lesions harboring HCMV and EBV-1 demonstrated a more rapid progression to periodontal disease compared with lesions containing only bacteria (12). It is evident that periodontal tissue destruction is heightened in herpesvirus infected vs. non-infected sites. In lesions in which herpesviruses were readily detected,

there was an increased incidence of oral periodontopathic gram-negative anaerobic bacteria including *Porphyromonas gingivals, Fusobacterium nucleautum, Actinobacillus actinomycetemcomitans,* and *D pneumosintes* (13). Oral bacteria, *P.gingivalis* and *F.nucleatum*, secrete proteases, lipopolysaccharide (LPS), and short chain fatty acids (SCFA), such as valproic acid and butyric acid, as a result of their metabolism. *P.intermedia* is a gram-negative oral anaerobe that has an LPS but releases markedly reduced levels of SCFA compared to *P.gingivalis* and *F.nucleatum* (29). Gram-positive bacteria, *S Mutans*, *P.streptococcus* and *S.Aureus,* do not share the same metabolites as gram-negative bacteria but release Lipotechoic acid (LTA).

Herpesviruses have two distinct modes of replication; a latent infection and lytic infection. Both infection states are essential for long-term herpesviral persistence. Herpesviral gene expression is tightly regulated as evidenced by the few viral genes that are expressed during viral latency (6, 9, 25). Upon herpesvirus reactivation, genes are expressed temporally. The cascade begins with the expression of the immediate early genes, the viral transcriptional transactivators. These gene products in turn activate early gene expression, which encode proteins necessary for replicating viral DNA. Late genes encode the structural proteins of the virus. In latently infected cell lines, lytic reactivation can be induced by treatment of the cells with a variety of chemical compounds, most notably phorbol esters such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and Histone Deacetylase (HDAC) inhibitors such as sodium butyrate. TPA promotes the expression of immediate early genes through activation of the cellular transcription factor, AP1, an end point of the protein kinase C signaling pathway (11, 20). HDAC inhibitors have been

shown to alter repressive chromatin structures on immediate early gene promoters (1, 2, 5, 27). SCFA, such as valproic acid and butyric acid in salt forms are used to stimulate herpesvirus reactivation by HDAC inhibition (10, 19, 39). LPS and LTA stimulation of toll like receptors 4 and 2 respectively, cause the activation by receptor-mediated phosphorylation, or dephosphorylation of proteins such as protein tyrosine kinases (PTKs)/Ras/IκB kinase/Raf-1/MEKK/MEK/MAPK. This leads to the translocation of AP-1 and NF-κB to nucleus and subsequent modulation of gene expression (15, 16, 43, 46, 50, 53). AP-1 binding to immediate early promoters is a more potent inducer of EBV reactivation than HDAC inhibition (37).

Little is known about reactivation of herpesviruses *in vivo*, except that viral shedding increases following stressful stimuli, UV radiation, and immune suppression (3, 22, 23, 36, 47, 48). We hypothesized that metabolic endproducts and components of commensal and pathogenic bacteria copresent with viruses facilitate reactivation of herpesviruses in the oral cavity. This report demonstrates herpesviral reactivation following incubation of latently infected cell lines with bacterial spent media. Genome morphology, and increased viral gene expression indicated lytic reactivation following treatment of latently infected cells. Bacterial metabolites activated herpesvirus immediate early promoters, as well as induced epigenetic changes, and transcription complexes on promoters. This is the first report demonstrating that oral bacterial metabolites differentially reactivate HSV-1, EBV, and KSHV, providing a molecular basis for viral reactivation in the setting of polymicrbial infection.

Materials and Methods

Cells, Media, and Organisms. BCBL-1 cells and B958 cells are lymphoma cell lines that were maintained in RPMI media supplemented with 10% FBS, 5% Pen/Strep, L-glutamine, and Sodium Pyruvate. HEK-293 cells, and HeLa Cells were maintained in DMEM media supplemented with 10% FBS, and 5% Pen/Strep.

Treatments. *Porphyromonas gingivalis* A7436, *Fusobacterium nucleatum* 1594, *Staplococcus aureus* ATCC 25923, *Prevotella intermedia* CB24, and *Streptococcus mutans* 10449 were harvested at a late exponential phase of growth. *P.gingivalis, F.nucleatum, P.intermedia,* and *S.mutans* were grown anaerobically in Wilkins Chalgrens anaerobic broth (Oxoid) in an atmosphere of 5% CO_2, 10% H_2, and 85% N2 at 35^{o}C. *S.aureus* was grown aerobically in WC broth at 37^{o}C. The resultant spent media were filtered and added to cells at final dilutions of 1/10, 1/20 or 1/50. Purified commercial *P.gingivalis* LPS (Invivogen) was added to cells in culture to a final concentration of 1ug/mL. Polymyxin B treatments were performed at a concentration of 10ug/mL.

Gardella Gel Analysis Gardella gel analysis was performed according to the methods described by Gardella et al (21). Gardella gels were run in duplicate. One set of gels was transferred to nitrocellulose and probed with a ^{32}P UTP riboprobe specific for the terminal repeat region of KSHV. For modified Gardella gel analysis, individual lanes were cut into sequential 0.5cm slices and DNA was extracted from the slices using the

Qiagenmethod(14).The ORF 26gene

(5'ATCACGAGGAATTCAGCCGAAAGGATTCCACCAT'3),

(5'ATCACGAGAAGCTTTCCGTGTTGTCTACGTCCAG'3) of KSHV was amplified from the DNA extracted from gel slices and run out sequentially from left to right on a 1% agarose gel.

HDAC activity assay The HDAC activity assay (Upstate Cell Signaling) is a fluorimetric assay in which spent media from bacteria were incubated with provided HeLa nuclear extract that was provided with the kit. Extracts were then subjected to fluorimetric analysis and HDAC activity was measured

Plasmids and Reporter Assays. - pICPO-luc was described previously (30)(31). PORF50luc promoter construct and pPANluc were made by inserting DNA isolated from BCBL-1 PCR of ORF 50 and Pan of KSHV into pGL2-Basic vector (Promega). Primers for ORF 50 were as follows 5'pORF505'CATGGGCGGGTGGGTGACAGTC'3, and 3'pORF50 5'CGATAAGCTTTTGTGGCTGCCTGGACAGTATTC'3. The 3' primer contains HindIII restriction site. Primers for KSHV PAN are as follows 5'kppPANATGTGGTACCTAGACGAGTCCGACAATCACAC', which contains a Kpn1 restriction site. The 3' primer for KSHV PAN promoter contains a Sac1 site, 5'CATTGAGCTCAGTCCCAGTGCTAAACTGACTC'3. Constructs were transfected into HeLa or HEK 293 cells in 6 well plates with 0.2ug of reporter plasmid and 0.2ug of B-galactosidase expression plasmid using Effectene kit (Qiagen). The relative light units were measured in a luminometer. The BRLF1 promoter reporter plasmid was a gift from

Dr. Shannon Kenney. βgalactosidase activity was measured as an internal control.

Protein Kinase C activity Assay-BCBL-1 cells were treated with 10ng/ml TPA or 1/50 dilution of bacterial spent media for 5 hours. Following incubation cells were lysed by sonication and lysate was mixed with Promega Kinase Substrate Relative flourimetric units were measured.

Quantitative Real time PCR analysis- RNA isolated from treated BCBL-1 cells using Qiagen kit was DNAse treated using RQ1 DNAse (Promega) and cDNA was created using oligodT primers (Invitrogen). 100ng of cDNA was used for quantitative real time analysis and amplified with Light Cycler 480 Syber Green 1 Master (Roche). Primers for KSHV K8.1 primers were previously described by Fakhari(18).

Immunoblotting. BCBL-1 and B958 were treated with 10ng/mL of TPA, or .3mM Sodium Butyrate (NaB), or 1/50 dilution of bacterial spent media. Lysates were prepared by RIPA buffer and 50ug of protein was used for SDS-PAGE. Protein samples were transferred to nitrocellulose, blocked and probed with a virus specific monoclonal antibodies, EAD, and vIL6, as previously described (38). Densitometry was done using NIH Image version 1.63 and Statistical analysis were achieved with Graph Pad Prism version 4.02.

ChIP (Chromatin Immunoprecipitation) Assay. Latently infected herpesvirus cell

lines, BCBL-1 and B958 were treated with .3mM NaB, 10ng/mL of TPA, 1:50 dilution of spent media from oral bacteria and media alone (WC media) and incubated for 24 hours. Assay was carried out as previously described by Lu et al(32). Eluted DNA was subjected to PCR using primers within the ORF 50 promoter region of the immediate early KSHV gene, ORF 50, ORF50F (5'GGTACCGAATGCCACAATCTGTGCCCT'3), and ORF50R (5'ATGGTTTGTGGCTGCCTGGACAGTATT'3). Primer sequence for EBV Rta Rp promoter sequence are as follows BRLF1F (5' AGGCCGGCTGACATGGATTA'3) and BRLF1R, (5'TATAGCTACATCACCACCCC'3). HSV ICP0 promoter primers are as follows, ICPOPT (5' CCGCCGACGCAACAG'3) and ICPOPB (5' GTTCCGGGTATGGTAATGAGTTTCT'3

Results

Spent media from Gram Negative Bacteria induces KSHV reactivation.

Gardella gel analysis was performed on latently infected KSHV cell line, BCBL-1, to determine the presence of episomal (latent) vs. linear (lytic) genomes following treatment with bacterial spent media. Southern blot analysis detected both linear and episomal genomes in BCBL1 cells treated with *P.gingivalis* and TPA while only episomes were detected in the untreated cells (data not shown). Following modified Gardella gel analysis of BCBL1 cells treated with spent media from *P.Gingivalis* and *F.nucleatem,* linear genomes were consistently detected, while only episomal viral genomes were present in BCBL-1 cells treated with gram positive, *S.aureus*, spent media or media alone (Figure

1).

Real time PCR of viral DNA detected a significant increase in KSHV DNA upon exposure to *P.gingivalis* spent media and TPA (data not shown). Quantitative reverse transcriptase polymerase chain reaction (qRT PCR) of BCBL1 total RNA detected increased expression of KSHV lytic gene, K8.1 in mRNA from cells treated with (TPA), sodium butyrate, *P.gingivalis* and *F.nucleatum* spent media. *P.intermedia* and *S.aureus*, both minimal SCFA secretors, resembled media alone (WC) and uninduced cells (29). The levels of the latency associated nuclear antigen (LANA) mRNA, ORF 73 remained unchanged regardless of treatment (Figure 2a). Western Blot analysis of BCBL-1 cells treated with *P. Ginvigalis* and *F.nucleatum* spent media also detected increased expression of KSHV lytic protein. v-IL6, an early protein was expressed at levels

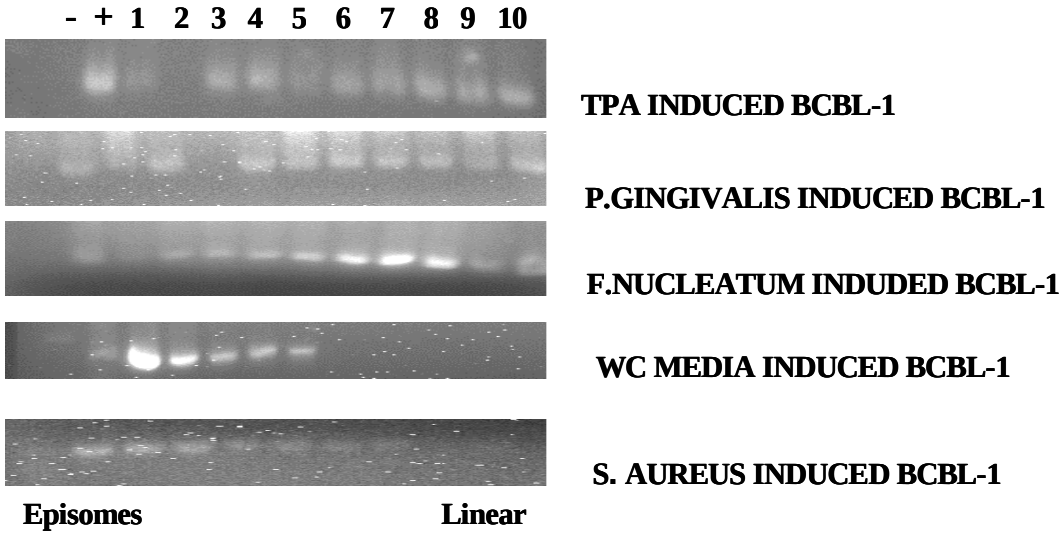

Figure 1-Spent media from oral gram-negative bacteria induce linear KSHV genome morphology. DNA extracted from sequential Gardella gels slices was PCR amplified for KSHV ORF 26 and products were run in order from 1 to 10 (top to bottom) compared to negative water control (-) and positive control (+) BCBL-1 DNA. Linear genomes were detected toward the right of the gel and episomes were detected towards the left. Reactivation was detected in TPA induced BCBL-1, *P.gingivalis* induced BCBL-1 cells, and *F.nucleatum* Induced BCBL-1. Latency was detected in the form of episomes on the left side of the gel following incubation of BCBL-1 cells with media alone (WC) and gram-positive bacteria (*S.aureus*).

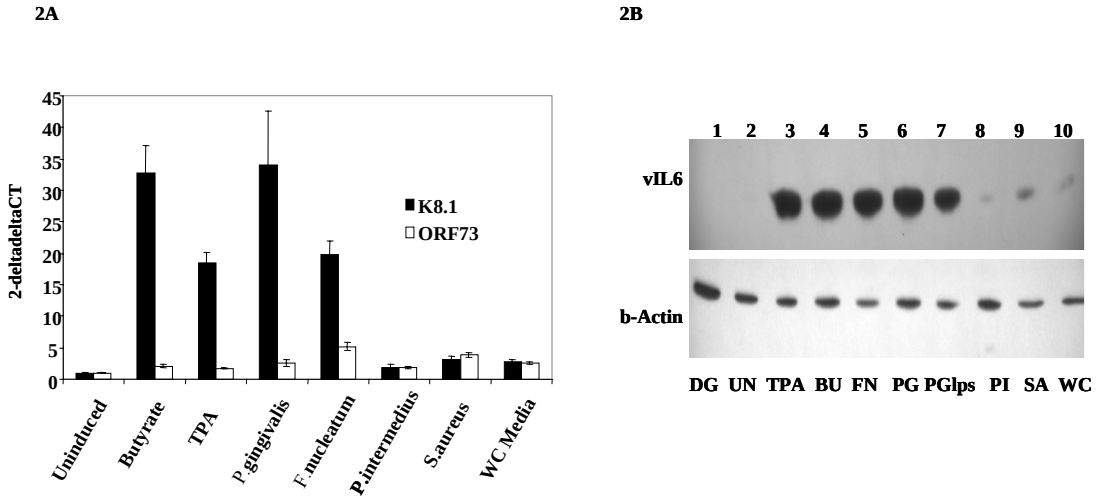

Figure 2-Spent media from oral gram-negative bacteria induce KSHV lytic gene expression. A. Real time PCR analysis of BCBL-1 cells treated with TPA, spent media from oral gram negative periodontopathic bacteria, *P.gingivalis*, and *F.nucleatum* detected comparable levels of lytic KSHV K8.1 mRNA transcription (black bars) while LANA transcription remained unchanged by treatment (Black/white bars). Treatment with *P intermedius*, *S Aureus* and media alone did not result in increased K8.1 transcription. B. Increased vIL6 expression was detected by Western blot following treatment with sodium butyrate, TPA and treatment with *P.gingivalis, F.nucleatum* spent media, and *P.gingivalis* LPS. Minimal vIL6 expression was detected in cells treated with *P.intermedia*, *S.aureus*, and media alone.

comparable to that of control inducers of KSHV such as TPA and sodium butyrate (Figure 2b). Robust v-IL6 expression was detected following *P.gingivalis* and *F.nucleatum* crude spent media treatment (Figure 2b). Taken together, these results demonstrate that spent media from SCFA secretors, *P.gingivalis* and *F.nucleatum* are inducers of KSHV reactivation.

Increased HDAC inhibition and Kinase activity following Spent media Treatment from Oral Bacteria

To determine the mechanism of reactivation following incubation with bacterial crude spent media, several analyses were performed. First, in order to determine if bacterial spent media affected HDAC activity, a fluorimetric based HDAC activity assay was performed using HeLa nuclear extract, which harbors peak endogenous transcriptional and HDAC activity. Following incubation with spent medias from the oral bacteria, HeLa nuclear extract had significantly less HDAC activity than HeLa nuclear extract alone and had HDAC levels comparable to the known HDAC inhibitor, sodium butyrate (Figure 3a). In contrast, WC media alone, and spent media from minimal SCFA secretors, *P.streptococcus* and *S.aureas,* exhibited minimal HDAC inhibition activity (Figure 3a). Bacterial spent media was also assayed for substrate based protein kinase C activity. Increased kinase activity was detected following treatment of BCBL-1 cells with TPA, and with each of the spent medias from *P.gingivalis, F.nucleatum*, and *S.aureus* (Figure 3b). Each of these organisms have an LPS or lipoteichoic acid (LTA)

that are known to activate TLRs, which could subsequently reactivate virus. Interestingly the presence of increased kinase activity following *S.aureus* treatment did not impact on KSHV reactivation as indicated by episomal viral morphology, mRNA, and protein expression (Figures 1 and 2). This suggests that SCFA present in crude spent media from gram-negative anaerobes, *P.gingivalis* and *F.nucleatum*, are more effective in inducing KSHV reactivation than bacterial components like LTA present in *S.aureus* spent media.

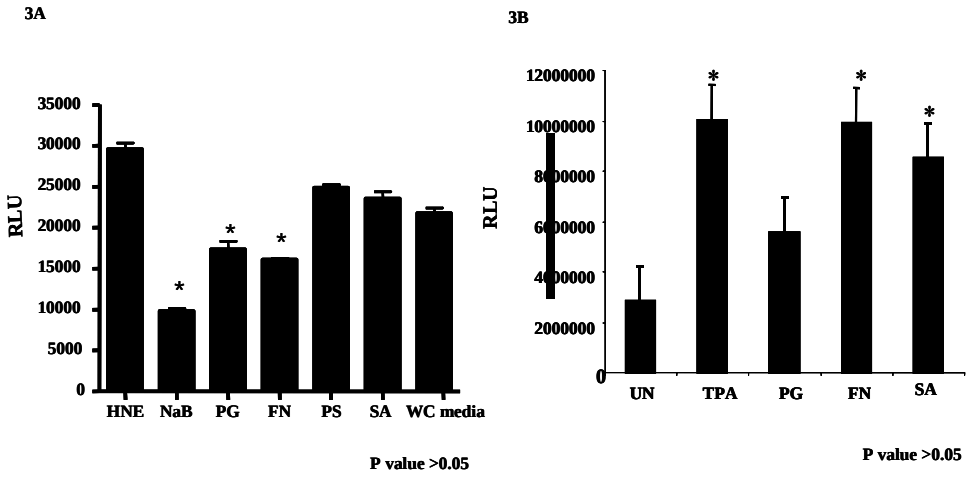

Figure 3. Bacterial Spent media activate PKC and HDAC inhibition. A. HDAC activity was decreased in HeLa Nuclear Extract (HNE) treated with Sodium Butyrate (NaB), *P.gingivalis* (PG), *F.nucleatum* (FN). HDAC activity in extracts treated with *S.aureus* (SA), and, *P.streptococus* (PS) was comparable to media alone (WC). B.*F.nucleatum* (FN), *P.gingivalis* (PG), and *S.aureus* (SA) treated BCBL-1 cells exhibited increased kinase activity comparable to TPA treated cells. This increase was statistically significant when compared to untreated BCBL-1.

Spent medias from Oral Bacteria activate KSHV butyrate responsive herpesvirus promoters

HDAC inhibition has been shown to activate the KSHV immediate early gene, ORF 50,` (Rta) and early genes of KSHV. The ORF 50 promoter becomes hyperacetylated following treatment with HDAC inhibitors such as TSA and sodium butyrate (32) Transcriptional modulation was assessed in cells that were treated with bacterial spent media that were transfected with luciferase reporter constructs containing either the immediate early KSHV ORF 50 or early KSHV Pan promoter.. The ORF 50 and KSHV Pan promoters were activated by 1/50 dilution of *P.gingivalis* and *F.nucleatum* spent medias but not *S.aureus* spent media to levels comparable to control inducer sodium butyrate (NaB) (Figure 4a and 4b).

To determine whether treatment with bacterial metabolites containing SCFA resulted in an increase in acetylation at the ORF 50 promoter, ChIP (chromatin immunoprecipitation) analysis was performed. Using an antibody specific for acetylated histone 3, and sequences specific for the KSHV ORF 50 promoter, BCBL-1 cells treated with spent media from oral bacteria, *F.nucleatum*, and *P. intermedia*, had increased binding of acetylated Histone 3 to the KSHV ORF 50 promoter, 13 fold and 5 fold respectively(Figure 4c lanes 4-6). Significantly less binding of acetylated H3 at the ORF 50 promoter was detected following *S.aureus* and WC media treatment. These data demonstrate that spent media from gram-negative anaerobic bacteria activate immediate early and early KSHV promoters as well as induce modifications of the ORF 50 promoter favorable for reactivation.

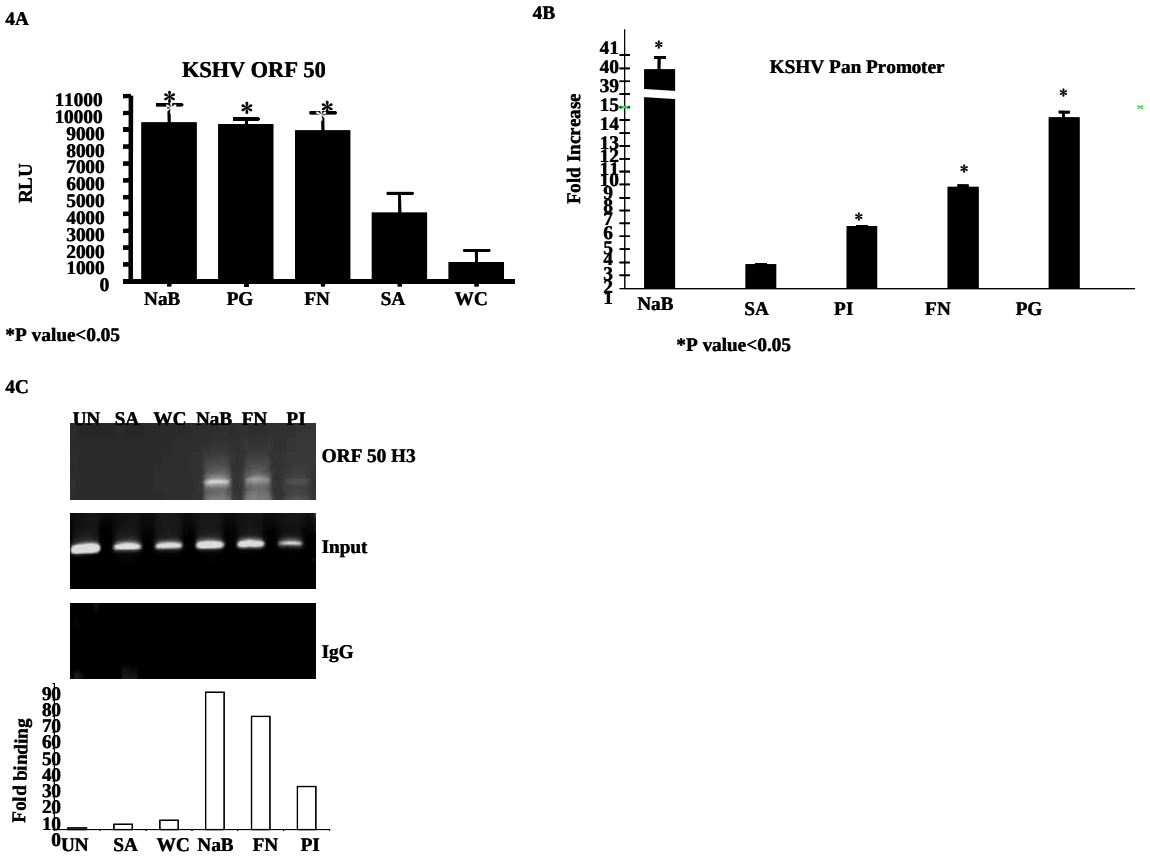

Figure 4. Spent media from oral bacteria activates KSHV immediate early and early viral promoters by histone acetylation. (A and B) Luciferase reporter constructs containing KSHV immediate early and early viral promoters, KSHV ORF 50(A) and KSHV PAN (B) respectively, were transfected into HeLa cells. Sodium Butyrate (NaB) and spent media from *P.gingivalis* (PG) and *F. nucleatum* (FN), activate immediate early and early promoters whereas spent media from Gram-positive bacteria, *S.aureus* (SA) and media alone (WC) did not. C. ChIP analysis of BCBL-1 cells treated with Sodium Butyrate (N) and *F.nucleatum* (F) indicated increased binding of acetylated histone 3 at the ORF 50 promoter while immunoprecipitation of aceytlated histone 3 was not detected in extracts from cells that were Untreated (U) or treated with spent media from *S. aureaus* (S),or WC media alone (WC)

Spent media from oral bacteria activates HSV-1 promoter

To determine whether bacterial metabolites had similar effects on other herpesviruses, Herpes Simplex Virus (HSV) promoter activity was assayed following treatment with bacterial spent media. Increased acetylation of ICP0 promoter has been detected in explanted mouse trigeminal ganglia early after sodium butyrate treatment (24, 41). In order to determine ICP0 promoter responsiveness to bacterial spent media, HeLa cells transfected with ICP0 promoter were treated with spent media from *P.gingivalis, F.nucleatum*, and *S.aureus*, and media alone. Following treatment with sodium butyrate and F.nucleatum and P.gingivalis spent media, a two fold to four-fold activation of the ICP0 was detected as compared to media alone (Figure 5A). ChIP analysis was performed on 293 cells transfected with the ICP0 promoter then treated with TPA, sodium butyrate, and bacterial spent media. Increased acetylation of H3 at the ICP0 promoter was detected following treatment with NaB, TPA, and spent media from both gram-negative bacteria. *F.nucleatum* and *P.gingivalis,* (Figure 5b). Spent media from *P.intermedia*, *S.aureus* and media alone did not result in increased acetylation at the ICPO promoter (Figure 5b).

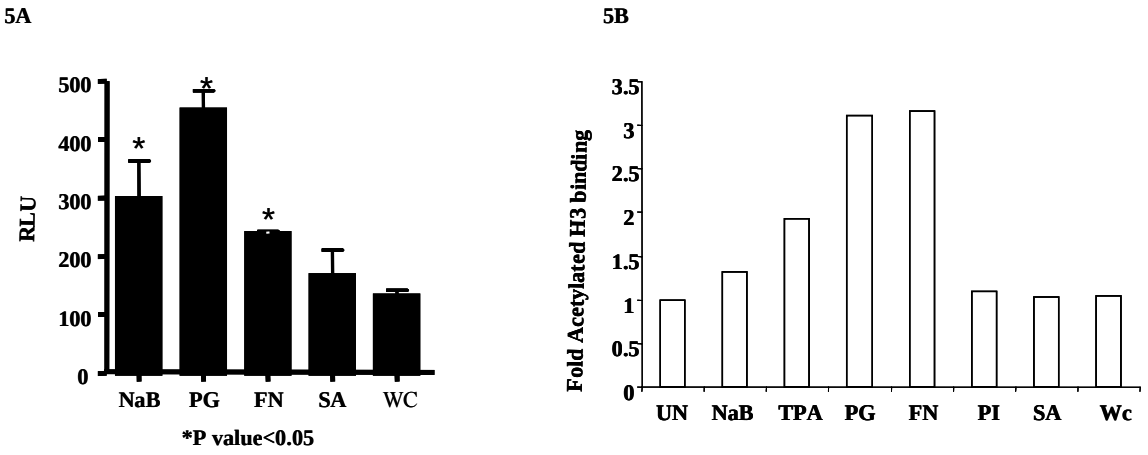

Figure 5. Spent media from oral bacteria activates immediate early promoter of HSV-1 ICP0. A. Sodium Butyrate (NaB) and spent media from oral bacteria, *P.gingivalis* (PG) and *F. nucleatum* (FN), activate luciferase reporter construct for immediate early promoter of HSV-1, ICP0, transfected into 293 cells whereas spent media from gram positive bacteria, *S.aureus* (SA) and media alone (WC) do not. B. ChIP analysis of 293 cells transfected with luciferase reporter construct, ICP0, and treated with sodium Butyrate (NaB), TPA, *P.gingivalis*, and *F.nucleatum* (F) exhibit increased acetylation of Histone 3 at the ICP0 (lanes 1-4). There is minimal immunoprecipitation of acetylated histone 3 detected in *S. aureaus* (S), *P.intermedia* (Pi) and WC media alone (W) (lanes 5-7) compared to input control and immunoprecipitation isotype control (IgG) (Figure 5b). Quantitative values of fold binding of Acetylated histone 3 are shown

Spent media from Oral Bacteria activates EBV Immediate Early BRLF1 promoter

We next sought to determine the effect of bacterial end products in EBV reactivation. Real time PCR of viral DNA detected a significant increase in EBV DNA upon exposure to *P.gingivalis* spent media and TPA (data not shown). BRLF1 is an immediate early promoter of EBV and is the positional homologue of KSHV ORF 50 Rta. HEK 293 cells transfected with BRLF1 luciferase promoter reporter plasmid were treated with TPA, NaB, bacterial spent media, and *P.gingivalis* LPS. TPA caused a 78fold increase in BRLF1 promoter activity while sodium butyrate treatment resulted in a 8-fold increase. Spent media containing LPS and SCFA from *F.nucleatum* and *P.gingivalis* activated the promoter 6 fold over uninduced. Ultrapure *P.gingivalis* LPS and spent media from S.aureus and *P.intermedia* activated BRLF1 2 fold, each of these increases was statistically significant (Figure 6a). ChIP analysis of latently infected EBV cell line, B958, detected a 3 fold increase in binding of the AP-1 complex on BRLF1 promoter following treatment with spent media from *P.gingivalis.* There was a 6 fold increase following *F.nucleatum P.intermedia*, and purified *P.gingivalis* LPS treatment compared to media alone (Figure 6b). These data demonstrate that bacterial metabolites containing LPS, LTA, and SCFA result in activation of the EBV immediate early promoter, BRLF1.

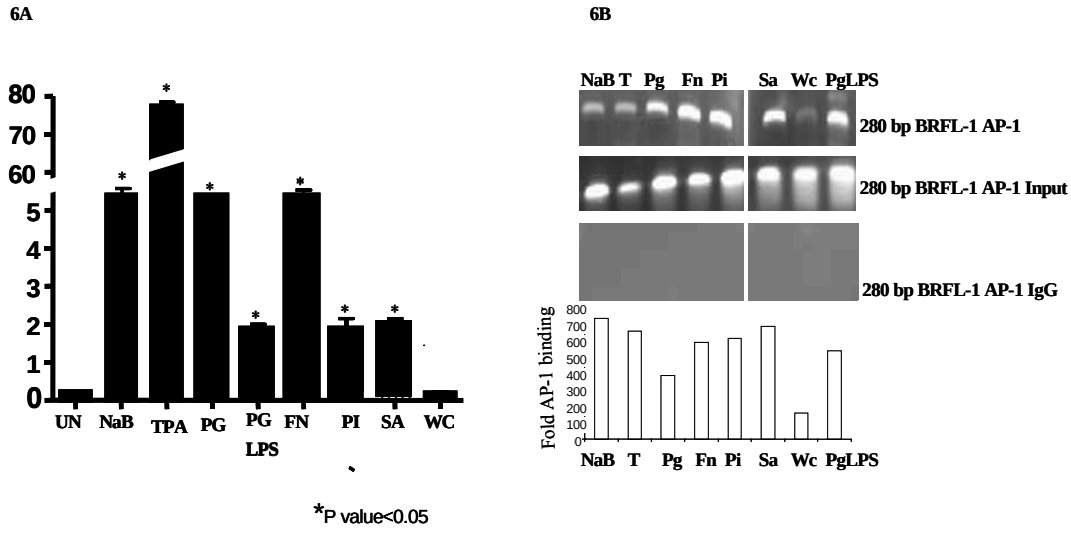

Figure 6. Spent media from oral bacteria activates immediate early promoter of EBV BRLF1. A. 293 cells transiently transfected with BRLF1 luciferase reporter plasmid were treated with Sodium Butyrate (NaB), TPA and spent media from oral bacteria (*F.nucleatum*, *P.gingivalis*, *P.intermedius*, and *S.aureus*), *P.gingivalis* LPS (PG LPS), and media alone (WC). All bacterial treatments activated the reporter construct while media alone did not. B. ChIP analysis of EBV positive B958 cells that were treated with Sodium Butyrate (NaB), TPA (T) and spent media from *F.nucleatum* (Fn), *P.gingivalis* (Pg), *P.intermedius* (Pi), *S.aureus* (Sa), and *P.gingivalis* LPS exhibit increased AP-1 at EBV BRLF1 immediate early promoter (lanes 1-6, and lane 8). There is minimal AP-1 present on BRLF1 immediate early promoter following WC media treatment (W) (lane 7). Loading control Input and negative control for IP Isotype control (IgG) are shown (Figure 6A)

Inhibition of TLR Signaling diminishes Bacterial Induced EBV Reactivation

To determine if spent media from oral bacteria increase EBV reactivation, B958 cells were incubated with bacterial spent media. Western blot analysis detected a robust increase in EBV EAD protein following treatment with TPA, Sodium butyrate, *P.intermedia*, and *F.nucleatum*. (Figure 7a). In order to determine the role of LPS in bacterial induced EBV reactivation, spent media was incubated with polymyxin B, the TLR 4 antagonist. We hypothesized LPS inability of binding to TLR receptor would diminish EBV reactivation. The B958 cells treated with TPA, P.gingivalis spent media, and P.gingivalis/Polymyxin B cotreatment demonstrated no difference in EAD expression. This was perhaps due to the presence of the SCFA, which may induce EBV replication even in the absence of LPS (Figure 7b). Interestingly in the absence of SCFA differences were detected. Comparisons of purified *P.gingivalis* LPS alone or P.gingivalis LPS/Polymyxin B cotreatment revealed a decrease in LPS mediated reactivation of EBV. These results highlight the importance of TLR signaling in EBV reactivation.

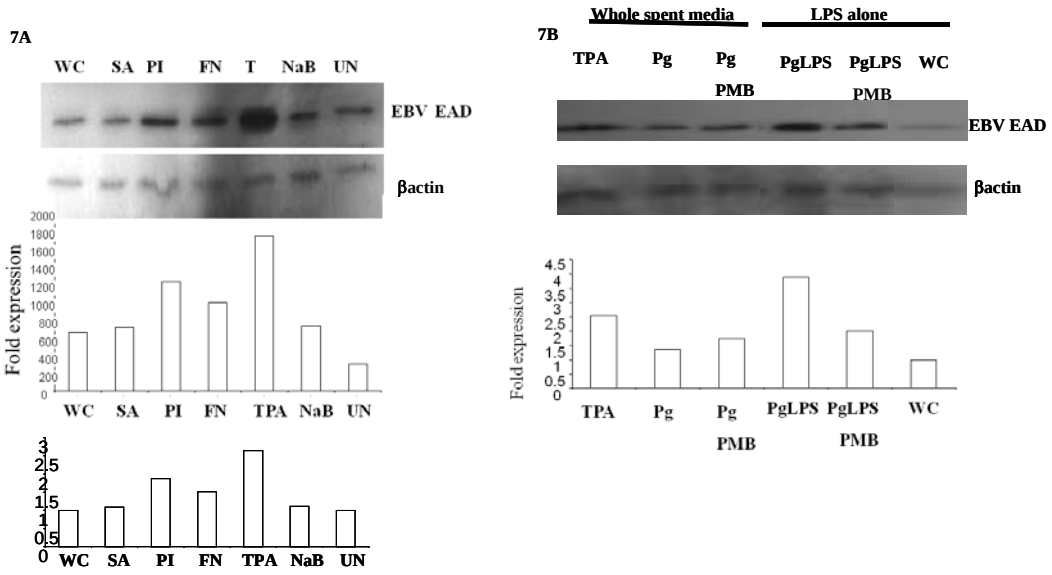

Figure 7. Toll like receptor signaling is important for bacterial mediated reactivation EBV. A. EBV positive B958 cells treated with *P.gingivalis* (Pg) spent media only exhibited increased EAD expression and whereas co-treatment with Polymyxin B (PMB),an inhibitor of LPS, did not decrease EAD expression (top). *P.gingivalis* LPS (Pg LPS) treatment of B958 cells increased EAD expression and cotreatment with Polymyxin B (PMB) with *P.gingivalis* LPS decreased EAD expression (bottom). Figures 7a top and bottom results are compared to media alone control WC. B Western blot analysis of B958 cells treated with NaB, TPA, and spent media from *F.nucleatum*, *P. intermedia*, *S.aureas* and media alone WC.

Discussion

While it has been postulated that stress, immune status, and indigenous natural products are important for reactivation, herpesviral reactivation *in vivo* is poorly understood (40, 54). In this study, we demonstrate for the first time multiple mechanisms by which bacterial metabolites induce herpesviruses reactivation. The data consistently showed that spent media containing metabolites and other bacterial components induce viral reactivation of KSHV, EBV and HSV. These bacterial-viral relationships are of key importance since polymicrobial infections harboring both viruses and bacterial infections exist in multiple body compartments including the mouth, the gut and the genitourinary tract. Within the polymicrobial setting it has been suggested that the presence of the herpesvirus potentiates bacterial pathogenesis by decreasing the host resistance against oral bacterial colonization and increasing the presence of bacterial pathogens (45).

The abundance of bacteria results in a rich reservoir of bacterial metabolites, among them LPS, LTA and short chain fatty acids. These metabolites in close proximity to latently infected lymphocytes present during a bacterial incited inflammatory response may result in herpesvirus reactivation in vivo in the oral cavity during periodontal disease. Similarly in the gut it has been demonstrated that circulating microbial products such as LPS derived from microbes of the gastrointestinal tract result in heightened HIV replication (7). While correlations were made, the underlying mechanisms were not explored. LPS, LTA and short chain fatty acids share biological properties with HDAC

inhibitors and PKC agonists that are commonly used to induce viral replication *in vitro* (16, 46, 53) .

Demethylation and acetylation are crucial for viral gene expression of many herpesviruses since they allow for the latent to lytic transition induced by changes in chromatin structure and covalent histone modifications found in association with herpesvirus genomes during latent infection (1, 28, 51). We have previously shown that like sodium butyrate, the p38 pathway mediates bacterial mediated histone acetylation to result in KSHV reactivation (38). In this report we demonstrate that crude spent media from oral bacteria, *P.gingivalis* and *F.nucleatum*, that secrete short chain fatty acids had HDAC inhibition and resulted in robust reactivation of KSHV, and HSV and to a lesser extent EBV. HDAC inhibition resulted in epigenetic changes at immediate early KSHV (ORF 50) and HSV-1 (ICP0) viral promoters providing favorable conditions to induce viral expression as evidenced by acetylation of histone 3 on the respective immediate early promoters. These data are consistent with published literature showing both KSHV and HSV immediate early promotes are modulated by HDAC inhibitor associated epigenetic changes (32, 41). Likewise, others have found that low concentrations of epigenetic modifying agents, 5-aza-2'-deoxycytidine (5-aza-CdR) or trichostatin A (TSA), induced the expression lytic EBV gene expression, in an EBV-positive gastric cancer cell line (26). Those bacteria that were minimal butyric acid secretors were unable to reactivate KSHV and HSV immediate early promoters to the same level. Interestingly, both secretors and non-secretors were capable of EBV reactivation. Each of these bacteria also expresses LPS and LTA.

P. gingivalis LPS, *F.nucleatum* LPS and *Streptococcos mutans* LTA are pathogenic components important to the initiation and development of oral disease (4). Lipopolysaccharide (LPS) and Lipoteichoic acid (LTA) are bacterial membrane components that are present along with SCFA in metabolic end products of gram negative and gram-positive bacteria. LPS shed from the surface of the bacterium binds to CD14, forms the LPS/CD14/LBP complex at the membrane This complex results in toll like receptor 4 mediated cellular activation of protein kinase C and mitogen-activated protein kinase to reactivate virus via NF-kb and AP-1 (MAPK) and stimulates inflammatory cytokine production (16, 20, 43, 46). In this report LPS and LTA containing spent media treated BCBL-1 cells demonstrated kinase activity comparable to TPA (Figure 3a). We demonstrate that bacterial crude spent media activates kinase activity and is capable of activating virus through PKC pathway, a downstream effector of toll like receptor signaling (8).

Herpesviruses were differentially induced. In BC-1 cells, a cell line dually infected with KSHV and EBV, TPA potently reactivated latent EBV through PKC while reactivation by HDAC inhibition by sodium butyrate was not as effective (37). Bacterial spent media containing SCFA capable of HDAC inhibition had robust effects on KSHV and HSV-1 replication, yet bacterial spent media containing LTA, and LPS in the absence of SCFA did not exert impressive reactivation. *S.aureus* and *P.intermedia* spent media both devoid of SCFA were unable to reactivate latent KSHV or activate HSV-1 ICP0 promoter (Figures 4a and 4b). Alternatively *S.aureus* and *P.intermedia* exhibited increased AP-1 complexes at BRLF1 promoter, which is considered favorable for EBV

reactivation. Polymyxin B, a LPS toll like receptor 4 antagonistdecreased LPS mediated activity by *F.nucleatum*, *P.gingivalis*, and *P.intermedia* and EBV lytic gene expression was significantly diminished by polymyxin B. These data further support that EBV in B958 cells is preferentially reactivated by PKC pathway activation by *P.gingivalis* LPS. Taken together these data suggest that different components of bacterial spent media have distinct mechanisms of viral induction in cells latently infected with different herpesvirus. Spent media containing LPS and LTA mediated reactivation of latent EBV through toll like receptor signaling while SCFA present in bacterial spent media reactivated latent HSV-1 and KSHV by means of epigenetic changes induced by HDAC inhibition.

Bacterial components have unique reactivation affects on different herpesviruses utilizing both TLR mediated pathways and histone acetylation to result in reactivation. This is the first report to demonstrate a favorable relationship between bacteria and the reactivation of multiple herpesviruses, highlighting the importance of polymicrobial infection to viral reactivation in vivo.

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Chapter 3

Bacterial Metabolites Decrease the Type 1 Interferon Anti-viral Response

Abstract

. Innate immunity represents the first line of defense against invading microbial pathogens. It has recently been demonstrated that histone deacetylase (HDAC) inhibition is critical for down modulation of innate antiviral type 1 interferon gene expression. Previous studies from our laboratory, have determined that bacterial end products are capable of inducing HDAC-associated chromatin changes. Based on these data we hypothesize that bacterial metabolites decrease type 1 interferon antiviral responses thus enhancing viral pathogenesis in the setting of polymicrobial infection. In this report it was determined usinggene expression studies and promoter based assays that both Interferon Regulatory factor 3 (IRF 3) and interferon alpha mediated induction of interferon stimulated gene transcription decreased subsequent to treatment with bacterial spent media. Bacterial metabolites dose dependently decreased activation of the interferon beta promoter. Chromatin immunoprecipitation (ChIP) analysis detected decreased binding of RNA polymerase II on the IFNβ promoter following bacterial spent media treatment. Upon viral challenge, there was decreased secretion of IFNβ from 2fTGH cells in the presence of bacterial spent media. Interestingly, the presence of bacterial LPS in the spent media did not affect the ability of metabolites to decrease innate antiviral immune responses. Spent media treatment upregulated the expression of NLRX1, an antagonist of the innate mitochondrial antiviral signaling (MAVS) response. Collectively, these data demonstrate that the polymicrobial environment may potentiate viral reactivation by decreasing the innate antiviral response and enhancing viral pathogenesis.

Introduction

Microorganisms rarely grow in isolation in nature but rather persist in communities or in niches with other organisms as part of a polymicrobial infection. Polymicrobial diseases themselves are acute and chronic in nature and result from various combinations of viruses, bacteria, fungi and parasites. Severe periodontal disease is a pathopnuemonic polymicrobial infection with multiple bacteria and herpesviruses present. It has been postulated that in this setting herpesviruses are activated by the bacterial metabolites and that herpesviruses aid in both bacterial colonization and modulation of cellular immunity (4, 17, 32, 33, 43).

Herpesvirus reactivation is orchestrated by both short chain fatty acid (SCFA) containing bacterial metabolites that act as HDAC inhibitors and bacterial lipopolysaccharide (LPS)/ lipoteichoic acid (LTA) that mediate PKC pathway activation (30). Previous reports have also demonstrated the p38 pathway is essential for viral reactivation by bacterial metabolites (23). Subsequent to reactivation stimuli, herpesvirus replication occurs in a temporal ordered cascade with the end result being fully assembled, infectious virions.

In order to combat disseminating viral infections, multiple host factors contribute to the establishment of an antiviral state in cells. Human interferons (IFNs) are cellular proteins important in innate immune defense that exhibit antiviral, immunoregulatory, and growth inhibitory functions (5, 28, 29). Secreted interferons bind and activate the

type 1 interferon receptor (a heterodimer of IFNAR1 and IFNAR2) in an autocrine and paracrine manner. The binding of secreted interferon to the receptor leads to the translocation of ISGF3, a trimer of signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2), and IFN regulatory factor 9 (IRF 9), which enters the nucleus, binds to conserved IFN-stimulated response element (ISRE) sequences on the promoters of IFN-α/β-stimulated genes (ISG) and increases their transcription rates (2, 7, 9, 13, 14, 18, 24, 45). ISGs are responsible for causing the antiviral state initiated by secretion of interferons.

The innate immune system has developed two pathways for the recognition of virus associated dsRNA in the cytosol and the extracellular space (1). Toll like receptors (TLRs) recognize conserved molecular patterns of microorganisms, which are not generated by the host and are essential for the microorganism's survival. TLR 3, localized in the endosome, detects dsRNA released as a result of virally infected cell death or necrosis that has been phagcytosed from the extracellular space (35). TLR-3 recognizes viral infections of both RNA and DNA viruses as well as treatment with polynucleotides like Poly I.C. resulting in the phoshorylation of interferon regulatory factor 3 (IRF-3) by Tank Binding Kinase 1 (TBK1). IRF-3, in its active phosphorylated form, then translocates to the nucleus and initiates the transcription of the interferon beta gene (19, 33, 46, 47).

A second pathway for detection of dsRNA utilizes the dsRNA dependant protein kinase (PKR). Viral dsRNA activates RLH receptors, RIG-I and MDA-5 that affect the mitochondrial antiviral signaling (MAVS) protein. MAVS functions downstream of

RIG-I, upstream of IkB, and upstream of IRF-3 phosphorylation. This signaling results in activation of both NF-κB and IRF-3 with subsequent upregulation of IFNβ expression (36). The recently described NLR protein, NLRX1, has been shown to inhibit MAVS-mediated activation of IFNβ and Nf-κB (22).

The type 1 interferon response is not only anti-viral but also has been shown to be anti-bacterial since lipopolysaccharide (LPS) and lipotechoic acid (LTA) stimulation of toll like receptor 4 has been shown to increase expression of IFNβ through MyD88 dependant and independent mechanisms (12). The MyD88 independent signaling after LPS/LTA stimulation of TLR 4 occurs through TRIF related adaptor molecule (TRAM) and TIR domain containing adaptor inducing IFNβ (TRIF)(48). The MyD88 dependant pathway is triggered by LPS and signaling via the adaptor molecule TIRAP also known as Mal (48). Both MyD88 dependant and independent pathways signal transductions originate from the intracellular Toll/interleukin-1 receptor (TIR). TIR associates with MyD88 or other adaptor molecules such as TRIF/TRAM or TIRAP to cause the phosphorylation and nuclear translocation of activated IRF-3 resulting in IFNβ. expression LPS/LTA stimulation also leads to the expression of ISGs through the IFNα/β positive feedback loop.

Recently it has been demonstrated by Nusinzon et al. that type 1 interferon gene expression is dependant on histone deacetylases (HDACs), particularly HDAC 1(25). Chromatin modifications and nucleosome arrangement are important in the maintenance of stable gene expression patterns. The concerted efforts of HDACs, acetyl groups and methyl groups control gene expression (10, 39, 40). HDAC inhibitors such as TSA

decreased the formation of the enhancesome at the interferon beta promoter as well as decreased the secretion of type 1 interferons from 2fTGH cells such that cells were not able to protect themselves subsequent Sendai virus challenge (26). HDAC inhibitors decreased the ability of HDAC 1 to induce interferon alpha mediated ISGF3 expression thus the expression of ISGs (25). These data exhibit compelling evidence that HDACs are required to mediate type 1 inteferon expression.

We have previously demonstrated that bacteria capable of secreting short chain fatty acids (SCFA) , mediated reactivation of latent herpesvirus via HDAC inhibition(23, 30). Since SCFA decrease HDAC activity it was important to determine whether bacterial spent media likewise decreasedtype 1 interferon responses to aid viral pathogenesis and reactivation. In this report, we demonstrate that bacterial spent media despite the presence of LPS decreased the antiviral response of interferon alpha and IRF-3 mediated ISGs transcription. Further IFNβ promoter activity was diminished in a dose dependant manner. Finally, we demonstrate that bacterial spent media decreased the innate antiviral response of 2fTGH cells to viral challenge and increased expression of the MAVs inhibitor NLRX1. Collectively, these data demonstrate that by decreasing the innate antiviral response bacteria may potentiate viral reactivation and pathogenesis.

Materials and Methods

Cells, Media, and Treatments. HEK-293 cells, 2fTGH cells, and HeLa Cells were maintained in DMEM media supplemented with 10% FBS, and 5% Pen/Strep. Human salivary gland cell line, HSG, were grown in McCoy's 5A media supplemented with 10% FBS and 5% Pen/Strep. BCBL-1 cells were maintained in RPMI media supplemented

with 10% FBS and 5% Pen/Strep. Oral gram-negative anaerobic bacteria were grown in anaerobic chambers in Wilkins Chalgrons (WC) media. Bacterial spent media was harvested at late log phase to ensure the production of the desired metabolic short chain fatty acids. Bacteria were centrifuged at 5500 rpm for 30 minutes and the supernatant containing metabolic end products were filtered through a .45uM filter to remove any cells. WC media that was used as a negative control was filtered as well. Treatment of HeLa cells with poly(I) · poly(C) (dsRNA; Amersham) was performed by direct addition to the medium at a final concentration of 100 μg/ml. Sodium Butyrate (NaB; Sigma) was added at specified concentrations. Treament of cells with *E. facealis* LTA (Sigma) was achieved at a final concentration of 20ng/mL.

Plasmids and Reporter Assays - The expression plasmids for pIRF-3, pPoly IC, and luciferase plasmid pIFNβLuc were described previously (36). The pISRE-luc construct was purchased from BD Biosciences/Clontech. Transfections were carried in 12 well plates with .8ug of total plasmid, which included a B-galactosidase expression plasmid as a internal control. Transfections were carried using Fugene according to manufacturers protocoal (Promega), and the relative light units were measured in a luminometer.

Quantitative Real time PCR analysis RT-PCR- RNA from treated cells was isolated using Rneasy kit (Qiagen) according to manufacturer. Contaminating DNA was removed by Dnase Treatment (Promega). cDNA was synthesized according to manufacturers instructions using Oligo dT (Invitrogen).cDNA was subjected to Quantitative Real Time

PCR analysis using Roche LightCycler 480 Syber Green 1 Master as detector. Primers are as follows; ISG54T AATGCCATTTCACCTGGAACTTG, ISG54B GTGATAGTAGACCCAGGCATAGT, 2'5'OAST5'AGGCAGAAAGGGATTTTATC'3, 2'5OASB,5'TAACCTAGGTCTCGACTCCA'3.IFNBgF5' GAGCTACAACTTGCTTGGATTCC'3, IFNBgR 5'CAAGCCTCCCATTCAATTGC'3. Real time PCR was conducted in Roche Light Cycler 480. NLRX1 studies were normalized to 18S RNA and were described previously (22).

ChIP Immunoprecipitation Assay. HeLa cells were treated with Poly I Poly C ds RNA (Amersham) as outlined above and cotreated with either NaB at .3mM or 5mM concentrations, with spent media at a 1:2 dilution of bacterial spent media or with media alone (WC media) and incubated for 2hr at 37oC. Following incubation cells were treated with formaldehyde to a final concentration of 1% for 15 minutes at 37oC and then treated with .125 M Glycine. Complexes were immunprecipitated using Pol II antibody (Santa Cruz). ChIP analysis experiments were performed according to chIP protocol by Upstate Cell Signaling.

Viral Infection and ELISA Assays Antiviral responses of 2fTGH cells were measured as a function of cytopathic effect. Cells were cultured in 96-well plates and were infected with Sendai virus that was serially diluted from 300 HA units/mL The virus was serially diluted to 9.37 HA units/mL in the presence of a .3mM and 5mM concentration of

sodium butyrate (NaB) or a 1:10 dilution of bacterial spent media and WC media alone for 2 hours in serum free DMEM. After the 2hour incubation to allow for viral absorption, media was removed and replaced with supplemented DMEM for 22 hours. After the twenty-four hour incubation, supernatants were collected for IFN□ ELISA analysis (US Biologicals). ELISA analysis was done according to manufacturers instructions.

Reverse Phase HPLC

Preparative reverse-phase HPLC was performed on a Varian ProStar 320 chromatography workstation configured with two PS-215 pumps fitted with 50 mL pump heads, Bonus-RP Zorbax Rx Bonus RP column (9.4 mm × 150 mm, 5 μm), PS-320 variable wavelength UV-vis detector, and a PS-701 fraction collector. Mobile phases consisted of mixtures of CH_3CN (0-94%) in water containing formic acid (40 mM) and ammonium formate (10 mM). Flow rates were maintained at 4 mL/min. Pooled fractions were evaporated using a TurboVap LV Nitrogen Evaporator, resuspended in 1 ml RPMI medium containing pen/strep, L-glut, Na pyruvate, BME, FBS and used for induction experiments. Cells were harvested 6 hrs following induction for protein and RNA isolation.

Results

Interferon Stimulated Gene Expression is decreased in the presence of bacterial spent media.

It was previously determined that short chain fatty acid containing bacterial metabolites inhibit HDAC regulated viral promoters (30). In order to determine whether bacterial spent media likewise decreased transcription of HDAC inhibitor responsive interferon stimulated genes (ISGs), 293 cells were treated with interferon alpha in the presence or absence of sodium butyrate (NaB) or bacterial spent media. Quantitative real time PCR analysis of ISGs, ISG54 and 2'5'OAS, revealed that interferon alpha treatment induced high expression levels of both ISGs, however the in the presence of the HDAC inhibitor, sodium butyrate (NaB), induction of both ISG54 and 2'5 OAS dramatically decreased. This decrease was also detected in interferon alpha induced cells that were co-treated with spent media from the bacteria F.nucleatum, and S.aureaus (Figure 1).

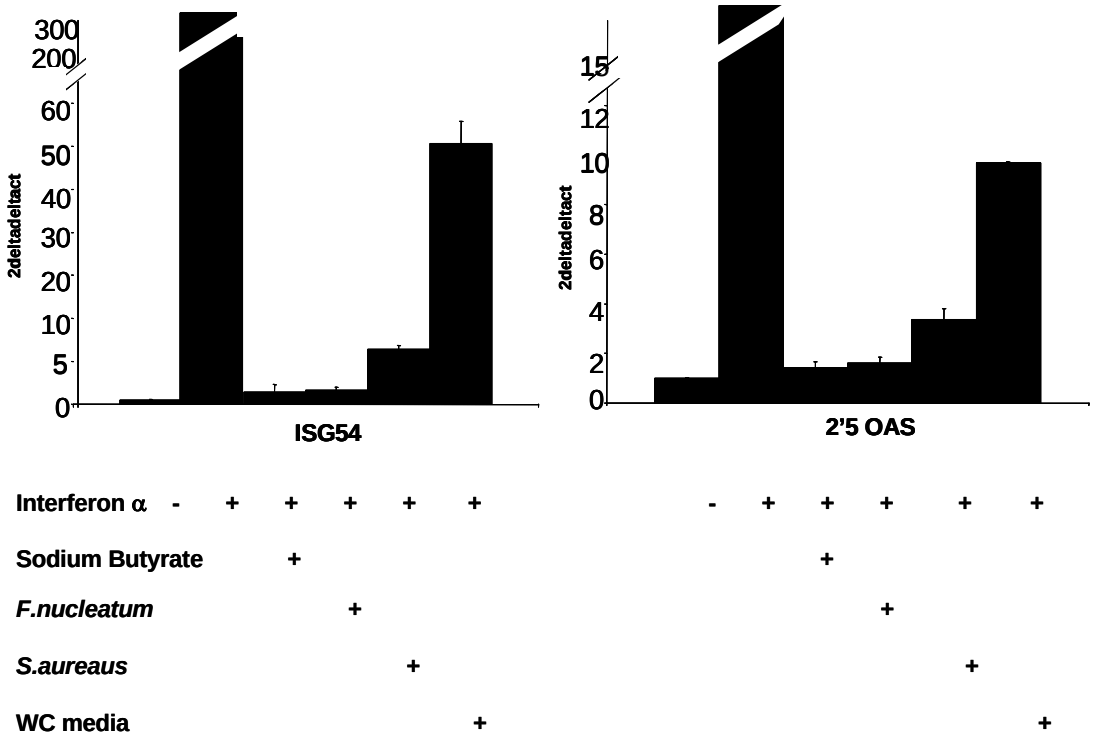


Figure 1 Bacterial spent media decrease interferon alpha mediated transcription of Interferon Stimulated Genes (ISGs) Quantitative Real time PCR of ISGs, ISG54 and 2'5 OAS, was performed on cDNA created from HEK 293 cells treated with interferon alpha alone or cotreated with .3mM sodium butyrate (NaB), 1:10 dilution of *F.nucleatum* spent media, 1:10 dilution of *S.aureaus* spent media or media alone (WC media). Cotreatment of interferon alpha and .3mM sodium butyrate (NaB) and bacterial spent media decreased transcription of ISG54 and 2'5 OAS. Cotreatment with media alone did not decrease the transcription of ISGs to the same level.

In order to determine whether bacterial spent media decreased ISGF3 controlled transcription, an ISRE (interferon stimulated response element) luciferase promoter reporter plasmid was transfected into 293 cells. Treatment of transfected cells with interferon alpha resulted in increased luciferase activity whereas co-treatment with sodium butyrate (NaB) and bacterial spent media decreased ISRE activation. This decrease was dose dependant with the most profound effect detected at a 1:2 dilution (average p value 0.0089) and the least at a 1:50 dilution (average p value 0.0186) compared to media alone (WC) (average p value 0.03) (Figure 2a). IRF-3 mediated activation of an ISRE luciferase reporter was assayed in the presence of bacterial spent media compared to media alone. Spent media and sodium butyrate decreased IRF-3 activation of ISRE luciferase reporter 2 fold (Figure 2b). Taken together these data demonstrate that bacterial spent media decreases interferon alpha activation of ISGF-3 transcription as well as IRF-3 activation of the ISRE promoter.

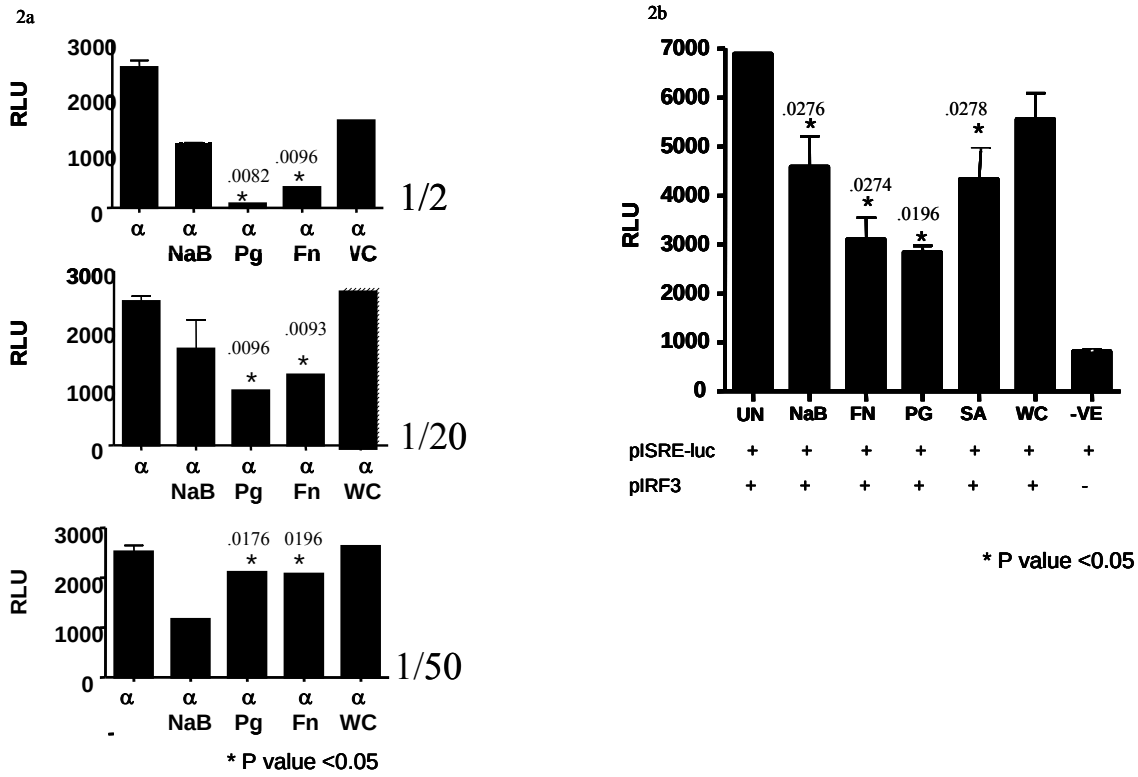
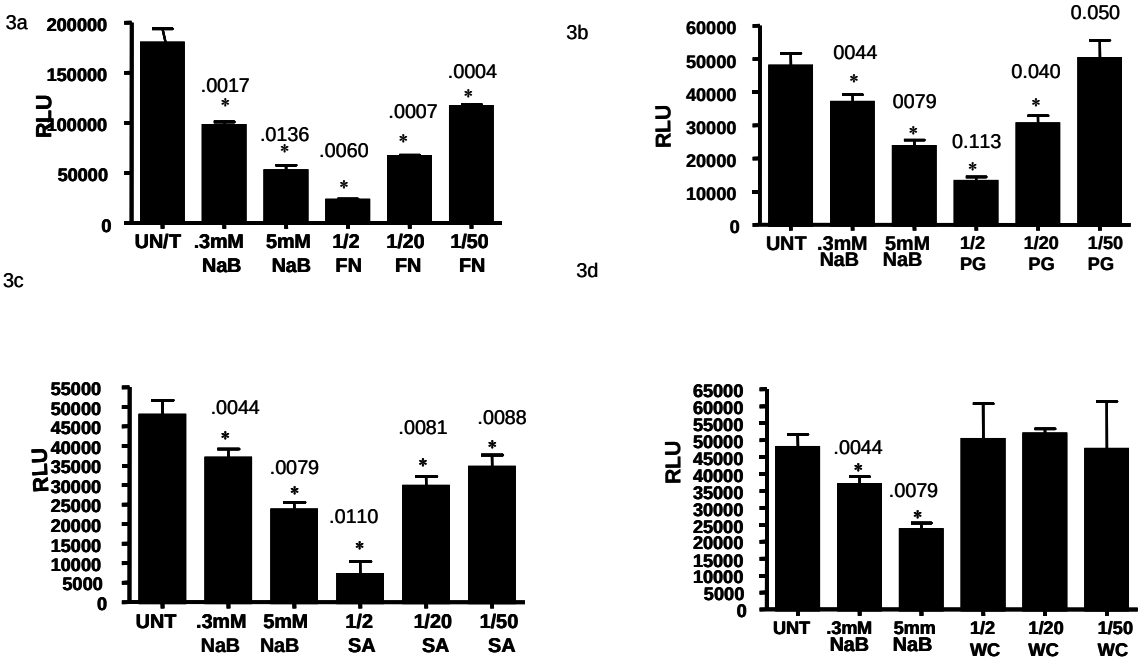


Figure 2 Bacterial spent media dose dependently decreased interferon alpha and IRF-3 activation of ISRE promoter HEK 293 cells transfected with interferon stimulated response element (ISRE) luciferase plasmid, pISRELuc, were co-treated with interferon alpha and spent media at multiple dilutions, 1:2, 1:20, and 1:50, from *F.nucleatum* (FN), *P.gingivalis* (PG) and media alone. Bacterial spent media dose dependently decreased interferon alpha activation of pISREluc. Treatment with WC media alone did not decrease luciferase activity like treatment with bacterial spent media (Figure 2a). The expression plasmid pIRF-3 and pISREluc plasmid were contransfected into HEK 293 cells. Bacterial spent media from *P.gingivalis, F.nucleatum*, and *S.aureus* decreased interferon regulatory factor 3 (IRF-3) mediated activation of the ISRE promoter comparable to .3mM sodium butyrate (NaB). WC media treatment did not

reduce luciferase activity to the same level as treatment with spent media from the oral bacteria (Figure 2b)

Bacterial spent media decreases IFNβ activation.

The antiviral innate immune response is often triggered by host cell detection of double stranded RNA. The observation that bacterial spent media decreased interferon alpha's activation of ISGF3 transcription, led us to determine whether bacterial spent media decreased IFNβ transcription activation following expression of the dsRNA homolog, Poly I:C. HEK 293 cells cotransfected with both IFNβ luciferase promoter reporter plasmid and Poly IC expression plasmid and treated with different dilutions of bacterial spent media and WC media alone. Spent media from *F.nucleatum*, *P.gingivalis*, and *S.aureus* dose dependently decreased Poly IC activation of IFNβ promoter. (Figure 3a, b, c, and d). Transfected 293 cells that were treated with WC media at different doses maintained IFNβ promoter activity.



* P value <0.05

Figure 3- Bacterial spent media dose dependently decreased activation of IFNβ promoter HEK 293 cells cotransfected with luciferase interferon beta promoter, pIFNβLuc, and poly I:C expression plasmid were treated with 1:2, 1:20, and 1:50 dilution of bacterial spent media, media alone control (WC), .3mM sodium butyrate (NaB) or 5mM NaB. Spent media from *F.nucleatum*(3A), *P.gingivalis*(3B) and *S.aureus* (3C) dose dependently decreased IFNβ promoter activation. Control sodium butyrate dose dependently decreased IFNβ promoter activation unlike WC media.

In order to determine whether bacterial spent media decreased the presence of the enhanceosome at the IFNβ promoter, a chromatin immunoprecipitatin (chIP) assay was performed on HeLa cells treated with Poly IC alone or cotreated with bacterial spent media, and WC media alone. In the presence of .3mM and 5mM of the HDAC inhibitor sodium butyrate there was decreased binding/immunoprecipitation of Pol II at the IFNβ promoter. This indicates deterred RNA polymerase loading onto the IFNß promoter subsequent to deacetylase inhibition as shown previously by others using another HDAC inhibitor TSA (26). Similarily, Pol II binding was decreased in the presence of bacterial spent media where as cells that were cotreated with media alone still had appreciable Pol II levels present at the IFNβ promoter (Figure 4). Taken together these data suggest that HDAC inhibitors/short chain fatty acids and other factors present in bacterial spent media decreased enhancesome presence and activity of the IFNβ promoter.

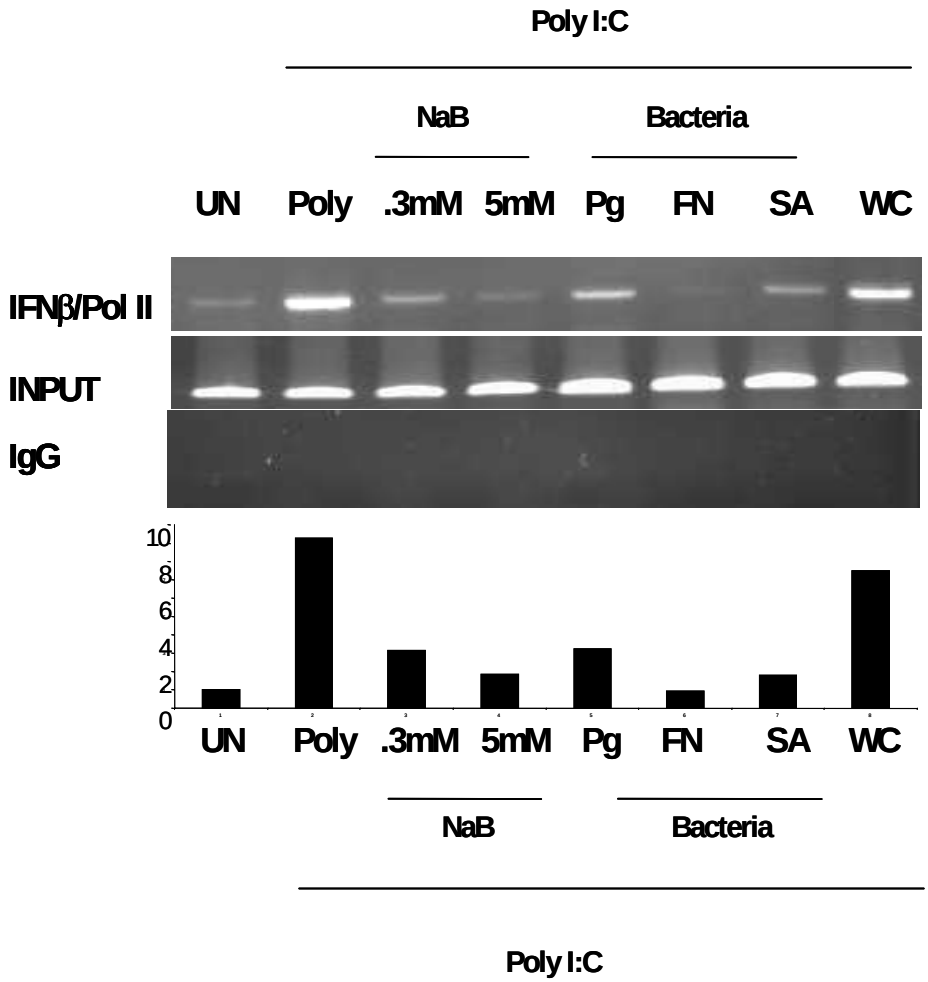


Figure 4- Pol II binding to the IFNβ promoter decreased with Bacterial spent media treatment ChIP analysis was performed on HeLa cell lysates to determine the amount of RNA polymerase II bound to the IFNβ promoter subsequent to treatment with poly I:C alone or cotreatment with .3mM NaB, 5mM NaB, 1:10 dilution of *P.gingivalis* spent media , 1:10 dilution of *F.nucleatum* spent media , 1:10 dilution *S.aureus* spent media or WC media alone. Poly I:C cotreatment with NaB and spent media from *P.gingivalis, F.nucleatum*, and *S.aureus* decreased RNA polymerase II presence at the IFNβ promoter.

WC media did not decrease RNA polymerase II at IFNβ promoter to the same exent as spent media treatments.

Bacterial spent media decreased functional interferon beta transcription and secretion

To determine the effect of bacterial spent media on IFNβ protein secretion, Cytopathic effect assays were performed using Sendai virus that was serially diluted from 300 HA units/mL to 9.37 HA units/mL on 2fTGH cells that were treated subsequent to infection with sodium butyrate, spent media from oral bacteria and media alone. Cells that were treated with WC media, did not have much cytopathic effect whereas infected cells treated with sodium butyrate and bacterial spent media exibited a profound amount of cytopathic effect even in the presence of low amount of virus at 9.37 HA units/mL (Data not shown) Supernatants from 2fTGH cells that were challenged with 9.37 HA units/mL of Sendai virus and treated with .3mM sodium butyrate, 5mM sodium butyrate, and bacterial spent media had less IFNβ secretion than infected cells that were treated with media alone (Figure 5A). Interestingly, both the gram positive pathogens *S.aureaus* and *E faecalis* also consistently decreased IFNβ innate immune responses despite the fact that theses organisms do not secrete short chain fatty acid metabolites like the gram negative bacteria, *F.nucleatum* and *P.gingivalis* (16). Previous work from our lab showed that spent media from S.aureaus does not decrease HDAC activity so the mechanism for decreasing innate immune responses may be mediated by a metabolite distinct from the short chain fatty acids secreted by the anaerobe (30). LTA is a commonly expressed and

secreted by gram-positive bacteria. LTA from the gram positive organism, *E. faecalis* was found to decrease IFNβ secretion in the EBV latently infected cell line, BL41compared to negative controls that did not receive treatment. (Figure 5 B). Taken together these data demonstrate that short chain fatty acids in addition to bacterial components such as LTA decreased interferon beta secretion. Data from this section revealed that not only does the bacterial spent media not only impacts the innate antiviral response at the level of transcription; it also affects IFNβ protein secretion.

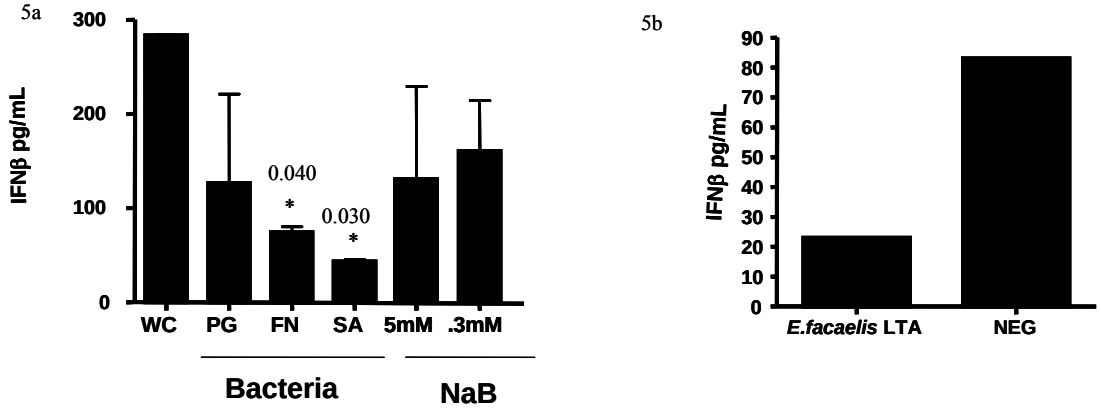


Figure 5 HDAC inhibitors present in bacterial spent media decreased antiviral defenses and IFNβ secretion. 2fTGH cells were infected with Sendai virus at a starting concentration of 300 HA units/mL in the presence of .3mM sodium butyrate, 5mM sodium butyrate, 1:10 dilution of spent media from P.gingivalis, F.nucleatum, or S.aureus, and WC media. Sendai virus was serially diluted to 9.37 HA units/mL. Supernatants from cells infected with 9.37 HA units/mL and treated with sodium butyrate and spent media treatments were subjected to IFNβ ELISA analysis. IFNβ secretion of 2fTGH cells decreased following treatment with spent media from P.gingivalis, F.nucleatum, and S.aureaus unlike cells that were treated with WC media alone (Figure 5a). Interferon beta ELISA analysis was performed on supernatants from Epstein Barr Virus (EBV) latently infected cell line, BL41, that either treated with *E.facaelis* lipoteichoic acid (LTA) or remained untreated. Cells that were treated with E.facaelis LTA had decreased IFNb secretion when compared to cells that were untreated (Figure 5b)

Despite the expression of LPS bacterial spent media decreased the activation of type 1 interferon.

LPS, LTA and HDAC inhibitors are secreted metabolites of oral bacteria. Stimulation of Toll like receptor 2 and 4 with LPS and LTA has been described to increase IFNβ expression in cells (8, 27, 31). The 293 and HeLa cell lines, used for previous experiments, lack toll like receptors 2 and 4. It was important to determine in a TLR containing relevant cell line whether stimulation of toll like receptors by LPS or LTA would modify bacterial spent media induced interferon beta expression. Toll like receptor 2 and 4 bearing human salivary gland cells, HSG, were treated with Poly I:C to activate the antiviral response along with spent media from *P.gingivalis*, *F.nucleatum,* and *S.aureus.* These conditions were compared to HSG cells treated with Poly I:C in addition to the control HDAC inhibitor sodium butyrate at .3mM and 5mM concentrations. Real time PCR analysis revealed that sodium butyrate dose dependently decreased Poly I:C induced transcription of IFNβ. Likewise, Poly I:C treated HSG cells that were co-treated with crude spent media from *F.nucleatum, S.aureus* and *P.gingivalis* spent media also demonstrated decreased IFNβ transcription (Figure 6). These data demonstrate that bacterial spent media of oral bacteria decrease IFNβ transcription even in the presence of the LPS inducer.

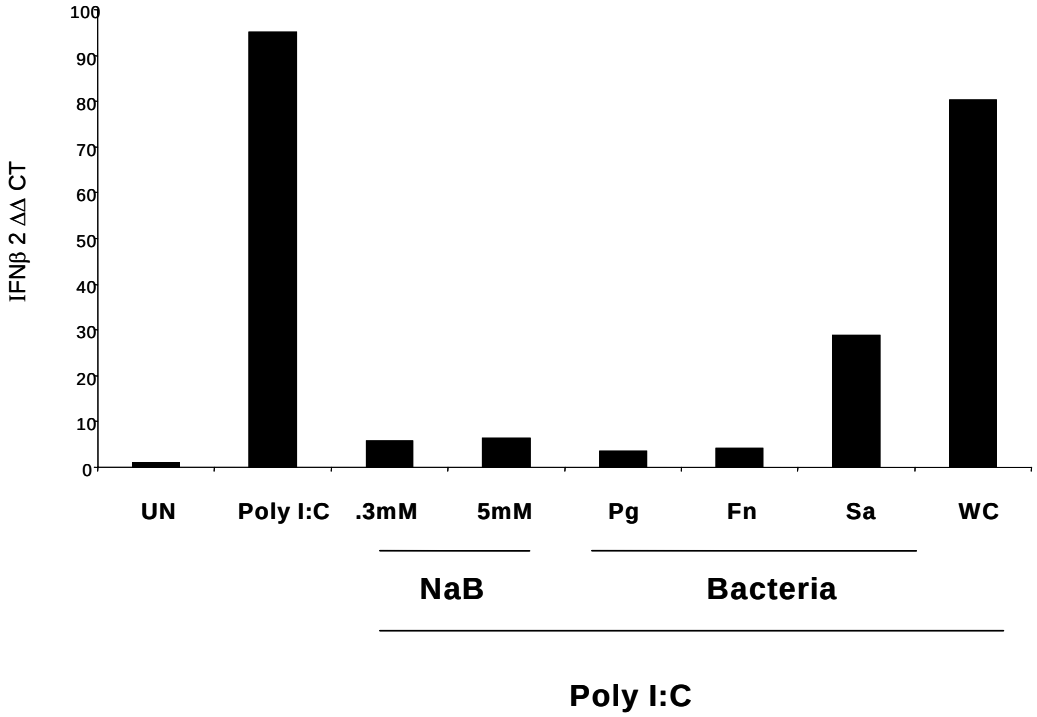


Figure 6: Despite LPS presence bacterial spent media decreased Interferon beta transcription. Quantitative real time PCR analysis was performed on cDNA created from HSG cells that were treated with Poly I:C alone or cotreated with .3mM sodium butyrate, 5mM sodium butyrate, 1:10 dilution of spent media from *P.gingivalis*, *F.nucleatum*, or *S.aureus* or WC media. Cells that were treated with Poly I:C alone exhibited increased transcription of IFNβ whereas the presence of sodium butyrate dose dependently decreased Poly I:C activation of IFNβ transcription. Similarly, spent media from pathogens decreased IFNβ transcription.

Spent media from *F.nucleatum* upregulates the expression of NLRX1.

A potential mechanism by which bacterial spent media diminish the type one interferon response is modulation of the mitochondrial antiviral (MAVS) pathway. NLRX1 has a recently been described as an inhibitor of MAVS activation of innate antiviral responses. Quantitative real time PCR analysis was used to determine the transcription of the NLRX1 gene subsequent to treatment of cells latently infected with Kaposi's sarcoma associated herpesvirus (KSHV), BCBL-1, with fractionated spent media from *F.nucleatum* and WC media. We have previously shown that treatment with *F.nucleatum* spent media results in viral reactivation of KSHV (23, 30). Spent media was fractionated by HPLC to determine which components resulted in reactivation, histone acetylation, and modulation of the innate immune response. It was determined that fractions 1 and 2 of F.nucleatum spent media most efficiently activated the expression of lytic proteins (KSHV vIL-6) and resulted in acetylation of histone H3.(23, 30). Likewise, control WC media was fractionated. Interestingly, treatment with F.nucleatum fractions 1 and 2 increased NLRX1 transcription compared to cells that were treated with fractions 1 and 2 of media alone (Figure 7). These data demonstrate that metabolites in *F.nucleatum* spent media can potentially decrease innate immunity by manipulation of innate antiviral immune defenses in the cytosol.

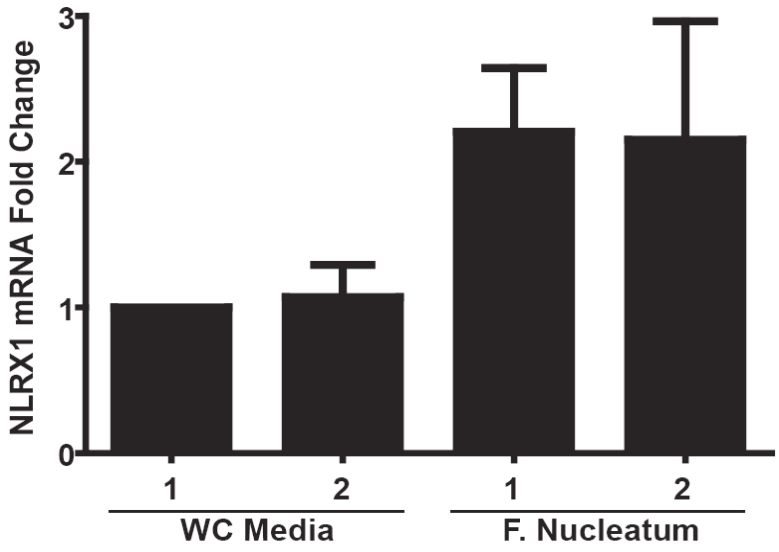


Figure 7: F.nucleatum increases NLRX1 transcription. Transcription of the NLRX1 gene was analyzed by quantitative real-time PCR analysis in KSHV latently infected cell line, BCBL, following treatment with spent media from F.nucleatum or WC media. Cells that were treated with F.nucleatum spent media exhibited increased transcription of NLRX1 gene compared to cells that were treated with media alone.

Discussion

Polymicrobial infections containing herpesviruses and pathogenic bacteria result in severe disease states (6, 20, 37, 38, 44). Recruitment of leukocytes in response to bacterial infections may result in the presence of cells containing latent herpesviruses. We have previously demonstrated that metabolites from periodontal pathogens, *F.nucleatum,* and *P.gingivalis,* aid in herpesvirus reactivation from latency by activation of the p38 pathway, HDAC inhibition, and activation of the PKC pathway(23, 30). Important to this reactivation in vivo would be the disarming, even if only transiently, of the innate immune response.

In this report, we demonstrate that bacterial spent media decreased the type I interferon response. In the setting of polymicrobial infection and viral reactivation this decreased antiviral activity would allow for the successful initiation and progress of viral replication prior to the expression of virally encoded immune inhibitors. We show that bacterial spent media diminish interferon alpha mediated transcription. Activation of interferon stimulated response elements (ISRE) by IRF 3 as well as IFN alpha stimulus was dose dependently decreased in the presence of bacterial spent media. Likewise, interferon beta luciferase promoter activation decreased dose dependently in the presence of bacterial spent media. As had been previously described by Nusinzon et al, in the presence of HDAC inhibitors enhanceosome formation on the Pol II promoter was diminished following treatment with bacterial spent media. Expression of interferon stimulated genes such as 2'5 OAS and ISG54 are critical to establish an antiviral state. Bacterial treatment also resulted in reduction of these genes as well.

The type 1 interferon response is not only anti-viral but also has been shown to be anti-bacterial since lipopolysaccharide (LPS) and LTA stimulation of toll like receptor 4 and 2 has been shown to cause the expression of IFNβ through MyD88 dependant and independent mechanisms (12). Both MyD88 dependant and indedendant pathways signal transductions originate from the intracellular Toll/interleukin-1 receptor TIR). TIR associates with MyD88 or other adaptor molecules such as TRIF/TRAM or TIRAP to ultimately cause the phosphorylation and translocation of activated IRF-3 to the nucleaus to cause the expression of IFNβ. Typically, the expression of IFNβ following stimulation of TLR3 with Poly I:C or TLRs 2 and 4 by *E.coli* LPS occurs pretty rapidly between 2 and 8 hours (8, 26). It was essential to determine if metabolites could decrease type 1 interferons in a relevant cell line, oral epithelial cells expressing TLR 2 and 4 were used (15, 41) Interestingly, at two hours post treatment, the presence of LPS and LTA in the bacterial spent media did not deter metabolite augmented decrease of IFNβ transcription in TLR 2 and 4 containing cells. Secretion of IFNβ in the supernatant decreased subsequent to spent media treatment comparably to known HDAC inhibitors. At 16 hours post treatment, this effect was not detected (data not shown). Spent media from the gram-negative periopathogen, *F.nucleatum*, increased the expression of the MAVS antagonist NLRX1, which would further disable the innate immune response. Data presented in this report demonstrates that bacterial metabolites have the capability to decrease antiviral innate immunity in the cytosol as well as the extracellular space.

Regulation of cellular immunity is essential to herpesviral lifelong persistence in a host. Cellular immune pressures force herpesviruses to establish primarily latent

infections of the host, however, following a reactivation stimulus, herpesvirus subvert host immune responses by encoding genes that regulate cellular immunity. Kaposi's Sarcoma associated herpesvirus (KSHV) encodes a myriad of cellular homologs including the viral IRFs (vIRF-1, vIRF-2, vIRF-3, and vIRF-4). These IRFs have all been shown to have antagonistic roles in type 1 interferon gene expression that range from deregulating type 1 interferon promoter activity to causing dysfunction in interferon mediated antiviral effects (4). Herpes simplex virus UsII gene product is able to deter eIF2a protein kinase PKR and 2'5'OAS activation by sequestering available dsRNA produced during viral infection (32). It has been reported in lymphoblastoid cell lines that are latently infected with Epstein Barr Virus (EBV) that interferon alpha's action on the interferon alpha/beta promoter is not carried out because the EBV gene LMP1 has altered the STAT1 DNA-binding complex (21). HCMV immediate-early 2 (IE2) gene product IE86 can effectively block the induction of IFN-beta during HCMV infection (42).

While most herpesviruses encode gene products that regulate type 1 interferon immune functions, decreasing innate immunity by bacterial metabolites prior to and/or simultaneous with viral reactivation would further aid in viral pathogenesis. The ability of bacterial metabolites to reactivate latent herpesviruses and to decrease innate type 1 interferon responses promotes a favorable situation for a lytic productive infection. Dampening the innate responses facilitate reactivation ultimately boosting expression of virally encoded modulators of innate immune responses. This combination of bacterial mediated activities may explain the increased presence of herpesviruses at the sites of

intense bacterial replication in severe periodontal lesions (34, 38). Kajita et al reported that in the setting of where there is intense bacterial infection while the expression of IFN-alpha1 mRNA was higher in periodontitis lesions compared with gingivitis lesions, the level was quite low (11). Based on data presented in this report it could be that the bacteria themselves are modulating this innate response. Polymicrobial infections occur throughout the body existing in the gut, genital-urinary tract, skin and the oral cavity. This report illustrates the importance of determining underlying host interactions modulated in the polymicrobial setting In this report microbial interference demonstrated provided by the bacteria aids the pathogenesis of herpesviruses in periodontal disease and highlights the importance of treating polymicrobial diseases as whole vs. separate entities. By modulating the innate antiviral response the bacteria sets up a favorable environment for viral reactivation and replication.

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Chapter IV

Bacterial Mediated Reactivation of Sexually Transmitted Viral Infections

Introduction

Polymicrobial interactions within the genital-urinary tract are abundant. Many epidemiological reports have described a correlation between viruses such as HIV and HSV-2 and STD bacterial infections. Pilcher et al consistently detected increased HIV replication concurrent with a sexually transmitted infection (STI). Data by Kaul et al demonstrated that persons with alterations in vaginal flora that are co-infected with viruses such as HSV-2 and HIV along with STD pathogens such as *Trichomonas vaginalis* had increased shedding of HIV and HSV-2 in the genital tract (11). Interestingly, HSV-2 reactivation induces the presence of proinflammatory cytokines and chemokines in the female genital tract, as well as increases nuclear factor κ–B, which have all been demonstrated to enhance infection by STD bacteria such as N. gonorrhoeae (16, 19). Further, women co-infected with HSV-2 and N. gonorrhea in the lower genital tract had increased odds of obtaining acute endometritis (3). Sexual transmission and shedding of other herpesviruses such as KSHV occur in the genital tract and pose a problem in endemic regions (6, 9).Taken together, these data suggest that the polymicrobial infections present in the genitourinary tract harboring both STD pathogens and viruses correlate with increase in viral shedding and worsened STD prognosis.

To date there is no explanation for the cause of increased viral shedding following a sexually transmitted infection (STI) event (4). One report demonstrates that end products from Bacterial vaginosis (BV) infection possess a heat resistant HIV inducing factor (HIF) (5). Similarly results from previous chapters of this report have demonstrated that end-products from oral bacteria stimulate viral reactivation by

activating immediate early and late viral promoters through means of HDAC inhibition and activation of PKC pathway activity (Chapter 2).These same end-products from oral bacteria decreased innate antiviral immune responses (Chapter 3). Based on previous data, it was proposed that metabolites from STD pathogens such as T.vaginalis and N. gonorrhea activate viral replication and decrease innate antiviral immune responses.

Data in this report demonstrates that metabolites from STD pathogens, T.vaginalis and N.gonorrhea posses HDAC inhibition potential. The ability to decrease HDAC activity lends to the ability of metabolites from these pathogens to activate immediate early viral promoters that are activated by HDAC inhibition such as HSV-2 ICPO and KSHV ORF 50. Further, spent media from STD pathogens decrease initiation of innate antiviral responses by decreasing interferon regulatory factor 3 (IRF-3) activation of the interferon stimulated response element (ISRE) promoter. Taken together data from this report highlights a mechanism by which viral replication is enhanced following bacterial STI treatment.

Materials and Methods

Cells HeLa and HEK 293 cells were maintained in DMEM media supplemented with 10% FBS, and 5% Pen/Strep.

Treatments. Trichomonas vaginalis species (3023, 3024, 5018, 5014, 5013, and 5016) were grown in Diamonds media (Difco), while Neisseria Gonorrhea FA1090 was grown in GC broth (Difco). Spent media was harvested at late log phase of bacterial growth and

centrifuged for 30 minutes at 5500 rpm to pellet cells. The resultant supernatant was filtered through a .25 uM filter, and stored at –80^{o}C until use.

HDAC activity assay The HDAC activity assay (Upstate Cell Signaling) is a fluorimetric assay in which spent medias from different gonorrhea species and Trichomonas vaginalis were incubated with HeLa Nuclear extract that was provided with the kit. HDAC activity was measured by fluorimetric analysis following treatment.

Plasmids and Reporter Assays. - pICPO-luc was described previously (12, 13) . The pISRE-luc construct was purchased from BD Biosciences/Clontech PORF50luc promoter construct and pPANluc were made by inserting DNA isolated from BCBL-1 PCR of ORF 50 and inserted into pGL2-Basic vector. Primers for ORF 50 are as follows upper primer 5'CATGGGCGGGTGGGTGACAGTC'3, and lower primer 5'CGATAAGCTTTTGTGGCTGCCTGGACAGTATTC'3. The lower primer contains HindIII site. Primers for Pan are upper primer 5'ATGTGGTACCTAGACGAGTCCGACAATGACAC', which contains a Kpn1 restriction site. Constructs were transfected into HEK 293 cells in 6 well plates with 0.2ug of reporter plasmid and 0.2ug of B-galactosidase expression plasmid using Effectene kit (Qiagen). The relative light units were measured in a luminometer.

Results

Spent media from STD pathogens decrease HDAC activity of HeLa Nuclear Extract

We have previously determined that oral bacteria, *F.nucleatum* and *P.gingivalis*, possess HDAC inhibition activity. In order to determine the HDAC inhibition potential of crude spent media from STD pathogens, a flourimetric based HDAC activity assay was used. HeLa nuclear extract harboring peak transcriptional and HDAC activity was incubated with spent medias from different *Trichomonas vaginalis* species, and *N.gonorrhea*. The amount of HDAC activity following incubation was quantitated by fluorimetric analysis. HeLa nuclear extract incubated with spent media from different species of *T.vaginalis* and *N.gonorrhea* had significantly less HDAC activity than nuclear extract alone and was comparable to the known HDAC inhibitor sodium butyrate (Figure 1). Interestingly, *T.vaginalis* species 5014 and 5016 had the most HDAC inhibition activity out of the panel of *T.vaginalis* organisms. T.vaginalis 5014 was used for subsequent analysis.

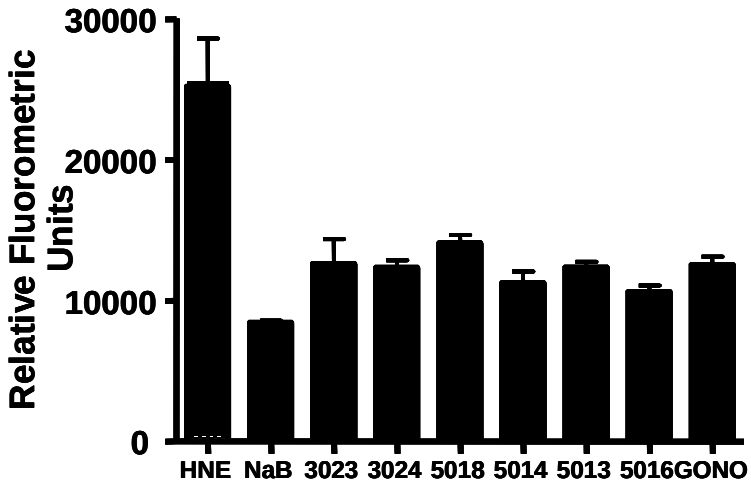

Figure 1. Spent media from STD pathogens T. vaginalis 5014 and N. gonorrhea decrease HDAC. HDAC activity was decreased in HeLa Nuclear Extract (HNE) treated with Sodium Butyrate (NaB), and spent media from *T.vaginalis* and *N. gonorrhea*. Spent media from all STD pathogens Spent media from *T.vaginalis* species 5014 had the most HDAC inhibition activity out of the panel of *T.vaginalis* organisms

Spent media from STD pathogens activated butyrate responsive immediate early viral promoters of herpesviruses

STD spent media activate butyrate responsive herpesviral immediate early promoters

STD spent media were assayed for their ability to activate HDAC responsive immediate early herpesvirus promoters, Kaposi's Sarcoma associated herpesvirus (KSHV) ORF 50 and Herpes Simplex (HSV) ICP0. Spent media from *T.vaginalis* species 5014 and N. gonorrhea was used to treat HEK 293 cells transfected with either KSHV (ORF 50) or HSV-1 (ICP0) immediate early viral promoters (14, 17). Spent media from STD bacteria activated immediate early KSHV promoter (ORF 50) and HSV immediate early ICP0 promoter were activated to about 25% of the level of sodium butyrate (NaB) positive control and were significantly higher than untreated controls (Figures 2a, and 2b respectively).

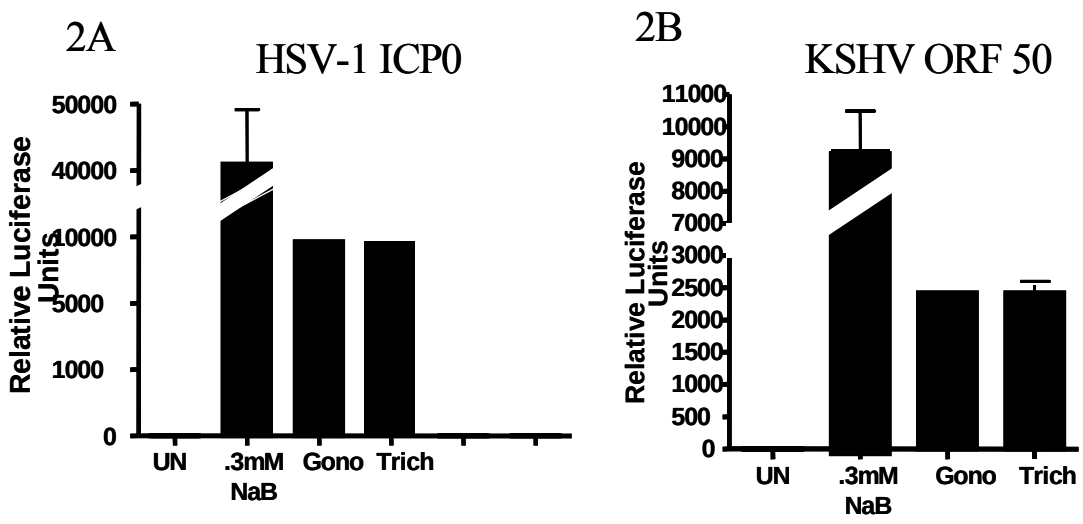

Figure 2. Spent media from STD bacteria activates KSHV and HSV immediate early viral promoters. HeLa cells transfected with Luciferase reporter constructs expressing KSHV (ORF 50) (A) and HSV (ICP0) (B) immediate early viral promoters, were treated with Sodium Butyrate (NaB) and spent media from *T.vaginalis* and *N. gonorrhea* 5014. Control HDAC inhibitor NaB and spent media from STD pathogens *T.vaginalis* and *N. gonorrhea* activated immediate early viral promoters.

Spent media from STD pathogens decreased Interferon regulatory factor 3 (IRF-3) mediated Interferon stimulated response element (ISRE) activation. Modulation of host immunity is a key factor for successful pathogenesis for bacterial and viral members of a polymicrobial community. HDAC activity is important to innate type 1 interferon antiviral responses. Nuiszon et al. determined that transcription that initiates at interferon stimulated response element (ISRE) containing promoters was decreased by HDAC inhibitors. As spent media from STD pathogens was capable of decreasing HDAC activity (Figure 1), its effect on interferon regulatory factor 3 (IRF-3) mediated activation of ISRE was determined. HEK 293 cells cotransfected with ISRE luciferase reporter plasmid and IRF-3 expression plasmid were incubated with spent media from STD pathogens, *T. vaginalis* 5014 and *N. gonorrhea*. In cells that were incubated with spent media from *T.vaginalis* 5014, N. gonorrhea and sodium butyrate a decrease ISRE promoter activation was consistently detected, while transfected cells that were incubated with media alone resembled untreated transfected cells (Figure 3).

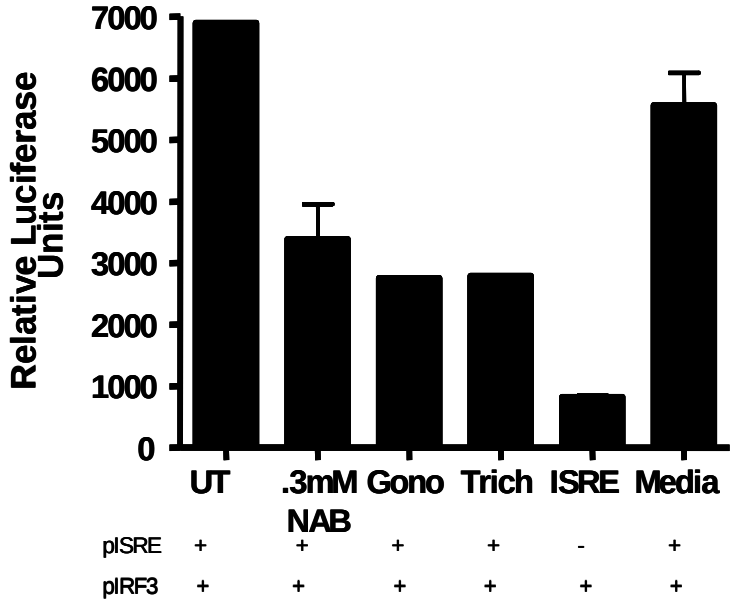

Figure 3. Spent media from STD pathogens decrease interferon regulatory factor 3 (IRF-3) activation of interferon stimulated response element (ISRE). HeLa cells transfected with ISRE luciferase reporter constructs and IRF-3 expression plasmids were treated with sodium butyrate (NaB) and spent media from STD pathogens, T.vaginalis 5014 and N. gonorrhea. Spent media from both STD pathogens decreased IRF-3 activation of ISRE comparable to sodium butyrate.

Discussion

In polymicrobial interactions, pathogen mediated epigenetic influences are important to both viral reactivation and pathogenesis (20, 21). During viral latency immediate early viral promoters are heavily methylated which constricts DNA to the histone core (1, 2, 15, 22). Inhibition of deacetylation of histones on immediate early viral promoters induces hyperacetylation of histone tails allowing DNA to be more accessible for transcription factors to bind (7, 10). In this report, we show that spent media from different species of *T.vaginalis* and *N.gonorrhea* strain FA1090 decreased HDAC activity (Figure1). This result was surprising since these STD pathogens have distinct metabolic differences from the oral bacteria mentioned in chapters 2 and 3 of this dissertation. Despite metabolic differences from oral bacteria, it appears that spent media from both Trichomonas vaginalis 5014 and Neisseria gonorrhoeae FA1090 activates two distinct herpesviral immediate early promoters. This ability could explain epidemiological reports, which have noticed increased HSV viral shedding with a concomitant bacterial STI.

Ornithine decarboxylase (ODC), the lead enzyme in polyamine biosynthesis, has been purified from Trichomonas vaginalis. ODC from T. vaginalis had a broad substrate specificity, decarboxylating ornithine (100%), lysine (1.0%) and arginine (0.1%)(24). Ornithine decarboxylase activity is common to all Trichomonad species but T.vaginalis has the most activity. This enzyme is important to the parasite since the arginine dihydrolase pathway generates ATP as well as putrescine from arginine. Trichomonas vaginalis secretes putrescine which is readily detected in vaginal secretions(23).

The ability to decrease HDAC activity extends to the modulation of the innate antiviral response. It has recently been shown that HDACs are important in interferon stimulated gene expression (18). Stimulation with interferon alpha on the interferon α/β promoters leads to the establishment of an antiviral state through the expression IFÑα/β-stimulated genes (ISGs). Activated interferon stimulated respone elements (ISRE) present on the promoters of IFN-α/β-stimulated genes (ISG) increase the transcription rates of ISGs. ISGs such as 2'5 OAS and PKR promote the antiviral state by preventing phosphorylation of cellular eIF2a thus halting translation in virus infected cells. Both T. vaginalis and N. gonorrhea decreased IRF-3 mediated activation of the type 1 interferon response. The ability of bacterial metabolites to reactivate latent viruses and to decrease innate type 1interferon responses promotes a favorable situation for a lytic productive infection. Dampening the innate responses facilitate reactivation ultimately boosting expression of virally encoded modulators of innate immune responses. This combination of bacterial mediated activities may explain the increased replication of sexually transmitted virus at the sites of intense bacterial replication within the genital-urinary tract (5).

Previous reports have demonstrated a correlation between virus and bacteria associated with STDs and worsened disease prognosis. Even though increases in viral shedding have been noted following STI events, a rationale for enhanced viral activation has not previously been described (8). This is the first report that describes a mechanism by which STD bacteria may set up a favorable environment for viral replication and pathogenesis in the genitourinary tract.

Further investigation into the metabolites of T.vaginalis species 5014 and N.gonorrhoeae FA1090 is needed to determine which components of the crude spent media from these organisms is responsible for the epigenetic changes that activate immediate early promoters and decrease IRF-3 activation of the interferon stimulated response element. It was interesting to see that STD pathogens that secrete distinct metabolites from oral bacteria *P.gingivalis* and *F.nucleatum* facilitate viral reactivation and decrease type 1interferon immune responses. These results demonstrate that secreted metabolites from pathogens could modulate microenvironments in a polymicrobial infection thus enhancing viral pathogenesis**.**

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Chapter V

Discussion

In the setting of polymicrobial infection, viral and bacterial contributions are additive ultimately worsening disease prognosis (6, 12, 16, 26, 29). These organisms set up favorable environment for others to propogate. This microbial interference results in increased disease severity at infection sites (3). Results from studies presented in this report demonstrate that bacterial metabolites from STD and oral pathogens enhance viral reactivation as well as decrease innate antiviral type 1 interferon immune responses. These data are important since they provide a mechanism by which herpesviruses are activated in the periodontal pocket and could explain the correlation of increased herpesvirus reactivation in the presence of abundant oral bacteria as a result of severe periodontal disease. Viral activation by bacterial metabolites is applicable to STD infections where many epidemiological studies have shown that there is correlation between an increase in HIV and HSV-2 shedding in the genital-urinary tract following a bacterial sexually transmitted infection (STI) event (5-7, 12, 16).

Components of bacterial metabolites facilitate viral reactivation by activating essential signaling pathways associated with viral reactivation. The key bacterial metabolite components that were tested in studies in this report were short chain fatty acids (SCFA) and bacterial cell membrane components such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA). Short chain fatty acids such as lactic acid, propionic acid, and butyric acid are metabolic endproducts of bacterial metabolism, which can

accumulate in the gingival crevice. Previous studies demonstrated that severely diseased subjects had a 10-fold increase in the mM concentration of SCFA acids when compared with mildly diseased subjects. Patients with severe periodontal disease secreted 9.5 +/- 1.8 mM concentration of SCFA compared to the mildly disease subjects who secreted SCFA at 0.8 +/- 0.3 mM concentrations (22). The concentration of the positive control HDAC inhibitor, sodium butyrate, was 0.3mM to ensure that experiments would reflect viral reactivation studies in the mouths of healthy or mildly diseased patients since most of the population has some form of gingivitis.

SCFA and bacterial components affect signaling pathways and chromatin structures to facilitate a productive viral infection. LPS and LTA stimulation of toll like receptors 4 and 2 respectively causes the activation by receptor-mediated phosphorylation or dephosphorylation of proteins such as protein tyrosine kinases (PTKs)/Ras/IκB kinase/Raf-1/MEKK/MEK/MAPK, which leads to the translocation of AP-1 and NF-κB to the nucleus and subsequent modulation of gene expression (8, 9, 30, 31, 36). AP-1 interaction with immediate early promoter binding sites is a key initiating factor for activating the temporal ordered cascade of viral gene expression during reactivation (21). Results from chapter 2; Figure 3b showed that oral bacteria that secrete LPS and LTA increased kinase activity of KSHV latently infected cell line, BCBL-1 compared to untreated cells.

Distinct bacterial pathogens reactivate EBV, KSHV, and HSV by two distinct mechanisms. A previous study examined reactivation of KSHV and EBV in the dually infected BC-1 cell line. This study demonstrated that EBV reactivation was more robust

following TPA treatment rather than treatment with HDAC inhibitors, which favored KSHV reactivation (21). Similarly, results from studies presented in chapter 2 demonstrate that various herpesviruses are reactivated uniquely by bacterial inducers. Epstein Barr virus (EBV) reactivation by bacterial metabolites occurs through TLR stimulation by LPS vs. HDAC inhibition by SCFA present in the spent media (Chapter 2; Figure 7b). Spent media without SCFA taken from LPS and LTA secreting bacteria, *S.aureaus* and *P.intermedia,* had more of a positive affect on EBV reactivation than on KSHV, since these spent media were unable to reactivate latent KSHV or activate HSV-1 ICP0 promoter (Chapter 2; Figures 4a and 4b). Alternatively *S.aureaus* and *P.intermedia* exhibited increased AP-1 complexes at BRLF1 promoter, which is favorable for EBV reactivation (Chapter 2; Figure 6b).

The salt form of SCFA butyric acid, butyrate, is a commonly used HDAC inhibitor, which facilitates reactivation by decreasing the presence of repressive epigenetic structures on the histones of immediate early promoters (11). Figure 3a of chapter two demonstrated that spent media from oral gram-negative bacteria have HDAC inhibition activity compared to media alone. Morris et al demonstrated that HDAC inhibition by metabolites from gram-negative oral bacteria such *P.gingivalis* and *F.nucleatum* facilitated KSHV reactivation by activating the P38 pathway, which is downstream of HDAC inhibition by co-treating BCBL-1 cells with P38 inhibitor, SB 202190 (4-(4 Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole. Data presented in Chapter 2 Figures 4a and 5a of this report showed that spent media harboring HDAC inhibition activity activated luciferase activity of transfected immediate

early promoters of KSHV (ORF 50) and HSV-1 (ICP0) and caused hyperacetylation of histone 3 at both immediate early promoters (Chapter 2; Figures 4c and 5b respectively).

The mechanism of viral activation by bacterial metabolites explains epidemiological STD data. Initial studies conducted by Kaul et al demonstrated that persons with alterations in vaginal flora that are co-infected with viruses such as HSV-2 and HIV along with STD pathogens such as *Trichomonas vaginalis* had increased shedding of HIV and HSV-2 in the genital tract (16). A previous studies demonstrated that there was a significant increase in the amount of short chain fatty acids like propionic acid and iso-valeric acid found in the culture fluids obtained at 72 or 120 hours after inoculation in Cysteine-Peptone-Liver infusion-Maltose medium (CPLM medium) of *Trichomonas vaginalis*. These short-chain fatty acids are considered to be produced in vitro and like butyric acid produced by oral gram negative anaerobes have a promoter-enhancing effect which could facilitate viral activation(15). Data from Chapter 4; Figure 1 demonstrated that spent media from *T. vaginalis* species and *N. gonorrhea* possessed HDAC inhibition activity which is a common mechanism utilized by SCFA to reactivate latent virus.

In retrospect, studies have demonstrated that when STD bacterial infections are treated, the shedding of virus in the genital urinary tract decreases. Studies by McClelland et al and Ghys et al demonstrated that treatment of infections caused by infection with STD pathogens such as gonorrhea, chlamydia, and trichomoniasis, decreased cervical shedding of HIV-1 (12, 18, 37). These data suggest that there is some sort of a viral activation factor that is enhanced by the presence of the bacteria. Similarly,

data from Chapter 4 Figure 2 demonstrates that metabolites of STD bacteria activated immediate early promoters that are responsive to HDAC inhibition such as ICP0 of HSV-1 and ORF 50 of KSHV. Taken together, data from chapters 2 and 4 of this report demonstrate that metabolites and components of oral and STD bacteria utilize HDAC inhibiton and activate the PKC pathway to facilitate viral reactivation. *In vivo* reactivation is thought to be spontaneous or occur following stressful stimuli or UV radiation (1, 13, 14, 20, 32, 33). These data provide a novel in vivo mechanism by which herpesviruses are activated and provides an explanation to the increased presence of herpesviruses in periodontal lesions.

The ability of oral and STD pathogens to secrete metabolites that decrease HDAC inhibition activity lends to the ability of these pathogens to dampen innate antiviral responses within endosomal compartments as well as antiviral activity in the cytosol. The ability of metabolites to dampen innate antiviral immune responses benefits herpesviruses prior to reactivation. Recent reports have demonstrated that HDACs are essential for the production of type 1 interferons as well as the establishment of an antiviral state by using HDAC inhibitors such as TSA (23, 24). In this report it was shown that metabolites present in the spent media from oral bacteria and STD bacteria decreased HDAC activity (Chapter 2; Figure 3a and Chapter 4; Figure 1 respectively). Oral bacteria that secrete butyric acid, *P.gingivalis* and *F.nucleatum*, were capable of decreasing IRF-3 and interferon alpha stimulation of interferon stimulated response elements (ISRE) dose dependently (Chapter 3; Figure 2a). Spent media from STD pathogens, *T.vaginalis* and *N.gonorrhea*, decreased IRF-3 activation of the ISRE

promoter (Chapter 4; Figure 3). The ability of both STD and oral pathogens to decrease the activation of the ISRE promoter demonstrates that metabolites of these bacteria have the ability to decrease the establishment an antiviral state of an infected cell, since the ISRE promoter elements are located in interferon stimulated genes and are activated by ISGF3 subsequent to interferon alpha stimulation of the interferon alpha/beta receptor. To further demonstrate that oral bacteria decrease interferon alpha's ability to activate the interferon alpha/beta receptor to initiate the antiviral state, it was shown that metabolites from oral bacteria, *F.nucleatum* and *S.aureaus*, decreased the transcription of integral interferon stimulated genes (ISGs) such as 2'5 OAS and ISG54, which are both important in inducing apoptosis of virus infected cells and deterring translation of viral proteins respectively (Chapter 3; Figure 1).

In addition to dampening autocrine antiviral response, spent media from oral bacteria decreased poly I:C activation of the interferon beta promoter dose dependently and enhanceosome formation at the IFNβ promoter compared to WC media alone (Chapter 3; Figures 3a-e). The secretion of the IFNβ cytokine decreased following treatment with spent media from oral bacteria comprable to cells treated with control HDAC inhibitor NaB at a .3mM and 5mM concentration (Chapter 3; Figure 5). Interestingly S.aureaus metabolites consistently decreased innate antiviral immune responses despite not having HDAC inhibition activity (Chapter 2; Figure 3a). The ability of S.aureaus spent media to decrease innate antiviral responses was not due to SCFA, but rather to the presence of LTA in the spent media (Chapter 3; Figure 5).

Metabolites of oral bacteria further decrease innate antiviral immune responses by increasing the expression of NLRX1. NLRX1 decreases cytosolic mediated immunity of mitochondrial antiviral signaling protein (MAVS)(28). F.nucleatum treatment of BCBL-1 cells increased NLRX1 transcription thus decreased MAVS expression. Data from Chapter 3 suggests that metabolites and bacterial components present in the spent media of oral bacteria are potent modulators of type 1 interferon immune responses at the extracellular level as well as within the cytosolic compartment.

Herpesviruses are skilled at subverting the host immune responses by encoding genes that regulate cellular immunity. For example, Kaposi's Sarcoma associated herpesvirus (KSHV) encodes a myriad of cellular homologs including the viral IRFs (vIRF-1, vIRF-2, vIRF-3, and vIRF-4). These IRFs have all been shown to have antagonistic roles in type 1 interferon gene expression that range from deregulating type 1 interferon promoter activity to causing dysfunction in interferon mediated antiviral effects (4). Herpes simplex virus UsII gene product is able to deter eIF2a protein kinase PKR and 2'5'OAS activation by sequestering available dsRNA produced during viral infection(27). It has been reported in lymphoblastoid cell lines that are latently infected with Epstein Barr Virus (EBV) that interferon alpha's action on the interferon alpha/beta promoter is not carried out because the EBV gene LMP1 has altered the STAT1 DNA-binding complex (19). HCMV immediate-early 2 (IE2) gene product IE86 can effectively block the induction of IFN-beta during HCMV infection (34). In addition to modulating cellular immune responses to augment bacterial pathogenesis, a recent report has demonstrated that the presence of HCMV

in periodontal lesions increases adherence of periodontal pathogen *A. actinomycetemcomitans* to cells (35).

As a whole results presented in chapters 2, 3, and 4 demonstrate that metabolites from oral and STD pathogens activate viral replication by means of HDAC inhibition and increasing PKC activity. Utilizing these means of viral reactivation, bacterial metabolites are capable of recruiting necessary players to the sites of immediate early promoters to initiate viral reactivation and successfully activate early and late viral genes. Components of spent media from gram-negative and gram-positive bacteria decrease type 1 innate antiviral immune responses subsequent to IRF-3 phosphorylation and activation of IFNβ and interferon alpha's activation of antiviral state of infected host cell. Further spent media decrease innate immune factors in the cytosol by increasing NLRX1 expression. Dampening of the innate antiviral immune response prior to reactivation augments viral pathogenesis. Once viral genes are expressed the virus is capable of further decreasing immune responses in the setting of a polymicrobial infection, which could aid bacterial replication and pathogenesis (Figure 1; model).

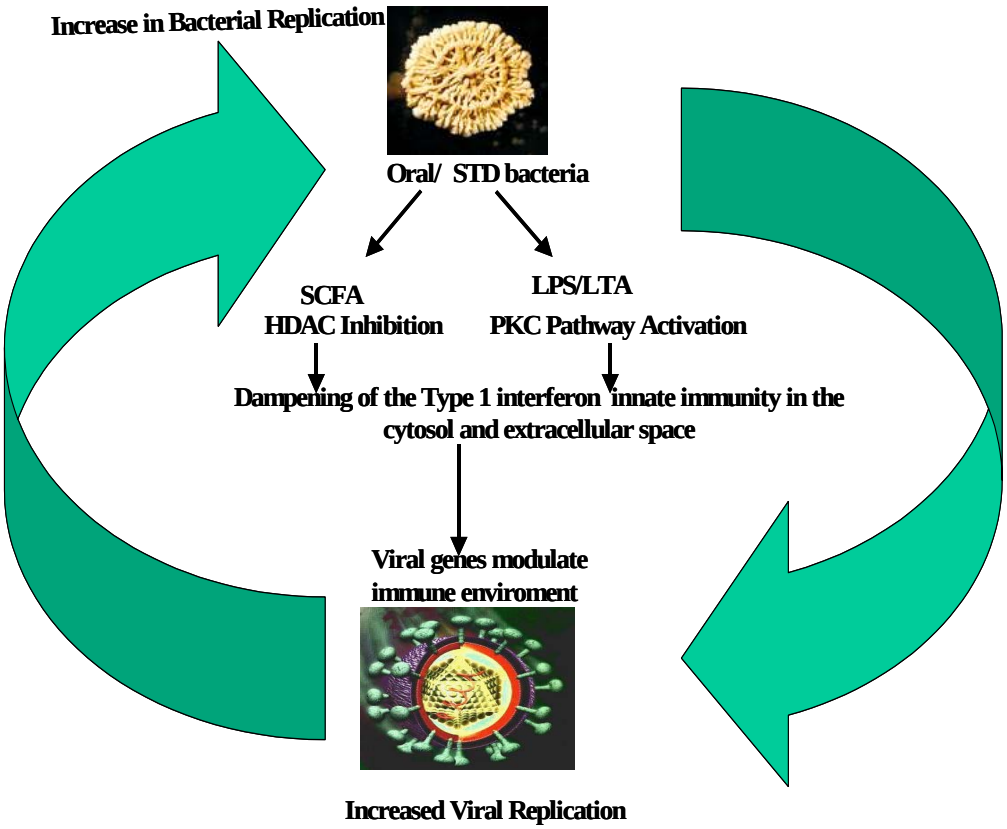

Figure 1Model of Bacterial mediated viral reactivation and pathogenesis Bacterial metabolites from oral and STD bacteria have the capability to increase viral reactivation by means of HDAC inhibition and activation of the PKC pathway. In turn the presence of these factors in the polymicrobial setting of periodontal disease and STDs decrease innate immune responses in the extracellular space and the cytosol, thus facilitating viral pathogenesis prior to reactivation. Following reactivation, viral genes that subvert host immunity are expressed which could enhance bacterial replication and pathogenesis.

These data highlight the importance of considering the dynamic environment of a polymicrobial infection, when treating oral diseases and STD infections. Oral issues of bacterial and viral origin that commonly affect immune compromised patients with AIDS include oral hairy leukoplakia, oral Kaposi's sarcoma, orolabial herpes simplex infection (HSV), oral herpes zoster infection (VZV), intraoral or perioral warts (HPV), and HIV-associated periodontal diseases. Although HAART has provided some anecdotal property to the problem of oral HIV associated disease, there are some individuals who fail treatment due to low CD4 counts and high viral titers. Evidence of failure of HAART therapy is noted by the presence of oral diseases such as oral candidosis (10). Immune reconstitution inflammatory syndrome (IRIS) represents another detriment of HAART therapy, which affects a small percentage of patients within a few weeks or months of initiating therapy and is caused by the inflammatory response to specific opportunistic pathogens. Based on data presented in this report, successful treatment for viral based oral HIV malignancies needs to be aimed at reducing the presence of periodontopathic gram-negative and gram-positive bacteria that secrete short chain fatty acids. If anti-microbials are used to reduce the presence of the bacteria then there should be a reduction in secretion of SCFA and bacterial components, which have been shown to increase viral dissemination and decrease innate antiviral immune responses.

Increased viral shedding in the genital-urinary tract has been linked to the presence of STD bacteria (7, 12, 18). Data from Chapter 4 of this report demonstrates that the cause for the increase in viral shedding following an STI is due to the presence of bacterial metabolites. Controlling viral dissemination in the genital urinary tract

should not only be aimed at treating the viral infection but also to treating the presence of bacteria. Like oral bacteria, STD pathogens secrete components that favor viral replication (15). Acyclovir treatment is the most successful therapy for decreasing symptomatic HSV-2 shedding in the genital urinary tract. It has been exhibited that prophylactic doses of Acyclovir therapy does not decrease the rate of asymptomatic shedding of HSV-2 (2, 25). Asymptomatic shedding of HSV-2 is problematic because it increases the rate of sexual transmission between partners and vertical transmission to neonates (17). The cause of asymptomatic for HSV-2 may be bacterial metabolites present in the genital urinary tract since studies have shown that high colonization of bacteria correlated with an increase in HSV-2 shedding (5).

Microcommunities housing viruses and bacteria within polymicrobial infection, facilitate and aid the pathogenesis of each member. Following the onset of periodontal disease or an STI there is an increased presence of bacterial replication, resulting in the production of an abundance of metabolites and components that augment herpesviral reactivation. Subsequent HDAC inhibition and activation of the PKC pathway result in herpesviral reactivation, and expression of viral genes that modulate cellular immunity. This not only facilitates viral pathogenesis but bacterial pathogenesis as well by modulating host immune responses. This positive feedback loop leads to increased bacterial replication and heightened hepesvirus reactivation. This may provide a disasterous scenario for immune suppressed persons. Polymicrobial environments present in the human body should not be ignoredsince these pathogens persist in communities in vivo and not in isolation. This body of

work demonstrates that bacteria impact viral reactivation and pathogenesis thus promoting a favorable environment for bacterial pathogenesis as well. When thinking about infections in vivo one must consider the entire polymicrobial infection as a whole its potential influence that these pathogens may have on each other and on their host.

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