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Influence of Epstein-Barr Virus on Systemic Lupus Erythematosus Disease Development

and the Role of Depression on Disease Progression

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A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

Influence of Epstein-Barr Virus on Systemic Lupus Erythematosus Disease Development and the Role of Depression on Disease Progression

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Doctor of Philosophy

Systemic Lupus Erythematosus (SLE) is an autoimmune disease affecting 20 to 250 individuals per 100,000 worldwide. Symptomology includes dermatological manifestations such as discoid lesions, acute cutaneous rashes, and oral and nasal ulcers, along with musculoskeletal, pulmonary, and renal complications. Abnormal T and B lymphocyte function and apoptosis, immune complex clearance, complement function, and nucleosome processing are typical of disease pathophysiology. SLE is the result of both environmental and genetic factors, which together create the conditions leading to disease onset and progression. Of these environmental factors, Epstein-Barr virus (EBV) infection is known to cause the genesis of cross-reactive antibodies in SLE prone individuals that can initiate disease activity. Viral infection and modulation of cellular genes is important in understanding the microenvironment that could lead to immune mis-regulation and the inception of lupus in those individuals at risk. During disease development, a variety of variables assist and detract from disease progression and the quality of life experienced by SLE patients. Research into EBV-infected naïve B lymphocytes revealed that EBV modulates the chemotactic receptor EBI2 during viral infection via the BRRF1 viral gene product Na. This likely changes B lymphocyte chemotaxis in secondary tissue in virally infected B cells. Current literature suggests this results in sequestration of cells to peripheral areas of the tissue and mis-regulation of the immune response.

It is not uncommon for SLE patients to have neuropsychiatric disorders due to lupus disease activity. With SLE patients being up to 6 times more at risk for depression, recognition and treatment of depression and anxiety have been shown to improve quality of life, pain, and treatment outcomes. Two studies investigate both clinical laboratory and psychosocial assessment variables that we suspect to be correlated with depression in patients with SLE. Univariate and multivariate analysis from our first study identified an array of variables that show strong associations with depression, including: Body Mass Index, Pain, Total Complement, fatigue assessments, and SF-36 scores. The second study found similar associations, but further found that serum IL-10 levels demonstrated a strong correlation with depression in SLE patients. In this final study SLE patients are compared alongside healthy, clinically depressed, and rheumatoid arthritis patients to provide evidence that increased depression in SLE patients is due more to disease pathology than a result of chronic inflammation.

Keywords: Lupus, Systemic Lupus Erythematosus, chemotaxis, SLE, BRRF1, EBI2, Epstein-Barr Virus, EBV, depression

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Abbreviations

7a,25-OHC: 7α,25-dihydroxycholesterol

Ab: Antibody

ACPA: Anti-citrullinated protein antibody

ACR: American College of Rheumatology

Ag: Antigen

AICc: Corrected Akaike Information Criterion

ANG: Angiogenin

APC: Antigen presenting cell

APRIL: A proliferation inducing ligand

A-RNP: A-ribonuclear protein

BCR: B cell receptor

BDI: Beck Depression Inventory

BLyS: B lymphocyte stimulator

BMI: Body mass index

CC2: C-protein fragment 2

CCL: Chemokine (C-C motif) ligand

CCR: C-C chemokine receptor

CFS: Chalder Fatigue Scale

CLIP: Class II-associated invariant chain peptide

CNS: Central nervous system

CRP: C-reactive protein

CSI: Couples Satisfaction Index

CXCL: Chemokine (C-X-C motif) ligand

CXCR: C-X-C chemokine receptor

DCM: Dilated cardiomyopathy

DNMT1: DNA methyltransferase 1

EAC: Experimental autoimmune carditis

EAE: Experimental autoimmune encephalomyelitis

EBI2: Epstein-Barr virus Induced gene 2

EBV: Epstein-Barr virus

FDA: US Food and Drug Administration

FSS: Fatigue Severity Scale

GADA: glutamic acid decarboxylase antibody

GAG: Glycosaminoglycan

GC: Germinal center

gG: Glycoprotein G

GHC1: Guanosine triphosphate cyclohydrolase-1

GILT: Gamma-interferon-inducible lysosomal thiol reductase

GMP: Granulocyte macrophage progenitor

GPCR: G protein-coupled receptor

GPCR: G-protein-coupled receptor

GTP: Guanosine triphosphate

HADS: Hospital anxiety and depression scale

HCAEC: Human coronary artery endothelial cell

hCMV: Human Cytomegalovirus

HHV: Human herpesvirus

HLA: Human leukocyte antigen

HN: Heymann Nephritis

HSPC: Hematopoietic stem/progenitor cell

HSV-1: Human Herpesvirus-1

HSV-2: Human Herpesvirus-2

IA-2: Islet cell antibody 2

IAA: Insulin auto-antibody

IBM: International Business Machines Corporation

ICA: Islet cell antibody

IDO: Indoleamine 2,3-dioxygenase

IL: Interleukin

INF: Interferon

IRF: Interferon regulatory factor

ISG: Interferon stimulated genes

KSHV: Karposi's Sarcoma-Associated virus

LANA: Latency-associated nuclear antigen

LCL: Lymphoblastoid cell line

MBP: Myelin basic protein

MHC: Major Histocompatibility complex

miR or miRNA: Micro RNA

MOG: Myelin oligodendrocyte glycoprotein

MS: Multiple Sclerosis

NK: Natural killer

NOD: Non-obese diabetic

NPSLE: Neuropsychiatric Systemic Lupus Erythematosus

ORF: Open reading frame

PBMC: Peripheral blood mononuclear cell

PLP: Proteolipid protein

PSQI: Pittsburg Sleep Quality Index

PTLD: Post transplant lymphoproliferative disorder

RA: Rheumatoid Arthritis

RAS: Relationship Assessment Scale

R-KO EBV: BRLF1/BRRF1 knock-out Epstein-Barr virus

RR-EAE: remitting-relapsing experimental autoimmune encephalomyelitis

SERT: Serotonin transporter

SLE: Systemic Lupus Erythematosus

SLEDAI: Systemic Lupus Erythematosus disease activity index

SmB: Recombinant Smith B protein

SMC: Smooth muscle cell

SmD: Recombinant Smith D protein

snRNP: small ribonuclear protein

SS: Sjogren's Syndrome

SV: Sedimentation velocity

T1D: Type-1 diabetes

TLR 7: Toll-like Receptor 7

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TSH: Thyroid stimulating factor

TSHR: Thyroid stimulating factor receptor

VEGF: Vascular endothelial growth factor

v-IL: Viral Interleukin

VZV: Varicella Zoster virus

Preface

To help the reader better understand the order and organization of this document, I will provide a brief explanation on that count. Due to the amount and variety of content contained in chapter 1, it has been organized slightly differently than chapters 2 through 5. This chapter has been dissected into three sections, each providing background information, as well as a review of the current literature pertinent to the research that will be explained in chapters 2 through 4. These three sections have further been organized into subsections labeled as such. For example, chapter 1, section 2, subsection 2 would be labeled 1.2.2 and so on. Section 3, which contains subsections of subsections would be labeled as 1.3.3.1, 1.3.3.2, and so on.

Chapters 2 through 4 have been organized like a scientific article, as that is how they were intended to be read. In these chapters a summary is provided followed by the introduction, methods, results, and discussion sections. Like in chapter 1, the chapter number is provided first and then the section number. For example, the summary for chapter 2 would be denoted 2.1. Chapter 5 is the concluding chapter and contains merely two parts addressing future potential experiments and providing final discussions of the research outlined in chapters 2 through 4.

It is also important to note that the content contained in chapter 1 sections 1 and 3 were published in peer reviewed journals (Cornaby, Gibbons, et al., 2015; Cornaby, Tanner, Stutz, Poole, & Berges, 2016). Chapter 2 was also published (Cornaby et al., 2017). Chapters 3 and 4 are currently under review in different peer-reviewed journals.

Chapter I. Introduction and Review of the Literature

Section 1: Introduction to Systemic Lupus Erythematosus and B Lymphocyte Involvement in Autoimmune Disease Pathophysiology[1]

Systemic Lupus Erythematosus

When healthy tissue is damaged due to an immune response against cellular markers or proteins that are mistaken as foreign antigens, this is termed autoimmunity. It is suspected that autoimmune diseases afflict 7.6 – 9.4 % of the world population and, in most cases, are more prevalent in females than in males of a given human population (Cooper, Bynum, & Somers, 2009; Gutierrez-Arcelus, Rich, & Raychaudhuri, 2016). About 140 – 150 in 100,000 people in the United States of America have Systemic Lupus Erythematosus (SLE) (Feldman et al., 2013). The incidence and prevalence of SLE differs greatly among populations due to sex, race, ethnicity, and socioeconomic status (Alarcon et al., 2004; Fessel, 1974; McCarty et al., 1995). Nearly 90% of SLE patients in the US are women, and the prevalence of SLE is twice as high in African Americans compared to the Caucasian population (Feldman et al., 2013). The highest prevalence of SLE observed in the continental United States was in the southern United States (Feldman et al., 2013).

SLE is a chronic autoimmune disease that affects multiple body systems often influencing the skin, joints, central nervous system, lungs, kidney, digestive tract, and

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[1] Most of the material comprising this section was published in the journal Immunology Letters, see Cornaby, Gibbons, et al., 2015.

hemopoietic system (Tiffin, Adeyemo, & Okpechi, 2013). This results in a diverse display of clinical signs and symptoms. Commonly exhibited symptoms include cutaneous manifestations such as malar rash, photosensitivity, discoid lesions, and oral ulcers, joint pain and swelling, serositis, renal disorders, hemolytic anemia, leukopenia, neuropsychiatric disorders and poor vascularization (C. Yu, Gershwin, & Chang, 2014). While the exact pathological mechanisms responsible for all the symptoms displayed by SLE patients is not yet well defined, the basic etiology of disease cause and progression has advanced quite rapidly in the last several decades. This being the case, there is still much that is not understood that if better defined could assist in the development of better patient care and treatment options.

B cell Involvement and Epitope Spreading in Autoimmune Disease

1.1.1 Introduction

Epitope spreading is the process by which T and B lymphocytes expand their receptor repertoire for one initial antigen (Ag) epitope to various other epitopes. This process is an important contributor to the efficiency and breadth of the immune system. However, epitope spreading is implicated in multiple autoimmune diseases (McCluskey et al., 1998; C. L. Vanderlugt & Miller, 2002). Epitope spreading is known to be a major component of systemic lupus erythematosus and bullous pemphigous, and is implicated in multiple other autoimmune diseases ranging from multiple sclerosis to diabetes (Table 1). In B cell epitope spreading, the antibody (Ab) specificity develops from the initial Ag that triggered the response to include other epitopes of these Ags, or other Ags altogether.

The contribution of epitope spreading in the progression of autoimmune disease was first recognized in print during the study of experimental autoimmune encephalomyelitis (EAE) in

Disease	Evidence of epitope spreading	Type of experiment	Type of B cell epitope spreading
Bullous pemphigoid	Ab diversification to epitopes of BP180 and BP230	Clinical study	Intramolecular and intermolecular
	Ab spread from collagen XVII to collagen VII	Clinical report	Intermolecular
	Bullous pemphigoid develops after pemphigus foliaceus	Clinical report	Intermolecular
Cardiomyopathy	Immunization with CC2 peptides induces EAC, then DCM	Lewis rat model	Intermolecular
Diabetes (Type 1)	Diversification of Abs to include ICA, GADA, IA-2, and IAA	Clincal study	Intermolecular
Graves' disease	Ab diversification from thyroglobulin AA2471–2490 to other epitopes	NZW rabbit model	Intramolecular
	Intramolecular epitope spreading on TSHR protein molecule	BALB/c mice model	Intramolecular
	Ab diversification on the TSHR protein molecule	Transgenic mice model	Intramolecular
Heymann nephritis	Ab spreading to various epitopes on Megalin protein	Lewis rat model	Intramolecular
Multiple sclerosis	Ab spreading from MOG peptide 92–106 to intramolecular epitopes	RR-EAE mice model	Intramolecular
	PLP Abs spread to target other myelin proteins	EAE mice model	Intermolecular
Pemphigus	Ab diversification to various regions of desmoglein 1 and 3	Clinical study	Intramolecular and intermolecular
	Development of paraneoplastic pemphigus from Lichenoid Dermatitis	Clinical report	Intermolecular
Rueumtoid arthritis	Abs diversify from ACPA to include anti-CCP	Serum collection analysis	Intermolecular
Scleroderma	Auto–Abs to differing centrosome domains were present in the same sera	Serum collection analysis	Intramolecular
Sjogrens syndrome	Ab spread from Ro/SSA to La/SSB and to unimmunized epitopes of Ro/SSA	BALB/c mice model	Intramolecular and intermolecular
Systemic lupus erythematosis	Ab diversification from 1.5 to 3 out of 7 auto-Ags screened	Serum collection analysis	Intermolecular
	Ab spread from SmD to SmB and A-RNP molecules	A/J mice model	Intermolecular
	Intermolecular more common than intramolecular epitope spreading	A/J mice model	Intramolecular and intermolecular

Table 1. Compiled evidence implicating B cell epitope spreading in various autoimmune diseases. The autoimmune diseases are listed with the completed cumulative research that provided provocative evidence for B cell epitope spreading. It is specified for each evidenced example of epitope spreading a sentence summary of the example, what type of experiment was performed to obtain the data, the type of epitope spreading involved, and the reference for that example.

Lewis rat and mouse models (Bernard et al., 1997; Steinman, 1995). In these studies, it was found that rats and mice injected with Myelin oligodendrocyte glycoprotein (MOG) induced a T cell encephalitogenic and B cell Ab immune response resulting in demyelination. During this study it was demonstrated that the Antigenic targets of the Abs changed and diversified during the course of EAE. It was later shown that epitope spreading was the cause of the Ab diversification. We now understand that B cell epitope spreading assists in the progression of many autoimmune diseases, acting as a two-edged sword for many that are affected by autoimmune diseases (McCluskey et al., 1998; C. L. Vanderlugt & Miller, 2002). Epitope spreading is essential for an effective adaptive immune response, at the same time contributing to the progression of self-targeting disorders. It is important to better understand epitope spreading due to its influence in autoimmune progression and the potential to develop more advanced strategies to assist in the treatment and prevention of autoimmune diseases.

While much research has been done to determine the significance of T cell epitope spreading in disease, far less is known about B cell epitope spreading. This review will address B cell epitope spreading and its involvement in the progression of various autoimmune diseases. The current understanding about factors contributing to B cell epitope spreading and intracellular mechanisms involved in this process will be discussed. Areas for future research and gaps in our understanding of B cell epitope spreading will also be commented upon. The purpose of this review is to provide a summary of our current understanding about B cell epitope spreading

mechanisms, involvement in autoimmune disease, and the importance of epitope spreading in treatment options.

1.1.2 Potential Mechanisms of B Lymphocyte Epitope Spreading

<u>Types of Epitope Spreading</u>

There are two different types of epitope spreading, intermolecular and intramolecular (C. J. Vanderlugt & Miller, 1996). The latter describes a type of spreading where the immune response is directed against different epitopes of the same molecule, while the former is a diversification of the immune response against two or more different molecules (Thrasyvoulides & Lymberi, 2003). By expanding the antigenic epitopes that our immune system recognizes, responses to these foreign Ags becomes optimized, allowing for neutralization via Abs, recognition by various immune cells, and clearance of the pathogen (Figure 1). Intermolecular epitope spreading is not dependent on intramolecular epitope spreading (Deshmukh, Bagavant, Sim, Pidiyar, & Fu, 2007); rather these different types of epitope spreading are independent of each other. Diversification takes place only after Ag recognition by cells of the immune system. B cell activation by CD 4+ T cells or by surface clustering immunoglobin bound to the specific Ag epitope initiate the beginning of this process. Through clonal expansion and affinity maturation, the spreading of epitopes for a given Ag expand and continue to broaden the longer the immune response continues. It has been shown that there are two ways to initiate B cell epitope spreading in autoimmune diseases. McCluskey et al. describe the first to be independent of a physical association with immune cells presenting the Ag, while the second is dependent (McCluskey et al., 1998). In an Ag presenting cell (APC) independent scenario, the inflammation and activation cytokines are sufficient enough to allow T cells to recognize cryptic epitopes and activate complementary B cells. This is observed in non-obese diabetic (NOD) and

EAE animal models (Kurien & Scofield, 2008). On the other hand, a dependent response happens typically when there is no tissue destruction, or it is delayed, relying on processing and presentation to achieve activated T cells that, through intrastructural T cell help, activate reciprocal B cells. In fine, the independent scenario exists when there are environmental conditions, such that T cells can recognize cryptic, or novel, epitopes without the processing and presentation by APCS, such as B cells and dendritic cells. The dependent circumstance relies upon APC processing and presentation to T cells in order for the immune system to recognize cryptic epitopes.

Intermolecular Epitope Spreading

Many of the Ags recognized in autoimmune diseases are part of multi-Antigenic complexes. For example, the spliceosome is a common target for auto-Abs in lupus, and consists of multiple proteins and nucleic acids that are associated with each other. T cells specific for one epitope of a multi-Antigenic complex can activate B cells that are specific for other Ags of the complex (Deshmukh, Bagavant, Lewis, Gaskin, & Fu, 2005). T cell help allows B cells to target a range of Ags to proliferate, differentiate, and produce Abs, even against Ags that were not originally involved in either the B cell or the T cell response (Fatenejad, Mamula, & Craft, 1993; Singh, 2004).

An experimental example of this concept was illustrated by Deshmukh et al. during their study of systemic lupus erythematosus (Deshmukh et al., 2007). To induce lupus-like symptoms in a mouse model, female A/J mice were immunized with various SmD peptides. This stimulates an immune response against the small nuclear ribonucleoprotein (snRNP) complex that is characteristic of systemic lupus erythematosus. Immunoprecipitation assays were used to determine Ab reactivity to SmD, SmB, and associated ribonucleoprotein (A-RNP molecules). It

was found that immunization with the SmD$_{52\text{-}66}$ lead to the development of Abs against not only regions of the SmD protein, but also to the U1 A-RNP. The Ab diversification from the SmD protein to the A-RNP is a demonstration of intermolecular epitope spreading. During their study of viral triggers leading to SLE, Poole et al. observed a similar example of intermolecular epitope spreading (B. D. Poole, Gross, Maier, Harley, & James, 2008). Immunization of rabbits with the protein peptide PPPGMRPP from Epstein-Barr virus (EBV) protein EBNA-1, lead to the development of antibodies to SmB, SmD, nRNPs and La/SSB.

Clinical studies and reports continue to provide evidence for this process. Maeda et al. described a case where a 64-year-old Brazilian male developed bullous pemphigoid twelve years after being diagnosed with pemphigus foliaceus (Maeda et al., 2006). He was diagnosed with bullous pemphigoid when subepidermal blistering was observed and immunoblotting assays revealed Ab activity against BP180. A congruous event was recorded by Peterson et al. where they observed similar results from a 86 year old African American male (Peterson, Chang, & Chan, 2007). This development of bullous pemphigoid after pemphigus foliaceus is a clinical manifestation of intermolecular spreading. Autoimmune targets resulting in pemphigus foliaceus are typically desmoglein proteins, particularly desmoglein 1. The cause of bullous pemphigoid is an autoimmune reaction against several bullous pemphigoid proteins (BP180 and BP230) of the hemidesmosome. In fine, the incidences reported above indicate an intermolecular epitope spreading event from the desmoglein proteins to the bullous pemphigoid proteins.

It does not take a large variety of T cells to drive a diverse B cell response leading to intermolecular epitope spreading. An example of this was shown by Milich et al. in using mouse T cells primed for the Hepatitis B nucleocapsid to invoke an immune response against the Hepatitis B virus. The result was Ab production against Hepatitis B surface Ags (Milich,

McLachlan, Thornton, & Hughes, 1987). This demonstrates CD 4+ T cells specific for priming recognition of just one region, leading to an immune response that diversified to include the surface Ag and nucleocapsid Ag of Hepatitis B. Intrastructural T cell help is necessary for B cell epitope spreading to elicit an immune response against pathogens and contributes during development of immune diseases(Craft & Fatenejad, 1997; Datta, Kaliyaperumal, & Desai-Mehta, 1997).

<u>Intramolecular Epitope Spreading</u>

After B cells are activated in response to Ag and T cell stimulation, they respond by progressing through clonal expansion and affinity maturation. Upon processing of the Ag, these cells can in turn present novel cryptic epitopes to CD 4+ T cells in the context of MHC class II molecules. Cryptic epitopes are understood to be epitopes that were previously un-recognized by the immune system. These cryptic epitopes allow for the priming of reciprocal T cells and the activation of complementary B cells. This process allows the immune response to recognize various epitopes from the same molecule, or associated molecules, and thus contributes to epitope spreading. Affinity maturation allows for selection of the clonally expanded B cell population for those cells that have a higher affinity for the Ag. Because of this selection, intramolecular epitope spreading can occur as B cells with higher affinity for a different epitope of the Ag are selected (Deshmukh et al., 2005). Endocytic processing and the subsequent MHC class II presentation is a further mechanism that can promote intramolecular epitope spreading. During endocytic antigen processing the antigen is endocytosed, selectively cleaved and loaded into a compatible MHC class II complex that is then displayed on the cell surface. This process allows for presentation of previously unrecognized epitopes and the broadening of the immune response to various epitopes on the endocytosed molecule.

A clinical study of pemphigus foliaceus in Brazil provided evidence that intramolecular epitope spreading contributes to autoimmune disease development (N. Li, Aoki, Hans-Filho, Rivitti, & Diaz, 2003). Sera from patients were collected pre-disease onset and while the patients were exhibiting clinical symptoms of skin lesions. Using immunoprecipitation assays to analyze the sera from the patients, several interesting observations came to light. Patients still in the
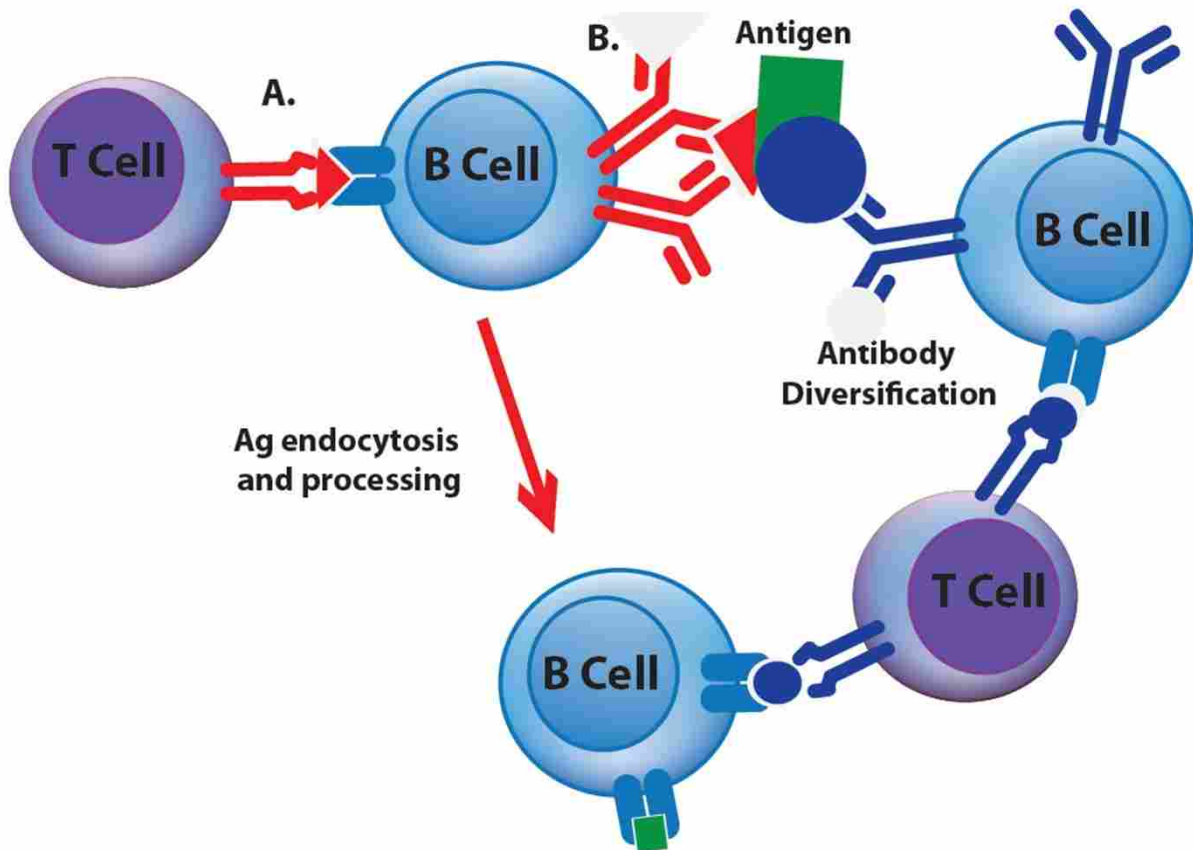
Figure 1. Intramolecular B cell epitope spreading.

(A) Once activated through T cell intrastructural help or (B) clustering of the BCRs attached to a specific epitope on the Ag, B cells endocytose the Ag, selectively cleave it, load selected peptides in MHC II molecules and display it in this context to T cells. Once a T cell is activated by the B cell for a new epitope presented via MHC II, it can then activate a reciprocal B cell which will then express Abs specific for that epitope. This process is known as intramolecular B cell epitope spreading.

pre-clinical phase of the disease developed Abs for the COOH-terminus region of the desmoglein 1 protein. During the study several patients progressed from the pre-clinical phase to the clinical phase of the disease. Testing of the serum during clinical onset of pemphigus foliaceus revealed Abs against the EC1 and EC2 domains of the NH_2 terminal region of the desmoglein 1 protein. Another example is presented by Poole et al. in a serum analysis study of SLE diagnosed patients collected at the Oklahoma Clinical Immunology Serum Repository, it was found that intramolecular epitope spreading contributed to the progression of SLE (B. D. Poole et al., 2009). Using autoantibody analysis of the extensive serum samples, it was observed that antibody diversification happened over time during disease progression to various epitopes on the nRNP-A and nRNP-C proteins. Anti-nRNP-A antibodies were found to bind to the N-terminus of the protein more frequently in later samples when compared to the initial samples. This diversification of Abs from one region of the protein to the other region demonstrates intramolecular epitope spreading and provides evidence of the role it plays in the progression of autoimmune disease.

Molecular Mimicry and Cross-Reactivity

B cell epitope spreading after molecular mimicry is a mechanism by which the body likely begins to break self-tolerance, leading to the development of autoimmune disease. Molecular mimicry has been indicated as a contributing factor in autoimmune diseases such as type I diabetes (Leech, 1998), rheumatoid arthritis, multiple sclerosis, glomerulonephritis (Cusick, Libbey, & Fujinami, 2012), and systemic lupus erythematosus.

Molecular mimicry exists when an auto-Ag is similar enough to an antigen from a pathogen or other environmental source that Abs generated against the pathogen will also bind to the autoantigen (Cusick et al., 2012). Cross-reactivity is a phenomenon that occurs in the

immune system when a single Ab reacts with multiple Ags varying in structure and composition. The immunochemical evidence in recent literature suggests that conformation as well as structure is essential for an effective Antigenic mimic (Ohtaki, Kieber-Emmons, & Murali, 2013). This was demonstrated by Marluzza et al. at the University of Maryland when they showed that the anti-idiotopic Ab E5.2 was a mimic for lysozyme in the way that it bound to D1.3 (Fields, Goldbaum, Ysern, Poljak, & Mariuzza, 1995). Marluzza's group further showed that it was a binding group mimic and shared no homologous sequence between the Ag and Ab E5.2 (Braden et al., 1996). These findings verified that the binding of Abs is not determined by amino acid sequence alone, but by the conformation of the epitope to which it binds. While certain amino acid sequences tend to display select conformations and structures, there is variability due to protein folding, molecular interaction, and binding. These findings indicate that an Ag, or auto-Ag, does not need to share homologous amino acid sequences in order to be an effective mimic.

The mechanisms of molecular mimicry and cross-reactivity are thought to be key contributors in B cell epitope spreading. One example of molecular mimicry was explored by Poole at al. in experiments tying Epstein-Barr Virus (EBV) infection to the development of the autoimmune disease systemic lupus erythematosus (SLE). The EBV nuclear Ag-1 (EBNA-1) contains a peptide sequence, PPPGRRP, that closely resembles the PPPGMRPP region on the Smith Ag (Sm) targeted by auto-Abs in SLE patients. Immunization with the PPPGRRP sequence from EBNA-1 led to development of cross-reactive antibodies that recognized both epitopes, as well as further epitope spreading leading to autoimmunity against Sm and nRNP complexes. Also, 5 out of the 6 rabbits immunized developed SLE like symptoms over the course of the experiment indicating that the mechanism of molecular mimicry involving EBNA-

1 can lead to development of systemic lupus erythematosus-like disease (B. D. Poole, Scofield, Harley, & James, 2006). Clinical evidence shows similar results with respect to the spreading of epitopes in SLE. After an individual loses tolerance for 60 kDa Ro/SSA and beings to produce Abs that specifically target Ro/SSA, later blood samples reveal Abs against Sm B', SmD1, nRNP A and C, and eventually La/SSB (B. D. Poole et al., 2009).

1.1.3 Intracellular Mechanisms of B Lymphocyte Epitope Spreading

<u>Endocytic processing and Ag presentation</u>

B cell endocytosis and Ag presentation to T cells contribute to epitope spreading. Endocytosis takes place after Ag binding to B cell receptors (Amigorena & Bonnerot, 1999a). During this process of B cell endocytosis, Ag processing and presentation determines what peptides will be loaded into MHC class II molecules and displayed on the cell surface. This determines what epitopes will be presented to T cells, ultimately identifying what epitopes should be targeted by the immune system. There are various circumstances that affect this processing (Figure 2).

The first part of endocytosis to affect processing is the BCR to which the Ag is bound (Amigorena & Bonnerot, 1999b; Binder et al., 2011). Binding to the BCR can affect the selection of epitopes presented to T cells (Watts, 1997), however, it is not known exactly how it impacts processing of Ags and the loading of class II MHC complexes (Lankar et al., 2002; McGovern, Moquin, Caballero, & Drake, 2004). Thanks to McGovern and Moquin et al. we now understand that the difference in Ag processing and presentation is due to BCR signaling and not due to the signaling induced changes in the biology of the cell (McGovern et al., 2004). It has been suggested that stability between the Ag and the surface immunoglobin (sIg) to which it is bound influences processing (Aluvihare, Khamlichi, Williams, Adorini, & Neuberger, 1997).
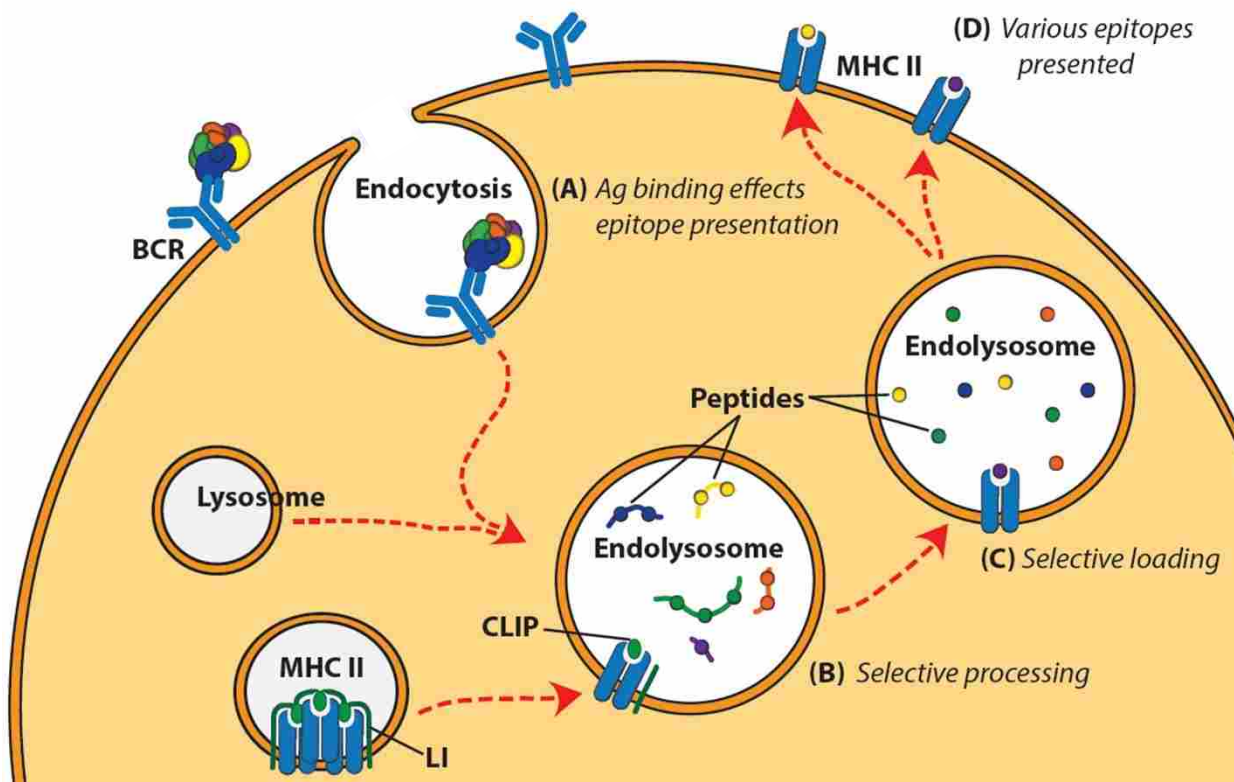
Figure 2. The endocytic pathway is a primary mechanism in B cell epitope spreading.
(A) The binding of the sIg to the Ag can affect the epitope that is selected for and loaded into MHC II. (B) The endosome fuses with the lysome, containing GILT, cathepsins, and other proteases. These enzymes selectively digest the Ag into peptide segments. This provides another point for selection of epitopes and the exclusion of others based on the cleavage sites. (C) With the disassociation of CLIP, peptides are selectively loaded into the MHC II molecule based on amino acid motifs, pH, HLA-DM association, among other enodsomal environmental factors (D) Once these various selective processes are accomplished the resulting epitope chosen is displayed via the MHC II on the surface of the cell. Because of the many factors involved, various peptides could be displayed.

A second point of processing selection is due to the selective cleavage of proteases contained in the lysosome. This selective processing of Ag into smaller peptide fragments depends on the proteases present in the lysosome, including their relative concentration and activity which is regulated by various endogenous cytokines and competitive inhibitors (Lennon-Dumenil, Bakker, Wolf-Bryant, Ploegh, & Lagaudriere-Gesbert, 2002). These competitive

Another proposed reason for the difference in the processing of Ags might be related to targeting as a consequence of the sIg cytoplasmic tail, the Igα/Igβ associated chains, or both (Amigorena & Bonnerot, 1998). These various components of the BCR play a role in internalization and processing, however, it is unknown how they affect this process in detail. It has been demonstrated that Igα and Igβ selectively target Ags to be loaded into MHC II (Bonnerot et al., 1995). inhibitors vie with protein that is being digested, regulating what proteases are more active in the cleaving of the endocytosed Ag. Cytokines can affect endocytic proteases by adjusting their activity, synthesis, or stability. Various cytokines have been shown to affect the endosomal pH, increasing or decreasing the ability of protease activity (Drakesmith et al., 1998; Fiebiger et al., 2001). In this way, cytokines can stimulate or inhibit enzymes involved in Ag cleavage and degradation, affecting what peptide segments are available to be loaded into MHC II complexes.

A final mechanism affecting the presentation of epitopes proposed is the loading of MHC II molecules. HLA-DM and the MHC II complex load only peptides that have an appropriate length and affinity. Peptides need to display specific amino acid motifs to be loaded (Rammensee, 1995). Several other factors affecting this loading include endosomal environment, including pH, and the HLA-DM (Escola, Grivel, Chavrier, & Gorvel, 1995; Griffin, Chu, & Harding, 1997). Age of the individual is also a factor that affects cellular efficiency. It is known that Ag presentation is better in younger individuals and declines with age (H. L. Clark et al., 2012). Recycled and newly synthesized MHC II molecules also play a role in selection. They are known to associate with different peptides (Lindner & Unanue, 1996), thus levels of recycled and newly synthesized MHC class II molecules could affect the peptides selectively loaded. Many questions remain unanswered about peptide selection for MHC class II loading and Ag

processing. Future investigation includes determining regulatory factors responsible for endocytic compartment targeting to lysosomes in the endocytic pathway and regulation of proteases in the cell, endogenous competitive inhibitors, and cytokines are other areas for research into selective processing of Ag.

It has also been demonstrated that through endocytosis, molecules that are associated together, non-covalently, can be presented in the context of MHC class II molecules as well (McCluskey et al., 1998). The event where an Ag and the associated molecule are processed and part of the Ag associated molecule is presented via MHC II to T cells has been termed intermolecular/intrastructural help (Lake & Mitchison, 1977). Such processing leads to intermolecular epitope spreading. Understanding of these events in concert with B cell processing and selection is essential for adequate comprehension of how and to what extent epitope spreading contributes to the human immune response, disorders, and diseases.

Somatic Hypermutation

During affinity maturation, B lymphocytes undergo somatic hypermutation (SHM) which can contribute to the spreading of epitopes (Deshmukh et al., 2005). This process takes place in germinal centers to improve B cell Ab affinity for an Ag. At this period of time SHM of the IgV gene region experiences single nucleotide substitutions. These happen at a frequency of about 10^3 per base pair in each B lymphocyte generation (Di Noia & Neuberger, 2007). The consequence of SHM results in Abs with a higher affinity for the Ag presented by dendritic cells in secondary lymphoid tissue during affinity maturation (Figure 3). It has been suggested that SHM also leading to B cell epitope spreading, contributes in the progression of autoimmune diseases. Auto reactive Abs can be generated against dsDNA through SHM in the development
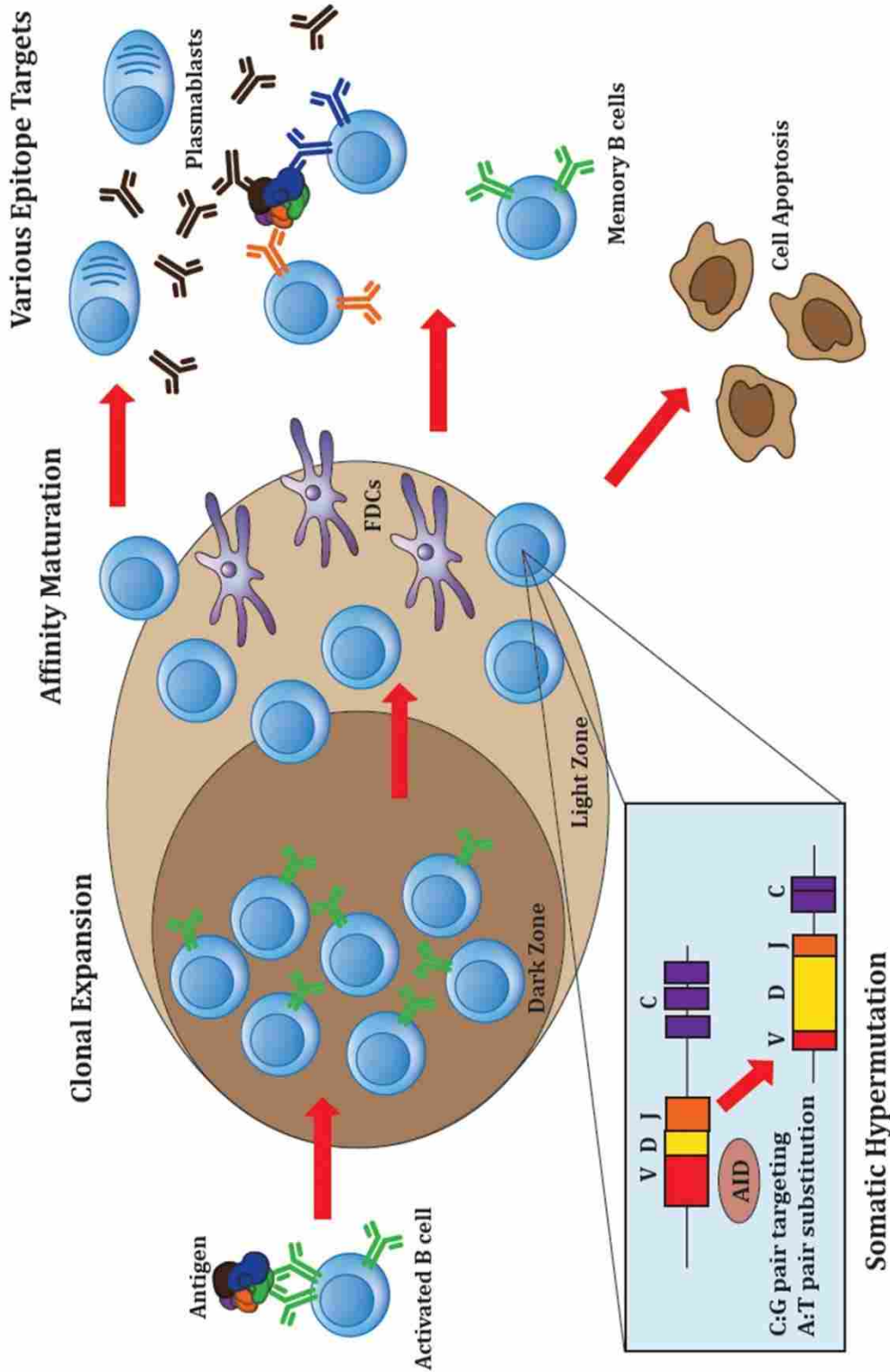
Figure 3. Somatic Hypermutation (SHM) as a mechanism of B cell epitope spreading.

of systemic lupus erythematosus (Ray, Putterman, & Diamond, 1996). It is uncertain to what extent SHM contributes to B cell epitope spreading. Investigating this question and determining if SHM contributes to other autoimmune diseases remains an area for future research that is lacking at this time.

1.1.4 B cell Epitope Spreading in Autoimmune disease

<u>Rheumatoid Arthritis</u>

The most common inflammatory arthritis worldwide, rheumatoid arthritis (RA) affects nearly 1% of the global population and can cause severe debilitation and joint deformity within the first few years of clinical symptoms due to joint inflammation and tissue degradation (McInnes & Schett, 2011; Schellekens, de Jong, van den Hoogen, van de Putte, & van Venrooij, 1998; Sokolove et al., 2012). While joint damage is the most characteristic and common symptom, the inflammation produced can also affect organs such as the cardiovascular and pulmonary systems (McInnes & Schett, 2011). Although the specific causes that lead to RA are unclear, it is thought to be the result of interplaying genetic factors, environmental agents, and chance, similarly observed among other autoimmune diseases. Certain Abs, including rheumatoid factor and Anti-Citrullinated Protein Abs (ACPA) are important contributors to RA (Schellekens et al., 1998). Rheumatoid factor is a high-affinity auto-Ab against the FC region of immunoglobulins (McInnes & Schett, 2011). These Abs often develop years before clinical disease, preceding the development of rheumatoid arthritis, with the number of targeted proteins gradually increasing until the time of diagnosis (Nielen et al., 2004; Rantapaa-Dahlqvist et al., 2003; Sokolove et al., 2012). Interestingly, increasing Ab targets correlate with increased inflammatory cytokine levels (Sokolove et al., 2012) and the disease course of RA. ACPAs can be directed against a multitude of targets (Snir et al., 2009). This wide range of targets arises

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because citrullinated proteins result when arginine residues in proteins are converted to citrulline residues due to a post-translational modification by peptidylarginine deiminase, a process that is not specific to a single protein (Kidd et al., 2008).

Epitope spreading is seen in the development of the ACPA repertoire. It is hypothesized that the neo-Ags that result from citrullination could evoke an initial immune response, which then develops into a polyclonal Ab response against the entire protein or protein-complex due to epitope spreading (Schellekens et al., 1998). This process is restricted mainly to genetically susceptible individuals, and the mechanism that triggers the initial citrullination is still unclear (Schellekens et al., 1998). Additionally, the reason why a systemic loss of self-tolerance leads to localized joint inflammation is also unclear (McInnes & Schett, 2011).

An investigation into United States Armed Services personnel clinically diagnosed with Rheumatoid Arthritis revealed unique information about Ab development and epitope spreading during the development of RA (Sokolove et al., 2012). Using clinical Ab assays and a novel multiplex auto-Ab assay, it was possible to process serum samples obtained from the Department of Defense Serum Repository and screen for various Abs unique to RA patients. The samples used predated and preceded clinical symptoms and diagnosis of RA. Upon analysis, it was noted that the Ab diversification increases with the progression of RA and occurs before the onset of clinical symptoms. ACPAs were observed prior to the development of anti-cyclic citrullinated peptides (anti-CCP). These findings indicate both intramolecular and intermolecular B cell epitope spreading during the progression of rheumatoid arthritis. It is hypothesized that this epitope spreading was initiated by B cells stimulated by citrullinated fibrinogen complexes (Sokolove et al., 2012). These examples indicate that B cell epitope spreading takes place during

disease progression in rheumatoid arthritis and that citrullination of peptides contributes to these epitope spreading events.

<u>Type 1 Diabetes</u>

Type I diabetes (T1D) results when an autoimmune reaction attacks and destroys the insulin producing beta cells of the pancreas. T cells specific for a number of diabetogenic auto-Ags are responsible for the destruction of the pancreatic islet cells. T1D is the most prevalent chronic disorder among children, and the loss of glucose level homeostasis that results can cause polydipsia, polyuria, weight loss, and potential complications of hypoglycemia such as hypoglycemic shock (Prasad, Kohm, McMahon, Luo, & Miller, 2012).

Autoimmunity may first be triggered against the B9-23 region of insulin (Prasad et al., 2012) and progression to overt disease is mediated by epitope spreading to an array of beta cell Ags. It was shown that non-obese diabetic (NOD) mice that lacked mature B cells could not develop this autoimmunity, suggesting a crucial role of B cells in the mechanism of epitope spreading. In this experiment, two to three-week old NOD mice were transfused with naive T and B cells, or only naïve T cells, and the T cell response to various beta cell Ags was measured at four and ten weeks after transfusion. The TB NOD mice demonstrated progressive autoimmunity development to glutamic acid decarboxylase (GAD), heat shock protein 277 (HSP277) and insulin B-chain while the T NOD mice showed no T cell response to any of the beta cell Ags tested. Researchers showed that NOD mice B cells have deficient tolerance to auto-Ags, and are relatively resistant to activation-induced cell death, which could explain why autoimmunity was able to develop (Tian, Zekzer, Lu, Dang, & Kaufman, 2006). In C57BL/KsJ db/db mice, it was found that the retroviral p73 protein, a group-specific Ag, acts as a molecular mimic of insulin (Serreze, Leiter, Kuff, Jardieu, & Ishizaka, 1988). It is speculated that

presentation of this retroviral Ag to T-helper cells may stimulate B-lymphocytes producing the cross-reactive anti-p73 Ab, which also bind insulin (Serreze et al., 1988). Once the initial self-tolerance to insulin is broken, epitope spreading can contribute to the development of autoimmunity. The currently proposed model is that an initial wave of activated T cells causes B cell activation through T-B cell interaction and cytokine release. This enhances the Ag presenting functions of the B cells, causing them to capture and present beta cell Ags to T cells, causing the expansion of T cell autoimmunity (Tian et al., 2006).

A clinical study further demonstrated epitope spreading as a mechanism leading to disease progression and clinical diagnosis (Brooks-Worrell, Gersuk, Greenbaum, & Palmer, 2001). During an average of 30 months, 25 volunteers at risk for type 1 diabetes donated blood for analysis. These individuals were classified as 'at risk' due to the detection of islet cell Abs (ICA), Glutamic acid decarboxylase Abs (GADA), islet cell Ab-2 (IA-2), or insulin auto-Abs (IAA). Serum samples obtained were tested for the four Abs using specific Ab assays for ICA, GADA, IA-2, and IAA. Out of the 25 at risk volunteers under observation, seven developed clinical type 1 diabetes. All seven of these volunteers displayed serological evidence of intermolecular B cell epitope spreading. One patient, during pre-clinical T1D only displayed ICA in the serum. By the end of the study the B cell repertoire had diversified to include all four Abs. Other at-risk volunteers not diagnosed with clinical type 1 diabetes also displayed evidence of intermolecular B cell epitope spreading (Brooks-Worrell et al., 2001).

Multiple Sclerosis

An autoimmune disease of the central nervous system, multiple sclerosis results from the degradation of myelin proteins that sheath the neurons. The demyelization is caused by the attacking immune cells, particularly T and B cells, causing inflammation and a subsequent

autoimmune response. With the destruction of the myelin sheath, biochemical electrical impulses from the brain are not transmitted efficiently. A variety of symptoms result including: fatigue, pain, movement, coordination, cognitive, and visual problems (Hemmer, Nessler, Zhou, Kieseier, & Hartung, 2006). Citrullination, via peptidylargenine deiminase, is thought to be caused by inflammation of target tissues and occurs in many circumstances normally. Myelin basic protein (MBP) is partially citrullinated in healthy brain tissue, but in those with MS there is an increase of citrullination. It is hypothesized that citrullination contributes to the progression of multiple sclerosis (Cao, Sun, & Whitaker, 1998). Auto-Abs derived during mouse model studies were between two and 20-fold more reactive against citrullinated MBP compared to non-citrullinated MBP (Kidd et al., 2008). In addition, these auto-Abs were found to be reactive against epitopes derived from αB-crystallin, the most abundant early gene transcript. Recent studies have shown that development of B cells into tertiary lymphoid tissue near CNS regions further contribute to the progression of MS (Kuerten et al., 2012).

The essential role of B cell epitope spreading in MS was demonstrated clearly in SJL/J mice with remitting-relapsing experimental autoimmune encephalomyelitis (RR-EAE) (Pollinger et al., 2009). These transgenic mice spontaneously develop RR-EAE affecting various central nervous system tissues and have a TCR specific for mouse myelin oligodendrocyte glycoprotein (MOG) peptide 92-106. The study showed that with the SJL/J mice, the endogenous B cells secreted Abs for new epitopes besides the peptide sequence the T cells targeted. Their findings also showed that there was no spontaneous EAE development in B cell deficient mice and that B cells enhanced the pathogenesis of EAE in the RR-EAE mouse model. The spreading of the B cell repertoire to other MOG epitopes besides the targeted amino acid sequence suggests that in

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EAE, the mouse model equivalent of multiple sclerosis, B cells are essential for the development of the disease, with a likely role for epitope spreading (Pollinger et al., 2009).

A different study conducted using EAE mice models contributed further to our understanding by showing that epitope spreading was found to directly correlate with disease progression (Kidd et al., 2008). In a mouse model, proteolipid proteins (PLP) where used to induce EAE. Various regions of these PLPs became targets for Abs, along with the targeting of additional epitopes on other myelin proteins, both citrullinated and non-citrullinated. This is indicative of both intramolecular and intermolecular B cell epitope spreading. These results add credence to previous research demonstrating the development of cryptic epitopes during disease development and provides an explanation for why this occurs (Lehmann, Forsthuber, Miller, & Sercarz, 1992). Epitope spreading has been shown to lead to the development of multiple sclerosis in mice during infection by Theiler's virus (Miller et al., 1997), indicating viruses as a potential trigger for the development of CNS autoimmune diseases.

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is an autoimmune disease caused by immune reacting with targets at diverse locations in the human body leading to a variety of clinical manifestations. SLE affects approximately 1 in every 2000 individuals with a 9:1 ratio for female vs. male diagnosis respectively (Klippel, Weyand, & Wortmann, 1997). Auto-Abs in SLE are often raised against double-stranded DNA as well as ribonuclear-proteins (RNPs) of the Sm complex found in spliceosomes (Klippel et al., 1997; B. D. Poole et al., 2006; C. J. Vanderlugt & Miller, 1996). This autoimmune response causes an accumulation of immune complexes and immune-mediated attack of various organs. Symptoms of SLE include: butterfly

rash, chest pain, sensitivity to light, joint pain, and kidney damage (Deshmukh et al., 2005; K. E. Taylor et al., 2011).

Arbuckle et al. explored the clinical evidences of B cell epitope spreading by comparing the presence of auto-Abs from the serum of pre-diagnosed and post diagnosed SLE patients (Arbuckle et al., 2003). This was made possible through The Department of Defense Serum Repository, containing over 30 million serum samples from members of the U.S. Armed Forces. Serum is collected at the time of enlistment and on average every year thereafter. This allowed Arbuckle et al. to identify SLE patients and then go back and observe the development of auto-Abs prior to SLE diagnosis. 130 SLE patients were selected and then tested for the presence of the major SLE autoantibodies including: anti-double-stranded DNA, anti-Ro, anti-La, anti-Sm, anti-nuclear ribonucleoprotein, and antiphospholipid. The presence of auto-Abs was confirmed via indirect immunofluorescence with HEp-2000 cells and ELISA assays. Arbuckle at el. observed that pre-diagnosis of SLE patients auto-Ab detection recognized on average 1.5 of 7 auto-Ags associated with SLE. At the time of clinical SLE diagnosis typically 3 of 7 auto-Ags were recognized, on average, by the Abs showing a spread of auto-Ab specificity over time (Arbuckle et al., 2003). These results indicate the occurrence of intermolecular B cell epitope spreading during the progression of preclinical to clinical autoimmune manifestation as well as during the progression of the pathogenesis of SLE.

Another investigation demonstrated the role of epitope spreading in SLE by studying auto-Ab diversification in response to small nuclear ribonuclear proteins (snRNP's) Ags (Deshmukh, Kannapell, & Fu, 2002). SLE patients often show diverse auto-Ab reactivity to snRNP's. To demonstrate this phenomenon, A/J mice were vaccinated with recombinant Smith D (SmD), Smith B (SmB), and A ribonuclear protein (A-RNP) and the specificity of the Abs that

developed were observed. Using western blots and immunoprecipitation assays to identify Ab specificity, it was observed that mice immunized with SmD also developed Abs specific against SmB and A-RNP. Likewise, mice immunized with SmB showed a development of Ab specificity against A-RNP and A-RNP immunized mice developed Abs against SmB as well as the 70-kDa protein A of the U1-snRNP. These results demonstrated that auto-Ab reactivity can spread from a single Antigenic site in a multi protein complex to associated complex proteins (Deshmukh et al., 2002).

Studies by Bavel et al. have investigated new antigenic epitopes presented after cell apoptosis that have been linked with SLE (van Bavel, Dieker, Tamboer, van der Vlag, & Berden, 2010). They found that acetylated epitopes of chromatin could allow for increased pathogenesis and epitope spreading. Scofield and associates studied a related line of research that indicate modification of Ro/SSA with lipid oxidation products, 4-hydroxy-2-alkenals in particular, increases the antigenicity and opportunity for B cell epitope spreading (Scofield, Kurien, et al., 2005). Research using A/J mice has also lead to the understanding that in some cases it is possible that intermolecular epitope spreading is more likely to occur than intramolecular epitope spreading (Deshmukh et al., 2007). As has been mentioned previously, environmental factors have been implicated leading to the breaking of tolerance and subsequent initiation of epitope spreading via molecular mimicry (B. D. Poole et al., 2006). Thanks to recent and past research, it is apparent that B cell epitope spreading plays a crucial role in the pathogenesis of SLE once self-tolerance is broken.

Sjogren's Syndrome

Sjogren's syndrome (SS) is an autoimmune disease that is associated with lymphocytic infiltration of the salivary and lacrimal glands resulting in dry mouth and eyes (Kurien et al.,

2013). The immune response can result in the damage and destruction of the glands. When only exocrine glands are involved in the autoimmune response it is considered primary Sjogren's syndrome, after other connective tissue becomes involved it is considered secondary Sjogren's syndrome. In SS, auto-Abs commonly develop against the Ro/SSA and La/SSB ribonucleoprotein particle linking SS to SLE through a common auto Antigenic site. SS is the second most common rheumatic disease but frequently goes without diagnosis due to its common symptoms. While a portion of the disease can be attributed to genetic risk factors, other stimuli are hypothesized to play a role in the development of SS, such as hormone imbalance and stress. Several environmental factors are thought to be triggers for SS including Epstein-Barr virus, Hepatitis C, tuberculosis, and malaria among others (Kivity et al., 2014).

Scofield et al. explored the role of epitope spreading in SS by vaccinating BALB/c mice with different amino acid sequences from 60-kDa Ro/SSA and observed whether auto-Abs against the entire 60-kDa Ro/SSA protein would develop (Scofield, Asfa, Obeso, Jonsson, & Kurien, 2005). In the experiment ten mice were vaccinated with Ro/SSA 480 and 10 separate mice were vaccinated with Ro/SSA 274. The results showed that mice developed Abs against their respective peptide sequence in 2-3 weeks and then over the course of several months developed auto-Abs that were reactive against the entire Ro/SSA and La/SSB complex. They further observed that mice vaccinated with 60-kDa Ro peptides not only developed SS associated auto-Abs but that the vaccinated mice developed salivary gland lymphocyte infiltrates and salivary gland dysfunction that resembled symptoms seen in human SS (Scofield, Asfa, et al., 2005).

It is important to continue research investigating risk factors related to the development of SS. Recent studies have reported HLA class II specific genetic markers as risk factor for SS and leading to B cell epitope spreading (Gottenberg et al., 2003; Kurien et al., 2013;

Paisansinsup et al., 2002). Routsias et al. recently suggested that intermolecular B cell epitope spreading in SS may begin via molecular mimicry in the RRM region of La/SSB (Routsias, Kyriakidis, Latreille, & Tzioufas, 2010). Further studies on possible risk factors and mechanisms of epitope spreading are needed to better understand the initiation and advancement of SS. Increased understanding in these areas would allow for the potential development of therapeutic treatments.

Graves' Disease

Also known as Basedow disease, Graves' disease is a condition where Abs develop that bind thyrotrophin receptors of the thyroid causing their activation and a subsequent up regulation of cyclic adenosine monophosphate and thyroid hormone synthesis (B. M. Genovese, Noureldine, Gleeson, Tufano, & Kandil, 2013; Prabhakar, Bahn, & Smith, 2003). This stimulation leads to hyperthyroidism and hyperplasia of the thyroid. Pathological symptoms include goiter development, insomnia, pretibial myxedema, muscle weakness, itching and Graves' ophthalmopathy (B. M. Genovese et al., 2013; Lazarus, 2012).

B cell epitope spreading has been related to thyroid diseases in clinical settings. Kim et al. found that in the serum analyzed during their study, Ab targets changed during disease progression. Since that time various experimental animal models have provided evidence of B cell epitope spreading in Graves' disease. Using New Zealand White rabbits in a study to determine if B cell epitope spreading plays a role in the pathogenesis of Graves' disease, Thrasvoulides et al. immunized their rabbits with three different peptides from thyroglobulin (Thrasyvoulides & Lymberi, 2003). Using affinity chromatography and ELISA assays, they confirmed that when they immunized the rabbits with peptide AA2471-2490, after a period of

several weeks there was intramolecular epitope spreading resulting in Abs binding to other epitopes on the thyroglobulin protein other than the initial peptide used during immunization.

Using Balb/c mice, evidence was found indicating intramolecular B cell epitope spreading (Vlase et al., 1995). In later mouse model studies, similar results were noted. When Balb/c and C57BL/6 mice where immunized with TSH receptor protein (TSHR), the Ab diversified from recognizing only the N-terminus domain to additional epitopes including the TSHR ectodomain (Schwarz-Lauer et al., 2003). Using more specific methods, Inaba et al. immunized breeding stock mice transgenic for HLA-DR3 and HLADR2 with 41 different TSHR peptides (Inaba et al., 2009). When mice where immunized with peptides AA70-88, AA83-102, or AA105-118 there was an Ab diversification outside of the original peptide epitope presented. More recently, it has been demonstrated that B cell epitope spreading occurs more rapidly than T cell epitope spreading (Inaba et al., 2013). The variety of research using animal and clinical means establishes B cell epitope spreading as a participant process in the development of Graves' disease.

Scleroderma

Scleroderma, or systemic sclerosis, is an autoimmune disease that involves connective tissue damage, most notably endothelial cells and fibroblasts of the extracellular matrix (Chung & Utz, 2004). Scleroderma is characterized by fibrosis of various organs throughout the body. The hardening of the organs is thought to be caused by an overproduction of collagen. An early symptom indicative of scleroderma, termed Raynaud's phenomenon, is when extremities such as fingers or toes, experience an exaggerated response to temperatures and distress. These areas will often feel numb or pained and may change color. B cells have often been shown to over express CD19, an activating receptor, during autoimmune progression. Auto-Abs for various targets are

seen in patients with scleroderma including anti-endothelial cell, anti-fibroblast, anti-MMP, and anti-fibrillin-1 among others, which involve targeting many DNA binding proteins including DNA topoisomerase (Chung & Utz, 2004; Henry et al., 2000). These autoimmune responses results in inflammation and tissue damage in the affected regions (Asano, Ihn, Yamane, Kubo, & Tamaki, 2003).

In scleroderma, B cell epitope spreading is initiated for a variety of reasons such as molecular mimicry or displays of previously cryptic domains which are displayed by enzymatic degradation, in which portions of the protein are degraded and novel domains are available for recognition. Furthermore, modification of target Ags may occur, such as the modification of U1-70 kd Ag modified by reactive oxygen species (Chung & Utz, 2004; Kurien & Scofield, 2008). Cryptic domains may also be exposed after vascular spasms, and the release of reactive oxygen (Gavanescu, Vazquez-Abad, McCauley, Senecal, & Doxsey, 1999). Few studies in recent years have investigated into epitope spreading and the development of scleroderma. In two studies, auto-Abs to differing centrosome domains were present in the same sera, suggesting intermolecular spreading (Gavanescu et al., 1999).

B cell epitope spreading has been observed as novel Ags are identified in patients with scleroderma. Of particular note is anti-PM/Scl Abs, antinucleolar Abs. Though further studies are necessary, it is thought that novel auto-Ags are targeted during disease progression. In a study conducted by Henry et al, auto-Abs were shown to have higher incidences to certain auto-Ags than to other target auto-Ags (Henry et al., 2000). For example, reactivity towards Scl-70 was much higher than topoisomerase I epitopes. These differences were concluded to occur because of the greater number of conformational determinants, which are larger than linear domains. This could explain the development of auto-Ags later in disease development resulting

from the protein preference and subsequent epitope spreading events. The continued identification of novel auto-Ags and how B cell epitope spreading contributes to the pathogenesis of scleroderma remain areas for substantial future research.

<u>Cardiomyopathy</u>

A disease resulting in the weakening and enlarging of the myocardium, dilated cardiomyopathy (DCM) is a fatal disorder causing various cardiovascular problems and a frequent culprit of heart failure. Dilated cardiomyopathy generated as an autoimmune response is a consequence of Abs reactive to heart associated protein (Rose, 2009). Anti-myosin IgG Abs are commonly observed in patients with autoimmune cardiomyopathy along with other auto Abs including anti-C protein Abs (Caforio et al., 2013; Neumann, Burek, Baughman, Rose, & Herskowitz, 1990; Nishimura et al., 2001). A clinical familial study conducted by Catorio et al. found that the production of cardiac specific Abs usually precludes disease development (Caforio et al., 2007). The occurrence of dilated cardiomyopathy is due to several factors both genetic and environmental. Potential triggers for dilated cardiomyopathy include enteroviruses and other myopathic diseases (Caforio et al., 2013; Miller et al., 1997).

One of the few examples showing the involvement of B cell epitope spreading in cardiomyopathy, Matsumoto et al., observed this event in Lewis rats immunized with C protein fragment 2 (CC2) (Matsumoto, Park, & Kohyama, 2007). During their study they immunized Lewis rats with various peptides derived from CC2, peptide segments P1 through P12. All CC2 peptide mixes induced experimental autoimmune carditis (EAC) and lead to DCM, with the exception of P12, which only lead to the development of EAC. Mixes of P1 to P3, P4 to P6, P7 to P9, P10 to P12, and several individual peptide segments were used to inoculate the Lewis rats. After immunization, it was observed that the Ab response diversified to include not only the Abs

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against the mix of segments used to immunize the rats, but also against various other peptide segments on CC2. All of the Lewis rats immunized P1 through P11 demonstrated this diversification and eventually progressed from EAC to DCM. While those Lewis rats immunized with CC2P12 only developed Abs against that region and did not develop DCM, but remained expressing EAC symptoms(Matsumoto et al., 2007). The study evidences the influence that B cell epitope spreading plays in the progression of dilated cardiomyopathy.

<u>Heymann Nephritis</u>

Heymann Nephritis (HN) disease models using Lewis rats are one of the most valuable tools for researchers to use in understanding the immunopathology of glomerular subepithelial immune deposit formation and mechanisms by which such deposits injure glomeruli. The Lewis rat model of induced active Heymann Nephritis first described in 1959 closely resembles that of glomerular injury and nephropathy in humans. Mechanisms of renal sodium retention, edema formation, and metabolic abnormalities have been observed as a guide to treating primary and secondary human glomerulonephritis diseases (Raychowdhury, Niles, McCluskey, & Smith, 1989; Salant, Quigg, & Cybulsky, 1989). Inducing active HN in rats is conducted by immunizing susceptible strains of rats with certain fractions of homologous or heterologous proximal tubular brush border. Granular glomerular capillary wall deposits of rat IgG and subepithelial electron-dense deposits characterize the disease after three to four weeks. Primary manifestations of HN include proteinuria (developing in 30-80% of induced rats), changes in glomerular hemodynamics, tubular function, and alterations in renal hormone production. These disease manifestations are closely related to human nephropathy disease manifestations, and demonstrate that active HN is indeed an autoimmune-induced disease (Raychowdhury et al., 1989; Salant et al., 1989; Shah, Tramontano, & Makker, 2007).

Megalin, or more specifically termed gp330, was found in 1982 and determined to be the glycoprotein and target Ag within the pits of glomerular and proximal tubular epithelia. The pathogenesis of HN includes the binding of circulating Abs to glomerular components. Megalin and RAP (receptor associated protein – 44kd) are the target Ags for nephritic activity and it was determined that RAP binds to Megalin (Farquhar, Saito, Kerjaschki, & Orlando, 1995; Shah et al., 2007).

Megalin is a complex Ag with four discrete ligand-binding domains (LBDs) that may contain epitopes to which pathogenic auto-Abs are directed. In a recent study a 236-residue N-terminal fragment (termed "L6") that spans the first LBD was shown to induce auto-Abs and severe disease in Megalin-immunized HN rats (Shah et al., 2007). Sera obtained from test results of rats that had received an L6 fragment showed reactivity only with the first LBD after a 4-week period. However, after 8 weeks the same L6 fragment showed reactivity with all four recombinant LBDs. The study demonstrated that the L6 immunogen did not contain the epitopes responsible for reactivity with the LBD fragments and therefore suggest intramolecular epitope spreading as the key variant to the increased severity and reactivity of HN rats. Further correlation of epitope spreading to the pathogenesis of HN disease indicated by the study was the onset of proteinuria. Proteinuria in rats immunized with L6 was seen at 6 to 8 weeks. ELISA of antisera indicated peak titers against L6 at weeks 4 through 6, however reactivity of LBDs arose after week 6 and had maximal values at week 9. Thus induction of proteinuria with serum reactivity was indicative of epitope spreading (Farquhar et al., 1995; Shah et al., 2007). Treatment in HN Lewis rats with DNA vaccinations resulted in lowered proteinuria and promise for future treatment (Y. Wang et al., 2013; H. Wu, Walters, Knight, & Alexander, 2003).

Pemphigus

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Pemphigus is classified as a rare form of skin blistering caused by an autoimmune response against desmosomal adhesion proteins. These proteins are responsible for the homeostasis and attachment of epidermal cell layers (Galichet, Borradori, & Muller, 2014). There are four distinct forms and each is classified according to clinical signs and symptoms. Pemphigus vulgarius is the most common and is diagnosed by the presence of anti-Desmoglein 3 protein IgG Abs, with the initiation of sores in the oral mucosa and spreading to blisters on the face, limbs, and trunk of the body (Groves, 2009). Pemphigus foliaceus is characterized by Desmoglein 1 auto-Abs and superficial blister formation on the extremities. P. foliaceus tends to be one of the least severe forms of Pemphigus. Immunoglobulin A pemphigus is identified by the characteristic IgA Abs directed against desmoglein, and in some cases, desmocollin, proteins. The final and most severe form, Paraneoplastic pemphigus, is often associated with a secondary malignancy. The product is a wide spread autoimmune response causing severe cutaneous blistering in potentially any epidermal or mucosal tissue of the body. The cause of the autoimmune response in this case is unknown, although it is suspect that disease initiation may be related to insect bites (Groves, 2009).

To demonstrate that intermolecular and intramolecular epitope spreading occurs in Pemphigus, Valerie K. Salato, Mong-Shang Lin, and their associates at the Medical College of Wisconsin collected and analyzed sera from Pemphigus Vulgaris (PV) positive volunteers (Salato, Hacker-Foegen, Lazarova, Fairley, & Lin, 2005). These samples were taken at various times during the course of disease progression. In their study 14% of their volunteers transitioned from mucosal PV to mucocutaneous PV. They evaluated their Ab profiles during the progression of PV by immunoprecipitation and competition indirect immunofluorescence assays. Giving an example of one of these patients, early assays and detailed epitope mapping showed that they had

Abs autoreactive for region AA405-566 of Desmoglein 3. Several years later that same patient transitioned to produce auto-Abs that reacted with regions AA1-88 and AA87-566 of Desmoglein 3, demonstrating the spread of the B cell repertoire and offering evidence of intramolecular spreading. This same patient also showed evidence of intermolecular epitope spreading when they developed Abs that were auto-reactive with human skin cells. Samples collected earlier in the study showed no human skin auto-Abs while six years later, after the patient had transitioned to mucocutaneous PV, samples taken exhibited these auto-Abs. This hypothesis of intermolecular spreading was further supported when they confirmed the Abs auto-reactive for desmoglein 1in later samples (Salato et al., 2005). Their study demonstrates how B cell epitope spreading contributes to the progression of Pemphigus Vulgarius from displaying strictly mucosal legions to cutaneous legions.

Further observations give credence to the phenomenon of epitope spreading and its role in the development of Pemphigus when Bowen et al. described the development of Paraneoplastic Pemphigus from patients displaying Lichenoid Dermatitis (Bowen et al., 2000). They speculate that the intramolecular spreading could be due to lichenoid sores and the subsequential inflammation predisposing patients via a heightened immune response. This theory was further compared to other leading ideas as to the cause for disease progression in Paraneoplastic pemphigus and was suggested as a possible area for future research (Chan, 2000). In a study done in a Brazilian community it was proposed that epitope spreading was the cause for the development of Pemphigus Foliaceus in patients diagnosed with Fogo Selvagem, loosely translated as "wild fire" disease (N. Li et al., 2003). In their study, Li et al. proposed that a genetic predisposition may contribute along with epitope spreading leading to Pemphigus Foliaceus. In a unique case, clinical evidence suggests that B cell epitope spreading can

contribute to the progression of Pemphigus to Bullous Pemphigoid (Peterson et al., 2007). B cell

epitope spreading has been observed to play a role in the progression of Pemphigus and

contribute in the development to more severe symptoms.

<u>Bullous Pemphigoid</u>

Similar to Pemphigus, Bullous Pemphigoid is a cutaneous blistering autoimmune disease.

Patients suffering from Bullous Pemphigoid do not typically experience mucosal lesions and the

disease is often marked by pruritic blisters on the trunk of the body, epithelial areas of the joints,

and the extremities. The autoimmune response in this case is characterized by the development

of Abs against Bullous Pemphigoid Ag 1 and 2 (BP230 and BP180 respectively), also known as

BPAG 1 and BPAG2, which compose part of the hemidesmosome and assist with cell to matrix

adhesion in the epithelium (Chan et al., 1998; Stanley, Hawley-Nelson, Yuspa, Shevach, & Katz,

1981). As a result, autoimmune response to differing collegen types is characteristically seen in

bullous pemphigoid. It is documented to have a higher incidence among the elderly and is

estimated to have an incidence in the general population of 6-7 cases per million. This estimate

increases to 150-330 cases per million in the population older than 80 years of age

(Kershenovich, Hodak, & Mimouni, 2014; Schmidt & Zillikens, 2013). With respect to the

environmental or genetic factors that could initiate an autoimmune response, there remains little

information on triggers. Our current understanding indicates that triggers for Bullous

Pemphigoid could include vaccines, medications, and possibly a variety of infections (Walmsley

& Hampton, 2011).

A clinical study was conducted by Giovanni Di Zenzo and his international associates

with the specific aim to appraise the development of IgG Abs during the course of Bullous

Pemphigoid development (Di Zenzo et al., 2011). Thirty-five patient volunteers diagnosed with

Bullous Pemphigoid were selected for this multicenter study along with a control group of fifty volunteers including both Bullous Pemphigoid patients and healthy individuals. All volunteers were assessed at the initiation of the study as well as one, three, six, and twelve months during the study while undergoing treatment. ELISA and immunoblotting assays specific for various domains on BP180 and BP230 were utilized to assess the Abs present in patient sera. Their study found that in three of the patients, BP180 Abs recognized the ectodomains of the protein before developing Abs specific for the intracellular domains. Intermolecular epitope spreading was also observed to occur during the course of the study. 17.6 percent of the volunteers developed an IgG response that initially recognized either BP180 or BP230, to recognizing both proteins. Analyzing the data collected during the study, it was observed that epitope spreading incidence was higher during the first three months after diagnosis compared to any other time during the study. It was noted that the spreading from ectodomains to intracellular domains and the incidence rates are correlated with the severity of Bullous Pemphigoid (Di Zenzo et al., 2011).

During a previous clinical study, Epitope spreading was observed in the Ab response progressing from collagen XVII to collagen VII (Fairley, Woodley, Chen, Giudice, & Lin, 2004). As has been previously mentioned, bullous pemphigoid has been observed to develop after diagnosis of pemphigus foliaceus (Maeda et al., 2006; Peterson et al., 2007). Another clinical report was submitted several years later with similar observations of intermolecular epitope spreading resulting in the development of bullous pemphigoid from pemphigus foliaceus, but with the additional information that the patient exhibited only IgG3 auto-Abs, not IgG1 (Recke et al., 2009). Antibody isotype switching was not noted during the 13-year course of clinical observation as normally would be the case. This remains an area for further research with regards to the role isotype switching, or the lack there of, has in epitope spreading.

1.1.5 Treatment Strategies for B cell Epitope Spreading: Current Concepts and Perspectives

Currently there exist few options for treatment of autoimmune diseases. Clinicians and researchers rely on generalized immunosuppressive treatments that are designed to block a broad-spectrum of potential risk factors associated with such diseases. Consideration of both efficacy and side effects of such treatments is vital. In a review published by Vanderlugt et. al. they discuss the role of tactics that can be designed to slow and/or neutralize the effects of epitope spreading in autoimmune function (C. L. Vanderlugt & Miller, 2002). Due to the unknown biologic pathway and change in antigenic specificity, it is difficult to design drugs that can treat epitope spreading. However, some of the available treatments have been demonstrated to affect epitope spreading.

The use of various drugs to control and inhibit both B and T cell activity is currently the most commonly used approach to autoimmune diseases, however these drugs typically suppress the entire immune system and are non-specific in their approach (Hauser et al., 2008; C. L. Vanderlugt & Miller, 2002). T-cells play a major role in recognizing foreign and self-Ags and thus promote a cytokine response due to changes in epitope specificity leading to autoimmune dysfunction. Vanderlugt et. al., utilizing EAE (experimental autoimmune encephalomyelitis) induced SJL mice and focusing on co-stimulatory signaling centers of CD28-CD80/86 and CD154-CD40, established anti-CD80 Ab fragments and monoclonal anti-CD154 Abs that were administered during peak phase or during relapse of EAE. Both anti-CD80 and anti-CD154 Abs showed significant inhibition of disease relapse. Epitope spreading has been thoroughly studied in EAE mice, which serve as a model for multiple sclerosis. Other drugs such as interferon pathway regulatory agents could provide a further targeted approach to management of autoimmune activity (Dreyfus, 2011; Lichtman, Helfgott, & Kriegel, 2012). Most recently

researchers are working to develop Th2 bystander suppression drugs to combat epitope spreading, disease-specific immunologic drugs, and targeted cytokine inhibitors (C. L. Vanderlugt & Miller, 2002). These treatments are not in common use at this point, and there is very little available data regarding their effects on epitope spreading.

Rituximab and B Cell Depletion Therapy

Rituximab is becoming a commonly used immunosuppressive. It utilizes a CD20 binding protein designed to deplete mature B cells. In one report discussing the multi-type efficacy of Rituximab, patients with pemphigus, a severe autoimmune disease resulting in blisters of the skin and skin and mucosal erosion, were treated with rheumatoid arthritis (RA) dosing schedules of Rituximab. At 6 months of dosage treatment 90% of patients achieved remission. At 22 months there was a 67% relapse rate with patients, however Global CD4+T cell numbers were preserved up to 3 months after treatment (Leshem et al., 2014). In conjunction with corticosteroids and other cytotoxic agents, Rituximab has also been used to treat SLE and other Ab mediated autoimmune diseases (Bekar et al., 2010; Leandro, Cambridge, Edwards, Ehrenstein, & Isenberg, 2005).

Other B cell depleting therapies include ocrelizumab and epratuzumab, which bind CD20 and CD22 respectively (Kamal, 2014). Use of B-cell depletion therapy may be helpful for patients that don't respond to other immunosuppression therapies (Leandro et al., 2005; Looney et al., 2004; C. L. Vanderlugt & Miller, 2002). Use of rituximab could potentially inhibit or impede B cell epitope spreading due to the depletion of B cells during the course of therapeutic treatment. However, there is no current evidence to support that B cell depletion significantly disrupts B cell epitope spreading.

Belimumab and Cytokine Inhibition Therapy

A promising treatment for SLE, Belimumab is a monoclonal Ab that binds B lymphocyte stimulators (BLyS). This type of therapy prevents the differentiation and survival of B cells. Used in various clinical studies, Belimumab has become recognized as a treatment with high efficacy and tolerability (R. Furie et al., 2011; Kamal, 2014; La Cava, 2010). A large multicenter phase III study performed on Belimumab by Furie et al. demonstrated the efficacy of the therapy. 819 anti-nuclear Ab or anti-double stranded DNA Ab positive patients participated in the randomized placebo controlled study over a period of 52 weeks. Those treated with non-placebo participants were randomly given a high or low dose of Belimumab, 10 mg/kg and 1mg/kg respectively. At one-year post treatment start of the study it was found that the risk of severe flares was reduced by 34% (P=0.023) for the low dose group and 23% (P=0.13) for the high dose group. It was also calculated that the SRI rates were 44%, 51% (P=0.0129), and 58% (P=0.0006) for the placebo, low, and high dose groups respectively (R. Furie et al., 2011). Similar results have been observed in other studies and suggest that Belimumab could be a good alternate treatment option for SLE patients.

Blisibimod and Tabalumab are two other BLyS targeting Abs that are currently in clinical trials and show promise for the treatment of SLE. In a recent randomized, placebo controlled clinical phase II study, 547 SLE patients were treated with Blisibimod at one of three dose levels and monitored over a period of 24 weeks. It was reported that the most effective dose was the highest at 200 mg/kg and that it significantly reduced levels of anti-double stranded DNA Abs (p<0.001) after 24 weeks. This dosage also improved levels of C3 and C4 (p<0.01 and p<0.001, respectively). It was also observed that the treatment significantly lowered levels of B cells (p<0.001). The results of the study suggests that at 200 mg/kg, Blisibimod is most efficacious for patients with severe SLE (R. A. Furie et al., 2014).

Atacicept is a biologic treatment option that is in clinical trials and has been studied for the treatment of SLE, RA, and MS (M. C. Genovese, Kinnman, de La Bourdonnaye, Pena Rossi, & Tak, 2011; Isenberg et al., 2014; Kappos et al., 2014). Due to its fusion protein construction it inhibits both BLyS and A proliferation inducing ligand (APRIL), which results in the prevention of symptoms similar to other BLyS targeting therapies, inhibiting B cell differentiation. A phase II clinical study published by Genovese et al. reported that Atacicept showed no significant efficacy compared to the placebo group used in the study (M. C. Genovese et al., 2011). But, they did demonstrate that Atacicept lowered rheumatoid factor and immunoglobulin levels even though it did not decrease the levels of anti–citrullinated protein Abs. It was observed in a recently published double blind, randomized, placebo controlled clinical phase II study of Atacicept for the treatment of MS, that the treatment adversely affected the patients treated compared to the control group (Kappos et al., 2014). The results of a randomized, double blind, placebo controlled clinical study to determine the efficacy in treating SLE patients were recently published by Isenberg et al. In this 52-week study 461 SLE patients were treated with 75 mg/kg, 150 mg/kg, or the placebo. They reported that the high dose group, 150 mg/kg, demonstrated lower flare rates verses the placebo group (P=0.002). Both the low and high dose treated groups showed reduced levels of total Ig and anti-double stranded DNA Abs (Isenberg et al., 2014). The results of these recent studies suggest that Atacicept could be a possible biologic treatment option for SLE. There is no published evidence to demonstrate that BLyS and APRIL therapeutic strategies inhibit or impede B cell epitope spreading, aside from the lack of clinical signs and symptoms demonstrated by patients during the clinical trials. It does stand to reason that such treatment options would prevent or disrupt epitope spreading, inhibiting disease progression.

Abatacept and Co-receptor Inhibition

A further strategy of treatment is utilized by Abatacept, which inhibits T cell activation by binding the CD28 receptor on B cells. Approved by the FDA in 2005, Abatacept is currently used in the treatment of RA and to slow the progression of joint damage. In a recent phase III clinical study comparing Abatacept to Adalimumab, a tumor necrosis factor α (TNF- α) inhibitor approved for the treatment of RA in 2002, demonstrated that subcutaneous Abatacept produced similar results in the treatment of RA (Weinblatt et al., 2013). In a multicenter, phase IIb, randomized, double-blind, placebo controlled study of Abatacept in the treatment of SLE it was observed that patients treated experienced a similar frequency of adverse events, and that these adverse events were typically worse in the treatment group compared to the placebo group (Merrill et al., 2010). While there is no evidence demonstrating this treatment inhibits epitope spreading, such a strategy shows promise as an effective way to inhibit disease progression and possibly impede B cell epitope spreading in patients with RA (Mease et al., 2011).

1.1.6 Summary

Over the past two decades our understanding of B cell epitope spreading has broadened substantially. It is well understood how the process of epitope spreading contributes to pathogen clearance and several factors that contribute to its effectiveness. Molecular mimicry and intrastructural T cell help allow B cells to expand their Ab repertoire, which diversity results in higher affinity Abs and a more effective immune response. Through various intracellular processes, stimulated B cells can generate novel target epitopes and present them in the context of MHC class II molecules to T cells. During B cell endocytosis, an Ag is endocytosed after stimulation of sIg molecules on the cell surface. GILT, endocytic proteases, and lysosomal cathepsins selectively process the Ag. After which a digested peptide is selectively loaded into the MHC II molecule post LI degradation and CLIP dissociation, allowing for various epitopes

to be displayed by MHC class II molecules. Through somatic hypermutation a variety of higher affinity Abs are produced granting opportunity for further diversification of epitope targets.

While this process contributes positively to our systemic defense against pathogenic organisms and macromolecules, it is also responsible for the progression of autoimmune diseases after self-tolerance is broken. Evidence from clinical studies and reports, a variety of animal models, and serological collection analysis has yielded substantial evidence indicating such. Both intermolecular and intramolecular epitope spreading are observed to happen congruously with the progression of autoimmune disease. Potential auto-Ags are more likely to become Antigenic targets to initiate epitope spreading after modification by reactive oxygen species (Chung & Utz, 2004; Kurien & Scofield, 2008) and lipid oxidation products(Scofield, Kurien, et al., 2005) or abnormal apoptosis (van Bavel et al., 2010) and citrullination (Kidd et al., 2008). Various other environmental triggers for initiating epitope spreading in autoimmune afflictions have been identified including assorted viruses, bacterial infections, and stress (Kivity et al., 2014; B. D. Poole et al., 2006). Currently we lack treatments that can effectively stop detrimental B cell epitope spreading. It can be considered that various treatments, B cell depletion and immune repression included, partial impede epitope spreading (Choi, Kim, & Craft, 2012).

There are many aspects that still remain to be explored and would contribute to future understanding of epitope spreading while the role of T cell interaction in epitope spreading is fairly well understood, less work has been done investigating the role of B cells in epitope spreading. Future research opportunities regarding B cell endocytosis include investigating into the currently unknown regulatory factors that are involved in the loading of MHC class II molecules and competitive inhibitory processes that regulate the selective processing of Antigenic peptides by cathepsins. It is also unclear how various compartments in the endocytic

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pathway target each other. The extent to which B cell epitope spreading contributes in the progression of many autoimmune diseases is still not well understood and could be better researched. Comprehension of patterns involved in epitope spreading to allow the prediction and potential therapy of epitope spreading in autoimmune diseases is also lacking. Treatments to inhibit epitope spreading or eliminate the detrimental aspects of epitope spreading are areas where future research could be conducted.

Section 2: Known Effects, Indicators, and Prevalence of Depression in Lupus Patients

Systemic lupus erythematosus is a widespread, often devastating autoimmune disease. It is characterized by a diverse symptomatology, including arthritis, sun sensitivity, kidney damage, hematologic manifestations, immunological manifestations, and neuropsychiatric involvement. Symptoms can vary widely between patients. Genetic, environmental and hormonal factors all contribute to lupus etiology.

Neuropsychiatric manifestations (NPSLE) are common in lupus. Major depression, obsessive-compulsive disorder, and generalized anxiety disorder are the most common manifestations of mood and anxiety disorders found in lupus, occurring in up to 47% of patients (Postal et al., 2016; Uguz, Kucuk, Cicek, Kayhan, & Tunc, 2013). Depression and fatigue due to lupus are among the most important factors in lowering the quality of life for lupus patients (S. T. Choi et al., 2012; Hanly et al., 2010; Yilmaz-Oner et al., 2015). These symptoms normally present early in the disease progress and are frequently found at diagnosis (De Marcaida & Reik, 1999). Some studies show a correlation between neuropsychiatric symptoms and disease activity, with depression and anxiety being more common during active disease (Nery et al., 2007; Segui et al., 2000). Other studies have not found associations between disease activity and depression (van Exel et al., 2013), although still finding high prevalence of neuropsychiatric symptoms.

The depression associated with lupus is thought to be dependent on psychosocial factors, iatrogenic effects and effects related to immunological anomalies associated with autoimmunity (Agmon-Levin, Shaye, & Shoenfeld, 2009). Depression assessment and adequate treatment is still a challenge given the emotional burden of the disease (Fonseca, Bernardes, Terroso, de

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Sousa, & Figueiredo-Braga, 2014) but represents an opportunity for optimized clinical management and health system efficiency. A recent review (Palagini et al., 2013) concluded that although psychosocial factors are the most frequently reported, identification of specific biomarkers of depression is needed, since emerging evidence demonstrates an etiologic role for immune activation and pro-inflammatory cytokines in depression (Leonard, 2010). For instance, a study performed in patients with Multiple Sclerosis reported a positive correlation between depression and IFN-γ production, and showed that antidepressants administration decreased IFN-gamma production (Mohr, Goodkin, Islar, Hauser, & Genain, 2001).

1.2.1 Type 1 Interferon

Type one interferons are primarily antiviral cytokines. Stimulation of any of a variety of pattern-recognition molecules such as toll-like receptors leads to activation of Interferon regulatory factors, which activate interferon expression. Interferon then binds to Interferon receptors (such as IFNAR1), leading to signal transduction and cellular responses, especially an antiviral state.

There are multiple lines of association between type 1 interferon and systemic lupus erythematosus (Niewold, Clark, Salloum, & Poole, 2010). Genetic factors, clinical findings, and studies in lupus mouse models all point towards an association between IFN and lupus. Lupus genetics is a complex area, with at least forty different loci being associated with risk for lupus (Ghodke-Puranik & Niewold, 2013). Many of these are associated with the IFN pathway (Ghodke-Puranik & Niewold, 2013). These include the transcription factors IRF5 (Graham et al., 2006; Graham et al., 2007; Kawasaki et al., 2008; Niewold, Kelly, et al., 2008; Reddy et al., 2007), IRF7 (International Consortium for Systemic Lupus Erythematosus et al., 2008; Salloum et al., 2010; Suarez-Gestal et al., 2009), IRF8 (Lessard et al., 2012), the signal transducers

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STAT4 (Abelson et al., 2009; Sigurdsson et al., 2008), Tyk2 (Sigurdsson et al., 2005), the toll-like receptor 8 and 9 (Armstrong et al., 2009; C. J. Xu et al., 2009), and the intracellular sensor IF1H1 (Zouk, Marchand, & Polychronakos, 2010).

One of the most striking findings of expression profiling in the peripheral blood of lupus patients is the presence of a marked interferon signature (Crow, Kirou, & Wohlgemuth, 2003; Crow & Wohlgemuth, 2003; Feng et al., 2006; Kirou et al., 2004; Kirou et al., 2005), where the cells express genes that are activated by interferon. IFNα regulates some of the most overexpressed genes observed in SLE patients (Crow et al., 2003). These IFNα induced genes are observed to be significantly higher in SLE patients compared to other interferon inducible genes (IFIGs), such as those regulated by IFNγ (Kirou et al., 2004). Expression studies using the MRL/lpr lupus mouse model is consistent with these conclusions (Kwant & Sakic, 2004; Y. Li et al., 2015). Other studies have similar findings, identifying type I IFNs as the main triggers for IFIGs and showing that these increased levels correlate with disease development in SLE (Dall'era, Cardarelli, Preston, Witte, & Davis, 2005; Kirou et al., 2005).

Serum levels of IFN are often higher in lupus patients than controls (Niewold, Adler, et al., 2008; Niewold, Hua, Lehman, Harley, & Crow, 2007; Niewold, Kelly, et al., 2008). The IFN signature and serum IFN levels have been shown to correlate with disease activity (Dall'era et al., 2005), although they are not a consistent predictor of disease flare (Mackay et al., 2016). There is certainly is a range of IFN levels displayed by SLE patients with some demonstrating an extremely high IFN profile while others seem closer to that of control patients in cohort studies. Niewold et al found that high IFNα levels were a heritable trait (Niewold et al., 2007). Their findings indicate that elevated expression of IFNα can be considered a risk factor for SLE.

1.2.2 Contribution of IFN to SLE-associated depression

It is easy to hypothesize IFN as a cause of NPSLE. Administration of therapeutic IFN to treat viral infections or cancer can cause neuropsychiatric symptoms (Hoyo-Becerra, Schlaak, & Hermann, 2014; Raison, Demetrashvili, Capuron, & Miller, 2005). Genetic disease that result in excess IFN causes several neuroinflammatory disorders (McGlasson, Jury, Jackson, & Hunt, 2015).

IFN has been associated with SLE neuropathology perhaps as early as IFNα has been correlated with SLE disease activity (Hooks et al., 1979; Ytterberg & Schnitzer, 1982). Several studies identified IFN in the spinal fluid and central nervous system (CNS) of SLE patients that were displaying neuropsychiatric manifestations (Lebon, Lenoir, Fischer, & Lagrue, 1983; Schaefer et al., 2002; Winfield et al., 1983). The presence of IFN during neuropsychiatric episodes and the subsequent lack of IFN once the neuropsychiatric symptoms passed is evidence for the hypothesis that IFN plays an important role in SLE associated psychiatric conditions (Isshi, Hirohata, Hashimoto, & Miyashita, 1994; Lebon et al., 1983). One study, conducted by Shiozawa et al. investigated further the IFNα detected in the CNS of SLE patients (Shiozawa, Kuroki, Kim, Hirohata, & Ogino, 1992). After comparing IFN levels of patients exhibiting neuropsychiatric symptoms and their SLE psychiatric control groups, it was observed that only those SLE patients exhibiting psychosis had significantly elevated levels of IFNα (Shiozawa et al., 1992). Other studies have further confirmed that IFNα levels are associated with chronic neuropathology, including in SLE (Jonsen et al., 2003; Morris, Berk, Galecki, Walder, & Maes, 2016). A recent study performed by Mostafavi et al. used a whole transcriptome gene expression approach to study major depressive disorder (MDD) (Mostafavi et al., 2014). Their study used 463 identified MDD patients and 459 control patients who had never experienced a 2-week depressed mood and two or more of the MDD criteria. Using RNASeq methods and analyzing

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whole blood to reflect the physiological state of the patient participants, their study found a significant association with genes in the IFNα/β signaling pathways (Mostafavi et al., 2014). While other IFNs may play a role in SLE-associated depression, it seems that type I IFNs play the largest role in the neuropathology of depression in SLE patients.

Several studies have shown little or no direct evidence for a role for IFN in NPSLE (Brey et al., 2002; Kirou et al., 2004; Morris et al., 2016; Uguz et al., 2013). A clinical study conducted by Kellner and colleagues investigated the relationship between IFN, based on type I IFN stimulated genes (ISGs) measured via pPCR, and depression (Kellner et al., 2010). Their cohort consisted of 58 patients that met the American College of Rheumatology classification criteria for SLE, and twenty other volunteers, chosen as healthy controls. Depression was measured using the Beck Depression Inventory (BDI) system. BDI levels did not correlate with disease severity indicators including SLE disease activity index (SLEDAI) scores, organ-specific symptoms, complement levels, ACR criteria count, or autoantibody profiles. There was a significant increase in ISGs from SLE patients in the study, as would be expected compared to the control group.

To analyze if SLE patient IFN levels correlated with depression, a composite IFN score was derived for each patient based on the measured ISGs. An IFN score 2 standard deviations higher than the control was considered abnormally high. They categorized patients into two groups, those with high IFN scores and those with normal (or close to normal) IFN. These two groups were then compared. They found no significant difference in the BDI scores between the two groups. To further test the hypothesis, they performed a longitudinal study since IFN levels do fluctuate and could be the reason no significant difference was found between the high and low IFN groups. This longitudinal analysis consisted of 22 patients observed over a period of 3

to 6 months. While IFN levels and BDI scores did fluctuate during that period, there was still no significant difference observed between the IFN high and IFN low SLE groups. Kellner et al concluded based on their observations that type I IFN did not correlate to SLE-associated depression in their study (Kellner et al., 2010). Jarpa et al. while investigating major depression in SLE patients also found no correlation between depression and disease severity (Jarpa et al., 2011).

Although these studies indicated that there is no association between IFN and depression in lupus, there may be other ways IFN can contribute to NPSLE. Perhaps IFN plays a contributing role but is not one of the main factors contributing to SLE-associated depression. This principle was explained by Bertsias et al. as they described that it is not always possible to distinguish between inflammatory and thrombotic processes that contribute to depression (Bertsias & Boumpas, 2010). It is further possible that the healthy controls were experiencing elevated levels of IFN, as healthy individuals can still have elevated levels of IFN, raising the threshold for 'normal' IFN scores (Niewold et al., 2007). However, to account for these types of variables with a human cohort is either unpractical now, would require a greater comprehension of factors contributing to SLE-associated depression, or demand a longer-term analysis of an SLE cohort.

It may be the case that IFN causes neurological damage, which then persists, and is not influenced by fluctuations in cytokines or disease activity. This would provide a role for IFN in NPSLE but explain why ongoing levels of IFN do not correlate with psychiatric symptoms. Mouse models have found that treatment of disease after lupus has begun does not affect the neurological symptoms (Stock et al., 2015). Perhaps the damage is done early and persists despite subsequent changes in cytokine levels. Other mechanisms through which IFN could

modulate NPSLE include through stimulation of other cytokines (J. L. Taylor & Grossberg, 1998) and modulation of the hypothalamus/pituitary/adrenal axis (Gisslinger et al., 1993).

1.2.3 Mechanisms of IFN-induced psychological symptoms

The mechanisms responsible for IFN-induced NPSLE-associated depression may be similar to those observed in depression stemming from the administration of INFα as a therapeutic agent (Figure 4). IFNα causes an increase in indoleamine 2,3-dioxygenase (IDO) expression and overactivation in macrophage and microglial cells (Wichers & Maes, 2004). IDO is one of the enzymes that catalyze the rate limiting and first step of the kynurenine pathway. By diverting L-tryptophan, a serotonin precursor, into the kynurenine pathway- IDO lowers the overall production of serotonin (Meszaros, Perl, & Faraone, 2012). Further, IFNα is thought to increase guanosine triphosphate cyclohydrolase-1 (GHC1) levels via IFNγ in PBMCs (Hoyo-Becerra et al., 2014; Schoedon, Troppmair, Adolf, Huber, & Niederwieser, 1986). This enzyme converts GTP into BH2, lowering the amount of BH4 produced and causing lower levels of serotonin and dopamine to be produced. Type I IFN also enhances the reuptake of serotonin from the intracellular space by increasing the amount of serotonin transporter (SERT) that is expressed (Tsao et al., 2008). Thus, increased levels of IFNα could cause the neuropathology observed in SLE associated depression by lowering the amount of serotonin and dopamine that are produced by various cells and increasing the uptake of serotonin from the extracellular space (Figure 4).

To summarize, the connection between IFN and lupus presents an attractive hypothesis: that IFN is responsible for the psychiatric manifestations of lupus. IFN is known to cause depression, in both endogenous states and after therapeutic IFN administration. Therefore, it would make sense that IFN, which is abundant in lupus patients, could cause the depression,

anxiety, and other psychological symptoms associated with lupus. There is some support for this

hypothesis, specifically the finding of elevated levels of IFN in the cerebrospinal fluid of lupus

patients undergoing psychiatric problems and not others. However, subsequent studies

Figure 4. Pathways induced by INFα contributing to depression.
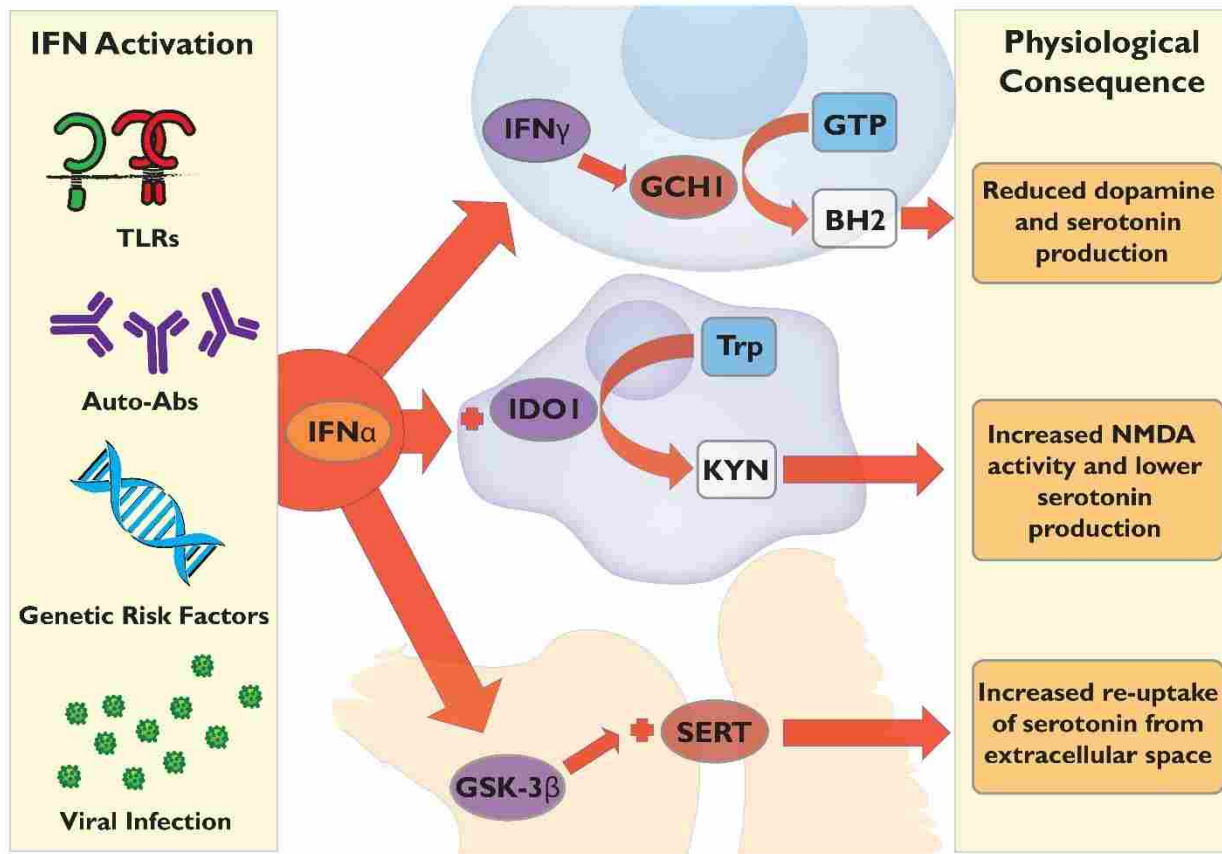
INFα induction in SLE patients is mediated a variety of ways including by auto-antibodies, genetic risk factors (i.e. IRF5, etc.), viral infection, and toll like receptors (TLRs) (i.e. TLR9, TLR7, etc.). INFα induces INFγ production and the subsequent conversion of Guanosine triphosphate (GTP) to 7,8-dihydroneopterin (BH2) by guanosine triphosphate cyclohydrolase-1 (GHC1). Increased production of indoleamine 2,3-dioxygenase (IDO1) is a result of IFNα, which leads to the sequestration of Tryptophan (Trp) to kynurenine (KYN) and the KYN pathway. Further, INFα induces increased production of serotonin transporter (SERT) via the glycogen synthase kinase (GSK)-3β-dependent pathway. These pathways result in lowered production and depletion of serotonin, as well as, decreased production of dopamine.

investigating such a link using peripheral levels of IFN or IFN response have for the most part not shown an association between levels of IFN and psychiatric symptoms. While the hypothesis that type I IFN contributes to SLE related depression is still worth pursuing, current data suggest that these IFNs would not be reliable biomarkers for neuropsychiatric disorders such as depression (Jarpa et al., 2011).

Several inhibitors of interferon, including antibodies and small molecule inhibitors, are currently in Phase II or III trials for treatment of lupus (Mathian, Hie, Cohen-Aubart, & Amoura, 2015). These treatments will provide the experimental basis for a definitive answer to the question of IFN involvement in NPSLE. If inhibition of IFN ameliorates neuropsychiatric symptoms more than traditional treatments, it will finally provide strong evidence for a role of IFN in lupus-associated psychological symptoms.

Section 3: Introduction to Epstein-Barr Virus and Herpesvirus Influence Upon Cellular Chemotaxis[2]

1.3.1 Epstein-Barr Virus

Prevalently infecting over 90% of the adult population, Epstein-Barr virus (EBV) is a common, often unnoticed companion (Amon & Farrell, 2005). Only able to replicate in human beings, this gamma-herpesvirus is genetically adept at immune evasion and persistence. These attributes allow it to be a near universal part of the human experience. The only certain exceptions are those with the genetic condition of X-linked agammaglobulinemia who lack mature B cells and cannot be infected by the virus (Faulkner et al., 1999).

EBV is an amorphic shaped enveloped virus which contains a layer of tegument protein surrounding a viral capsid housing the double stranded, 172 kb DNA genome. Typically, the virus infects B lymphocytes, but has the ability to infect certain types of endothelial cells (Toussirot & Roudier, 2008). The viral glycoprotein 350 binds to cellular CD21 and allows for viral penetration. In B cells, the MHC class II molecules act as cofactors during viral infection (Q. Li et al., 1997). The glycoproteins and cofactors allow the viral envelope to fuse with the cell membrane and release the tegument proteins and viral capsid into the cell cytoplasm. Upon entry, tegument proteins that are packaged as part of the viral particle, assist in degrading the capsid, avoiding immune detection, and transporting and circularizing the EBV DNA in the cell nucleus.

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[2] The majority of this section, comprising subsection 3.3, was published in the Journal of General Virology, see Cornaby, Tanner, Stutz, Poole, & Berges, 2016.

Often spread orally, EBV infects nasopharyngeal epithelial cells and mucosal B cells in the associated tissue (J. I. Cohen, 2000). During the immune response to clear the infection many infected cells are detected and killed, however a portion of infected cells remain. Once intracellularly contained in B cells the virus sets up a latent infection in the memory B cell population. By this means the virus can spread to the rest of the body as reactivation occurs and other B cells become infected. It also aids the virus in establishing a lifelong persistence in the host.

Latency and reactivation

Depending on the stage of maturation of the B cell infected, EBV expresses one of four different programs when establishing latency. In the latency 0 program, no genes are expressed. During latency I only one EBNA-1 is expressed. EBNA-1 binds to viral DNA and maintains the circularity of the genomic episome while binding to the OriP site to regulate replication of the episome during cell division (J. I. Cohen, 2000). In latency program II EBNA-1 and latent membrane proteins (LMP-1, LMP-2A, and LMP-2B) are expressed. LMP-1 is an oncogene that codes for a protein that mimics CD40 and binds to several tumor necrosis factors (Uchida et al., 1999). This causes an up regulation of adhesion molecules and several cytokines, while inducing B cell proliferation. LMP-2 prevents viral reactivation by blocking tyrosine kinase phosphorylation (Kutok & Wang, 2006; Merchant et al., 2001). In the final type of latency, type III latency, a total of nine proteins are expressed including all from the previous latency programs and EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-LP. EBNA-2 assists in up regulation of LMP-1 and LMP-2 expression and is the major viral transcription activator while EBNA-3A, 3B, and 3C play important roles in the transformation of B cells (Abbot et al., 1990; J. I. Cohen, 2000; White et al., 2010). It is suggested that they are active in repressing

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genes that would impede B cell proliferation. It is known that EBNA-LP acts as a co-activator with EBNA-2 and is responsible for switching on both the EBNA promoter and the LMP promoters (Ling et al., 2005; Tierney, Kao, Nagra, & Rickinson, 2011). Also expressed during all of the programs are the non-polyadenylated RNA transcripts EBER1 and EBER2 which assist in immune evasion (Maruo, Nanbo, & Takada, 2001; Young & Rickinson, 2004).

Infection of naïve B cells results in their transformation into lymphoblast B cells, expressing a type III latency program (Babcock & Thorley-Lawson, 2000). Later the viral expression pattern changes to that of a type II latency as the B cell differentiates and is induced by LMP-1 and LMP-2 to form germinal centers (Casola et al., 2004). After B cell differentiation to become a memory B lymphocyte, the viral proteins expressed will come to be more restricted as the virus exhibits a type I or type 0 latency program (Babcock & Thorley-Lawson, 2000; Thorley-Lawson, 2001). Upon latency set up inside memory B cells, they circulate undetected in the peripheral blood (Amon & Farrell, 2005). During infectious mononucleosis, it has been observed that only latently infected memory B cells are typically found in the peripheral blood (Hochberg, Souza, et al., 2004). It has been suggested that this could be due to the immune response in eliminating all infected lymphoblastoid cells prior to entering the peripheral blood and not purely due to the change of viral latency programs (Amon & Farrell, 2005). During regular B cell maturation to become a memory B cell, the cell is activated by foreign antigen and differentiates according to the signals received from various immune cells and tissue. An EBV formed memory B cell mimics this process, but the necessary signals are provided to the cell by the virus, not by necessarily by the extracellular environment (Y. J. Liu & Arpin, 1997; MacLennan, Liu, & Ling, 1988).

Reactivation of the virus occurs during activation of memory B cells, such as becoming plasma cells in response to a foreign antigen (Hochberg, Souza, et al., 2004). The viral episome shares similar nucleosome positioning and spacing as human DNA. Because of this it comes as no surprise that viral reactivation is regulated heavily by host proteins (Mansouri, Wang, & Frappier, 2013). It has been shown that the EBV EBNA-1 recruits several histone chaperone proteins including template-activating factor Iβ (TAF-Iβ), bromodomain-containing protein 4 (Brd4), and nucleosome assembly protein 1 (NAP1) to assist in activating BZLF1 by controlling histone dimethylation and acetylation (A. Lin, Wang, Nguyen, Shire, & Frappier, 2008; S. Wang & Frappier, 2009). BZLF1 as a homo-dimer is then able to activate various viral protein genes involved in the lytic cycle by binding to the AP-1 and BZLF1 response element sites on the EBV episome (Wille et al., 2013). BRF1, once expressed, assists BZLF1 in activation of the lytic cycle having a similar capability to bind many of these promoter sites (Wille et al., 2013).

Immune evasion

In order to establish a successful latent infection, EBV has many factors that contribute to its capacity to avoid immune detection and the subsequent death of the host cell. An effective strategy used by EBV to prevent detection of viral infection is to inhibit the processing of proteins and loading of MHC class I molecules. These MHC I molecules are constantly loaded with endogenous proteins processed from the proteome. During cellular infection, foreign proteins are processed and loaded in the MHC I molecules where they are transported to the surface of the cell. They are then scanned by cytotoxic T cells that recognize non-self-protein epitopes. Viral BNLF2a prevents the loading of the MHC I by blocking TAP, the protein that loads peptides into the MHC I, from binding to peptides and ATP (Horst et al., 2009). Due to this, there is a lower quantity of MHC I expressed on the cell surface. Other viral proteins

prevent their own loading into MHC I molecules, such as EBNA-1 (Yin, Manoury, & Fahraeus, 2003).

Several viral proteins also impair the cell from initiating or completing the caspase cascade that would result in cell apoptosis. BHFR1 inhibits cell apoptosis by acting as a homologue of the human B-cell lymphoma-2 protein (bcl-2), which inhibit cell death (Henderson et al., 1993). LMP-1 up regulates host bcl-2 and A20 to further inhibit this process (Kulwichit et al., 1998). EBV also influences production of pro-inflammatory cytokines. By lowering or keeping the inflammatory cytokine levels low during infection, less immune cells are recruited to assist in clearance of the infection. The viral protein BARF1 acts as a soluble receptor for human colony-stimulating factor 1 (CSF1) (J. I. Cohen & Lekstrom, 1999; Hsu et al., 1990). By binding up CSF1, BARF1 lowers the efficiency of CSF1 to induce a higher expression of interferon-α (INF-α). Another example of this type of viral manipulation is BCRF1, which mimics IL-10, an anti-inflammatory cytokine that inhibits interferon-y (INF-y) (K. W. Moore et al., 1990). It has also been observed that viral proteins manipulate cell chemotaxis, possibly to avoid detection (Ehlin-Henriksson et al., 2009; Gatto & Brink, 2013).

1.3.2 Epstein-Barr Pathogenesis and Disease

Mononucleosis

Most EBV infections occur in the first few years of life of an individual and are asymptomatic. However, some are exposed only later in life and experience an infection with a more pronounced immune response, resulting in infectious mononucleosis. Symptoms typically include fever, pharyngitis, and lymphadenopathy. The most likely cause of infectious mononucleosis according to several studies is kissing (Balfour et al., 2005). This conclusion is

reached based on the high titer of virus still orally shed while the disease was in convalescence and the patients were asymptomatic. During viral infection, those who experience the clinical symptoms have a heightened response of cytotoxic T cells and a subsequent cytokine mediated immune response (Callan et al., 1996). In this scenario, CD8+ cytotoxic T cells specific for the virus secrete INF-y and IL-2, driving the inflammatory response to the viral infection (McAulay et al., 2007; Sitki-Green, Edwards, Covington, & Raab-Traub, 2004). Studies also indicate that symptoms relating to the immune response are influenced by infection with more than one strain of EBV (Sitki-Green et al., 2004). Studies further indicate that certain MHC class I polymorphisms predispose individuals for infectious mononucleosis during viral infection (McAulay et al., 2007).

Malignancies

While Epstein-Barr virus is directly responsible for the symptoms elicited during viral infection in infectious mononucleosis, the virus is implicated in various other carcinomas, lymphomas, and disorders. It many cases it is unknown exactly how EBV is involved in the resulting conditions or to what extent, we do however know that EBV is associated and contributes to disease progression. Since EBV was discovered in Burkitt's Lymphoma cells, it has been suspected that EBV plays some role in the development of the disease (Hochberg, Middeldorp, et al., 2004; Young & Rickinson, 2004).

Burkitt's Lymphoma is a B cell lymphoma were the MYC oncogene has been translocated to be under control of the immunoglobulin chains, heavy or light (Z. Li et al., 2003; Polack et al., 1996). Many tumors also have mutations of the TP53, which inhibits cell division (Lindstrom & Wiman, 2002). EBV has been found in all patients with endemic Burkitt's Lymphoma (Young & Rickinson, 2004). It is suspected that these lymphoma cells originate in

germinal centers due to the profile bearing so much similarity with germinal centroblast cells (Chapman, Mockridge, Rowe, Rickinson, & Stevenson, 1995; Harris, Croom-Carter, Rickinson, & Neuberger, 2001). While it is not known what viral gene products contribute to the translocation or mutagenesis of lymphoma cells, several are suspected in various studies including EBNA-1 and the EBER RNAs (Kutok & Wang, 2006; Young & Rickinson, 2004). Burkitt's Lymphoma is also common among HIV carriers with 30-40% of tumors from AIDS patients being EBV positive (Davi et al., 1998; Young & Rickinson, 2004).

Another EBV associated disorder is Hodgkin's lymphoma. This is characterized by extremely large lymphocytes derived from B cells, called Reed-Sternberg (RS) cells (Kutok & Wang, 2006). These cells all have a mutated IgV sequence which contributes to the characteristics of the lymphoma and are also thought to have arisen from germinal center B cells (Kuppers, Hansmann, & Rajewsky, 1998; Kuppers & Rajewsky, 1998). About 40% of all lymphomas are EBV positive and in those tumors that are EBV positive, all of the RS cells are infected with EBV (Kutok & Wang, 2006; Young & Rickinson, 2004). EBV infection in these cells expresses a type II latency profile (Deacon et al., 1993). It is suspected that LMP-1 and LMP-2 could contribute to the mutagenesis of the IgV, but if EBV assists in tumor genesis besides this is unknown.

During epithelial cell infection, a possible outcome is the generation of malignant cells as a result of the viral infection. Nasopharyngeal carcinoma and gastric carcinoma are both potential cancers due to epithelial infection. Nasopharyngeal carcinoma contains malignant mucosal epithelial cells of the pharynx with infiltrating lymphocytes, which seem to be vital for the continued development of the cancer (Young & Rickinson, 2004). In these EBV malignant cells, EBV expresses EBNA-1, LMP-2A and 2B proteins along with BamHI transcripts (Raab-

Traub, 2002). Some EBV positive cells also show expression of LMP-1. It has been suspected that there is a genetic predisposition as those of Chinese descent have a higher incidence rate of the disease, regardless of the geographical region (M. C. Yu & Yuan, 2002). Gastric carcinomas also share a high rate of EBV positive tumors, with 10% of all gastric carcinoma malignancies being EBV positive (Shibata & Weiss, 1992). EBV gene expression in EBV gastric carcinoma is similar to that seen in nasopharyngeal carcinoma cells that are EBV positive. It is thought that EBV infection in gastric carcinogenesis is a later event during the malignancy formation.

Another possible malignancy can occur in transplant patients. Post-transplant lymphoproliferative disorder (PTLD) is an aggressive cancer for the immunosuppressed transplant patients. Most PTLDs are due to EBV infected B cells from the donor (Moosmann et al., 2010). If B cells are not removed from the donor transplant or if the T cell population is depleted, the risk of PTLD increases. Further EBV associated malignancies include Leiomyosarcoma, as well as, T cell and natural killer (NK) cell lymphomas.

1.3.3 Herpesviruses Influence Cellular Chemotaxis

1.3.3.1 Introduction

Cells respond to a variety of cytokines and chemokines that allow them to migrate in different areas in the body depending on where they are needed. This process is essential for appropriate tissue maintenance, homeostasis, formation, repair, and pathogen clearance (Turner, Nedjai, Hurst, & Pennington, 2014; Zhou et al., 2014). Dysregulation of the delicate balance of cellular signals and/or improper positioning could impede these processes. Aside from being related to a range of diseases, viral-induced chemotaxis contributes to the epidemiology and persistence of human herpesviruses. These viruses regulate a multitude of cellular genes that

direct cellular chemotaxis, thereby manipulating these genes for the benefit of the invading virus. Herpesviruses also produce various chemokines and chemokine receptors from genes in the viral genome, further affecting cellular chemotaxis. In essence, viral infection results in the piracy of cellular function as it directs cell movement in both infected and uninfected cell types.

The family *Herpesviridae* is divided into various subfamilies including *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* (Flint & American Society for Microbiology., 2009; Yoshida & Yamada, 2006). The nine human herpesviruses (HHVs) include herpes simplex virus type 1 (HHV-1 or HSV-1) and 2 (HHV-2 or HSV-2), Varicella Zoster virus (HHV-3 or VZV), Epstein-Barr virus (HHV-4 or EBV), human cytomegalovirus (HHV-5 or hCMV), human herpesvirus 6A (HHV-6A) and 6B (HHV-6B or Roseola virus), human herpesvirus 7 (HHV-7) and Kaposi's sarcoma-associated herpesvirus or human herpesvirus 8 (HHV 8 or KSHV) (Siakallis, Spandidos, & Sourvinos, 2009). All of these viral species share similar structural characteristics with a genome composed of double stranded DNA, an icosahedral capsid, an envelope studded with a variety of viral and host proteins, and viral tegument proteins in an amorphous layer between the capsid and envelope (Flint & American Society for Microbiology., 2009). Herpesviruses are able to remain latently infected in host cells for the life of the individual, during which time viral particles are undetectable but viral nucleic acids can be found and viral gene expression is very limited. Various stimuli can cause viral reactivation, wherein viral gene expression re-commences, and infectious particles can be detected and shed to new hosts. Herpesviruses encode a complex assortment of proteins that manipulate cellular functions during infection in order to promote viral persistence. Human herpesviruses are an integral part of human existence with over 90% of adults persistently infected with at least one or more of these nine herpesviruses in their lifetimes. Although the

incidence of serious herpesvirus-induced diseases is rare in most cases, the prevalence of infection is so high that the overall disease burden takes a toll on society.

It has been hypothesized that several herpesvirus species affect development or progression of diseases through interference with cellular chemotaxis, including lymphomas, atherosclerosis, autoimmune disorders, and disruption of angiogenesis (Coupland, 2011; Ehlin-Henriksson et al., 2009; Franciotta, Salvetti, Lolli, Serafini, & Aloisi, 2008; Rosenkilde & Schwartz, 2004; Stern & Slobedman, 2008; Streblow, Orloff, & Nelson, 2001; Velaga et al., 2009). In this review we will elaborate on the known human herpesvirus mechanisms and pathways that influence cellular chemotaxis during viral infection. Strategies for why herpesviruses potentially evolved these mechanisms will be presented as well as the resulting potential for their roles in disease development.

1.3.3.2 Alphaherpesvirinae

Herpes simplex virus 1 (HSV-1), Herpes simplex virus 2 (HSV-2), and Varicella Zoster virus (VZV) encompass the human pathogens of the *Alphaherpesvirinae* subfamily, typically showing lytic replication in epithelial cells and harbored as a latent infection in neuronal cells. HSV-1 is quite common in industrialized countries with a seroprevalence of around 90% (Viejo-Borbolla et al., 2012) in the adult population of industrialized countries. Symptoms of viral infection include cold sores and redness of the skin; however, many infections are asymptomatic. HSV-1 transmittance only occurs when viral production has increased during a lytic outbreak. The most common methods of transferring HSV-1 include direct skin contact and via saliva. Similar to HSV-1, HSV-2 can mask its presence from the host's immune system, demonstrating a preference to lay dormant in the sacral ganglia (HSV-1 in trigeminal ganglia) and manifest occasional lytic outbreaks, typically in the genital area. HSV-2 is one of the most common

Table 2. Alphaherpesviruses change cellular receptors/chemokines

Cell type	Receptor or chemokine	Virus	Amount or functionality	References
Monocyte	CXCR4	HSV-1, HSV-2	Increase	Bellner *et al.* (2005); Viejo-Borbolla *et al.* (2012)
Neutrophil	CXCR4	HSV-1, HSV-2	Increase	Bellner *et al.* (2005)
Epithelial	CXCL9	HSV-2	Increase	Huang *et al.* (2012)

sexually transmitted diseases with a seroprevalence of 12-20% in the United States. HSV-2 infection is of greater concern in developing countries where seroprevalence is much higher (Weiss, 2004; F. Xu et al., 2006). VZV primary infection results in the common childhood disease varicella (chickenpox) after which the virus establishes latency in the ganglia of a variety of neurons (Gilden, Nagel, & Cohrs, 2014). Reactivation of the virus results in zoster (shingles) and other chronic pain diseases, which can be manifest in various places on the epithelium (Gilden et al., 2014).

Until recently, not much was known about herpes simplex virus and how it affects chemotaxis (see Table 2). In 2012, Borbolla and associates (Viejo-Borbolla et al., 2012) showed that a secreted form of viral glycoprotein G (SgG) from both HSV-1 and HSV-2 binds chemokines with high affinity. Membrane-bound glycoprotein G (gG) was shown to be necessary for chemokine binding activity. They found that HSV SgG in both HSV-1 and HSV-2 increased chemotaxis of monocytes in infected individuals towards CXCL12 and that gG attaches at the surface of cells to glycosaminoglycans (GAGs) without negative effects on G-protein-coupled receptors (GPCRs). Another more recent study further investigated the mechanism by which viral SgG enhances chemotaxis. It was found that gG binds to GAGs which induces lipid raft clustering leading to increased CXCR4 incorporation. The conformational change causes an increase in functional chemokine-receptor complexes at the cell surface (Martinez-Martin et al., 2015). CXCL12 is the natural ligand for CXCR4 and is secreted

constitutively in a variety of tissues including the lymph nodes, bone marrow, lungs, and adrenal glands (Alkhatib, 2009; Luker & Luker, 2006). It is also known that CXCR4 signaling is important in modulating the survival of neuronal cells and modulating synaptic function(Nash & Meucci, 2014). The increased functionality of CXCR4 could potentially allow infected cells to migrate to these areas *in vivo*. By migrating to areas secreting CXCL12, infected cells could migrate to areas where cell movement could allow viral exposure to more target cells. Similar results had been observed by Bellner et al. when they tested the chemotactic ability of HSV-2 glycoprotein G (gG-2p20) (Bellner et al., 2005). Using isolated human neutrophils and monocytes, they found that these cell types followed a gradient of gG-2p20 via binding of the formyl peptide receptor (FPR) on the surface of these cells. While the chemoattractant properties have never been displayed using the full-length gG2 protein, several speculations can be made based on the findings which suggest that neutrophils and monocytes could be attracted to areas with infected cells expressing gG-2p20. This could possibly be beneficial for HSV-2 infection. Attracting a large number of phagocytic cells would increase tissue damage and activated cells, potentially enabling viral spread and propagation (Bellner et al., 2005). It was shown that gG-2p20 is an FPR-activating agonist. Activation of FPR *in vivo* led to the downregulation of other chemotactic receptors. These observations suggest the possibility that the change in expression could lead to impaired clearance of HSV-2 during infection (Bellner et al., 2005). In summary, a variety of studies have demonstrated the effectiveness of HSV-1 and HSV-2 in manipulating CXCR4 in infected cells.

HSV-2 has demonstrated the ability to manipulate chemotaxis via a host chemokine as well. A study performed by Huang and colleagues measured elevated expression of CXCL9 in the cervical mucosa of HSV-2-positive women (Huang et al., 2012). Further research confirmed

that HSV-2 regulated the expression of CXCL9 in human cervical epithelial cells by inducing the phosphorylation and translocation of C/EBP-β to the nucleus where it transactivates CXCL9. The known receptor for CXCL9 is CXCR3, which is expressed predominantly in non-resting T cells (Van Raemdonck, Van den Steen, Liekens, Van Damme, & Struyf, 2015). Expression has also been observed in epithelial, endothelial, fibroblast, and smooth muscle cells (Billottet, Quemener, & Bikfalvi, 2013; Van Raemdonck et al., 2015). This upregulation of CXCL9 was shown to result in increased migration of activated peripheral blood leukocytes (PBLs) and CD4+ T lymphocytes (Huang et al., 2012). Huang and associates postulate that HSV-2 is responsible for upregulating CXCL9, however, it was not shown what viral protein induced the expression or if the increase in CXCL9 expression was a cellular response to viral infection. The viral benefits for inducing migration of CD4+ T cells and PBLs to sites of infection are unclear. The ability of HSV-2 in regulating CXCL9 could be investigated more in depth as this is the only study demonstrating this type of subversion in epithelial cells.

Past research has also suggested that VZV could utilize glycoproteins as chemoattractants, inducing migration of polymorphonuclear leukocytes (Ihara, Yasuda, Kamiya, Torigoe, & Sakurai, 1991). No other recent research has been conducted to determine if VZV affects chemotaxis of other infected cell types, although several studies and a case study do provide evidence for how VZV might influence cellular chemotaxis(Desloges, Schubert, Wolff, & Rahaus, 2008; Shavit, Shehadeh, Zmora, Avidor, & Etzioni, 1999; Steain, Gowrishankar, Rodriguez, Slobedman, & Abendroth, 2011) We now understand that HSV-1 and HSV-2 can manipulate monocytes through increasing the functionality of CXCR4 by making lipid rafts with the viral SgG protein. HSV-2 can further change the migration of cells by increasing the

expression of CXCL9 in infected epithelial cells, potentially attracting CD4+ T cells and PBLs to sites of infection.

1.3.3.3 Betaherpesvirinae

<u>Human Cytomegalovirus</u>

Also known as human herpesvirus 5, human cytomegalovirus (hCMV) is a prominent member of the *Betaherpesvirinae* subfamily. With a seroprevalence worldwide ranging from 45-100%, hCMV is a common human pathogen that is often asymptomatic in infected adults and children (Cannon, Schmid, & Hyde, 2010; Chen, Jiang, Lee, Liu, & Zhou, 1999; McGavran & Smith, 1965). hCMV has gained public scrutiny and awareness due to further understanding of its prevalence in causing congenital infections leading to birth defects (Bialas, Swamy, & Permar, 2015). In the United States it is a more common cause of birth defects than many others including fetal alcohol syndrome, Down syndrome, spina bifida, HIV/AIDS, Haemophilus influenza type B and congenital rubella syndrome (Cannon & Davis, 2005). Like other herpesviruses, hCMV is associated with various post-transplant complications and is a main viral cause of solid organ transplant and hematopoietic stem cell transplant morbidity and mortality (Ariza-Heredia, Nesher, & Chemaly, 2014; Gandhi & Khanna, 2004). It is also known to cause severe disease in other immunocompromised individuals such as AIDS patients. Viral shedding can occur via saliva, urine, breastmilk, semen, and tears. hCMV is known to infect various cell types including epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, dendritic cells, and lymphocytes, typically remaining latently infected in the latter cell type for the life of the host.

Using a variety of viral proteins to manipulate migration of host cells and potential target cells, hCMV uses both surface receptors and secreted chemokines (see Table 3 and 4). Among

Table 3. Human herpesvirus-encoded chemokine receptors

Virus	Viral receptor	Result	References
hCMV	US27	Potentiates CXCR4, increases migration to various tissues	Arnolds *et al.* (2013)
	US28	Migration of infected cells to areas of inflammation	Streblow *et al.* (1999); Vomaske *et al.* (2009)
	UL33 and UL78	Prevents migration to sites of inflammation and certain tissues	Tadagaki *et al.* (2012); Tschische *et al.* (2011)
HHV-6A	U51	Prevents NK cell interaction and prevents apoptotic signals	Catusse *et al.* (2008); Fitzsimons *et al.* (2006)
HHV-6B	U12	Migrates to inflammatory and T cell-rich zones	Isegawa *et al.* (1998)
HHV-7	U51	Migration of infected cells to T cell-rich and inflammatory areas	Nicholas (1996); Tadagaki *et al.* (2005)
	U12		
KSHV	KSHV-GPCR	Increases cell survival	Couty *et al.* (2009); Pati *et al.* (2001); Shepard *et al.* (2001)

the viral chemokines secreted by hCMV-infected cells are the products of the UL128 and UL146 genes. Numerous studies have been performed demonstrating how these viral gene products affect the migration of hCMV-infected cells. It had been noted that hCMV-infected monocytes demonstrated a reduced chemotactic ability due to a downregulation of CCR1, CCR2, and CCR5 (Frascaroli et al., 2006). A similar downregulation of various chemokines was observed along with an increase in migratory inhibitory factor (MIF) in hCMV-infected macrophages, resulting in a lack of motility (Frascaroli et al., 2009). Later it was demonstrated by Frascaroli and associates that in the presence of UL128 there was a resulting downregulation of CCR1, CCR2 and CCR5 in monocytes (Straschewski et al., 2011). Because of this impairment, monocytes could no longer migrate following the chemokines CCL5 and CCL2 which are ligands of the aforementioned receptors. CCL2 and CCL5 are known to be involved in the recruitment of monocytes and T cells and are secreted as pro-inflammatory cytokines in response to tissue damage or viral detection (Ansari, Kamarulzaman, & Schmidt, 2013; Soria & Ben-Baruch, 2008). Recently, using a UL128 transfected cell line (CHO-UL128) to produce UL128, Gao et al. studied the effects of this β chemokine on cell migration (H. Gao, Tao, Zheng, Xu, & Shang,

2013) (H. Gao et al., 2013). They found that UL128 acted as a chemoattractant for peripheral

blood mononuclear cells (PBMCs) *in vitro* and functioned similarly to CCL3 as a

chemoattractant. These results suggest that UL128 could act to prevent chemotaxis of monocytes

following other gradients, such as CCL5 and CCL2, and could use a separate receptor to attract

the monocytes to areas of infected cells (H. Gao et al., 2013). This increases the cells available to

 be infected by hCMV, potentially furthering viral spread. The other known chemokine

produced, UL146, codes for an α chemokine and viral homologue to CXCL1 (vCXCL1)

(Penfold et al., 1999). Several studies demonstrated that vCXCL1 could induce the chemotaxis

of neutrophils *in vitro* (Luttichau, 2010; Penfold et al., 1999). It was found that vCXCL1 was a

ligand for CXCR1 and CXCR2 using calcium mobilization, chemotaxis, and

phosphatidylinositol turnover assays. CXCR1 and CXCR2 are both expressed on neutrophils and

it is expected that hCMV-infected endothelial cells express vCXCL1 as a chemoattractant to

increase the numbers of neutrophils and assist in viral spread to other endothelial cells

(Luttichau, 2010). In a study conducted by Smith et al. it was observed that hCMV-infected

Table 4. Human herpesvirus-encoded chemokines

Virus	Viral chemokine	Result	References
VZV	GP I and GP II	Chemoattractant for polymorphonuclear leukocytes	Ihara *et al.* (1991)
HSV	gG2	Chemoattractant for monocytes	Bellner *et al.* (2005)
hCMV	UL146	Chemoattractant for neutrophils	Lüttichau, (2010); Penfold *et al.* (1999)
hCMV	UL128 and UL146	Attracts PBMCs and prevents monocyte migration	Frascaroli *et al.* (2006); Gao *et al.* (2013); Straschewski *et al.* (2011)
HHV-6A	U83A	Chemoattractant for T cells, monocytes and immature dendritic cells	Catusse *et al.* (2007); Dewin *et al.* (2006)
HHV-6B	U83B	Chemoattractant for monocytes	Clark *et al.* (2013); Lüttichau *et al.* (2003)
KSHV	vCCL1	Chemoattractant for monocytes	Nakano *et al.* (2003); Weber *et al.* (2001)
KSHV	vCCL2	Prevents Th1 T cell chemotaxis and attracts monocytes	Nakano *et al.* (2003); Weber *et al.* (2001)
KSHV	vCCL3	Inhibits chemotaxis of Th1 T cells and NK cells	Lüttichau *et al.* (2007)
KSHV	vIL-6	Attracts endothelial cells	Wu *et al.* (2014)

monocytes induced transendothelial migration *in vitro*, although the viral mechanism is unknown (Smith, Bentz, Alexander, & Yurochko, 2004).

Regulating host cell chemokines can also result in chemotactic changes. hCMV UL144 is a viral protein that activates NF-κB (E. Poole, King, Sinclair, & Alcami, 2006) (see Table 5). This leads to a cascade of multiple pathways, including induced expression of host CCL22 which acts as a chemoattractant for Th2 and regulatory T cells (T_{regs}). By recruiting these cells to sites of viral infection it is possible to suppress T helper and CD8+ T cells, tapering the immune response (Fielding, 2015). It has also been found that granulocyte macrophage progenitors (GMPs) latently infected with hCMV demonstrate increased expression of CCL2 (Stern & Slobedman, 2008). CCL2 is a pro-inflammatory cytokine that acts as a chemoattractant to monocytes, macrophages, dendritic cells and T cells expressing CCR2. This increase in CCL2 acts to attract CD14+ monocytes to latently infected GMPs (Luther & Cyster, 2001; Rollins, 1997; Sozzani, 2005; Stern & Slobedman, 2008). This behavior of latently infected GMPs is likely a viral strategy employed to recruit new leukocytes to be infected, however, too little is known about *in vivo* hCMV reactivation to know if this spread and reactivation occurs before or after GMPs develop into macrophages. Further research could be done into the manipulation of hCMV infected GMPs as there is currently just one study demonstrating this change in chemotaxis.

There are a variety of hCMV chemokine receptors shown to affect cell migration including US27, US28, UL33, and UL78 (Fielding, 2015), all of which are homologous to human GPCRs. US27 is expressed late during lytic infection and has no known ligand (Fraile-Ramos et al., 2002; Stapleton, Arnolds, Lares, Devito, & Spencer, 2012). However, it was found to potentiate CXCR4-mediated chemotaxis, increasing the expression and amount of surface

CXCR4 (Arnolds, Lares, & Spencer, 2013). As previously explained, CXCR4 is a seven membrane-spanning GPCR that allows the cell to follow the chemokine gradient of its natural ligand, CXCL12 which is secreted constitutively in a variety of tissues including the lymph nodes, thymus, bone marrow, lungs, and adrenal glands (Alkhatib, 2009; Luker & Luker, 2006). The potentiation of CXCR4 resulted in increased migration to CXCL12 during *in vitro* migration assays (Arnolds et al., 2013). It has been speculated that increased CXCR4 levels at appropriate times could allow hCMV-infected cells to migrate to bone marrow or lymph nodes where there would be an increased opportunity to spread to susceptible cells (Arnolds et al., 2013).

US28 was first shown to affect migration in vascular smooth muscle cells (SMCs) (Streblow et al., 1999). It was found that US28 directed cell migration following the chemokines CCL2 and possibly CCL5. In the absence of CCL2, there was no migration of hCMV-infected SMCs (Streblow et al., 1999). This would allow infected SMCs to migrate to areas of inflammation, potentially providing opportunity for viral spread to leukocytes. Later it was demonstrated that US28 acted to control migration of both infected SMCs and infected macrophages. Kledal and associates found that US28 also bound CX3CL1 (Kledal, Rosenkilde, & Schwartz, 1998), which is a chemokine that is found on the cell surface and extracellularly in a secreted form; this work was later followed up by others (Murphy, Caplice, & Molloy, 2008; Vomaske et al., 2009). CX3CL1 is only known to be produced by endothelial cells and results in the recruitment of inflammatory cells (Bazan et al., 1997; Vomaske et al., 2009). They found that the presence of CX3CL1 inhibited the migration of hCMV-infected SMCs, but induced the migration of hCMV-infected macrophages. It was also demonstrated that the inverse was true, in the presence of CCL5, hCMV-infected macrophages US28-mediated migration was inhibited, but hCMV-infected SMCs demonstrated normal chemotaxis as expected (Vomaske et al., 2009).

This makes the viral GPCR US28 unique in that it is chemokine and cell-type specific. It seems important for the virus to control cellular migration during hCMV infection. Evidence for how US28 functions was provided by Tschische et al. when they found that hCMV chemokine receptors heteromerize with each other (Tschische, Tadagaki, Kamal, Jockers, & Waldhoer, 2011). It was observed that UL33 and UL78 heteromerization resulted in silencing of US28 mediated activation of the NF-κB pathway. Tadagaki et al. investigated UL33 and UL78 and found that these two GPCR homologues formed heteromers with CCR5 and CXCR4 on the surface of infected THP-1 cells (Tadagaki et al., 2012). This was found to prevent cell chemotaxis utilized via CCR5 and CXCR4 *in vitro*. CCR5 allows the cell to follow a variety of chemokines including CCL3, CCL4, or CCL5, these being the best agonists, while CXCR4 is known to be chemoattracted to CXCL12 (Alkhatib, 2009). The majority of chemokines that act as CCR5 ligands are pro-inflammatory.

During hCMV infection the virus is able to regulate host receptors in various ways to prevent chemotaxis. It has been demonstrated that hCMV prevents CCR7 expression in monocyte-derived dendritic cells, preventing chemotaxis following CCL19 and CCL21 chemokine gradients *in vitro* (Moutaftsi, Brennan, Spector, & Tabi, 2004). hCMV-infected Langerhans cells also demonstrate reduced chemotaxis in response to lymphoid chemokines (Lee et al., 2006). After these observations it was found by Wagner et al. that hCMV UL18 inhibited chemotaxis of dendritic cells *in vitro* (Wagner et al., 2008). The extracellular UL18 is expressed late in hCMV infection and binds the LIR-1 molecule on the surface of dendritic cells. This results in various changes including reduced chemotaxis, increased pro-inflammatory cytokine production, upregulation of CD83 and inhibition of CD40 (Park et al., 2002; Wagner et al., 2008). It was also observed that hCMV-infected dendritic cells showed downregulated

chemokine expression and inhibited maturation due to vIL-10, a gene product of the hCMV

UL111A gene. Dendritic cells that were able to mature during hCMV-infection showed an

increase in chemotactic ability to follow the lymph node homing chemokine (W. L. Chang,

Baumgarth, Yu, & Barry, 2004). It has further been observed that chemotaxis is disrupted in

infected endothelial cells. Reinhardt et al. demonstrated how hCMV-infected human coronary

artery endothelial cell (HCAEC) chemotaxis to vascular endothelial growth factor (VEGF) is

inhibited (Reinhardt et al., 2014). HCAEC migration is important for repair post-vascular injury

(Deanfield, Halcox, & Rabelink, 2007; Waltenberger, 2007). While the observation explains how

hCMV can play a role in contributing to pro-atherosclerotic phenotypes, the viral strategy for

inhibiting HCAEC migration remains unknown. A further way to inhibit chemotaxis of cells is

by secreting chemokine binding proteins. hCMV-produced UL21.5 acts in this capacity by

binding CCL5, acting as a chemokine sink or decoy receptor (D. Wang, Bresnahan, & Shenk,

2004). This would prevent cellular receptors from being able to bind CCL5 and follow the

chemoattractant. hCMV also utilizes miR-UL148D to silence CCL5 protein synthesis in infected

cells(Kim et al., 2012). These studies emphasize the importance of CCL5 regulation during

hCMV infection. Preventing immune cell production and detection of CCL5 would assist in

preventing the attraction of monocytes and T cells to areas of hCMV infection. While a certain

number of monocytes would be beneficial for viral spread, an overabundance of monocytes and

the presence of T cells could result in the impairment of viral spread.

To better enhance viral spread, hCMV uses virally encoded chemokines UL128, UL146,

and vCXCL1 to attract target immune cells. The piracy of host chemokine CCL22 further assists

in this process. By upregulation of CCL22 in infected monocytes, T_{regs} are attracted and could

assist in downregulation of an immune response to viral infection. By increasing the

functionality of CXCR4, chemotaxis of virally-infected cells to other tissues could be encouraged. Manipulating host chemokine receptor CCR7, hCMV can avoid migration to primary and secondary lymph tissue, evading possible detection. The dysregulation of viral infected cell movement appears to allow hCMV the edge in evading immune detection and increase the opportunity for viral spread (see Figure 1). Future studies investigating the function of viral chemokines and chemokine receptors could examine their effects in vivo utilizing animal models as has been done with hCMV US28(Bongers et al., 2010).

Human Herpesvirus 6 (HHV-6)

Human herpesvirus 6 (HHV-6), initially named human B lymphotropic virus, was first discovered in 1986 in patients with lymphoproliferative disorders (Salahuddin et al., 1986). HHV-6A and HHV-6B were recognized as different variants of the same species in 1992 and in 2012 the International Committee on Taxonomy of Viruses classified them as two distinct viruses (D. Ablashi et al., 1993; Adams & Carstens, 2012). Because classification as two distinct viruses has come relatively recently, it makes it difficult to distinguish between HHV-6A and HHV-6B in some of the early literature. The seroprevalence of HHV-6 in adults worldwide is 83-100% (Hall et al., 2006).

HHV-6A

Human herpesvirus 6A is a betaherpesvirus that has primary tropism for CD4+ T cells and can also infect CD8+ T cells, natural killer (NK) cells, gamma/delta T cells, human neural stem cells, human progenitor-derived astrocytes, and oligodendrocyte progenitor cells (D. Ablashi et al., 2014; Lusso et al., 1991; Lusso, Garzino-Demo, Crowley, & Malnati, 1995; Lusso et al., 1993). It has also been shown to lytically infect B cells that have been immortalized with EBV (D. V. Ablashi et al., 1989). HHV-6A can alter the expression of different cellular markers
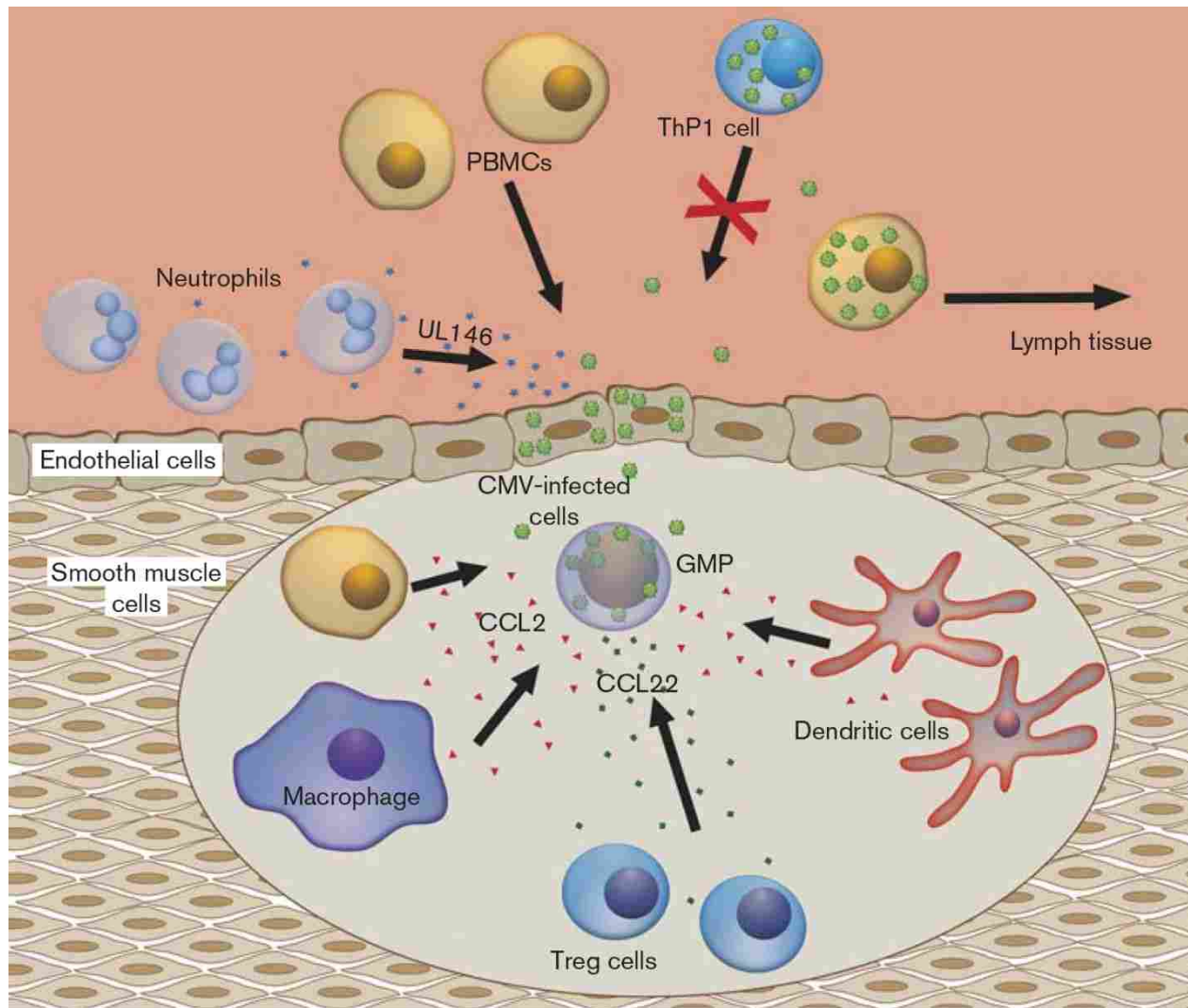
Figure 5. Changes in cellular chemotaxis resulting from hCMV infection.

hCMV uses virally encoded chemokines UL128, UL146 and vCXCL1 to attract target immune cells such as neutrophils and PBMCs. Increases in CCL2 expression further assist in attracting monocytes, macrophages and dendritic cells. Upregulation of CCL22 in infected monocytes attracts Treg cells, which can assist in downregulation of an effective immune response to viral infection. Increasing the functionality of CXCR4 allows virally infected cells to migrate to other tissues, including secondary lymphoid tissues.

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Table 5. Betaherpesviruses change cellular receptors/chemokines

Cell type	Receptor or chemokine	Virus	Amount or functionality	References
Dendritic cell	CCR7	hCMV	Decrease	Moutaftsi *et al.* (2004)
	CCR1	hCMV	Decrease	Varani *et al.* (2005)
	CCR5	hCMV	Decrease	Varani *et al.* (2005)
GMP	CCL2	hCMV	Increase	Stern & Slobedman (2008)
Monocyte	CCR1	hCMV	Decrease	Frascaroli *et al.* (2006, 2009);
	CCR2	hCMV	Decrease	Straschewski *et al.* (2011)
	CCR5	hCMV	Decrease	
	CCL22	hCMV	Increase	Poole *et al.* (2006)
T cell	CCL5	HHV-6A	Decrease/increase	Cerdan *et al.* (2001)
	CCR7	HHV-6	Increase	Hasegawa *et al.* (1994)
	CXCR4	HHV-6	Decrease	Yasukawa *et al.* (1999)
	CXCR4	HHV-7	Decrease	Yasukawa *et al.* (1999)
	CCR7	HHV-7	Increase	Hasegawa *et al.* (1994)

involved in cellular homing and trafficking which causes significant disruption to immune cell function and viability. The virus has been implicated in a number of diseases including multiple sclerosis, Hashimoto's thyroiditis, and AIDS. *In vitro* studies show that HHV-6A causes upregulation of CD4 on cells that do not typically express this marker, making these cells susceptible to HIV infection and possibly contributing in the progression to AIDS (Lusso et al., 2007; Lusso et al., 1991).

HHV-6 has one functional chemokine-like protein, U83 (see Table 4). The viral chemokine U83A from HHV-6A is involved in chemoattraction and has selective specificity for receptors CCR1, CCR4, CCR5, CCR6, and CCR8. These are found on T cells, monocytes/macrophages and activated T lymphocytes (CCR1, CCR5, CCR8), skin-homing T lymphocytes (CCR4, CCR8), immature dendritic cells (CCR1, CCR6), and skin-homing T lymphocytes and NK cells (CCR8) (D. Ablashi et al., 2014; Catusse et al., 2008; Dewin, Catusse, & Gompels, 2006). The difference in specificity of U83A (from HHV-6A) and U83B (from HHV-6B) to attract diverse cell types could account for the tropic variability of the two viruses

(D. J. Clark, Catusse, Stacey, Borrow, & Gompels, 2013). U83A is found in a full-length form as well as a truncated splice variant (French et al., 1999). It is thought that because of the different forms of the peptide, U83A could both block innate and adaptive immune responses, as well as attract the cells involved in these responses for further infection (Dewin et al., 2006). U83A induces chemotaxis and morphological changes in cells expressing CCR5 in a manner similar to CCL4, but with a significantly delayed internalization of CCR5 compared to that of CCL4. Interestingly, binding of U83A to CCR5 has been shown to inhibit CCR5 tropic HIV-1 infection (Catusse, Parry, Dewin, & Gompels, 2007).

HHV-6 has two GPCRs, U12 and U51, which encode chemokine receptors (see Table 3). U51, known to affect migration in HHV-6A infected cells, is expressed at early time points post-infection, whereas U12 is expressed late and influences chemotaxis of HHV-6B infected cells. HHV-6A U51A has novel specificity for CCL5 and can also bind CCL2, CCL11, CCL7 and CCL13. This makes U51A unique from other viral and cellular receptors in that it overlaps activity with CCR1, CCR2, CCR3, and CCR5 in the binding of CCL5 (Catusse et al., 2008). There is also overlap with CCR2, CCR4, US28, UL12, D6, and Duffy in the binding of CCL2; CCR3 and E1 in the binding of CCL11; CCR1, CCR3, US28, and D6 in the binding of CCL7; and CCR2 and CCR3 in the binding of CCL13. Unlike many viral GPCRs that have constitutive signaling, U51A has been shown to perform both inducible and constitutive signaling (Catusse et al., 2008; Fitzsimons et al., 2006).

U51A expression has been shown to cause a reduction of CCL5 expression using the Hut78 human CD4+ T lymphocyte cell line. U51A has high relative affinity for XCL1, which normally binds human receptor XCR1 found on NK cells and T lymphocytes. This binding could have a number of effects including: prevent infected cells from interacting with NK cells, induce

chemotaxis to T lymphocytes which could spread infection, and prevent apoptotic signals within infected cells (Cerdan, Devilard, Xerri, & Olive, 2001). CCL19, normally bound by human receptor CCR7, can also be bound by U51A. This could cause infected cells to migrate to the T cell-rich lymph node, again promoting further infection. HHV-6A U83A chemokine does not bind U51A. Expression of U51A ligands in the brain could also allow migration of infected cells into the central nervous system. Damaged epithelial lung cells and airway parasympathetic nerves express CCL2 and CCL11, which both bind U51A, and could promote migration of the infected cells to these areas to be transmitted from host to host.

CCR7, which is expressed in various lymphoid tissues, is another receptor that is modulated by herpesviruses (see Table 5). HHV-6A and HHV-6B upregulate CCR7 expression in CD4+ T cells (Hasegawa, Utsunomiya, Yasukawa, Yanagisawa, & Fujita, 1994). CCR7 is specific for CCL19 and CCL21 and plays roles in cell migration and proliferation (Tadagaki, Nakano, & Yamanishi, 2005). This upregulation of CCR7 could be an important aspect of HHV-6 pathogenesis as upregulation of CCR7 promotes migration of T cells and dendritic cells to the paracortex in lymph nodes (where T cell priming occurs) and the periarteriolar lymphoid sheath in the spleen, both of which are T cell-rich (Comerford et al., 2013).

As mentioned previously, HHV-6A can also downregulate cellular receptors. Along with downregulation of CD46 (its cellular receptor) and CD3 (Grivel et al., 2003), CXCR4 is downregulated by HHV-6A in primary CD4+ T lymphocytes and Jjhan T cells which affect the chemotactic response of the cells to CXCL12, the natural ligand of CXCR4 (Yasukawa et al., 1999). The disruption of CXCR4/CXCL12 signaling by downregulation of CXCR4 by HHV-6A could prevent the retention of hematopoietic stem/progenitor cells (HSPC) and more mature leukocytes in the bone marrow allowing these cells to be mobilized and enter into circulation

(Karpova & Bonig, 2015). The migration of CXCR4-expressing thymocytes out of the thymus was shown to occur in a CXCL12-dependent manner (Poznansky et al., 2002; Weinreich & Hogquist, 2008), so the downregulation of CXCR4 by HHV-6A could be another way the virus prevents migration away from areas where target cells are present. Additionally, the downregulation of CXCR4 by HHV-6A could prevent homing of bone marrow-derived precursor cells to the thymus (Calderón & Boehm, 2011), possibly preventing positive and negative selection from occurring in these cells.

HHV-6 has been shown to cause modulations to CCL5. This chemokine has selective chemoattractive activity on resting CD4+ memory T cells (Hasegawa et al., 1994) and has been shown to be upregulated by HHV-6 in an *ex vivo* study where human tonsil blocks were infected with both HHV-6 and HIV-1. This upregulation of CCL5 was shown to suppress HIV-1 CCR5-tropic variants and possibly to stimulate replication of CXCR4-utilizing variants, which gives evidence that HHV-6 may play a role in HIV pathogenesis by promoting the switch between CCR5-tropic to CXCR4-tropic HIV-1 (Grivel et al., 2001). In contrast, CCL5 expression in epithelial cells is downregulated by U51A from HHV-6A (Milne et al., 2000). Epithelial cells expressing U51A also had morphological changes and exhibited increased spreading and flattening, which could increase the ability of HHV-6 to spread to uninfected cells as it is primarily spread by cell to cell contact (Milne et al., 2000). As has been observed with other viral chemokines and chemokine receptors, their functions could be multipurpose in attracting cells to the area of infection, and also in evading the immune cells of the host so replication and latency can take place.

As described above, HHV-6A alters the expression of different cellular markers. Many of these markers are involved in cellular homing and tracking to specific areas of the body, and

when altered, can cause significant disruption to immune cell function and viability. Further research into HHV-6A effects on cellular trafficking could serve as a critical guide for developing new treatments to prevent these disease-causing disruptions.

HHV-6B

HHV-6B causes exanthem subitum (roseola) (Yamanishi et al., 1988) and is found in ~95-100% of adults worldwide. Unlike HHV-6A, HHV-6B has very little to no ability to infect CD8+ T cells, NK cells, and gamma/delta T cells (Grivel et al., 2003; Martin, Schub, Dillinger, & Moosmann, 2012). The cellular receptor for HHV-6B is CD134 which, like the cellular receptor for HHV-6A, CD46, is expressed on almost all human cells (Tang et al., 2013), indicating that other factors are at play for an effective viral infection to take place.

The HHV-6B viral chemokine U83B is specific for CCR2 and can cause chemoattraction of CCR2 expressing cells (classical and intermediate monocytes) for infection (D. Ablashi et al., 2014; D. J. Clark et al., 2013; Lüttichau et al., 2003) (see Table 4). U83 from HHV-6B induced transient calcium mobilization and efficient migration in THP-1 cells (a monocyte cell line derived from monocytic leukemia) (Zou et al., 1999). U83B has been shown to have a different specificity from U83A as U83B chemoattracts CCR2-expressing monocytes, whereas U83A has a broader but still selective specificity as mentioned previously (Catusse et al., 2008; Dewin et al., 2006). The specificity of U83B for CCR2 appears to be due to its N-terminal region. Human chemokines can induce rapid internalization of CCR2 upon binding, whereas *in vitro* experiments show U83B does not cause CCR2 internalization. This finding is similar to the delayed internalization of CCR5 observed with U83A. CCR2 expression is induced in pro-inflammatory conditions and interestingly HHV-6B is associated with inflammatory diseases such as encephalitis and myocarditis (D. J. Clark et al., 2013).

The HHV-6B GPCR U12 efficiently binds CCL2, CCL5, and CCL4 so it has overlapping activity with the receptors for CCL2 and CCL5 as in HHV-6A, but also has overlapping activity with the receptors for CCL4 (Balkwill, 2004; Isegawa, Ping, Nakano, Sugimoto, & Yamanishi, 1998) (see Table 3). The exact role of chemokine receptors with these viruses are still unknown but could be multipurpose in that they could have been developed for immune evasion to intercept chemokines that would otherwise be attracting immune cells to the area of infection, to attract uninfected cells that could then be infected, to induce latency, or to transition from latency to active replication.

Similar to HHV-6A, HHV-6B was shown to downregulate CXCR4 in CD4+ T lymphocytes as well as MT-4 cells. This downregulation impaired the chemotactic response of the cells to the natural ligand, CXCL12 (Yasukawa et al., 1999). Similar to HHV-6A, this could induce mobilization of HSPCs into the circulation as well as prevent migration of cells out of the thymus, both of which aid in the propagation and survival of the virus.

Human Herpesvirus 7

As part of the same subfamily as HHV-6A and 6B, human herpesvirus 7 (HHV-7) shares similar characteristics, including also being a T lymphotropic virus, although it can infect other cell types (D. V. Ablashi et al., 1995; Ward, 2005). Like other human herpesviruses, once HHV-7 is acquired, the host is infected for life. The virus is shed in saliva and spread through this route of transmission. Compared to the other human herpesviruses, much less research has been conducted on HHV-7 infection and pathogenesis. Clinically it has been associated with the development of pityriasis rosea, post-infectious myeloradiculoneuropathy, encephalopathy, and other syndromes. There is some speculation on how involved HHV-7 is in the development and

progression of these diseases (Chuh, Chan, & Zawar, 2004; Mihara et al., 2005; van den Berg et al., 1999). HHV-7 infections can have a variety of symptoms including fever, rash, febrile respiratory problems, vomiting, and diarrhea (D. A. Clark et al., 1997; van den Berg et al., 1999). Infections typically occur in children and are most often asymptomatic (Ward, 2005).

HHV-7 has been shown to influence migration in human cells in a variety of ways (see Tables 2 and 5). Yasukawa and associates showed that it downregulated transcription and surface expression of CXCR4 in CD4+ T cells (Yasukawa et al., 1999). As described before, CXCR4 is the receptor for CXCL12 which is secreted by various cells in the lymph nodes, bone marrow, etc. With CXCR4 assistance, T cells can follow a CXCL12 gradient to sites of inflammation (Domanska et al., 2013). After infection with HHV-7, Yasukawa and associates tested the migration and intracellular levels of Ca^{2+} of CD4+ T cells. It was found that infected cells demonstrated less migration following the CXCL12 gradient and decreased levels of intracellular Ca^{2+} compared to the mock infected cells used as controls. It is currently unknown what viral factor(s) contribute to the downregulation of CXCR4. It has been demonstrated that lower levels of CXCR4 in HHV-7 positive T lymphocytes prevents infection by T lymphocyte trophic HIV (Yasukawa et al., 1999). Future research could explore how HHV-7 manipulates CXCR4 in infected cells and further confirm the findings of Yasukawa and associates as theirs is the only study investigating this change in chemotaxis.

While CXCR4 is a cellular GPCR that is influenced post-viral infection, HHV-7 has two known viral chemokine receptors, products of the HHV-7 genes U12 and U51. These genes were identified as GPCR homologs and later Tadagaki et al. investigated the functionality of the protein products of these genes (Nicholas, 1996; Tadagaki et al., 2005). They verified that these proteins do accumulate on the surface of the cell. Further, they verified that they could act as

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functional chemokine signal receptors. Cells expressing U12 and U51 expressed heightened levels of intracellular Ca^{2+} after appropriate signaling through the U12 and U51 GPCRs. Testing the chemotactic effect of the expression of these proteins in the Jurkat T cell line using microchannel migration techniques, it was found that cells expressing U12 migrated effectively following a gradient of CCL19 and CCL21. This would make U12 a viral homolog of the cellular GPCR CCR7, as it also responds to both CCL19 and CCL21. Both of these chemokines are strongly expressed in the T cell zone of secondary lymph tissue and are important in lymphocyte homing and migration (Nomura, Hasegawa, Kohno, Sasaki, & Fujita, 2001). It has also been observed that CCR7 expression is upregulated during HHV-7 infection (Hasegawa et al., 1994). While the strategy behind the manipulation of cellular chemotaxis following these ligand chemokines is still unclear, it could be speculated that migration to such areas could be beneficial for HHV-7 transmission as T cells are preferential targets of infection. Tadagaki et al. have also speculated that expression of these viral proteins could aid in immune evasion and viral replication (Tadagaki et al., 2005). Further research in murine L1.2 cells showed that U12 and U51 products could respond to CCL22 and CCL19 respectively (Tadagaki, Yamanishi, & Mori, 2007). Gene products U12 and U51 could act with CCR4 and CCR7, respectively, to direct migration in this cell line in response to CCL22 and CCL19 (Luther et al., 2002). If this were to hold true in human cells infected with HHV-7, then infected cells would be expected to migrate more to areas of inflammation, as CCL22 is a pro-inflammatory chemokine secreted by a wide variety of cells, and areas of high T cell density as CCL19 is constitutively expressed by stromal cells in the T cell zone (Luther et al., 2002). These areas would be attractive locations for the viral spread of HHV-7.

1.3.3.4 Gammaherpesvirinae

Epstein-Barr virus

Table 6. Gammaherpesvirus changes cellular receptors/chemokines

Cell type	Receptor or chemokine	Virus	Amount or functionality	References
B cell	CXCR4	EBV	Decrease	Ehlin-Henriksson *et al.* (2006, 2009);
	CCR7	EBV	Decrease/increase	Nakayama *et al.* (2002)
	CCR6	EBV	Increase	Nakayama *et al.* (2002)
	CCR10	EBV	Increase	
	CXCR5	EBV	Decrease	Ehlin-Henriksson *et al.* (2009); Nakayama *et al.* (2002)
	EBI2	EBV	Increase	Birkenbach *et al.* (1993); Kelly *et al.* (2011)
Endothelial	CXCL8	KSHV	Increase	Wang *et al.* (2004b)
	CCL2	KSHV	Increase	Pati *et al.* (2001); Xu & Ganem (2007)
	CCL5	KSHV	Increase	
	CXCL7	KSHV	Increase	
	CXCL16	KSHV	Increase	

The main cause of viral mononucleosis, Epstein-Barr virus (EBV) infects nasopharyngeal epithelial cells and B lymphocytes (Balfour et al., 2005; J. I. Cohen, 2000). Viral spread is accomplished through shedding in saliva (Balfour et al., 2005). EBV gains access to appropriate host cells by using viral gp350 to bind CD21 (a type 2 complement receptor) on the cell surface, the viral envelope then fuses with the cell membrane releasing the viral capsid and associated tegument proteins into the cytoplasm (Toussirot & Roudier, 2008). The virus uses MHC class II molecules as cofactors when infecting B lymphocytes (Q. Li et al., 1997). During its latent infection of host B cells, EBV expresses one of four possible latency programs depending on cellular development and conditions (Young & Rickinson, 2004). It is likely that reactivation *in vivo* of latent virus is due to the differentiation of infected memory B lymphocytes (Amon & Farrell, 2005; Hochberg, Souza, et al., 2004). EBV is associated with a variety of malignancies due to its ability to regulate cell proliferation, including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, gastric carcinoma, and post-transplant lymphoproliferative disorder (PTLD) (Hochberg, Middeldorp, et al., 2004; Kutok & Wang, 2006; Shibata & Weiss, 1992; Young & Rickinson, 2004).

During infection of B cells, EBV controls the expression of various endogenous chemokines and chemokine receptors (see Table 6). One such manipulated receptor that is shown to affect migration is CXCR4. As previously described it is the receptor for CXCL12 which is secreted by various cells in a number of organs including the lymph nodes, lungs, liver, kidneys, heart, and bone marrow (Teicher & Fricker, 2010). Henriksson et al. demonstrated that tonsillar B cells infected with EBV showed reduced expression of CXCR4 (Ehlin-Henriksson, Mowafi, Klein, & Nilsson, 2006). Assays of chemotactic migration further showed that infected tonsillar B cells had decreased ability to migrate towards CXCL12. This decreased expression and the subsequent lack of chemotaxis was demonstrated in EBV-immortalized B cells as well (Nakayama et al., 2002). The inability to follow the CXCL12 gradient would prevent infected B cells from migrating to tissues expressing only this chemokine. CCR7 is another host receptor that is virally regulated during EBV infection. In a later study done by Henriksson and associates, it was found that CCR7 is downregulated in tonsillar B cells post-infection. This change in expression led to decreased migration following the natural chemokine ligand CCL21 (Ehlin-Henriksson et al., 2009). CCL21 is produced by stromal cells in primary and secondary lymphoid tissues and lymphatic endothelial cells in the peripheral tissue (Comerford et al., 2013). It is critical for directing the formation of secondary lymphoid tissues such as spleen, Peyer's patches, and lymph nodes (Ohl et al., 2003). It is has also been surmised that CCR7 ligands are influential in tertiary lymphoid organs (Comerford et al., 2013). Immortalized B lymphoblast cell lines (LCLs) have been shown to have an increased expression of CCR7 compared to uninfected cells. This upregulation resulted in increased migration following a CCL21 gradient in assays of chemotaxis (Nakayama et al., 2002). While these results may seem to be paradoxical, it is possible that the difference in expression could be a result of a different

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Figure 6. Changes in cellular chemotaxis resulting from EBV infection.

EBV decreases expression of cellular CXCR4 and CCR7 to prevent migration to certain tissue areas, probably to avoid immune detection. Increases in host chemokine CCR6 allow infected cells to migrate to areas of inflammation. CCR10 function is also pirated, allowing infected cells to migrate toward epithelial cells, such as mucosal epithelial cells. Downregulation of CXCL10 and CXCL11 could help in immune avoidance by suppressing the ability of infected cells to attract T lymphocytes.

latency program or stage of viral infection. Henriksson and colleagues used harvested tonsillar B cells and measured the CCR7 expression and migration 7 days post-infection. In contrast, LCLs are a result of EBV immortalization of B lymphocytes, the process taking several weeks to establish the cell line and expressing a type III latency program (Young & Rickinson, 2004). The difference in expression could be a result of either the length of infection or the latency program employed by the virus post-infection.

In that same study of LCLs, it was found that they expressed increased amounts of CCR6 and CCR10, the natural ligands of which are CCL20 and CCL28, respectively. Migration assays confirmed that this change resulted in increased chemotaxis towards CCL20 and CCL28 chemokine gradients (Nakayama et al., 2002). CCL20 is an inflammatory chemokine involved in the recruitment of dendritic cells, CD4+ T lymphocytes, and B lymphocytes (Zhao, Xia, Wang, & Xu, 2014). CCL28 is secreted by epithelial cells that line the mucosa and is used to recruit IgA+ plasma cells (Vazquez, Catalan-Dibene, & Zlotnik, 2015; Wilson & Butcher, 2004). CCL28 expression is highest in the salivary glands (G. X. Liu, Lan, Sun, Hu, & Jiang, 2012). It would be in the best interest of EBV to regulate these receptors, allowing the virus to migrate to mucosal tissues, such as the salivary gland, for effective viral spread. Chemotaxis to sites of inflammation could result in viral reactivation and increased targets for further infection. A final cellular receptor that is downregulated during infection effecting a change in chemotaxis is CXCR5. The inability to migrate due to lowered levels of CXCR5 was observed in LCLs and infected tonsillar B cells (Ehlin-Henriksson et al., 2009; Nakayama et al., 2002). CXCR5 allows B cells to migrate in response to CXCL13 (Carlsen, Baekkevold, Morton, Haraldsen, & Brandtzaeg, 2004). CXCL13 is an important chemokine for secondary lymphoid tissue development, and the main cells responsible for secretion of CXCL13 are follicular dendritic

cells (Cyster et al., 2000; Legler et al., 1998). It is expressed in vascular tissue, Peyer's patches, and inflamed lymphoid tissue (Ebisuno et al., 2003; Mazzucchelli et al., 1999; Okada et al., 2002; Shi et al., 2001). A recent study of murine B lymphocyte positioning in CXCR5-negative mice demonstrated that CXCR5 is important for the retention of B cells in Peyer's patches (Schmidt & Zillikens, 2013). While avoiding tissue types expressing CXCL13 could be beneficial for the virus, possibly assisting in immune avoidance, the exact reason for regulating CXCR5 is still unclear. Another receptor thought to be influenced by EBV is Epstein-Barr virus-induced gene 2 (EBI2). Infected B cells display a heightened expression of EBI2 (Birkenbach, Josefsen, Yalamanchili, Lenoir, & Kieff, 1993; Kelly, Pereira, Yi, Xu, & Cyster, 2011). While it remains unknown how EBV manipulates EBI2 expression in B lymphocytes, it has been observed that EBI2+ cells migrate following a 7α-OHC gradient (Preuss, 2014). 7α-OHC is the natural ligand of EBI2 and is expressed by stromal cells of secondary lymph tissue, assisting in directed migration during cell chemotaxis in these areas (Gatto & Brink, 2013; Hannedouche et al., 2011). Exaggerated expression of EBI2 by EBV could result in migration to the outer follicular zone in secondary lymph tissue, preventing migration toward T cell zones and germinal centers (Cyster, 2010).

The viral regulation of host lymphocytes extends to controlling various chemokines produced during infection, resulting in a change in the chemotaxis of uninfected cells. EBNA-3C, a viral product essential in establishing latency and immortalization of B cells, acts to regulate two host produced chemokines, CXCL10 and CXCL11 (McClellan et al., 2012). EBNA-3C has been found to interact with both transcriptional corepressors and coactivators (Cotter & Robertson, 2000; Radkov et al., 1999; Touitou, Hickabottom, Parker, Crook, & Allday, 2001). Using the EBV-negative cell line, BJAB, McClellan et al. showed that expression

of EBNA-3C reduces expression of these two chemokines. The result is decreased migration of CXCR3+ cells (McClellan et al., 2012). Cells that express and migrate in response to CXCL10 and CXCL11 via CXCR3 include various T lymphocytes, including CD8+ T cells. CXCL10 and CXCL11 are typically expressed to attract Th1 cells in response to infection. EBV has also been also demonstrated the ability to influence the expression of chemokines via microRNAs. miR-BHRF1-3, an EBV-produced miRNA, has the ability to silence CXCL11 protein synthesis (Xia et al., 2008). Downregulation of these chemokines suggests that immune avoidance could be a reason behind viral manipulation. Repression of CXCL11 would prevent attraction of cytotoxic T cells that might recognize virally infected B lymphocytes.

The chemokine receptor CXCR4 is a popular target for manipulation and EBV, like other herpesviruses, uses it to prevent cell migration to certain tissue areas, probably to avoid immune detection. To achieve this same purpose, EBV also downregulates CCR7. During infection the virus increases the host chemokine CCR6, allowing infected cells to more readily migrate to areas of inflammation. CCR10 function is also pirated, allowing infected cells to migrate toward epithelial cells, such as mucosal epithelial cells. This is likely vital for the spread of EBV. Reduction in expression of CXCL10 and CXCL11 could help in immune avoidance by suppressing the ability to attract T lymphocytes via these chemokines (see Figure 2).

Kaposi Sarcoma Herpesvirus

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is named after Moritz Kaposi who originally described a unique skin lesion in the 1870s. The discovery of the association of herpesviral DNA sequences in Kaposi's sarcoma (KS) did not occur until 1994 (Y. Chang et al., 1994; Ganem, 2010). KS presents as tumors most often found in the dermis but can also be found in lungs, liver, and intestines (P. S. Moore & Chang,

2003). KSHV is also linked to primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Avey, Brewers, & Zhu, 2015; Cesarman, Chang, Moore, Said, & Knowles, 1995; Soulier et al., 1995).

KSHV encodes three secreted chemokines; vCCL1 (ORF K6 or vMIP-I/MIP-1a), vCCL2 (ORF K4 or vMIP-II/MIP-1b), and vCCL3 (ORF K4.1 or vMIP-III/BCK) which activate CCR8, CCR3, and CCR4 respectively (see Table 4). This set of chemokines antagonizes the recruitment of Th1 and NK cells. This re-directs the immune response from a Th1-like response towards a Th2 profile. vCCL2 has also been shown to prevent CCL5-mediated chemotaxis of Th1-like lymphocytes (P. S. Moore & Chang, 2003; Stebbing, Portsmouth, & Bower, 2003; Weber et al., 2001). The receptor XCR1, which normally binds the ligand XCL1 and is involved in T-cell recruitment, is selectively activated by vCCL3 but is also blocked by vCCL2. The opposing function and differing time of expression of the two viral chemokines could indicate the importance of the regulation of the XCR1 receptor in KSHV infection and pathogenesis. Neutrophils have high levels of XCR1 and vCCL3 chemoattracts these cells, which may indicate that neutrophils play a role in viral spread (Lüttichau, Johnsen, Jurlander, Rosenkilde, & Schwartz, 2007). vCCL1 and vCCL2 expression were also shown to induce migration of monocytes. This could play a role in the process of tumor development in Kaposi's sarcoma as circulating monocytes could be recruited to KSHV-infected cells, thus propagating the infection (Nakano et al., 2003).

KSHV encodes a GPCR (vGPCR or ORF74) that is homologous to CXCR2 and has a high level of constitutive activity (Arvanitakis, Geras-Raaka, Varma, Gershengorn, & Cesarman, 1997; Cesarman et al., 1996; Hensbergen et al., 2004; Pati et al., 2001) (see Table 3). Constitutive expression of ORF74 in microvascular lung endothelial cells inhibits migration and

increases cell survival. This inhibitory effect on migration can be reversed by endogenous chemokines CXCL10 and CXCL12. These act as inverse agonists of ORF74 as seen in an *in vitro* wound closure assay where CXCL10 increased migration of ORF74-expressing cells. Limiting migration of infected cells may aid in immune evasion and KSHV survival. Constitutive expression of ORF74 has also been shown to attract uninfected endothelial cells which could then be infected and propagate the infection (Couty, Lupu-Meiri, Oron, & Gershengorn, 2009).

ORF74 has been shown to activate the transcriptional activators NF-κB and activator protein 1 (AP-1), leading to the downstream production of signals including IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and CCL5 (Pati et al., 2001; Schwarz & Murphy, 2001; Shepard et al., 2001). Elevated levels of CXCL8 are observed in KS patients and can activate KSHV-infected cell growth and induce chemotaxis (J. F. Wang et al., 2004). CXCR1 and CXCR2, receptors that bind CXCL8, have been found in KS lesions. CXCR4 has also been found expressed on cells in these lesions, which is important as this receptor acts as a co-receptor for CXCR4 tropic strains of HIV (Masood et al., 2001; Pati et al., 2001; J. F. Wang et al., 2004). These combined effects of KSHV-GPCR could stimulate the proliferation, migration, and chemotaxis of endothelial cells in KS.

KSHV encodes a homologue of IL-6, vIL-6, that has been shown to promote migration of endothelial cells in both an autocrine and paracrine fashion. Inhibition of this migration can be specifically inhibited by a DNA methyltransferase 1 (DNMT1) inhibitor, suggesting that the mechanism of vIL-6 is dependent on enhancing expression of DNMT1. As the control of DNA methylation is crucial for gene expression and other cellular processes, disruption of methylation could be a mechanism for KS tumorigenesis (J. Wu et al., 2014).

There are a number of different cellular chemotactic proteins shown to be upregulated by KSHV, including: CCL2, CXCL7, CCL5, GM-CSF, CXCL16, and angiogenin (ANG) (Y. Xu & Ganem, 2007) (see Table 6). Some of these, such as CCL5 and GM-CSF, likely increase migration of endothelial cells toward KSHV-GPCR-expressing KS cells (Bussolino et al., 1989; Pati et al., 2001). In contrast, CXCL16 appears to play an indirect role in tumor growth and expansion through migration of activated T cells (Y. Xu & Ganem, 2007). KSHV also causes downregulation of certain genes. The KSHV microRNA (miRNA), miR-K12-10a, downregulates the cytokine receptor tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) receptor (TWEAKR). This inhibits the proinflammatory response and also provides protection from TWEAK-induced apoptosis (Abend, Uldrick, & Ziegelbauer, 2010).

Latency-associated nuclear antigen 1 (LANA-1 encoded by ORF73), a latently expressed gene, has been shown to hinder neutrophil chemotaxis which interferes with recruitment of neutrophils to infected areas and could be a way in which latent KSHV survives host-induced acute inflammation. Neutrophil recruitment is restored in LANA-1 knockdowns, although not to the level of uninfected cells, indicating that other factors of KSHV play a part in repressing neutrophil recruitment (X. Li, Liang, Lin, Robertson, & Lan, 2011).

As seen with other herpesviruses, KSHV can induce or inhibit cellular migration, according to what is most beneficial for viral infection at the given stage. It not only interferes with cellular marker expression, it also induces increased production of specific chemokines and cytokines which leads to other issues in cellular function and trafficking.

1.3.3.5 Summary

With the exposure to human herpesviruses being very high, it is important to understand how these infectious human pathogens influence infected and uninfected cell types. The *Alphaherpesvirinae* use glycoprotein G to increase the functionality of CXCR4, leading to increased chemotaxis to a variety of tissues while being able to manipulate cellular chemokines, such as CXCL9, to attract PBLs. *Betaherpesvirinae*, also capable of producing and manipulating chemokine and chemokine receptors, influence a variety of cells during infection. hCMV inhibits migration in infected monocytes and potentially attracts monocytes, PBMCs, macrophages, dendritic cells, and regulatory T cells to sites of infection. Similarly, through its ability to attract target cells, HHV-6 is able to induce chemotaxis of T lymphocytes, monocytes, immature dendrocytes, and NK cells to areas of infected cells using viral U83. T cells infected with HHV-6 are further manipulated as viral and cellular GPCRs allow cells to migrate to sites of inflammation and areas rich in T cells. Also manipulating T cells, HHV-7 prevents infected T lymphocytes from migrating to various organs and tissues by downregulating CXCR4. However, it too potentially encourages migration to inflammatory sites and locations high in T cells by inducing cells to follow chemokine gradients of CCR7, CCL21, CCL22, and CCL19.

Further masters of cellular piracy, *Gammaherpesvirinae* also influence cellular chemotaxis to avoid immune detection and spread viral infection throughout the host until latency can be established. To prevent newly infected cells from potentially migrating to lymph tissue and other organs, EBV reduces expression of CXCR4, CCR7, and CXCR5. By downregulating the chemokines CXCL10 and CXCL11, EBV could prevent infected cells from attracting cytotoxic T cells. To regulate chemotaxis of cells during infection, KSHV regulates the attraction or avoidance of neutrophils and monocytes by several viral chemokines vCCL1, 2, and

3. KSHV would be able to induce the chemotaxis of uninfected endothelial cells by upregulating various cellular chemokines and activating the NF-κB pathway, enabling viral spread.

Though our current understanding of how human herpesviruses affect host cell migration during infection is rather expansive, there still remain various areas for future research opportunities. In this review we have elaborated on the cells potentially affected by virally-encoded and virally-induced chemokines. However, the full range of cells affected by these chemokines remains to be tested and investigated further. Several virally-regulated cell chemokine receptors suspected of influencing viral spread and immune avoidance are in need of confirmatory scientific inquiry. While various human herpesviruses, including the *Alphaherpesvirinae* and HHV-7 have not been studied as much in terms of how they affect cellular chemotaxis. These areas leave a variety of opportunities for future research that could contribute to our understanding of how these viruses lead to disease pathogenesis and progression.

Chapter 2. In Naïve B Lymphocytes, Epstein-Barr Virus Induced Gene 2 (EBI2) is Controlled by Epstein-Barr Virus (EBV) During Viral Infection[3]

2.1 Summary

Epstein–Barr virus-induced gene 2 (EBI2) is an important chemotactic receptor that is involved in proper B-cell T-cell interactions. Epstein–Barr virus (EBV) has been shown to upregulate this gene upon infection of cell lines, but the timing and mechanism of this upregulation, as well as its importance to EBV infection, remain unknown. This work investigated EBV's manipulation of EBI2 expression of primary naive B cells. EBV infection induces EBI2 expression resulting in elevated levels of EBI2 after 24 h until 7 days post-infection, followed by a dramatic decline (P=0.027). Increased EBI2 expression was not found in non-specifically stimulated B cells or when irradiated virus was used. The EBV lytic gene BRRF1 exhibited a similar expression pattern to EBI2 (R^2=0.4622). BRRF1-deficient EBV could not induce EBI2. However, B cells transduced with BRRF1 showed elevated expression of EBI2 (P=0.042), a result that was not seen with transduction of a different EBV lytic transfection factor, BRLF1. Based on these results, we conclude that EBI2 expression is directly influenced by EBV infection and that BRRF1 is necessary and sufficient for EBI2 upregulation during infection.

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[3] The content of this chapter was published in the Journal of General Virology, see Cornaby et al, 2017.

2.2 Introduction

Epstein-Barr virus (EBV), a human B-lymphotropic herpes virus, infects over 90% of adults (Amon & Farrell, 2005; J. I. Cohen, 2000; Henle, Henle, & Lennette, 1979). Primary EBV infection usually occurs in childhood (Toussirot & Roudier, 2008). Although most infections are asymptomatic, delayed infection into adolescence commonly causes infectious mononucleosis (McAulay et al., 2007; Sitki-Green et al., 2004). In this case the virus is spread via saliva and infects permissive epithelial cells of the oropharynx (Shannon-Lowe, Neuhierl, Baldwin, Rickinson, & Delecluse, 2006). During this time the virus also infects mucosal B cells, however, once B cells are infected the virus enters a stage of latency. This results in the expansion of an LCL-like population of B lymphocytes in the tonsils (Hislop, Taylor, Sauce, & Rickinson, 2007). This also allows the virus to avoid immune detection and remain with the host for the rest of their life, persisting in the B lymphocyte population. Infection with EBV is also associated and suspect of being responsible for several types of cancers (Robertson, 2012). It is a strongly associated agent of Burkitt's lymphoma, as well as various types of non-Hodgkin's lymphomas, nasopharyngeal carcinoma, immunoproliferative disorders and is associated as an environmental trigger in autoimmune diseases such as Systemic Lupus Erythematosus (Cornaby, Gibbons, et al., 2015; Hochberg, Middeldorp, et al., 2004; Kawa, 2000; Moosmann et al., 2010; B. D. Poole et al., 2008). Post infection, EBV is adept at controlling lymphocyte expression, likely manipulating EBV-Induced Gene 2 (EBI2) expression, among others, as a means of controlling B lymphocyte migration.

EBI2 is a member of the rhodopsin-like subfamily of seven-transmembrane G protein-coupled receptors (GPCRs). Sequence alignments have grouped EBI2 with lipid receptors and it shows highest similarity with GPR18 (Norregaard, Benned-Jensen, & Rosenkilde, 2011;

Rosenkilde et al., 2006). EBI2 expression is found to be naturally highest in secondary lymphoid tissues (Hannedouche et al., 2011; Rosenkilde et al., 2006). Chemotaxis of naïve B cells within the follicle is mediated through the expression of EBI2, CCR7, and CXCR5. Varying expression levels of EBI2 affect the positioning and migration of the B cells throughout the follicle (Gatto, Paus, Basten, Mackay, & Brink, 2009; Pereira, Kelly, Xu, & Cyster, 2009). When EBI2 expression is highest, the B cell migrates to the outer areas of the B cell follicles (Pereira et al., 2009). Likewise, low levels of EBI2 expression lead B cells to accumulate in the FDC-dense region in the center of the follicle or the interface of the T cell zone (Gatto et al., 2009). These expression patterns are kept in a fine balance as positioning within the follicle, directly affects both B cell proliferation and differentiation (Pereira, Kelly, & Cyster, 2010).

EBV controls expression of EBI2 upon infection. This control could affect EBV infected cells in several ways. These include B cell chemotaxis, improper positioning of B cells within the follicle and immune deficiency or other disruptions to cell hematopoiesis and function (Casola et al., 2004). Altered positioning and chemotaxis of B cells is also thought to be necessary for the development and progression of lymphomas (Coupland, 2011). Understanding how EBV controls lymphocyte positioning could allow for improved control of infection and response to EBV-induced malignancies. To enhance our understanding of EBV infection, we investigated the expression profile of EBI2 during EBV infection and potential viral mechanisms responsible for tampering with EBI2 expression.

2.3 Material and Methods

Generation and Harvesting of viral stocks

Viral stock was generated using the Bac B95-8 EBV producing HEK 293 cell line as previously constructed and described by Delecluse et al. (Delecluse, Hilsendegen, Pich, Zeidler, & Hammerschmidt, 1998). To summarize, the strain has been engineered with an F factor origin of replication, partitioning proteins A and B, chloramphenicol-resistance gene, hygromycin-resistance gene, and an EGFP reporter. Cells were seeded in T-75 flasks (Corning) to attain a confluence of 50 to 60 percent. The cells were then transfected with pUltra+BZLF1, pUltra+BRRF1, and pUltra+BRLF1 plasmids using calcium phosphate transfection. Media was changed 16 hours post transfection to RPMI and cells were allowed to incubate for 7 to 10 days. Viruses were harvested in the RPMI media, filtered using a 0.45 μm filter, collected in 15 mL conical vials, and stored at -80 °C. The titer of Epstein-Barr viral infectious units was determined using green Raji cell assays following a previously described protocol (Hong et al., 2004). To summarize, $2x10^5$ Raji cells were suspended in 0.5 ml of viral supernatant. The cells were allowed to incubate for 3 hours and then 1.5 ml of RPMI media was added to the wells. At day 2 post infection, sodium butyrate and Phorbol-12-myristate-3-acetate (PMA; ACROS) were added to a final concentration of 3 mM sodium butyrate and 50 ng of PMA/ml. The cells were allowed to incubate another 16 to 24 hours and then the GFP positive cells were quantified by fluorescence microscopy.

The BRLF1 and BRRF1-deficient Epstein-Barr virus (R-KO EBV) was kindly was given to us by Dr. Henri-Jacques Delecluse from the German Cancer Research Center, having been described and used in previous studies (Hagemeier, Barlow, Kleman, & Kenney, 2011; Hong et al., 2004; Hong et al., 2005). For specific information on the construction and testing of this virus, the reader is referred to previous publications by Hong et al, 2004; Hagemeier et al, 2011; and Feederle et al, 2000 (Feederle et al., 2000; Hagemeier et al., 2011; Hong et al., 2004). R-KO

EBV viral stocks were generated by transfecting HEK 293 cells containing the R-KO EBV-Bac with pUltra+BZLF1, pUltra+BRRF1, and pUltra+BRLF1 plasmids using calcium-phosphate transfection. Seven to ten days' post transfection viral supernatant was collected and filter sterilized as described for the B95-8 viral stock. The titer of viral infectious units was determined using green Raji cell assays following a previously described protocol (Hong et al., 2004) and as described.

The lentiviruses (LVs) pUltra+BZLF1, pUltra+BRRF1 and pUltra+BRLF1 DNA were grown in DH5alpha *Escherichia coli* cells and extracted using plasmid extraction kits (Qiagen). The plasmid DNA was then transfected into the PHX cell line using calcium-phosphate transfection. The supernatant was harvested and filter sterilized using a 0.45 µm filter. Lentiviral vector concentrations were quantified by placing 100 µl of lentiviral media with one ml of Raji cells at a concentration of $1x10^5$ cells per ml. Cells were allowed to incubate for 48 hours and then counted by fluorescence microscopy to determine the LV titer.

UV irradiation of EBV

EBV supernatants in 15 ml centrifuge conical tubes were exposed to ultra violet light (200-280 nm) in a biosafety cabinet hood for a period of 4 hours. The UV irradiated virus was then used to infect isolated B cells.

Human B Cell Isolation

Human naïve B cells were isolated using lymphocyte separation media (Cellgro) and EASYSEP negative selection magnetic separation (STEMCELL Technology). Peripheral blood mononuclear cells (PBMC)s were isolated from 30 ml of peripheral blood collected from healthy volunteers after informed consent using lymphocyte separation medium (Cellgro). PBMCs were

then re-suspended in PBS + 2% FBS with 1 mM EDTA added. Naïve B cells were separated from other lymphocytes by magnetic cell separation using the Human B cell enrichment kit (STEMCELL Technology) following the EASYSEP protocol.

Infection assays and RNA extraction

Using standard twelve well plates, 2×10^6 naïve B cells were placed in each well in one mL of medium. One mL B95-8 EBV viral stock was added to each well. Media without EBV was added to the negative controls. Cells were collected by centrifugation. Using the RNAqueous-Micro RNA extraction kit (Ambion) and procedure, RNA was extracted and suspended in elution solution.

For infection with R-KO EBV, isolated naïve B cells at a concentration of 2×10^6 cells per ml were incubated with R-KO EBV supernatant at an MOI of 15. At each time point samples were pelleted and treated as previously described for RNA extraction.

For lentiviral transduction, either pUltra+BRRF1 or pUltra+BRLF1, at an MOI of 15, was added to isolated naïve B cells at a concentration of 2×10^6 cells per ml. At 24 hours post-infection, the cells were pelleted and treated as previously described for RNA extraction.

Quantification of gene expression by Q-PCR

Reverse-transcriptase quantitative PCR was performed using StepOne Plus software and equipment with Power SYBR Green PCR master mix (Applied Biosystems). Samples were analyzed using GAPDH as the housekeeping gene. Q-PCR Primers for all target genes were designed using Primer Express 3.0 (Applied Biosystems). Primer sequences can be found in figure 4.6.

Statistical analysis

In all cases two-tailed paired t-tests and an alpha value of 0.05 was used to determine significant differences in relative mRNA expression levels. To analyze correlations between the expression of EBV genes and EBI2, linear regression analysis was performed using JMP Pro 10 statistical analysis software.

LCL cell line generation

PBMCs were re-suspended at $2x10^6$ cells/ml in complete RPMI. 5 ml of cells suspended in media and 5 ml of B95-8 EBV cell culture supernatant were placed together in a T-25 flask with cyclosporin A or actinomycin D. Cells were incubated for 3 weeks, pipetted weekly to break up cell clumps. LCL cell lines are maintained in complete RPMI and passaged frequently.

Lentivirus generation

The lentiviral vector pUltra+BRRF1 was constructed using pUltra, a 3rd generation lentivirus obtained from Addgene. The BRRF1 gene was PCR amplified from wild type B95.8 EBV using forward (TCTAGAATGGCTAGTAGTAACAGAGGAAATG) and reverse (TGATCATTATTTGTATTGCATGGCAGAACAGT) primers with an XbaI and BclI restriction site extensions added respectively. BRRF1 was then cloned into pUltra cut with XbaI and BclI restriction enzymes. The ligated pUltra+BRRF1 was then transfected into DH5alpha *E. coli* cells. Using a BRRF1-specific forward primer and a pUltra specific reverse primer, the colony containing the complete pUltra+BRRF1 plasmid was verified by sequencing. The plasmid used for transfecting EBV producing HEK cells pUltra+BZLF1 was produced using pUltra with the BZLF1 gene cloned into the construct. Forward (GTCGACTCAAAGAGAGCCAACAGGAAG) and reverse (GAATTCAAAGGGGAGATGTTAGACAGGT) primers with SalI and EcoRI

restriction site extensions added respectively. The PCR amplified BZLF1 gene was cloned into pUltra cut with SalI and EcoRI. The ligated pUltra+BZLF1 was transfected into DH5alpha *E. coli* cells. Using a BZLF1 specific forward primer and a pUltra specific reverse primer, the colony containing the complete pUltra+BZLF1 plasmid was verified. The lentiviral vector pUltra+BRLF1 was constructed using the same pUltra lentivirus as afore mentioned. The BRLF1 gene was PCR amplified from wild type B95.8 EBV using forward (TCTAGAATGGTCGGGCATTTCCTCTG) and reverse (TGATCACCAAAAGAGGAGGAGGCAGT) primers with XbaI and BclI restriction site extensions added respectively. The PCR amplified BRLF1 gene was cloned in to pUltra cut with XbaI and BclI. The ligated construct of pUltra+BRLF1 was then transfected into DH5alpha *E. coli* cells. All primers for cloning the desired the PCR products were designed using Primer 3 software. All lentiviral constructs were verified by sequencing and expression of the cloned genes were verified by RT-Q-PCR. Q-PCR efficiency and primers used during this study can be found in Figure 18.

Western Blot

One ml of Raji cells at a concentration of $2\text{x}10^6$ cells per ml were infected with EBV at an MOI of 15. At time of sample collection, the cells were pelleted and re-suspended in lysis buffer (Thermo Scientific). Cells were then vortexed and passed through a 25-gauge needle, followed by incubation in Laemmli sample buffer (BIO RAD) and 5% 2-Mercaptoethanol (Sigma) for 5 minutes at 95 °C. The samples were then subject to electrophoresis in a 12% polyacrylamide gel and transferred to a nitrocellulose membrane (Thermo Scientific). Blots were blocked with 2.5% (w/v) non-fat dry milk. EBI2 polyclonal goat anti-human IgG obtained from Santa Cruz Biotechnology at a diluted 1:1000 was used as the primary Ab. Rabbit anti-goat IgG-

HRP obtained from Santa Cruz Biotechnology was used as the secondary Ab. ECL Plus (GE Healthcare) Lumigen reagents and C-DiGit blot scanner (LI-COR) were used to image the Western blot.

2.4 Results

Time course of EBI2 expression after EBV infection

EBI2 is vital in directing migration of primary B cells to areas in secondary lymph tissue where they can potentially detect antigenic proteins (Cyster, 2010). To investigate the way EBI2 expression was influenced by viral infection, naive human B cells were infected with EBV. Levels of EBI2 expression were measured and compared to uninfected control naive B cells (Figure 8). EBV-infected cells showed, on average, a threefold higher relative mRNA expression of EBI2 than uninfected cells. When the EBI2 expression found in infected B cells is compared to the expression in cell lines that contain EBV, there is a significant difference of gene expression. Freshly isolated naive B cells demonstrated a much higher expression of EBI2 when compared to the expression observed in the EBV- positive LCL (P=0.012) and Raji cell lines (P=0.027), which are primarily in a latent state.

To determine the pattern of EBI2 expression during the course of EBV infection, isolated naive B cells were infected with EBV and EBI2 expression was measured at intervals over a 21-day period of time. An expression time line was constructed starting with pre-infection and ending 21 days post infection Between 3 and 6 h post infection, there was a significant decrease in EBI2 expression (P=0.002). There is also a significant increase in expression at 24 h post infection (P=0.027) (Figure 8). This heightened level of EBI2 expression persisted for as long as

Figure 7. EBI2 expression is modulated during the course of EBV infection.

(a) Twenty-four hours after infection, EBI2 expression in infected isolated naive B cells increased by a mean of threefold over that of uninfected B cells. Expression of EBI2 in EBV-infected B cells was significantly higher than in the EBV-containing LCL B-cell-derived cell lines (***P=0.012) and the EBV-containing Ramos cell line (****P=0.027), n=4. (b) Isolated naive B cells demonstrated significantly decreased expression of EBI2 by 6 h p.i. with EBV. After this initial downregulation, EBI2 expression increased to remain at a heightened level of expression for several days (*P<0.007, **P0.027). Seven days p.i., EBI2 expression decreased to levels equal to or lower than those observed prior to EBV infection. (c) Non-specific gene regulation was controlled by measuring the expression of the REEP5 gene at all time points, n=5. Error bars indicate standard error.

7 days post infection At this point, there was a large variance in the samples. This could be indicative of EBV induction of EBI2 ending prior to this time in some samples received from different donors while others are still maintained. However, by days 8 and 9 post infection, the levels of EBI2 expression have decreased to levels equal to or lower than those observed in uninfected control naive B cells (Figure 8). Non-specific gene regulation in infected cells was controlled for by measuring the expression of the REEP5 gene at every time point. This gene expression was stable throughout the time course of infection, except that it decreased between 3 and 6 h post infection, as did EBI2. This suggests that some genes are downregulated immediately upon EBV infection, and the initial dip in EBI2 may be part of an overall pattern of gene expression that is separate from the increase seen by 24 h.

To determine if increased RNA expression corresponded to increased protein levels, naive B cells were infected with EBV and proteins were harvested at 24 h post infection Western blots were performed to determine the ratio of EBI2 protein between the control and infected cells (Figure 9). At 24 h post infection, the EBV-infected cells displayed more than twice the amount of protein compared to uninfected cells (P=0.015).

Cell activation alone is not sufficient to increase EBI2 expression

It is important to establish if EBI2 expression could possibly be induced by a mechanism such as B-cell activation due to viral infection. For this purpose, isolated naive B cells were treated with imiquimod. Imiquimod binds to Toll-like receptor 7 (TLR7), which normally recognizes ssRNA, and leads to B-cell activation (Yang et al., 2005). We used imiquimod to stimulate naive B cells instead of using other methods, such as CD40 and IL-4, because we wanted to investigate whether the TLR pathway could be responsible for the induction of EBI2 (H. Wang et al., 2006; K. Zhang, Clark, & Saxon, 1991). The imiquimod treatment activated the

B cells, as shown by increased IL-1 and IL-6 transcription after treatment, but no increase in EBI2 expression was noted (Figure 9).

To further establish that upregulation of EBI2 expression was due to EBV genes expressed during infection, naïve B cells were treated with UV-irradiated EBV. Media containing EBV was UV irradiated as described and then used to treat naive B cells. The irradiation inactivates viral particles, preventing replication after entering the cell due to DNA damage. After incubation with the UV-irradiated EBV, EBI2 expression was measured. The relative mRNA expression in the B cells treated with the UV-irradiated EBV did not differ from that found in uninfected naive B cells (Figure 9).

BRRF1 demonstrates a similar pattern of expression as EBI2 during EBV infection of B cells

EBV uses various viral proteins to regulate cellular gene expression (Lu et al., 2011; Price et al., 2010). Significant regulation of EBI2 was observed beginning between 3 and 6 h post infection, indicating that an immediate early or early gene product would most likely be responsible for regulating EBI2 expression. Various EBV genes that are expressed during this period were selected as candidates for investigation. These included BARF1, BHRF1, BRRF1, BMLF1, LMP1, LMP2, BRLF1 and BZLF1. Analysis was first begun on these genes, with the intention of examining other viral genes if strong correlation values could not be found upon comparing the expression pattern of these EBV genes to EBI2.

Expression of these genes at three different time points during naive B-cell infection by EBV was measured using reverse-transcriptase quantitative PCR (RT-pPCR) and a pattern of expression was established for each one (Figure 10). These time points were chosen due to the
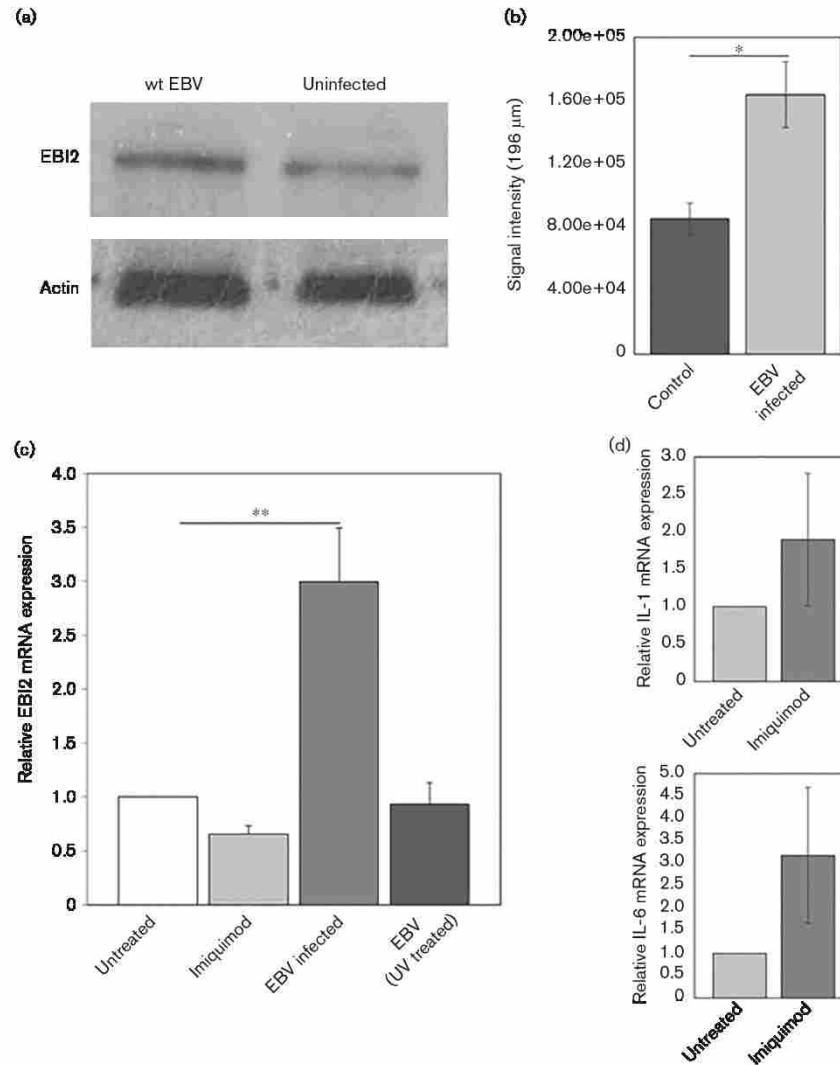
Figure 8. EBI2 protein expression is highly upregulated only by infection with infectious EBV.

(a) EBI2 protein levels correlate with RNA levels. Twenty-four hours p.i. of naive B cells infected with EBV demonstrate heightened protein levels compared to uninfected naive B cells. (b) EBI2 protein signal measured was twofold higher than the uninfected cells as observed by Western blot (*P=0.015), n=4. EBI2 was normalized to b-actin, which was used as a loading control. (c) Both Toll-like receptor 7 stimulation with imiquimod and incubation with UV-irradiated EBV showed expression levels of EBI2 similar to unstimulated naive B cells, while EBV-infected naive B lymphocytes demonstrated significantly higher levels of EBI2 mRNA expression (**P<0.028), n=3. (d) IL-1 and IL-6 mRNA expression was measured in all imiquimod-treated samples. Heightened mRNA levels indicate successful imiquimod stimulation compared to unstimulated cells. Error bars indicate standard error, n=3.

distinct difference of EBI2 expression observed during EBV infection. Epstein– Barr virus nuclear antigen 1 (EBNA1) was used to control for viral gene expression as it is expressed throughout EBV infection. It was also chosen as the control to standardize for viral infection, and even though the multiplicity of infection. used was the same for each experiment, there is still variation observed in the samples treated with EBV. Therefore, all viral gene levels are relative to EBNA1 in this experiment. Upon comparing the expression patterns of the viral genes to the expression pattern of EBI2, BRRF1 demonstrated a similar expression pattern with the highest R value (Figure 10). Linear regression analysis comparing EBI2 gene expression to the mRNA expression of the various viral genes revealed that BRRF1 shared more similarity than any other gene screened (R^2=0.4622). Between 3 and 6 hours post infection, there is a significant decrease in expression (P=0.033) and by 12 h post infection, expression had increased to a level similar to that observed at 3 h post infection These results suggest a possible connection between BRRF1 and EBI2 expression.

BRRF1 induces a heightened expression of EBI2 in B lymphocytes during EBV infection

BRRF1 is an early lytic gene product (Hong et al., 2004; Segouffin-Cariou, Farjot, Sergeant, & Gruffat, 2000) encoding a transcription factor (Na), which plays an important part in regulating between latent and lytic EBV infection (Hagemeier et al., 2011). In high enough concentrations, BRRF1 presence alone has been shown to induce EBV lytic gene expression (Hagemeier et al., 2011). To determine if upregulation of EBI2 was caused by BRRF1, naive B lymphocytes were infected with BRRF1-deficient (BRLF1/BRRF1 knockout, R-KO) EBV. The R-KO EBV was used and described in previous studies (Hagemeier et al., 2011; Hong et al., 2004). To summarize, the R- KO EBV strain is a bac engineered B95.8 Epstein–Barr virus (V01555). It lacks the ability to express both the BRLF1 and BRRF1 lytic genes resulting in the

Figure 9. BRRF1 demonstrates a similar pattern of expression to EBI2 in EBV infected B cells.

(a) EBI2 protein levels correlate with RNA levels. Twenty-four hours p.i. of naive B cells infected with EBV demonstrate heightened protein levels compared to uninfected naive B cells. (b) EBI2 protein signal measured was twofold higher than the uninfected cells as observed by Western blot (*P=0.015), n=4. EBI2 was normalized to b-actin, which was used as a loading control. (c) Both Toll-like receptor 7 stimulation with imiquimod and incubation with UV-irradiated EBV showed expression levels of EBI2 similar to unstimulated naive B cells, while EBV-infected naive B lymphocytes demonstrated significantly higher levels of EBI2 mRNA expression (**P<0.028), n=3. (d) IL-1 and IL-6 mRNA expression was measured in all imiquimod-treated samples. Heightened mRNA levels indicate successful imiquimod stimulation compared to unstimulated cells. Error bars indicate standard error, n=3.

absence of the subsequent gene products, Rta and Na, respectively. Without the BRLF1 gene, it would be predicted that the virus would not be able to induce expression of various genes that depend on Rta response elements in their promoters including BMLF1, BMRF1, BALF2, BARF1 and BLRF2 (Heilmann, Calderwood, Portal, Lu, & Johannsen, 2012). Rta is one of the main proteins involved in EBV reactivation and is thought to be essential for viral reactivation and lytic cycle induction, along with Zta. Na is known to act with Rta as a co-activator and assists in inducing transcription of BZLF1 and the subsequent protein synthesis of Zta (Hagemeier et al., 2011; Hong et al., 2004). It has been hypothesized that it helps regulate Rta transcriptional effects (Kenney & Mertz, 2014).

The EBI2 mRNA expression was measured at 0 and 24 h post infection in naive B lymphocytes with R-KO EBV (Figure 10). The mRNA was extracted from cells and quantified by pPCR. Following infection of naive B lymphocytes with Na- and Rta-deficient R-KO EBV, no significant change in expression of EBI2 was detected at 24 h post infection (Figure 10). To verify that our R-KO infection assays were not yielding low EBI2 levels of expression due to low R-KO EBV infection rate, we measured the mRNA expression of EBNA1 in samples that were EBV or R-KO EBV infected at 24 h post infection (Figure 10). If our R-KO EBI2 expression was a result of low viral infection, we would expect the measured EBNA1 expression to be less than that measured in samples from EBV-infected B cells. However, our results show that EBNA1 expression is not lower in samples from R-KO-infected B cells. This suggests that lower levels of EBI2 expression are not a result of a poor R- KO infection.

To confirm our findings from the R-KO EBV assay and verify that low EBI2 levels were a result of the lack of BRRF1 and not due to the lack of expression of other viral genes, such as BRLF1, we first transduced naive B lymphocytes with a lentivirus containing the BRRF1 gene
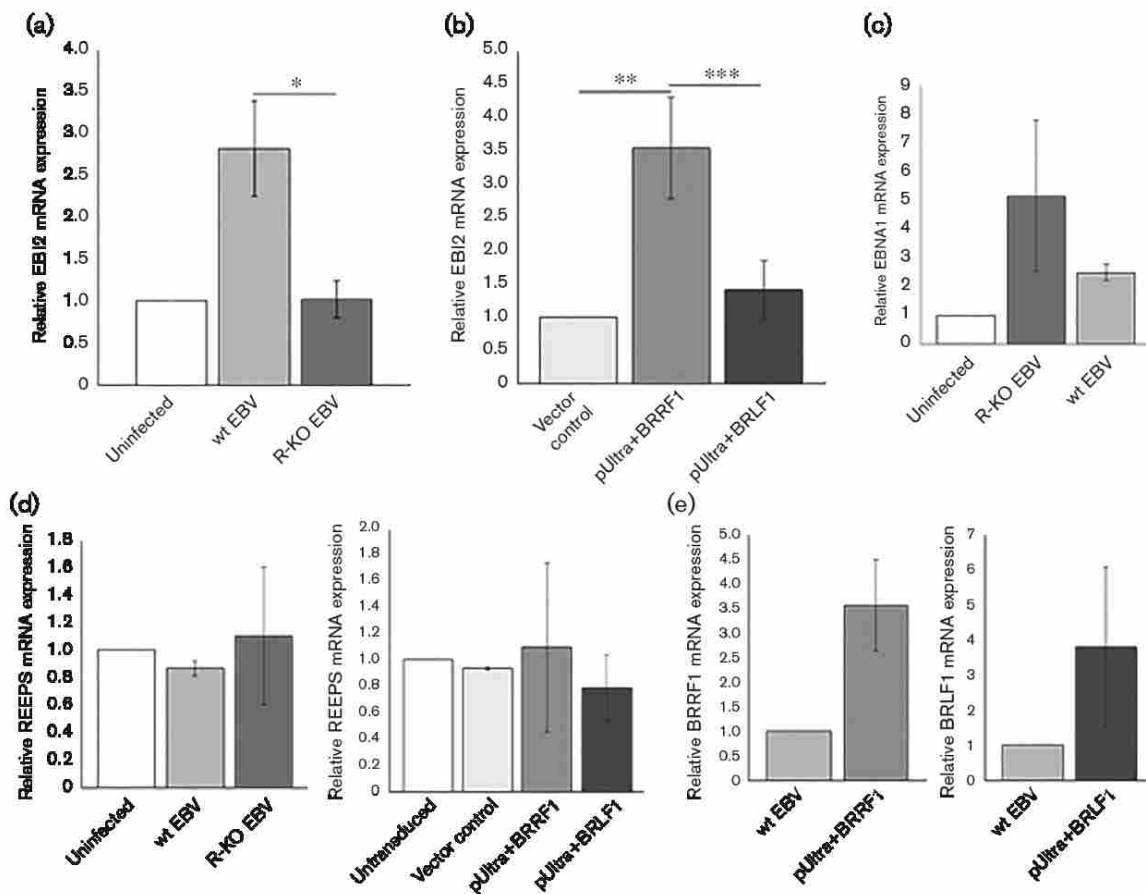
Figure 10. BRRF1 is necessary and sufficient to induce expression of EBI2 in B lymphocytes.

(a) EBI2 expression was measured in naive B lymphocytes infected with the BRRF1-deficient R-KO EBV. There was no significant change in expression at 24 h p.i. with the BRRF1- deficient EBV, n=6. (b) Naive B cells were transduced with BRRF1 using a lentiviral vector pUltra+BRRF1) and EBI2 expression was measured. There was a significant increase in EBI2 expression at 24 h (**P=0.042) post-lentiviral treatment with BRRF1. Naive B cells were transduced with BRLF1 using a lentiviral vector (pUltra+BRLF1) and expression was measured. There was no significant difference in EBI2 expression measured at 24 h post-transfection when BRLF1 was expressed. Error bars indicate standard error (*P=0.049; ***P=0.05), n=6. (c) Relative EBNA1 expression measured in EBV and R-KO EBV-infected B cells 24 h p.i. This demonstrates that the RKO EBV could establish an infection in the naive B cells, evidenced by the expression of viral genes, n=6. (d) As a negative control, REEP5 mRNA expression was measured and found to be similar in all experiments, demonstrating no significant difference, n=6. (e) BRRF1 and BRLF1 mRNA expression was measured in lentiviral-transduced naive B-cell samples and wild-type EBV-infected naïve B cells and compared. The levels of BRRF1 and BRLF1 mRNA expression in pUltra+BRRF1 and pUltra+BRLF1-transduced samples, respectively, are similar. Error bars indicate standard error, n=6.

(pUltra+BRRF1). The relative EBI2 expression was measured at 0 and 24 h (see Figure 10). Heightened expression of EBI2 was observed at 24 h post-transduction (P=0.042) when naive B lymphocytes were transduced with pUltra+BRRF1. Second, to test whether BRRF1 was specifically causing EBI2 upregulation and confirm that the increased levels of EBI2 expression were not the product of transactivation by a viral DNA-binding gene, the viral gene BRLF1 was transduced into naive B cells (pUltra+BRLF1). The relative EBI2 mRNA expression was measured at 0 and 24 h post-transduction. In contrast with BRRF1, there was not a significant increase in EBI2 expression after treatment with BRLF1 (Figure 10).

It was probable that most of our blood donor volunteers had previously been infected with EBV. It was not expected that EBV reactivation would contribute to the heightened EBI2 expression levels observed since EBV genomes are only present in approximately 1 in 106 circulating B cells. However, to verify that latent EBV from prior infection was not interfering with the results from our pUltra+BRRF1 and pUltra+BRLF1 transduced samples, BZLF1 was measured in the EBV-infected naive B cells and the lentivirus-transduced naive B cells. BZLF1 expression was greater, estimated at $2x10^4$ fold higher, in EBV-infected B- cell samples compared to the pUltra+BRRF1-transduced B-cell samples. In most cases, BZLF1 expression was not detectable in B cells transduced with the BRRF1-expressing lentivirus. This would suggest that potential reactivation of EBV by the pUltra+BRRF1 in EBV-positive primary B cells is not responsible for the resulting increase in EBI2 expression observed.

It could also be suggested that perhaps high expression of an EBV lytic gene could non-specifically induce EBI2 expression. To investigate this possibility, we measured BRRF1 transcripts in pU+BRRF1-transduced, EBV-infected and control samples. We found that there was about three-fold more BRRF1 expression in pU+BRRF1-transduced samples compared to

EBV-infected samples. This difference can be attributed to the effectiveness of the human ubiquitin promoter used to drive expression of BRRF1 post-transduction compared to the BRLF1 and BRRF1 promoter used by wild-type EBV. These results combined with the lack of upregulation in cells transfected with BRLF1 using the same promoter led us to conclude that BRRF1 expression alone can induce EBI2 and that EBI2 expression is not a result of non-specific binding or latent EBV reactivation.

2.5 Discussion

Regulation of EBI2 is crucial to B cell chemotaxis in secondary lymph tissue (Cyster, 2010; Gatto & Brink, 2013). Up-regulation of EBI2 generally occurs during cellular migration in secondary lymph tissue (Cyster, 2010). When expressed, EBI2 allows the cell to follow a 7a,25-dihydroxycholesterol (7a,25-OHC) gradient(Gatto & Brink, 2013; Hannedouche et al., 2011; C. Liu et al., 2011). B cells upregulate EBI2 at key times to maneuver the cell away from the follicular region to the outer and inter-follicular regions. It has been suggested that these movements allow the naïve B cells to potentially be exposed to any antigens that are present in that region (Figure 11) (Cyster, 2010). Down regulation of EBI2 allows for the naïve B cells to return to the follicular area and migrate to the T cell zone for a time before exiting the secondary lymph tissue. The migration of B cells through this pattern allows for exposure to areas with different antigens as well as the possibility to be primed by T cells.

EBV is known to manipulate cellular genes in order to avoid the immune system, control viral replication, and prevent apoptosis (Ehlin-Henriksson et al., 2009; Kulwichit et al., 1998; Thorley-Lawson & Allday, 2008). EBV had been previously shown to influence EBI2 (Birkenbach et al., 1993; Cornaby, Tanner, Stutz, Poole, & Berges, 2015; Kelly et al., 2011).
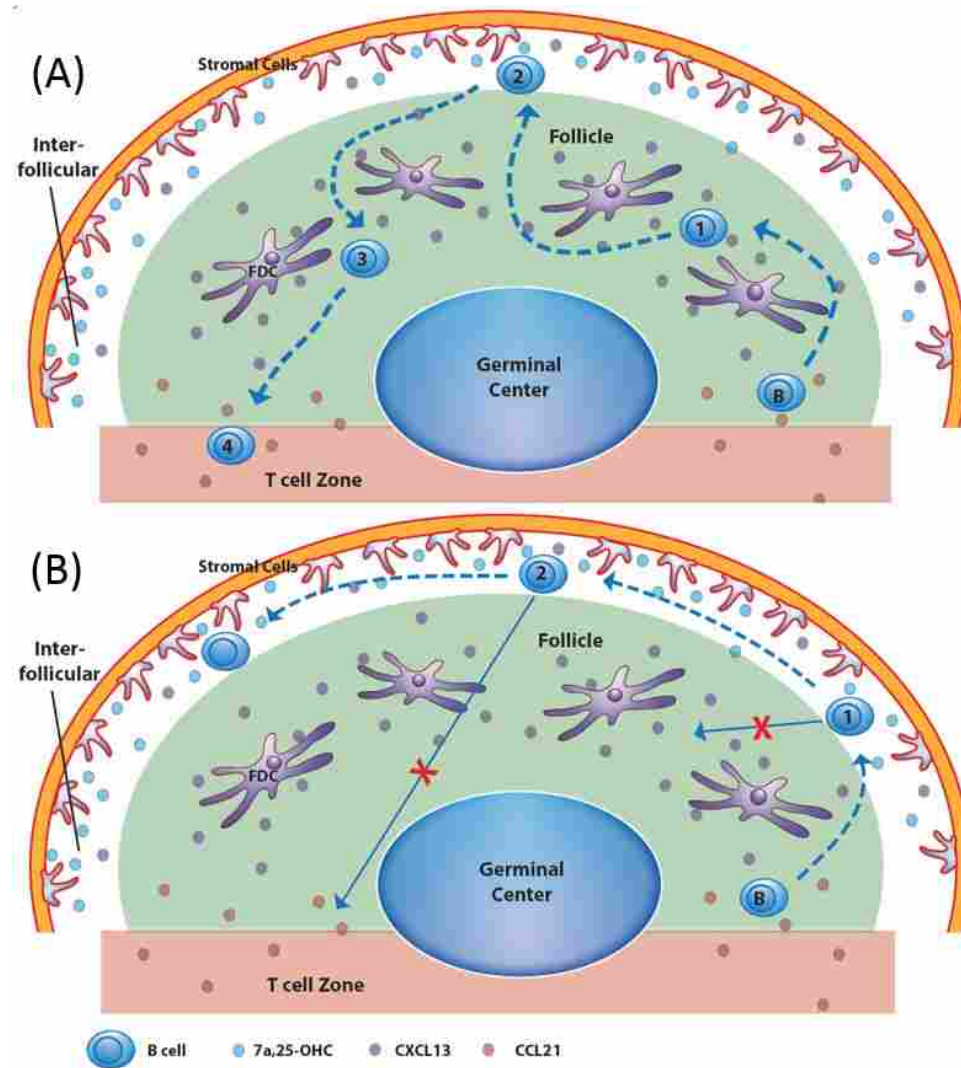
Figure 11. Proposed effects on B-cell migration during EBV infection due to modulation of EBI2 expression.

(A) This illustration demonstrates the regular pattern of naive B-cell migration in the lymph node. (1) CXCR5 is expressed, allowing cells to follow a CXCL13 gradient and enter the follicular region. CXCR5 is constitutively expressed to assist in cell migration. (2) EBI2 is upregulated and the cells follow a 7a,25-OHC gradient to the outer or inter-follicular region. (3) Downregulation of EBI2 allows the naive B cells to return to the follicular area and (4) upregulation of CCR7 permits the cell to follow the CCL21 chemokine gradient to the T-cell zone. If B cells remain inactivated, they will leave via the cortical sinus of the lymph node. If they become activated, they will be directed back to the follicular and germinal center area. (B) This figure depicts the predicted pattern of migration during EBV infection of naive B cells. (1) Upon entry into the lymph node, the B cell will follow the 7a,25-OHC gradient to the outer and inter-follicular region of the lymph node. (2) The cell will be unable to migrate to the follicular region.

Various studies have researched EBI2 expression during EBV infection; however, it has yet to be deter- mined if the virus induces EBI2 or if heightened EBI2 expression is a result of the cellular response to viral infection (Cahir-McFarland et al., 2004; Craig et al., 2007; Dirmeier et al., 2005; Rosenkilde et al., 2006). Previously, it has been hypothesized that EBI2 expression is a result of the immune response to viral infection since EBI2 has been shown to be induced during EBV latency program I. Normally, only EBNA1 is highly expressed (Birkenbach et al., 1993; Rosenkilde et al., 2006). It has also been hypothesized that the increase of EBI2 expression observed during EBV infection is a result of viral manipulation to promote a successful persisting EBV infection, allowing for the virus to direct infected cells to areas that would provide a better survival niche for viral persistence (Hannedouche et al., 2011; C. Liu et al., 2011). The results of this study provide evidence for the later explanation of why heightened EBI2 expression is observed during EBV infection.

It is possible that the EBV-induced upregulation of EBI2 could have been the result of non-specific activation, as a cellular response to EBV infection. However, the B-cell- stimulating agent imiquimod did not cause an upregulation in EBI2 expression, nor did incubation with inactivated EBV. These findings indicate that EBI2 upregulation is not likely due to cellular activation by TLR7, and that viral tegument or surface proteins are not responsible for the upregulation of EBI2.

Screening for viral gene candidates that could be responsible for the regulation of EBI2 post-EBV infection found that BRRF1 shares a similar mRNA expression pattern as EBI2. BRRF1 encodes a viral transcription factor, Na. Na is responsible for assisting in the activation of viral lytic genes in various latently infected epithelial cells (Hagemeier et al., 2011) and has been found to associate with human TNF receptor-associated factor 2 in a yeast two-hybrid assay

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(Calderwood et al., 2007). Due to its function as a transcription factor and its expression pattern being similar to that of EBI2, BRRF1 seemed the best candidate of those screened. To ascertain if BRRF1 expression could directly influence EBI2 expression, the BRRF1-deficient R- KO EBV was used to infect isolated naive B cells. R-KO EBV has been used in several studies and has the R gene, BRF1, knocked out as well as the promoter of BRRF1 resulting in EBV that cannot express BRRF1 (Hagemeier et al., 2011). R acts with BZLF1 to activate latent EBV and can bind to various EBV promoters (Gruffat et al., 1992; Gruffat, Manet, Rigolet, & Sergeant, 1990; Wille et al., 2013). The results show no upregulation of EBI2 expression upon infection with the BRRF1-negative EBV. This would suggest that BRLF1 or BRRF1 was inducing EBI2 expression. To further verify that BRRF1 could induce EBI2 expression, the pUltra+BRRF1 lentivirus was used to treat isolated naive B cells. This resulted in a significant increase in EBI2 expression. These results demonstrate that the expression of BRRF1 was necessary and sufficient to induce EBI2 expression. While the mechanism that BRRF1 uses to induce this expression in B cells is unknown, it is possible that Na acts as a transcription factor at the EBI2 promoter site. Further research needs to be performed in order to ascertain if this is the case.

It is probable that cell migration would be influenced by the EBV-induced regulation of EBI2. With a heightened concentration of EBI2 on the surface of the infected B cells, it is no stretch to infer that EBI2 might override other migratory signaling pathways and direct the cell to follow a 7a,25- OHC gradient, which is the natural ligand of chemoattractant receptor EBI2 (see Figure 11) (Hannedouche et al., 2011; C. Liu et al., 2011). 7a,25-OHC is only produced by stromal cells of secondary lymph tissue (Yi et al., 2012). Following this gradient would cause infected B cells to migrate and remain in the outer follicular and interfollicular regions of

secondary lymph tissue. This type of control over cell migration could allow the virus to avoid immune detection until latency has been established, assisting in immune evasion.

It is also possible that increased levels of EBI2 might influence B-cell proliferation. Benned-Jensen et al. (Benned-Jensen et al., 2011) found that overexpression of EBI2 in antibody stimulated murine B cells resulting in increased proliferation. By increasing the proliferation of infected B cells during a specific time, it would increase the chances of viral persistence in the infected host. The results of this study emphasize the importance of EBI2 regulation during viral infection. It is possible that the use of EBI2 antagonists, or inverse agonists, could be used as EBV antiviral treatment options or to disrupt the viral life cycle in an animal model (Ardecky et al., 2010; Benned-Jensen et al., 2011; Daugvilaite, Arfelt, Benned-Jensen, Sailer, & Rosenkilde, 2014).

During our study, we have established that EBI2 is controlled during EBV infection of B cells. Different EBV expression profiles result in different levels of EBI2 expression. Higher levels of EBI2 mRNA expression result in higher levels of EBI2 protein. We have further established that EBI2 is induced by an EBV gene, BRRF1. Potential research for the future can address to what degree heightened expression of EBI2 possibly changes migration of EBV-infected B cells. It would also be beneficial to verify the mechanism used by BRRF1 to induce EBI2 expression.

Acknowledgments

Gene	qPCR primer sequence
BARF1	For: TGGAGCCCGCACCAACT Rev: GCTCCCATCCTTTTCCTTCAT
BHRF1	For: CCTGTCAGTTCGTGGGATGTT Rev: TGTTGATGAATCCAACCATCCA
BRLF1	For: CTGTTGCCCGAGTCACTG Rev: GATGAACTCGAAAGTCTCCCG
BZLF1	For: AATGTCTGCTAGCTGTTGTCC Rev: AGAAGCACCTCAACCTGGAG
BRRF1	For: TGCCAGGAAGCGACTACACA Rev: ACCTATGGAGATGCCGTCGTA
BMLF1	For: TGCCCGGAGAGAGAATGG Rev: GTGACTGCCGAAGTGCATCA
LMP1	For: TCATCGTGGTGGTGTTCATCACTG Rev: ATCTCCTTTGGCTCCTGTTTC
LMP2	For: TGATTGTCGCTGGGATACTCTTCA Rev: CAGGCCACCGAGGCACATAA
EBI2	For: CTCCGCCCTCTGCAACTC Rev: TGGCCGTGGTGTGATGTG
GAPDH	For: TGCACCACAACTGCTTAGC Rev: GGCATGGACTGTGGTCATGAG
REEP5	For: CCGAGCCCTTCTAATGGGG Rev: GGCCTTGTCTTTAAGGTCCTTG

Figure 12. Primers and consistency of RT-pPCR analysis.

(a) This table contains the pPCR forward and reverse primer sequences used in the experiments described. (b) GAPDH cycle threshold (CT) scores for the various figures have been compiled to verify that they are similar. There is no significant difference when comparing the GAPDH CT scores from the different experiments.

Chapter 3. Correlation between physical markers and psychiatric health in a Portuguese systemic lupus erythematosus cohort: the role of suffering in chronic autoimmune disease

3.1 Summary

Background. Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that affects a large number of people throughout the world. Anxiety, depression and fatigue are common companions of SLE that substantially contribute to decreased quality of life. This study investigates the interplay between physical and psychiatric manifestations of lupus. To this end, an SLE patient cohort was examined for correlations between clinical presentation, laboratory tests, and psychological indicators.

Methods. Seventy-two lupus patients were evaluated for psychological status using a battery of instruments, including assessments for fatigue (CFS & FSS), depression (HADS), anxiety (HADS), overall health (SF-36 & PSQI) and intimate relationship satisfaction (RAS & CSI). Scores from these assessments were correlated with lupus clinical profiles and laboratory test values

Results. The prevalence of depression in the SLE patient cohort was 41.7 %, as measured by the hospital depression and anxiety scale. The study identified that pain ($p = 0.001$), body mass index ($p = 0.026$), Chalder's fatigue scale ($p < 0.001$), fatigue severity scale ($p < 0.001$), and anxiety ($p = 0.001$) are all positively correlated with depression in SLE patients. Total complement (CH50) ($p = 0.032$), and SF-36 physical and mental characteristic assessments are negatively correlated with depression. Longitudinal analysis indicated that the disease related

complaint alopecia (p = 0.008) and relationship assessment scale scores (p= 0.004) may also be correlated to depression in SLE patients. Multivariant scrutiny of the clinical and psychosocial characteristics identified the fatigue severity scale (p = 0.026), SF-36 physical function (p = 0.040), physical role function (0.030), and mental health (p = 0.002) as the best indicators directly correlated with depression for the SLE cohort.

Conclusion. These results reveal the influence of physical manifestations of lupus including fatigue, pain, body mass index and anxiety, as well as decreased physical and mental function, on depression. Fatigue is the strongest factor correlated to depression in SLE patients in the cohort. Both physical and social/psychological aspects likely contribute to the depression and anxiety in lupus.

3.2 Introduction

Systemic Lupus Erythematosus (SLE) is an autoimmune disease that can affect a wide variety of organ systems. Symptoms vary and can include fever, arthritis, fatigue, weight loss, lymphadenopathy, a characteristic "butterfly rash", renal disease and cytopenia, in a pleomorphic clinical presentation. Depression and fatigue are very common, early symptoms of lupus that are major contributors to diminished quality of life (S. T. Choi et al., 2012; Yilmaz-Oner et al., 2016). Despite the prevalence of these psychiatric manifestations in lupus, they are not well understood. Increased study of variables such as emotional stressors, culture, social and environmental factors, age, sex, and disease duration, as well as indicators of disease activity, may clarify the causes and contributions of depression and fatigue in lupus (Ad Hoc Committee on Systemic Lupus Erythematosus Response Criteria for, 2007).

Young women are predominantly affected by lupus, which brings significant costs into their personal, family and professional lives. Depressive symptoms can occur as the first manifestation of lupus, accompany more active stages or be regarded as a consequence of therapeutic interventions (H. X. Gao et al., 2009; Jorge et al., 2017). A complete understanding of the nature of depression in SLE is confounded by many factors that lupus can bring to a person's life, such as weight and activity changes, fatigue and sleep disturbances. Moreover, a depressive disorder must be differentiated from more transient depressive symptoms (Iverson, Woodward, & Green, 2001). Examination of the quality of depression, more than its prevalence, might yield more clues to the underlying mechanisms (Denburg, Carbotte, & Denburg, 1997)

For these reasons, the causes of depression in lupus are not clear. Biological mechanisms have been implicated because depression in lupus is associated with other CNS diseases, higher disease activity and more severe clinical manifestations (Nery et al., 2007; Segui et al., 2000). Brain inflammation, interaction of auto-antibodies with antigens on neuronal cells membrane, and cytokine expression triggering neurotransmitter dysfunction have been regarded as possible causes of depression in SLE patients and in rodent SLE models (Lapteva et al., 2006; Lawrence, Bolivar, Hudson, Mondal, & Pabello, 2007; Postal et al., 2016; Sakic et al., 2005; Stojanovich, Zandman-Goddard, Pavlovich, & Sikanich, 2007; Tsai et al., 1994). Cytokines are thought to modify neuroplasticity and neurogenesis, induce biochemical changes and decrease neurotransmitters' bioavailability causing depression (Braga & Campar, 2014). Peripheral proinflammatory cytokines levels were found to be correlated with the presence of depressive symptoms, and a reciprocal relationship has been consistently reported between TNF-α, IL-6, IL1 and major depression (Postal et al., 2016).

Fatigue and pain are common symptoms in SLE, affecting up to 90% of the patients, who rate these symptoms as severe manifestations of the disease (Petri et al., 2013; Ramsey-Goldman & Rothrock, 2010; Zonana-Nacach et al., 2000). Both are considered the result of a complex interplay between numerous variables – physical activity, sleep quality, cognitive function, mood, quality of life, medication and comorbid conditions. Disease activity and medication fail to predict self-reported levels of pain and fatigue (Jump et al., 2005). The progression of SLE as a chronic disease may be better monitored by clinicians studying fatigue and depression and its association with immune activation (Fonseca et al., 2014).

3.3 Methods

Patients

The studied population included 72 Caucasian lupus patients and 13 controls, recruited in northern Portugal. All SLE patients were previously diagnosed and followed at an outpatient unit. No selection was done besides willingness to participate. Diagnosis and stage of disease activity was established according to the American College of Rheumatology Criteria (ACR) and the duration of the disease was measured from the time when the patients first met at least the classification criteria. The control group of patients with depression but without SLE was undergoing treatment at a private psychiatric clinic at the time of the study.

To obviate inter-interviewer variation, psychiatric evaluation and psychometric markers were tested by one psychiatrist and one psychologist to establish the severity of depression. A convenience age matched sample of healthy women was also recruited. Exclusion criteria comprised history of substance abuse, personality disorders and or other major psychopathology than depression. Patients and controls were subsequently interviewed by phone by trained

interviewers. The literature corroborates phone interviews as valid and precise tools for psychological data collection (B. B. Cohen & Vinson, 1995; Rohde, Lewinsohn, & Seeley, 1997; Siemiatycki, 1979).

Participants' socio-demographic data included age, educational level, employment status (active/non-active) and marital status (Table 7). Laboratory and SLE clinical evaluations were obtained for the SLE patients through the clinical records (Table 8-12). Lab tests included leukocytes (10^9/L), lymphocytes (percentage), neutrophils (percentage), platelets (10^9/L), erythrocyte sedimentation rate (mm/h), anti-dsDNA antibody titer (IU/ml), C3 level (g/L), C4 level (g/L), CH50 level (U/ml), and C-reactive protein level (mg/dl). Other clinical symptomology also recorded from patients included cutaneous manifestations, photosensitivity, foot and mouth ulcers, arthritis, alopecia, headaches, kidney disease, neurological symptoms, pulmonary disease, musculoskeletal pathology, history of hypertension, and acute confusion syndrome (ACS). Smoking and alcohol consumption were also recorded.

The study was submitted and approved by the Ethical Committee of the São João Hospital IRB (EPE) according with the Declaration of Helsinki. All participants gave informed consent.

Psychosocial evaluation

Socio-demographic characterization included age, education measured as years of school, marital status and socio-economic class evaluation. Psychological evaluations were obtained through a battery of standardized instruments.

Fatigue Severity Scale (FSS)

The short form of the FSS allows evaluation of self-reported fatigue (Krupp, LaRocca, Muir-Nash, & Steinberg, 1989). The Portuguese version includes nine-items and is recommended as the instrument of choice for research purposes in studies involving patients diagnosed with SLE (Ad Hoc Committee on Systemic Lupus Erythematosus Response Criteria for, 2007).

The FSS demonstrates good psychometric properties (Cronbach's α= 0.89 and test-retest reliability 0.84). A final score is obtained from the mean of all scored items, with higher scores revealing higher severity of fatigue. Presence of clinical levels of fatigue was defined by a FSS score >3. The scale has proved to be sensitive to change and reliable for telephone interviewing.

Hospital Anxiety and Depression Scale (HADS)

The Hospital Anxiety and Depression Scale (HADS) is a self-rating scale with good psychometric properties (Cronbach's alpha coefficients of 0.94), designed to measure anxiety and depression in physically ill individuals (Zigmond & Snaith, 1983). Translated and adapted for Portugal (Ferreira, 2000; Pais-Ribeiro et al., 2007), it is subdivided in two subscales of 7 items that measure independently anxiety and depression. The partial result of each scale varies between 0 and 21. Scores ranging from 8 to 10 are considered mild, from 11 to 14 moderate and 15 to 21 severe(Marcolino, Suzuki, Alli, Gozzani, & Mathias, 2007) and the authors suggest 8 as the cutoff point, considering values below as indicating the absence of anxiety and depression(Zigmond & Snaith, 1983). It is important to note that the scale is indicative of depressive symptoms in the last week, and not necessarily clinical depression.

Pittsburgh Sleep Quality Index (PSQI)

This instrument presents good psychometric properties, with high reliability (Cronbach's alpha = 0.83) and validity. The seven components evaluated - sleep latency, sleep disturbances, sleep duration, sleep quality, sleep efficiency, use of sleep medications and daytime dysfunction allow the gathering of a global score varying from 0 to 21 (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989; Carpenter & Andrykowski, 1998). The PSQI is reliable for sleep quality assessment in telephone interviews and permits the identification of poor sleepers (score > 5) (Monk et al., 2013; Palmieri, Chipman, Canetti, Johnson, & Hobfoll, 2010).

Chalder Fatigue Scale (CFS)

The Chalder Fatigue Scale (Chalder et al., 1993), is an instrument with 11 items that evaluates the extent and severity of mental and physical fatigue in a four-point scale. Higher scores indicate higher fatigue.

Medical Outcomes Study Questionnaire Short Form 36 Health Survey (SF-36)

The SF-36 (Ware & Sherbourne, 1992), Portuguese version (Ferreira, 2000), is a 36 item questionnaire that measures functional health and well-being in eight domains: physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, mental health and reported health transition. The instrument allows a score for each domain, as well as a global score. Higher scores indicate better health.

Couples Satisfaction Index (CSI)

The CSI (Funk & Rogge, 2007) (Portuguese experimental version from Barbosa & Figueiredo-Braga, 2014) is a 32 item questionnaire that measures couple satisfaction in the relationship. Higher scores indicate greater satisfaction.

Relationship Assessment Scale (RAS)

The RAS (S., 1988) (Portuguese experimental version from Mesquita, Barbosa, & Figueiredo-Braga, 2014) is a 7 item instrument, with a five-point scale that measures general satisfaction with the relationship.

Statistical analysis

Differences in the demographic, clinical, and psychological variables between the total SLE subjects, SLE non-depressed, SLE depressed, and depressed control subjects was determined using the independent t-test, Fisher's exact chi-squared, Mann-Whitney U, Wilcoxon rank sum, or Welch's tests when appropriate. Fisher's exact chi-squared was used in place of the standard chi-squared test, which would typically be utilized, due to the smaller sample size of the groups compared. The statistical test used for the comparisons are indicated in the table legends. Multivariant analysis was performed using a generalized linear model. The best fit model was determined using the model with the appropriate number of variables (less than or equal to 8 variables) and the highest pseudo R^2 value with the lowest approximate AICc value. In the multivariate analysis, the HADS depression score for all SLE subjects was used as the dependent variable and all candidate models included the confounding variables of age, education in years (socioeconomic status indicator), and body mass index. Statistical analysis was performed using the statistical software R and SPSS (IBM). An alpha value less than or equal to 0.05 was considered significant.

3.4 Results

The systemic lupus erythematosus (SLE) patient cohort consisted of 72 females with a mean age of 44.31 years. 41.7% of SLE patients demonstrated pathological HADS depression

scores. A cohort of thirteen physically healthy females diagnosed and being treated for depression acted as a control group. This group was slightly older than the lupus cohort with a mean age of 57.69 years. For several analyses the SLE cohort was divided into two groups, those who demonstrated normal HADS depression scores (HADS ≤ 7) and those who recorded above normal HADS depression scores (HADS ≥ 8). To ensure that the depressed control group was comparable with the lupus depression group, despite the selection of the lupus depression group by HADS score, the depressed non-lupus group was also stratified by depression, and only the depressed patients with HADS scores of >8 were compared to the lupus depression group. This selection still significantly demonstrated the same patterns that were observed using the entire depressed patient cohort.

The SLE patients experiencing pathological depression display fewer years of formal education compared to the SLE patients with normal HADS scores (p = 0.005). When years of education are compared between SLE non-depressed patients and depressed controls, the depressed cohort is significantly lower (p = 0.030), but there is no significant difference between depressed SLE patients and depressed controls (p = 0.871) (Table 7). Aside from these sociodemographic differences, the SLE cohort sub groups and the depressed patient control cohort share similar marital and employment status distributions.

Clinical Manifestations

The laboratory data were similar between the SLE depressed and non-depressed groups save for the total complement (CH50) levels. These levels are significantly lower on average in the SLE depressed patients, who had a mean CH50 level of 9.81 (± 39.07) U/ml compared to the SLE non-depressed group that had a mean CH50 level of 45.88 (± 86.24) U/ml. This large difference is statistically significant (p = 0.032) (Figure 8).

TABLE 7. Sociodemographic Characteristics of the Patient Cohort

Characteristics	All Subjects (N=85)	Total SLE Subjects (N=72)	SLE, non-depressed (HADS Depression ≤ 7) (N=42)	SLE, depressed (HADS Depression ≥ 8) (N=30)	SLE, non-depressed to SLE, depressed p-value	Depressed (N=13)	SLE total to Depressed p-value	SLE, non-depressed to Depressed p-value	SLE, depressed to Depressed p-value
Gender, no. (%)									
Female	86 (100)	72 (100)	42 (100)	30 (100)		13 (100)			
Age, mean ± SD	46.35 ± 10.6	44.31 ± 9.9	41.55 ± 8.51	48.17 ± 10.53	0.004 a	57.69 ± 6.56	< 0.001 a	< 0.001 a	0.004 a
Education (years), mean ± SD	8.56 ± 3.642	8.8 ± 3.6	9.8 ± 3.18	7.43 ± 3.73	0.005 a	7.23 ± 3.75	0.154 a	0.018 a	0.871 a
Education Level, no. (%)					0.006 b		0.182 b	0.030 b	0.269 b
Primary	33 (38.8)	26 (36)	8 (19)	18 (60)		7 (53.8)			
Middle School	20 (23.5)	16 (22.2)	12 (28.6)	4 (13.3)		4 (30.8)			
High School	20 (23.5)	20 (27.8)	15 (35.7)	5 (16.7)		0 (0)			
College	11 (12.9)	9 (12.5)	6 (14.3)	3 (10)		2 (15.4)			
Marriage Status, no. (%)					0.667 b		0.050 b	0.111 b	0.132 b
Unmarried	11 (12.9)	11 (15.3)	8 (19.0)	3 (10)		0 (0)			
Married	65 (76.5)	53 (73.6)	30 (71.4)	23 (76.7)		12 (92.3)			
Divorced	8 (9.4)	8 (11.1)	4 (9.5)	4 (13.3)		0 (0)			
Widowed	1 (1.2)	0 (0)	0 (0)	0 (0)		1 (0)			
Employment Status, no. (%)					0.088 b		0.758 b	0.343 b	1.000 b
Employed	32 (37.6)	28 (38.9)	20 (47.6)	8 (26.7)		4 (30.8)			

[a] Independent samples t-test; [b] Fisher's exact chi-squared test; [c] Mann-Whitney U test; [d] Welch's t-test

TABLE 8. SLE Study Cohort Clinical Characteristics

Characteristics	SLE Subjects (N=72)	SLE, non-depressed (HADS Depression < 8) (N=42)	SLE, depressed (HADS Depression ≥ 8) (N=30)	SLE, non-depressed to SLE, depressed Odds Ratio (95% CI)	p-value
Disease Duration, mean ± SD	15.11 ± 8.47	14.57 ± 7.27	15.87 ± 9.99		0.526 a
Body Mass Index, mean ± SD	24.55 ± 5.64	23.16 ± 3.63	26.49 ± 7.24		0.026 d
Pain Score, median	5.5	4	7.5		0.001 c
Laboratory Results					
Leukocytes (10⁹/L), mean ± SD	6.77 ± 2.15	6.88 ± 2.36	6.62 ± 1.85		0.514 a
Lymphocytes (%), mean ± SD	28.98 ± 8.11	29.58 ± 8.37	28.14 ± 7.81		0.443 a
Neutrophils (%), mean ± SD	58.78 ± 7.10	58.43 ± 7.53	59.27 ± 8.49		0.665 a
Platelets (10⁹/L), mean ± SD	228.64 ± 82.33	217.06 ± 90.42	244.62 ± 67.92		0.172 a
Sedimentation Velocity (mm/h), mean ± SD	22.67 ± 20.04	22.84 ± 21.49	22.43 ± 18.25		0.935 a
Anti-dsDNA (IU/ml), mean ± SD	99.89 ± 111.73	99.63 ± 116.53	100.30 ± 106.28		0.983 a
C3 (g/L), mean ± SD	44.25 ± 55.19	42.11 ± 53.4	47.17 ± 58.38		0.710 a
C4 (g/L), mean ± SD	8.67 ± 11.36	8.49 ± 10.71	8.9 ± 12.38		0.881 a
CH50 (U/ml), mean ± SD	32.09 ± 73.63	45.88 ± 86.24	9.81 ± 39.07		0.032 d
C-Reactive Protein (mg/dl), mean ± SD	0.43 ± 0.58	0.37 ± 0.6	0.49 ± 0.53		0.453 a
Clinical Symptomology					
Cutaneous Manifestations, no. (%)					0.100 b
Yes	37 (51.4)	18 (42.9)	19 (63.8)	2.30 (0.88-6.03)	
Photosensitivity, no. (%)					0.227 b
Yes	65 (90.3)	36 (85.7)	29 (96.7)	4.83 (0.55-42.45)	
Foot & Mouth Ulcers, no. (%)					0.328 b
Yes	28 (38.9)	14 (33.3)	14 (46.7)	1.75 (0.67-4.58)	
Arthritis, no. (%)					0.063 b
Yes	60 (83.3)	32 (76.2)	28 (93.3)	1.89 (0.34-10.48)	
Alopecia, no. (%)					0.623 b
Yes	48 (66.7)	29 (69)	19 (63.3)	0.77 (0.29-2.08)	
Headaches, no. (%)					0.004 b
Yes	40 (55.6)	17 (40.5)	23 (76.7)	4.83 (1.70-13.76)	
Kidney Disease, no (%)					0.812 b
Yes	39 (54.2)	22 (52.4)	17 (56.7)	1.19 (0.46-3.05)	
Neurological Symptoms, no. (%)					0.289 b
Yes	19 (26.4)	9 (21.4)	10 (33.3)	1.83 (0.63-5.28)	
Smoke, no. (%)					0.537 b
Yes	13 (18.1)	9 (21.4)	4 (13.3)	0.56 (0.16-2.04)	
Alcohol, no. (%)					0.688 b
Yes	6 (8.3)	3 (7.1)	3 (10)	1.44 (0.27-7.70)	
Neuropsychiatric pathology, no. (%)					0.298 b
Yes	19 (26.4)	9 (21.4)	10 (33.3)	1.83 (0.63-5.28)	
Pulmonary disease, no. (%)					0.596 b
Yes	19 (26.4)	10 (23.8)	9 (30)	1.37 (0.48-3.94)	
Musculoskeletal Pathology, no. (%)					1.000 b
Yes	11 (15.3)	6 (14.3)	5 (16.7)	1.2 (0.33-4.37)	
Physically Active, no. (%)					0.204 b
Yes	24 (33.3)	17 (40.5)	7 (23.3)	0.45 (0.16-1.27)	
Thyroid Pathology, no. (%)					1.000 b
Yes	5 (6.9)	3 (7.1)	2 (6.7)	0.93 (0.15-5.93)	
History of Hypertension, no. (%)					1.000 b
Yes	17 (23.6)	9 (21.4)	8 (26.7)	1.33 (0.45-3.98)	
Acute Confusion Syndrome, no. (%)					1.000 b
Yes	3 (4.2)	2 (4.8)	1 (3.3)	0.69 (0.06-7.97)	

a Independent samples t-test; b Fisher's exact chi-squared test; c Mann-Whitney U test; d Welch's t-test

TABLE 9. SLE & Depression Study Cohort Clinical Characteristics

Characteristics	SLE Subjects (N=72)	SLE, non-depressed (HADS Depression ≤ 7) (N=42)	SLE, depressed (HADS Depression ≥ 8) (N=30)	Depressed (N=13)	total SLE Subjects to Depressed Odds Ratio (95% CI)	p-value	SLE, non-depressed to Depressed Odds Ratio (95% CI)	p-value	SLE, depressed to Depressed Odds Ratio (95% CI)	p-value
Body Mass Index, mean ± SD	24.55 ± 5.64	23.16 ± 3.63	26.49 ± 7.24	26.15 ± 5.38		0.343 a		0.025 a		0.884 a
Pain Score, median	5.5	4	7.5	5		0.491 c		0.074 c		0.379 c
Smoke, no. (%)										
Yes	13 (18.1)	9 (21.4)	4 (13.3)	0 (0)		0.203 b		0.096 b		0.297 b
Alcohol, no. (%)										
Yes	6 (8.3)	3 (7.1)	3 (10)	5 (38.5)	6.87 (1.70-27.75)	0.011 b	8.13 (1.61-41.20)	0.013 b	5.63 (1.10-28.84)	0.042 b
Neuropsychiatric pathology, no. (%)										
Yes	19 (26.4)	9 (21.4)	10 (33.3)	3 (23.1)	0.84 (0.21-3.37)	1.000 b	1.1 (0.25-4.86)	1.000 b	0.6 (0.13-2.68)	0.720 b
Pulmonary disease, no. (%)										
Yes	19 (26.4)	10 (23.8)	9 (30)	1 (7.7)	0.23 (0.03-1.91)	0.284 b	0.27 (0.03-2.31)	0.266 b	0.19 (0.02-1.73)	0.237 b
Musculoskeletal Pathology, no. (%)										
Yes	11 (15.3)	6 (14.3)	5 (16.7)	0 (0)		0.202 b		0.317 b		0.301 b
Physically Active, no. (%)										
Yes	24 (33.3)	17 (40.5)	7 (23.3)	7 (53.8)	2.33 (0.71-7.71)	0.212 b	1.72 (0.49-6.00)	0.525 b	5.83 (1.50-22.71)	0.077 b

a Independent samples t-test; b Fisher's exact chi-squared test; c Mann-Whitney U test; d Welch's t-test
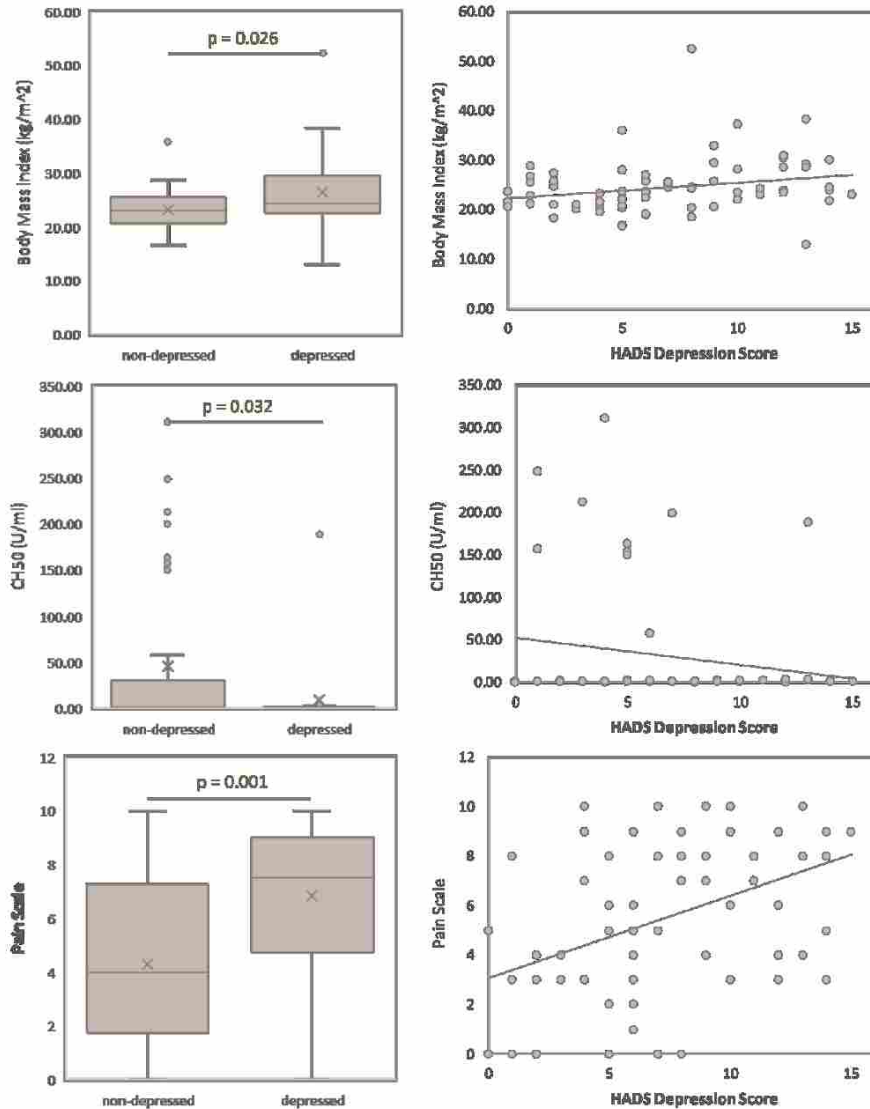
Figure 13. Lupus manifestations show correlation with HADS depression scores.

Body mass index, total complement (CH50), and pain show significant correlation with HADS depression scores. This correlation is displayed by comparing the SLE non-depressed and SLE depressed measurements. It is further demonstrated by displaying the variable measurements from all SLE patients on the y-axis of the scatter plots, with the HADS depression scores on the x-axis.

The SLE depressed patients reported higher pain scores than the SLE non-depressed subjects (p = 0.001). The SLE depressed group recorded a median pain score of 7.5, compared to 4 for the non-depressed SLE group. As HADS depression scores increase, the pain scores also increase (Figure 8). Along with the increase in pain, the SLE depressed patients further reported a higher frequency of headaches (p = 0.004) with 76.7 % compared to 40.5 % of the SLE non-depressed patients.

An increase in body mass index (BMI) was observed in the SLE depressed cohort (p = 0.026) (Figure 8). SLE non-depressed subjects had a mean BMI of 23.16 (± 3.63) compared to 26.49 (± 7.24) in the SLE depressed group. The control depressed subjects had a mean BMI of 26.15 (± 5.38) (Table 9). Interestingly, both the SLE depressed and control depressed subjects have significantly higher BMI than the SLE non-depressed group (p = 0.025). There was a higher percentage of subjects from the depressed group that regularly consumed alcoholic beverages compared to the SLE cohort, with 38.5 % compared 10 % and 7.1 %, for the SLE depressed and SLE non-depressed subjects respectively (p = 0.013 and p =0.042).

Psychosocial Function

Both psychological tools used to assess fatigue severity demonstrated significantly different levels of fatigue between the SLE depressed group and the SLE non-depressed patients (Table 10). Lower scores on the CFS indicate less fatigue, while higher scores indicate more fatigue. The SLE non-depressed subjects had less physical, mental, and overall fatigue than the SLE-depressed patients (p = 0.001, p = 0.001, p < 0.001, respectively) (Figure 2). The depression-only group CFS scores are slightly higher than the SLE non-depressed patients, but lower than the SLE depressed group and not statistically significant from either group.

TABLE 10. Study Cohort Psychological Characteristics

Characteristics	All Subjects (N=85)	SLE Subjects (N=72)	SLE, non-depressed (HADS Depression ≤ 7) (N=42)	SLE, depressed (HADS Depression ≥ 8) (N=30)	SLE, non-depressed to SLE, depressed Odds Ratio (95% CI)	p-value	Depressed (N=13)	SLE Subjects to Depressed Odds Ratio (95% CI)	p-value	SLE, non-depressed to Depressed Odds Ratio (95% CI)	p-value	SLE, depressed to Depressed Odds Ratio (95% CI)	p-value
Chalder Fatigue Scale, median	16	16	14	18		< 0.001 c	15		0.792 c		0.320 c		0.054 c
Physical Score	10	10	9	12		0.001 c	10		0.831 c		0.428 c		0.122 c
Mental Score	5	5	5	7		0.001 c	6		0.896 c		0.227 c		0.058 c
SF–36 Scores													
Physical Function, Median	22	22	24	17.5		< 0.001 c	23		0.301 c		0.605 c		0.003 c
Physical Role Function, Median	12	11.5	14	9.5		0.001 c	14		0.087 c		0.633 c		0.002 c
Emotional Role Function, Median	9	9	11	7.5		< 0.001 c	12		0.025 c		0.430 c		< 0.001 c
General Health, mean ± SD	12.68 ± 3.31	12.34 ± 3.27	13.32 ± 3.44	10.97 ± 2.46		0.002 a	14.6 ± 2.3		0.023 a		0.233 a		< 0.001 a
Health Transition, Median	3	3	3	4		0.001 c	3		0.296 c		0.958 c		0.030 c
Social Role Function, Median	7	7	8	6		< 0.001 c	7		0.873 c		0.272 c		0.069 c
Bodily Pain, mean ± SD	6.53 ± 2.78	6.32 ± 2.86	7.43 ± 2.93	4.75 ± 1.87		< 0.001 d	7.73 ± 1.97		0.091 a		0.735 a		< 0.001 a
Vitality, Median	10	10	11	7.5		< 0.001 c	12		0.029 c		0.530 c		< 0.001 c
Mental Health, Median	15	14.5	16.5	13		< 0.001 c	15		0.927 c		0.083 c		0.036 c
HADS Anxiety, mean ± SD	9.12 ± 3.86	9.31 ± 3.86	8.05 ± 3.75	11.07 ± 3.32		0.001 a	8.08 ± 3.88		0.294 a		0.981		0.014 a
HADS Anxiety Groups, no. (%)						0.001 b			0.352 b		1.000 b		0.014 b
normal (HADS ≤ 7)	29 (34.1)	23 (31.9)	20 (47.6)	3 (10)	0.12 (0.03-0.47)		6 (46.2)	1.82 (0.55-6.05)		0.94 (0.27-3.28)		7.71 (1.53-38.83)	
mild to severe (HADS ≥ 8)	56 (65.9)	49 (68.1)	22 (52.4)	27 (90)	8.18 (2.15-31.18)		7 (53.8)	0.55 (0.17-1.81)		1.06 (0.30-3.69)		0.13 (0.03-0.65)	
PSQI, median	8	8	8	9		0.076 c	9		0.185 c		0.056 c		0.749 c
PSQI groups, no. (%)						1.000 b			0.348 b		0.317 b		0.297 b
normal (PSQI ≤ 5)	10 (11.8)	10 (13.9)	6 (14.3)	4 (13.3)	0.92 (0.24-3.60)		0 (0)						
poor (PSQI ≥ 6)	75 (88.2)	62 (86.1)	36 (85.7)	26 (86.7)	1.08 (0.28-4.23)		13 (100)						
Relationship Assessment Scale, mean ± SD	24.26 ± 5.12	25.45 ± 4.19	25.48 ± 3.95	25.40 ± 4.56		0.869 c	18.50 ± 5.49		< 0.001 c		< 0.001 c		0.001 c
Fatigue Severity Scale, mean ± SD	4.57 ± 1.52	4.73 ± 1.55	4.12 ± 1.65	5.59 ± 0.85		< 0.001 a	3.68 ± 1.09		0.009 a		0.223 a		< 0.001 a

a Independent samples t-test; b Fisher's exact chi-squared test; c Mann–Whitney U test; d Welch's t-test

HADS: Hospital Anxiety and Depression Scale; PSQI: Pittsburg Sleep Quality Index
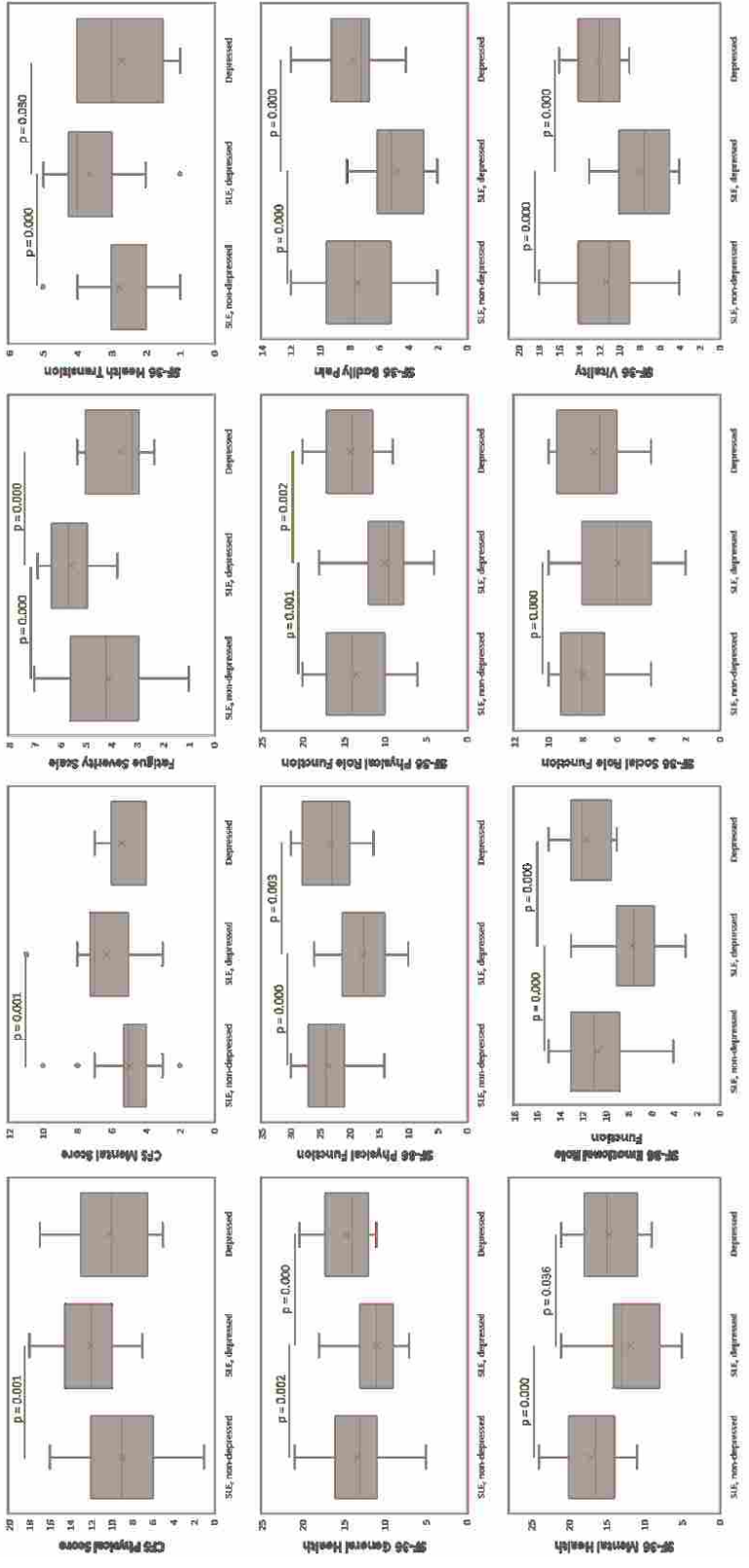
Figure 14. Psychosocial variables show correlation with HADS depression scores.

A variety of psychosocial assessments demonstrate a correlation with HADS depression scores including the Chalder Fatigue Scale (CFS), SF-36 sub categories, HADS anxiety, and fatigue severity scale (FSS). The box and whisker plots compare the SLE non-depressed, SLE depressed, and depressed control cohort's assessment scores.

The second fatigue assessment, the fatigue severity scale (FSS), also found statistical differences in fatigue between the SLE depressed and SLE non-depressed subjects ($p < 0.001$). The SLE non-depressed patients demonstrated a mean FSS score of 4.12 ($\pm$ 1.65) compared to the SLE depressed subjects (5.59 $\pm$ 0.85) (Figure 9). Interestingly, the SLE depressed subjects had higher levels of fatigue compared to the depressed control cohort (3.68$\pm$ 1.09) ($p < 0.0001$).

To assess the patients' perception of their general health, the SF-36 assessment was used. This psychological tool yields eight sub scores, each of which was found to be different between the SLE depressed and SLE non-depressed subjects (Table 10). The ranking of the SF-36 assessment gives a higher score for patients that feel healthier in that sub category, with lower scores indicating lower health. The SLE non-depressed cohort reported a median physical function score of 24, a median physical role function score of 14. The SLE depressed patients demonstrated significantly lower physical function and physical role function scores, with median scores of 17.5 and 9.5 respectively ($p < 0.001$ and $p = 0.001$, respectively) (Figure 14). The SLE non-depressed patients exhibited a mean general health score of 13.32 $\pm$ 3.44 and a median health transition score of 3, which was significantly higher than the SLE depressed patients, (general health 10.97 $\pm$ 2.46, health transition 4), ($p = 0.002$ and $p = 0.001$, respectively). The final sub score for physical health, bodily pain, in the SLE non-depressed cohort was had a mean of 7.43$\pm$ 2.93, with the SLE depressed patients recording a mean of 4.75

± 1.87. This difference in scores indicates a significantly higher level of perceived pain in the SLE depressed subjects (p < 0.001) (Table 10 & Figure 14).

The mental health assessment portion of the SF-36 consists of the variable sub scores including vitality, social role function, emotional role function, and mental health. SLE non-depressed patients exhibited higher vitality and social role function scores (p < 0.001 and p < 0.001, respectively) with a median score of 11 and 8 respectively, than the SLE depressed patients, which had a median score of 7.5 and 6, respectively. Emotional health assessed by the SF-36 recorded that the SLE non-depressed subjects had a median score of 11, while the SLE depressed patients had a lower median score of 7.5 (p < 0.001). The final sub score calculated for the SLE non-depressed cohort, mental health, had a median score of 16.5 and the SLE depressed patients had a median score of 13 (p < 0.001). Surprisingly, the depressed control subjects scores were quite similar to the SF-36 sub categorical scores of the SLE non-depressed cohort (Table 10). Upon comparing the depressed controls to the SLE depressed patients, distinct differences in all the SF-36 categories, save for that of social role function, were found. In these seven categories, the SLE depressed patients reported significantly worse scores (Table 10).

The SLE depressed patients were highly anxious, with 90% demonstrating heightened anxiety scores, compared to the SLE non-depressed subjects with 52.4 % of the patients with HADS anxiety scores exceeding what is considered normal (p=0.001). The depressed control cohort exhibited similar anxiety scores as the SLE non-depressed control with 53.8 % of the subjects having higher than normal anxiety scores. These values are significantly lower than those observed in the SLE depressed patients (p = 0.014).

Longitudinal Impact

TABLE 11. SLE subjects with reduced HADS scores

Characteristics	First Observation SLE (depressed)	One month later SLE (non-depressed) (N=5)	p-value
Pain Score, median	6	7	0.269 c
Cutaneous Manifestations, no. (%)			0.400 b
Yes	2 (40)	1 (20)	
Photosensitivity, no. (%)			0.500 b
Yes	5 (100)	4 (80)	
Foot & Mouth Ulcers, no. (%)			0.206 b
Yes	4 (80)	1 (20)	
Arthritis, no. (%)			1.000 b
Yes	4 (80)	4 (80)	
Alopecia, no. (%)			0.008 b
Yes	5 (100)	0 (0)	
Headaches, no. (%)			0.500 b
Yes	5 (100)	4 (80)	
Kidney Disease, no (%)			0.524 b
Yes	3 (60)	1 (20)	
Neurological Symptoms, no. (%)			1.000 b
Yes	2 (40)	3 (60)	
Smoke, no. (%)			1.000 b
Yes	1 (20)	1 (20)	
Alcohol, no. (%)			0.500 b
Yes	0 (0)	1 (20)	
Physically Active, no. (%)			1.000 b
Yes	3 (60)	3 (60)	
Laboratory			
Leukocytes (10^9/L), mean ± SD	6.39 ± 1.83	5.85 ± 2.66	0.905 a
Lymphocytes (%), mean ± SD	24.54 ± 5.68	22.5 ± 12.01	0.963 a
Neutrophils (%), mean ± SD	63.44 ± 6.36	65.93 ± 14.09	0.984 a
Platelets (10^9/L), mean ± SD	256 ± 137.81	296.5 ± 84.11	0.473 a
Sedimentation Velocity (mm/h), mean ± SD	20 ± 31.52	22.25 ± 30.08	0.423 a
Anti-dsDNA (IU/ml), mean ± SD	135.83 ± 162.04	145.1 ± 198.47	0.820 a
C3 (g/L), mean ± SD	21.85 ± 46.48	80.68 ± 54.02	0.185 a
C4 (g/L), mean ± SD	4.95 ± 10.65	17.55 ± 11.61	0.181 a
CH50 (U/ml), mean ± SD	1.49 ± 1.48	1.00 ± 0.11	0.500 a
C-Reactive Protein (mg/dl), mean ± SD	0.67 ± 0.37	7.05 ± 11.57	0.445 d
Psychological			
HADS Anxiety, mean ± SD	9.2 ± 2.86	6.6 ± 3.78	0.332 a
HADS Anxiety, no. (%)			1.000 b
normal (HADS ≤ 7)	1 (20)	2 (20)	
High (HADS ≥ 8)	4 (80)	3 (60)	
Relationship Assessment Scale, median	22.40 ± 1.52	30.60 ± 2.79	0.004 c
Fatigue Severity Scale, mean ± SD	5.73 ± 0.97	5.86 ± 0.86	0.911 a
PSQI, median	9	7.5	0.414 c
PSQI, no. (%)			0.500 b
poor (PSQI > 5)	5 (100)	4 (80)	

a Paired samples two-sided t-test; b Fisher's exact chi-squared test; c Wilcoxon signed-rank test; d Welch's t-test

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Approximately one month after the initial clinical and psychosocial assessment, patients from the SLE cohort were reassessed. Of thirty SLE patients that exhibited pathological HADS depression scores, five of them had normal HADS depression scores during this second assessment. While five patients compose a rather small group, these differences might be of interest in later studies. In the first assessment, every one of these patients exhibited alopecia as a disease related complaint, however, in the second assessment, all five no longer had alopecia ($p = 0.008$). A further difference identified was the strong increase in relationship assessment scale (RAS) scores ($p = 0.004$). The first assessment revealed a mean relationship assessment scale score of 22.40 ± 1.52, while the second assessment recorded a mean relationship assessment scale score of 30.60 ± 2.79 (Table 11).

TABLE 12. Multivariate Model for SLE Cohort

Variables	Odds Ratio (95% CI)	p-value
Age (years)	1.01 (0.99-1.02)	0.277
Education (years)	0.99 (0.96-1.02)	0.451
Body Mass Index	1.01 (1.00-1.02)	0.123
SF-36 Score		
Physical Function	0.97 (0.95-1.00)	0.040
Physical Role Function	1.04 (1.00-1.07)	0.030
Vitality	0.97 (0.93-1.01)	0.180
Mental Health	0.96 (0.93-0.98)	0.002
Fatigue Severity Scale	1.11 (1.01-1.22)	0.029
	pseudo R^2	AICc
Model Fit Summary	0.787	367.720

Multivariate Analysis

To determine what variables might play a role as the best indicators of increased HADS depression scores relative to the SLE cohort, we utilized generalized linear regression models for the multivariate analysis. A list of candidate models was compiled and compared to one another

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based on the number of variables included, pseudo R^2, and AICc values. Each model included the confounding sociodemographic variables that were identified as significantly different between the SLE depressed and SLE non-depressed subjects, namely age, body mass index, and years of education. All other variables that were found to be significantly different between the SLE non-depressed and SLE depressed patients were included as possible variables in the multivariant analysis. The only significant variable not included as a candidate variable was HADS anxiety scores. This variable was omitted due to the close correlation with the HADS depression score and because they are derived from the same assessment. The best fit model, identifies the best indicators of depression for this SLE cohort. Of the variables included in the model, several were considered significant. These include three SF-36 scores, physical function ($p = 0.040$), physical role function ($p = 0.030$), and mental health ($p = 0.002$), as well as fatigue severity scale scores ($p = 0.029$). Of these, the latter is likely the most sensitive to changes in HADS depression scores as indicated by the odds ratio of 1.11 (1.01-1.22), which is slightly higher than other significant variables.

3.5 Discussion

The overwhelming finding of this study is that lupus patients with depression are experiencing extreme suffering. They present higher number of depressive symptoms even than patients for whom depression is the primary diagnosis. They are also more prone to experience anxiety. In addition to being more depressed and anxious than primary depression patients, they suffer more physical symptoms than lupus patients without depression. These depressed lupus patients are living in the worst of both worlds.

The study examined the relationship between various clinical and psychosocial variables that could contribute to or indicate an increase in depression in patients with systemic lupus erythematosus. Fatigue is directly correlated to an increased risk for depression among lupus patients (Morris et al., 2016; van Exel et al., 2013; Wiseman et al., 2017; Yilmaz-Oner et al., 2016). This was observed in the univariate analysis, in the Chalder Fatigue Scale and Fatigue Severity Scale, and the multivariate analysis, making the measurement of fatigue an excellent indicator of depression in SLE patients (Table 10 & Figure 14).

Interestingly, the SLE depressed subjects and SLE non-depressed cohort did not show a significant difference in sleep quality. While the prevalence of poor sleepers is equal to or higher than that observed in the general population, it is does not appear to be correlated with depression indicating that a poor sleep quality is not causing depressive symptoms, and perhaps is not one of the significant factors contributing to such drastically high levels of fatigue among lupus patients (Inoue et al., 2017). Other studies have found that fatigue was strongly correlated with an increase in body mass index in SLE patients (Wiseman et al., 2017). The trends observed in past studies would suggest that as fatigue increases, depression and body mass index scores would also increase. The data supports this trend (Table 8 & Figure 14). Increased body mass index scores were found to correlate with increased levels of depression amongst in SLE cohort. These results emphasize the importance of assisting patients alleviate chronic fatigue, as fatigue seems to be associated with a variety of psychosocial and clinical aspects of SLE. The findings from the fatigue assessments demonstrate that the depressed lupus cohort suffers from both physical and mental fatigue. The need for continued research to better understand how to best treat both types of patient fatigue is necessary.

The data from the cohort analyzed further found that the SF-36 psychosocial assessments were also excellent indicators of depression for the lupus cohort. All the physical and mental categories were found to be negatively correlated with depression (Table 10 & Figure 14). Two of the SF-36 physical components were found to be directly correlated to depression scores in multivariate analysis, that of physical and physical role function. These findings support multiple studies that indicate SF-36 scores are negatively correlated with depression in SLE subjects (Mak, Tang, & Ho, 2013; Yilmaz-Oner et al., 2016). Interestingly, the depressed control group show significantly higher scores in these three categories and most of the other SF-36 categories when compared to the SLE depressed subjects. These results suggest that the SLE depressed patients decreased physical and mental health is likely a result of SLE disease activity as opposed to depression. Another hypothesis could be the cumulative burden of the disability imposed by an autoimmune and a mental disorder severely impinges quality of life.

Heightened pain is correlated with increased depression. This evidence confirms various studies demonstrating that increased pain is related to greater risk for depression in SLE patients (Karol, Criscione-Schreiber, Lin, & Clowse, 2013; Xie, Wu, & Chen, 2016). Also of interest is the higher frequency of headaches observed in the SLE depressed population compared to the SLE non-depressed patients. A final interesting clinical characteristic is the decreased levels of total complement (CH50) observed in the depressed patients. This supports previously observed changes in complement levels in patients with neuropsychiatric lupus and animal models (Alexander, Jacob, Bao, Macdonald, & Quigg, 2005; Karol et al., 2013; Stock, Wen, & Putterman, 2013). Low complement is a marker of inflammation and disease activity in lupus, and suggests that heightened immune dysfunction and associated disease activity correlates with more depression. Interestingly, other commonly used indicators of disease activity, such as C-

reactive protein levels or Sedimentation Rate, do not correlate with depression. These tests also failed to correlate with any lupus symptoms in this cohort.

From the assessment of patients, a month after their first contact, several significant differences indicating possible correlations were observed. All of these patients had alopecia on their first contact, but it was gone by their second assessment. Lastly, they also rated their relationships higher. This longitudinal observation of the data indicates a possible correlation between these variables and depression. It is the belief of the authors that the increased satisfaction with a close relationship in SLE patients is likely a protective factor resulting in decreased depressive symptoms. Supporting and gratifying relationships are known to alleviate depression associated with other chronic and debilitating diseases (Manne & Badr, 2010). However, with the small sample size and the nature of the experiment, no conclusive directionality can be imposed upon the data at this time.

In the SLE cohort we observed a high prevalence of depression, 41.7 %, above the threshold for depression as indicated by the hospital depression and anxiety assessment. A still higher proportion of SLE subjects reported above average anxiety scores, 65.9 %. This prevalence, while high, is not uncommonly so for SLE populations (Figueiredo-Braga et al., 2009; L. Zhang, Fu, Yin, Zhang, & Shen, 2017).

In summary, this cohort provides a variety of insights into the relationship between depression in SLE patients and clinical and psychosocial variables that are associated with both the neuropsychological and physiological symptoms. The findings highlight the importance of assisting patients with both physical and mental health care. Beyond clinical and laboratory monitoring of immune dysfunction, attentive clinicians are aware of the value of more discrete symptoms as fatigue, pain, anxiety and depression. It has been demonstrated in past studies that

treatment of the neuropsychological symptoms improves overall health, and appropriate treatment of physical symptoms assists in the improvement of psychological manifestations (Karol et al., 2013; E. H. Lin et al., 2003).

Chapter 4. Interrelation between the physical and the psychological in inflammatory disease: Comparison of clinical, immunological and psychosocial factors between Lupus and Rheumatoid Arthritis patients in a Portuguese cohort.

4.1 Summary

Objective To increase the understanding of the relationship between depression and various psychosocial, clinical, and immunological factors relevant to systemic lupus erythematosus (SLE) were examined in a cohort of SLE patients using healthy, depressed, and arthritic patients for comparison.

Methods The cohort consisted of seventy-seven Caucasian patients. Physiological, clinical, and psychosocial data was collected from all patients and compared using a variety of statistical analysis to determine correlation and identify similarities and differences between the SLE patients and control cohorts. ELISA was used to measure cytokine levels.

Results The prevalence of moderate to severe depression in the SLE cohort as determined by the Hospital Anxiety and Depression Index was forty percent with six times greater chance of having depression when compared to the healthy control subjects. Pain, IL-6, and Pittsburg quality sleeping index values were all significantly higher in SLE patients compared to the healthy control group ($p < 0.001$, $p = 0.038$, and $p = 0.005$, respectively). Anxiety levels were significantly higher in SLE patients compared to healthy and arthritic control patients ($p = 0.020$ and 0.011, respectively). Serum IL-10 concentrations, relationship assessment scale, and fatigue severity scale values were found to be directly correlated to depression among the SLE patients

(p = 0.036, p = 0.007, and p = 0.001, respectively). Relationship assessment and fatigue severity scale scores were found to be the best indicators of depression for the SLE patients (p = 0.042 and 0.028, respectively).

Conclusion Fatigue Severity, relationship satisfaction, and IL-10 concentrations are correlated with depression in the SLE patients and are potential indicators of depression in lupus patients. By comparing our clinical and psychosocial assessments from our lupus patients with our arthritic patients we were able to identify that inflammatory symptoms likely contribute to measured levels of IL-10 and fatigue, while the drastically decreased relationship satisfaction recorded from SLE patients is likely a psychiatric disease manifestation. These results emphasize the importance of clinical and psychological patient care in the treatment of SLE.

4.2 Introduction

Systemic lupus erythematosus (SLE) is a common autoimmune disease afflicting 1 – 12 people per 5,000 worldwide (Alarcon et al., 2004; Ghodke-Puranik & Niewold, 2015). Often characterized by autoantibody production, cutaneous epidermal manifestations, and various rheumatic symptoms, lupus pathophysiology is marked by abnormal T and B cell behavior, mis-regulated apoptosis, complement function, immune complex clearance, and nucleosome processing, among others (Cornaby, Gibbons, et al., 2015; Cornaby et al., 2017; Mak & Kow, 2014). These result in a multitude of physiological complications and organ involvement. Symptoms often include fever, arthritis, fatigue, weight loss, lymphadenopathy to a characteristic "butterfly rash", renal disease and cytopenia, in a pleomorphic clinical presentation. Early symptoms of lupus that contribute to diminished quality of life also include depression and fatigue.

Ninety percent of lupus patients are women with the disease typically manifesting in earlier years. With disease manifestation early on, it causes difficulties for the family, and the patients emotional and professional lifestyle. Because of these conditions, it is no surprise that SLE patients commonly suffer from psychological disorders (Nery et al., 2007; Nery et al., 2008; Uguz et al., 2013). Interesting, depressive symptoms can be one of the early manifestations of SLE disease activity(H. X. Gao et al., 2009; Jorge et al., 2017). Also, depression and anxiety seem to be more acute during disease activity (Nery et al., 2007; Segui et al., 2000). Higher rates of depression have been observed consistently in SLE populations worldwide (L. Zhang et al., 2017). Even with psychological disorders involved so often with SLE, little research has been invested in understanding the mechanisms involved in disorder development compared to end-organ failure and peripheral pathology (Y. Li et al., 2015).

Fatigue and pain are frequently rated by SLE patients as being severe manifestations of the disease (Petri et al., 2013; Ramsey-Goldman & Rothrock, 2010; Zonana-Nacach et al., 2000). Both disease indicators and medication fail to satisfactorily predict fatigue and pain in SLE patients, making it difficult to estimate patient discomfort and subsequently apply appropriate treatments in a timely manner (Jump et al., 2005). Laboratory and clinical relationship to fatigue and pain are still ambiguous. However, these symptoms are frequently associated with mood disorders, including depression.

It is likely the psychosocial impact of SLE contributes to the often-observed disease association with depression. With such a large number of confounding variables involved with disease progression including age, disease activity, weight, fatigue, sleep and physical activity, it is no simple matter to identify both psychological and physical factors that play into mood disorder involvement in SLE patients. Due to these reasons, the causes related to the high

prevalence of depression and anxiety in SLE is largely misunderstood. Increased disease severity, SLE related autoimmune dysfunction, and psychological stress of having a major chronic systemic disease are all hypotheses to explain this increased prevalence, however, there remain a large gap in the understanding that only further research into pathophysiology and psychosocial understanding of depression onset in SLE patients will sate (van Exel et al., 2013).

To better understand the relationship between depression in SLE patients and the variety of factors that could contribute to its prevalence, we investigated the involvement of these variables utilizing laboratory and clinical results alongside psychosocial assessments to aid in this endeavor. Further, the study also included several cohort subgroups to allow comparisons with the SLE patients, giving us a unique perspective into possible disease related factors that are correlated with and can predict depression among the SLE patients. By comparing our SLE cohort findings to those of arthritic patients, we can better comprehend what symptoms correlated with depression are likely the result of a chronic inflammatory disease. Our Depressed cohort further allows us to predict what variables are likely caused by psychological complications, rather than inflammatory disease.

4.3 Methods

Study Patients

The study included 77 total Caucasian patients, 15 patients with systemic lupus erythematosus (SLE), 21 rheumatoid arthritis (RA) patients, 20 healthy control subjects, and 21 depressed control subjects. All patients were recruited from northern Portugal. All SLE patients were previously diagnosed and followed at an outpatient unit. No selection was done except the patient's willingness to participate in the study. Diagnosis and stage of disease activity was

established according to the American Rheumatism Association Classification Criteria (ACR) and the duration of the disease was measured from the time when the patients first met at least fthe classification criteria. All Ra patients were also previously diagnosed prior to the study by a physician as defined by the ACR. The control group of depressed control subjects was undergoing treatment at a private psychiatric clinic at the time of the study.

To obviate inter-interviewer variation, psychiatric evaluation and psychometric markers were tested by one psychiatrist and one psychologist to establish the severity of depression. A convenience age matched sample of healthy women was also recruited. Exclusion criteria comprised history of substance abuse, personality disorders and or other major psychopathology than depression. Patients and controls were subsequently interviewed by phone by trained interviewers. The literature corroborates phone interviews as valid and precise tools for psychological data collection (B. B. Cohen & Vinson, 1995; Rohde et al., 1997; Siemiatycki, 1979).

Participants' socio-demographic data included age, educational level, employment status (active/non-active) and marital status (Table 13). Laboratory and SLE clinical evaluations were obtained for the SLE patients through the clinical records (Table 14-15). Lab tests included leukocytes (10^9/L), lymphocytes (percentage), platelets (10^9/L), erythrocyte sedimentation rate (mm/h), anti-dsDNA antibody titer (IU/ml), and C-reactive protein level (mg/dl). Serum collected from participants from the all members of the cohort was obtained and levels of IL-6 (pg/ml), IL-10 (pg/ml, and TNF-α (pg/ml) were measured using standardized ELISA assays, utilized per manufactures instructions (SIGMA). The confounding variables of smoking and alcohol consumption were also recorded. Further, physical activity of the patients was assessed based on patient involvement in active sport activities.

The study was submitted and approved by the Ethical Committee of the São João Hospital IRB (EPE) in accordance with the Declaration of Helsinki. The nature and the purpose of the study were explained to all participants who signed the informed consent form before they entered the study.

Psychosocial evaluation

Socio-demographic characterization included age, education measured as years of school, marital status and socio-economic class evaluation. Psychological evaluations were obtained through a battery of standardized instruments.

Fatigue Severity Scale (FSS)

The short form of the FSS allows evaluation of self-reported fatigue (Krupp et al., 1989). The Portuguese version includes nine items and is recommended as the instrument of choice for research purposes in studies involving patients diagnosed with SLE (Ad Hoc Committee on Systemic Lupus Erythematosus Response Criteria for, 2007).

The FSS demonstrates good psychometric properties (Cronbach's $\alpha = 0.89$ and test-retest reliability 0.84). A final score is obtained from the mean of all scored items, with higher scores revealing higher severity of fatigue. Presence of clinical levels of fatigue was defined by a FSS score >3. The scale has proved to be sensitive to change and reliable for telephone interviewing. The FSS has also been shown to be reliable across different patient populations (Johansson, Kottorp, Lee, Gay, & Lerdal, 2014).

Hospital Anxiety and Depression Scale (HADS)

The Hospital Anxiety and Depression Scale (HADS) is a self-rating scale with good psychometric properties (Cronbach's alpha coefficients of 0.94), designed to measure anxiety and depression in physically ill individuals (Zigmond & Snaith, 1983). Translated and adapted for Portugal(Ferreira, 2000; Pais-Ribeiro et al., 2007), it is subdivided in two subscales of 7 items that measure independently anxiety and depression. The partial result of each scale varies between 0 and 21. Scores ranging from 8 to 10 are considered mild, from 11 to 14 moderate and 15 to 21 severe(Marcolino et al., 2007) and the authors suggest 8 as the cutoff point, considering values below as indicating the absence of anxiety and depression (Zigmond & Snaith, 1983). It is important to note that the scale is indicative of depressive symptoms in the last week, and not necessarily clinical depression.

Pittsburgh Sleep Quality Index (PSQI)

This instrument presents good psychometric properties, with high reliability (Cronbach's alpha = 0.83) and validity. The seven components evaluated - sleep latency, sleep disturbances, sleep duration, sleep quality, sleep efficiency, use of sleep medications and daytime dysfunction allow the gathering of a global score varying from 0 to 21 (Buysse et al., 1989; Carpenter & Andrykowski, 1998). The PSQI is reliable for sleep quality assessment in telephone interviews and permits the identification of poor sleepers (score > 5) (Monk et al., 2013; Palmieri et al., 2010).

Relationship Assessment Scale (RAS)

The RAS (S., 1988) (Portuguese experimental version from Mesquita, Barbosa, & Figueiredo-Braga, 2014) is a 7 item instrument, with a five-point scale that measures general satisfaction with the relationship.

Statistical analysis

Significant differences in the demographical, clinical, and psychological variables between the SLE subjects, healthy controls, RA subjects, and depressed control subjects was determined using the independent t-test, Fisher's exact chi-squared, Mann-Whitney U, Wilcoxon rank sum, or Welch's tests when considered appropriate. Fisher's exact chi-squared was used in place of the standard chi-squared test, which would typically be utilized, this is due to the smaller sample size of the groups compared. The statistical test used for the comparisons are indicated in the table legends. Univariate analysis was accomplished by using generalized linear regression with HADS depression scores as the dependent variable and the individual variable suspected of showing a correlation to depression as the independent variable. Multivariant analysis was performed using a generalized linear model. The best fit model was determined using the model with the appropriate number of variables (less than or equal to 2 variables due to the small SLE cohort) and the highest pseudo R^2 value with the lowest approximate AICc value. In the multivariate analysis, the HADS depression score for the SLE subjects was used as the dependent variable. Statistical analysis was performed using the statistical software R and SPSS (IBM). An alpha value less than or equal to 0.05 was considered significant in all analysis.

4.4 Results

In the study cohort, we investigated the prevalence and levels of multiple symptomatic and clinical characteristics that are relevant to the lupus patients. by using several different

TABLE 13. Sociodemographic of the Study Cohort

Characteristics	All Subjects (N = 77)	SLE Subjects (N = 15)	Healthy Subjects (N = 20)	p-value	Depressed Subjects (N = 21)	p-value	RA Subjects (N = 21)	p-value
Sex - no. (%)				0.571 b		0.250 b		0.062 b
Female	67 (87%)	15 (100%)	19 (95%)		18 (86%)		16 (73%)	
Male	10 (13%)	0 (0%)	1 (5%)		3 (14%)		5 (24%)	
Age, mean ± SD	48.38 ± 12.17	49 ± 8.21	43.95 ± 11.77	0.165 a	47.81 ± 14.8	0.780 a	52.71 ± 11.17	0.282 a
Education Lvl (Years) ± SD	9.73 ± 4.8	7.6 ± 3.98	13.5 ± 3.15	< 0.001 a	9.76 ± 4.36	0.138 a	7.62 ± 5.08	0.990 a
Education Lvl - no. (%)				< 0.001 b		0.264 b		0.229 b
Primary ($\leq$ 4 yrs)	17 (22%)	4 (27%)	0 (0%)		4 (19%)		9 (43%)	
Middle School ($4 < 12$ yrs)	17 (22%)	7 (47%)	1 (5%)		4 (19%)		5 (24%)	
High School ($12 < 14$ yrs)	10 (13%)	1 (7%)	2 (10%)		5 (24%)		2 (10%)	
College ($14 \leq 18$ yrs)	33 (43%)	3 (2%)	17 (85%)		8 (38%)		5 (24%)	
Marital Status - no. (%)				0.123 b		0.495 b		0.818 b
Unmarried	17 (22%)	2 (13%)	8 (40%)		5 (24%)		2 (10%)	
Married	50 (65%)	12 (80%)	8 (40%)		13 (62%)		16 (76%)	
Divorced	8 (10%)	1 (7%)	4 (20%)		1 (5%)		2 (10%)	
Widowed	3 (4%)	0 (0%)	0 (0%)		2 (10%)		1 (5%)	
Unemployed - no. (%)				0.072 b		0.156 b		0.482 b
Yes	38 (49%)	10 (67%)	6 (30%)		8 (38%)		14 (67%)	
No	39 (51%)	5 (33%)	14 (70%)		13 (62%)		7 (33%)	

[a] Independent samples t-test; [b] Fisher's exact chi-squared test

groups as controls, we could identify potential indicators and factors that demonstrated correlations with depression in the lupus subjects. By comparing the SLE patients to another group of patients that have a similar chronic rheumatic inflammatory disease, in this case, rheumatoid arthritis, similarities would help us understand what is a possible result of chronic disease, and what is specific to lupus. When comparing the SLE patients to the healthy controls we identify factors that are different from a healthy population and could be a result of psychological or physical disease. Differences and similarities observed between the SLE subjects help identify characteristics that are potentially strictly related to psychological or physiological disease.

The study was comprised of seventy-seven Caucasian men and women with a mean age of 48.38 ± 12.17 years. The sociodemographic information gathered about the cohort is displayed in Table 13. Of the seventy-seven subjects, 15 were diagnosed systemic lupus erythematosus patients, 21 were diagnosed rheumatoid arthritis patients, 20 subjects were healthy controls, and 21 were individuals being treated for depression at a private psychiatric clinic. The lupus patients had an average age of 49 ± 8.21 years. The arthritis patients had an average age of 52.71 ± 11.17 years, while the healthy and depressed control subjects had a mean of 43.95 ± 11.77 and 47.81 ± 14.8 years, respectively. There is some variability between the mean ages of the groups, but it was not enough to be considered significant between any of the groups (Table 13).

The level of education received based on years in school is similar for the SLE, depressed, and RA subjects. However, the healthy control group has a higher percentage of well-educated members ($p < 0.001$). The distribution of marital status was similar for all the study groups with the majority of subjects currently in a marital relationship. There were some slight

differences in employment status between the cohort groups. The distribution between the SLE and RA patients is similar, with 33% of patients from both groups currently employed, while the healthy and depressed control groups had 70% and 62% of the study subjects currently employed, respectively. The differences between the employment status of the study cohort groups was not considered significantly different (Table 13).

Clinical manifestations

Table 14 and 15 contain the clinical and psychological assessment data from the cohort with SLE patient data compared to all other control groups in Table 14 and logistical regression comparison displayed from the SLE cohort in Table 15. The HADS depression score was used as the dependent variable with only the indicated characteristic as the independent variable. The SLE cohort had a mean disease duration of 17.80 ± 7.32 years, which was similar to the mean duration of disease among the rheumatoid arthritis subjects, 17.57 ± 10.24 (Table 14). The mean body mass index scores were fairly similar for all groups. It is important to note that the potential confounding variables of smoking, drinking, and physical activity are similar between the groups being compared in the study (Table 14). The SLE patients, not uncharacteristically, demonstrated higher pain scores on average than all of the other groups with the median score being 7.0. However, it was only significantly higher than the healthy control group ($p < 0.001$). Interestingly the depressed control cohort, while lower on average than the SLE patients, was not significantly different from the SLE patients. Further, univariate regression analysis found that none of these characteristics showed direct correlation with depression among the SLE patients (Table 15).

The SLE patients demonstrated typical levels of sedimentation velocity (mm/h), leukocyte concentrations (10^9/L), and lymphocyte percentage. These measurements were similar

TABLE 14. The Clinical, Laboratory, and Psychological Characteristics

Characteristics	All Subjects (N = 77)	SLE Subjects (N = 15)	Healthy Subjects (N = 20)	Odds Ratio (95% CI)	p-value	Depressed Subjects (N = 21)	Odds Ratio (95% CI)	p-value	RA Subjects (N = 21)	Odds Ratio (95% CI)	p-value
Disease Duration (years), mean ± SD	17.68 ± 4.15	17.80 ± 7.32	N/A			N/A			17.57 ± 10.24		0.942 a
Body Mass Index, mean ± SD	26.16 ± 5.65	27.89 ± 8.2	25.39 ± 4.71		0.304 a	24.94 ± 4.44		0.221 a	26.89 ± 5.32		0.660 a
Pain Score (0-10), median	5.0	7.0	0.0		0.000 c	5.0		0.783 c	5.0		0.213 c
Smoking - no. (%)					0.199 b			1.000 b			0.367 b
Yes	13 (18%)	1 (7%)	6 (30%)	0.17 (0.02-1.57)		2 (10%)	0.68 (0.06-8.25)		5 (24%)	0.23 (0.02-2.20)	
Drinking - no. (%)					0.627 b			0.424 b			1.000 b
Yes	17 (22%)	2 (13%)	5 (25%)	0.46 (0.08-2.79)		6 (29%)	0.38 (0.07-2.25)		4 (19%)	0.65 (0.10-4.14)	
Physically Active - no. (%)					0.506 b			0.736 b			0.175 b
Yes	32 (42%)	7 (47%)	12 (60%)	0.58 (0.15-2.26)		8 (38%)	1.42 (0.37-5.45)		5 (24%)	2.80 (0.67-11.67)	
Laboratory Analysis											
Sedimentation Velocity (mm/h), mean ± SD	17.94 ± 19.1	20.69 ± 20.1	N/A			N/A			15.84 ± 18.64		0.500 a
C-Reactive Protein (mg/L), mean ± SD	3.74 ± 10.11	0.27 ± 0.23	N/A			N/A			3.42 ± 4.25		0.010 d
Leukocytes (10^9/L), mean ± SD	7.25 ± 3.84	6.3 ± 2.13	N/A			N/A			7.97 ± 4.7		0.205 d
Lymphocytes (%), mean ± SD	26.95 ± 7.81	29.05 ± 6.77	N/A			N/A			25.34 ± 8.35		0.203 a
Anti-dsDNA (IU/ml), mean ± SD	N/A	635.54 ± 1504.14	N/A			N/A			N/A		
IL-6 (pg/ml), mean ± SD	3.25 ± 6.36	3.74 ± 4.16	1.11 ± 1.34		0.038 d	1.30 ± 2.23		0.060 d	10.48 ± 12.76		0.137 d
IL-10 (pg/ml), mean ± SD	6.06 ± 9.71	6.62 ± 4.04	3.07 ± 3.77		0.016 a	9.18 ± 15.66		0.482 d	4.12 ± 2.97		0.112 a
TNF-Alpha (pg/ml), mean ± SD	56.25 ± 95.73	73.52 ± 113.25	33.77 ± 53.08		0.241 d	38.44 ± 69.75		0.264 a	109.92 ± 151.85		0.507 a
Psychosocial Assessments											
HADS Depression Score, mean ± SD	6.32 ± 4.70	8.07 ± 4.37	4.15 ± 3.25		0.007 c	8.2 ± 5.33		0.947 c	5.06 ± 4.51		0.081 c
Depression Groups - no. (%)					0.046 b			0.736 b			0.122 b
low (HADS < 11)	58 (75%)	9 (60%)	18 (90%)	0.17 (0.03-1.00)		14 (67%)	0.75 (0.19-2.97)		17 (81%)	0.35 (0.08-1.58)	
high (HADS ≥ 11)	19 (25%)	6 (40%)	2 (10%)	6.00 (1.00-35.90)		7 (33%)	1.33 (0.34-5.27)		4 (19%)	2.83 (0.63-12.71)	
HADS Anxiety Score, mean ± SD	8.57 ± 4.50	10.93 ± 4.68	7.30 ± 3.48		0.020 c	9.58 ± 4.44		0.536 c	6.27 ± 4.32		0.011 c
Anxiety Groups - no. (%)					0.020 b			0.736 b			0.025 b
low (HADS < 11)	55 (71%)	8 (53%)	17 (85%)	0.20 (0.04-0.99)		12 (57%)	0.86 (0.23-3.25)		18 (86%)	0.19 (0.04-0.93)	
high (HADS ≥ 11)	22 (29%)	7 (47%)	3 (15%)	4.96 (1.01-24.37)		9 (43%)	1.17 (0.31-4.42)		3 (14%)	5.52 (1.07-25.70)	
Pitsburge Sleep Quality Index, mean ± SD	6.32 ± 3.52	7.67 ± 3.06	4.75 ± 2.29		0.005 c	6.43 ± 2.60		0.300 c	6.76 ± 4.97		0.233 c
Pitsburge Sleep Quality Index, no. (%)					0.920 b			1.000 b			0.728 b
normal (PSQI ≤ 5)	35 (45%)	5 (33%)	13 (65%)	0.27 (0.07-1.11)		7 (33%)	1.00 (0.25-4.08)		10 (48%)	0.55 (0.14-2.17)	
poor (PSQI ≥ 6)	42 (55%)	10 (67%)	7 (35%)	3.71 (0.90-15.26)		14 (67%)	1.00 (0.25-4.08)		11 (52%)	1.82 (0.46-7.18)	
Relationship Assessment Scale, mean ± SD	27.56 ± 5.38	24.75 ± 4.81	28.07 ± 4.62		0.050 c	26.24 ± 5.11		0.610 c	30.82 ± 5.69		0.015 c
Fatigue Severity Scale, mean ± SD	4.00 ± 1.71	4.6 ± 1.30	3.00 ± 1.30		0.002 c	3.84 ± 1.95		0.234 c	4.93 ± 1.62		0.497 c
Fatigue Severity Scale, no. (%)					0.071 b			0.296 b			1.000 b
low (FSS ≤ 4)	50 (65%)	7 (47%)	16 (80%)	0.22 (0.05-0.97)		15 (71%)	0.35 (0.09-1.40)		12 (57%)	0.66 (0.17-2.49)	
high (FSS ≥ 5)	27 (35%)	8 (53%)	4 (20%)	4.57 (1.03-20.35)		6 (29%)	2.86 (0.71-11.44)		9 (43%)	1.52 (0.40-5.78)	

a Independent samples t-test; b Fisher's exact chi-squared test; c Mann-Whitney U test; d Welch's t-test

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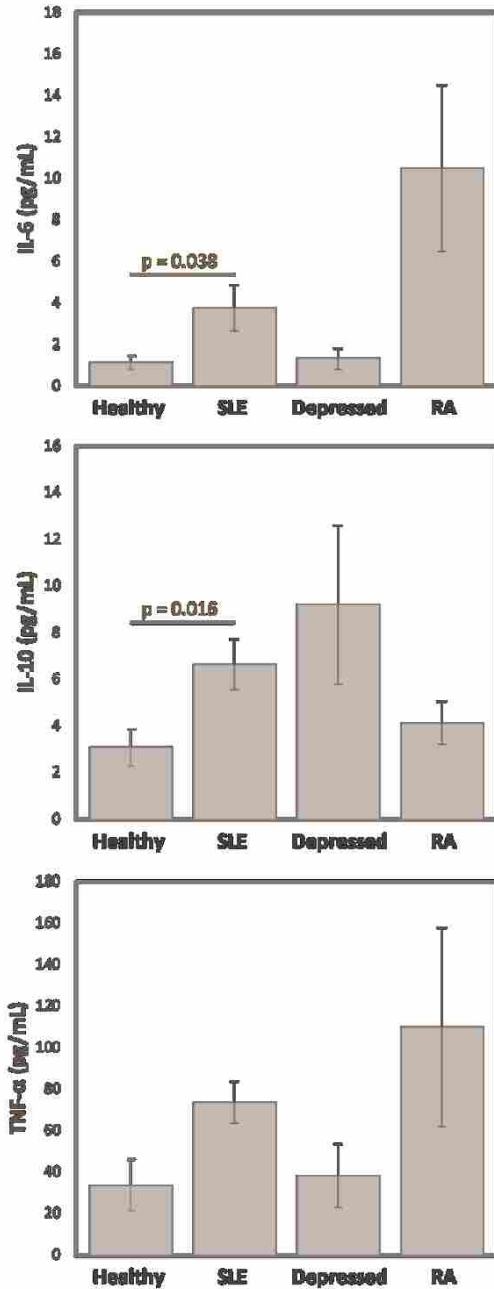
Figure 15. Cytokine serum concentrations differ between cohort subgroups.

SLE IL-6 and IL-10 serum concentrations are statistically higher than healthy subjects. RA patients demonstrate slightly higher average serum levels of pro-inflammatory cytokines IL-6 and TNF-α. Depressed and SLE patients demonstrate slightly higher concentrations of IL-10 on average than other cohort subjects.

between the SLE and RA subjects with no significant differences identified (Table 14).

However, RA patients had elevated levels of c-reactive protein compared to the SLE patients (p

= 0.010) (Figure 11). This is normally the case and has often been seen when comparing SLE

and RA laboratory data (Keenan, Swearingen, & Yazici, 2008). Serum levels of Il-6, IL-10, and

TNF-alpha were measured for all subjects (Figure 15). The SLE patients exhibited increased

levels of both IL-6 and IL-10 compared to the healthy subjects ($p = 0.038$ and $p = 0.016$,

respectively). While there are some slight differences between the levels of IL-6, IL-10, and

TNF-α between the other groups, these differences are not significantly different. Univariate

regression analysis demonstrated a direct correlation between IL-10 levels and depression in the

SLE subjects ($p = 0.036$) (Table 15) (Figure 15). Aside from IL-10, univariate analysis did not

show any of the other characteristics being directly correlated to depression in the SLE cohort.

Psychosocial function

Of the subgroups of the cohort, the SLE subjects had the highest percentage with

moderate to severe depression as measured by the hospital anxiety and depression assessment.

Forty percent of the SLE patients demonstrated HADS depression scores above 10 (Table 14). In

fact, the SLE patients showed a 6-fold greater likely hood than the healthy control group to have

moderate or severe depression. The SLE patients had significantly higher depression scores than

the healthy control subjects ($p = 0.007$), but not higher than the depressed or RA subjects. Not

surprising either was the fact that the SLE subjects experienced higher levels of anxiety

compared to both the healthy controls and the RA patients ($p = 0.020$ and $p = 0.011$,

respectively). However, the depressed group had similar anxiety scores.

Upon examination of the quality of sleep that the study patients were receiving, as

recorded by the Pittsburg Sleep Quality Index (PSQI) assessment, the SLE cohort had a mean
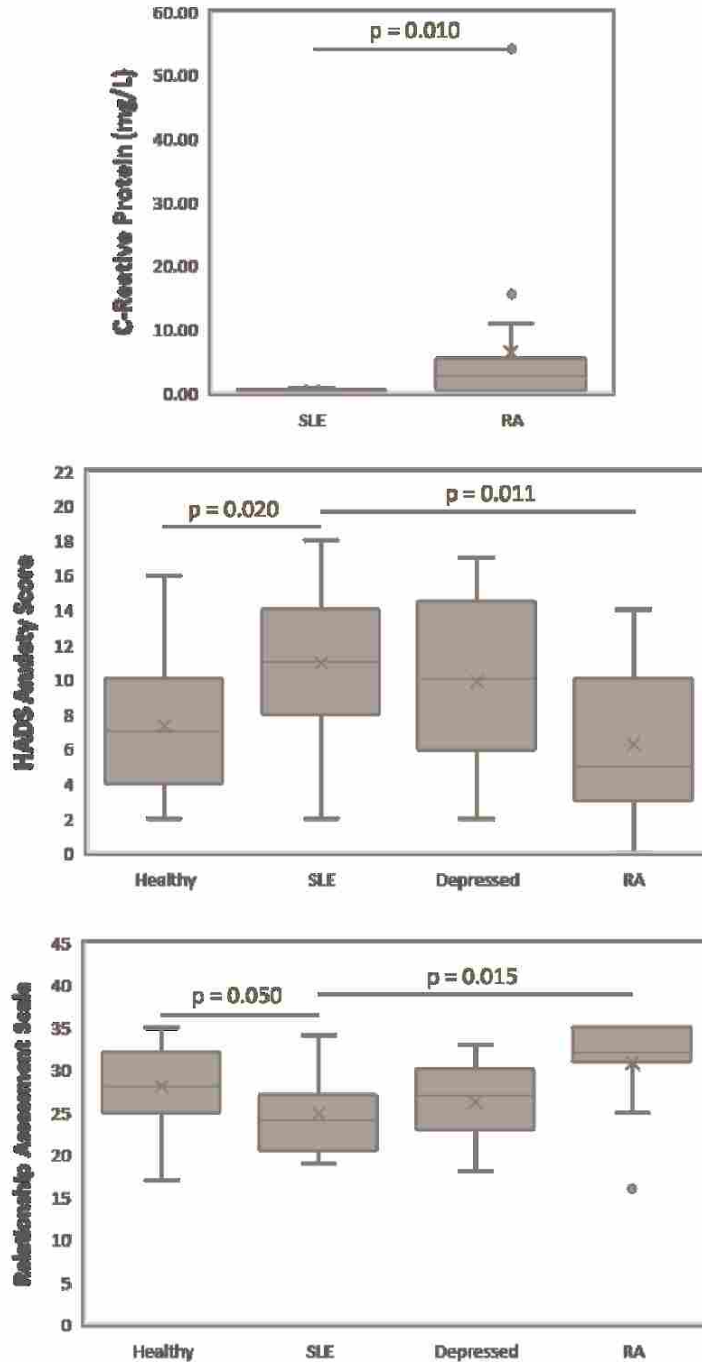
Figure 16. Clinical and Psychosocial Assessments differ between patient cohorts.

Measured serum levels of CRP are significantly higher in the RA patients compared to the SLE subjects. SE patients demonstrate elevated HADS anxiety scores compared to healthy and RA control cohorts. Further, SLE patients demonstrate decreased relationship assessment scale scores, indicating lower relationship satisfaction.

TABLE 15. Univariate Analysis of the SLE Cohort					
Characteristics	psuedo R^2	AICc	coefficient	Odds Ratio (95% CI)	p-value
Disease Duration (years), mean ± SD	0.008	99.73	0.004	1.00 (0.98-1.03)	0.736
Body Mass Index, mean ± SD	0.142	97.55	0.016	1.02 (1.00-1.04)	0.116
Pain Score, median	0.128	97.79	0.044	1.04 (0.98-1.11)	0.161
Sedementation Velocity (mm/h), mean ± SD	0.590	88.46	0.006	1.01 (1.00-1.01)	0.197
C-Reactive Protein (mg/L), mean ± SD	0.603	88.03	0.600	1.82 (0.79-4.06)	0.150
Leukocytes (10^9/L), mean ± SD	0.575	88.92	-0.051	0.95 (0.86-1.04)	0.294
Lymphocytes (%), mean ± SD	0.588	88.50	-0.017	0.98 (0.96-1.01)	0.203
Anti-dsDNA (IU/ml), mean ± SD	0.549	89.70	0.000	1.00 (1.00-1.00)	0.565
IL-6 (pg/ml), mean ± SD	0.575	88.92	0.009	0.95 (0.86-1.04)	0.680
IL-10 (pg/ml), mean ± SD	0.463	91.22	-0.057	0.94 (0.89-0.99)	0.036
TNF-Alpha (pg/ml), mean ± SD	0.380	93.25	-0.002	1.00 (1.00-1.00)	0.118
PSQI, mean ± SD	0.056	98.97	0.028	1.03 (0.97-1.09)	0.352
Relationship Assessment Scale, mean ± SD	0.926	68.96	-0.064	0.94 (0.89-0.98)	0.007
Fatigue Severity Scale, mean ± SD	0.565	87.35	0.273	1.31 (1.13-1.54)	0.001

index of 7.67 ± 3.06, which was higher than the healthy control subjects (p = 0.005). A higher

PSQI indicates a lower quality of sleep where as a higher quality of sleep is indicated by lower

scores. Normal PSQIs are reported being anywhere between zero and five. Any index above five

indicates poor sleep quality for the patient. In this case, the SLE cohort had 67% report poor

sleep quality based on the PSQI standard. This distribution of patients that experienced lower

quality of sleep is similar in the SLE cohort compared to the depressed, and RA subjects (Table

14).

All married and cohabitating cohort patients had their relationship satisfaction measured

based on the relationship assessment scale criteria. This scale provides a higher value based on

the patients perceived satisfaction with their significant other or spouse. Interestingly, the SLE

patients record having on average less satisfaction in their relationship than the averages

recorded for all the other subgroups, with a mean scale score of 24.75 ± 4.81 (Figure 11). This

score was significantly lower than both the healthy control and RA patients (p = 0.050 and p =
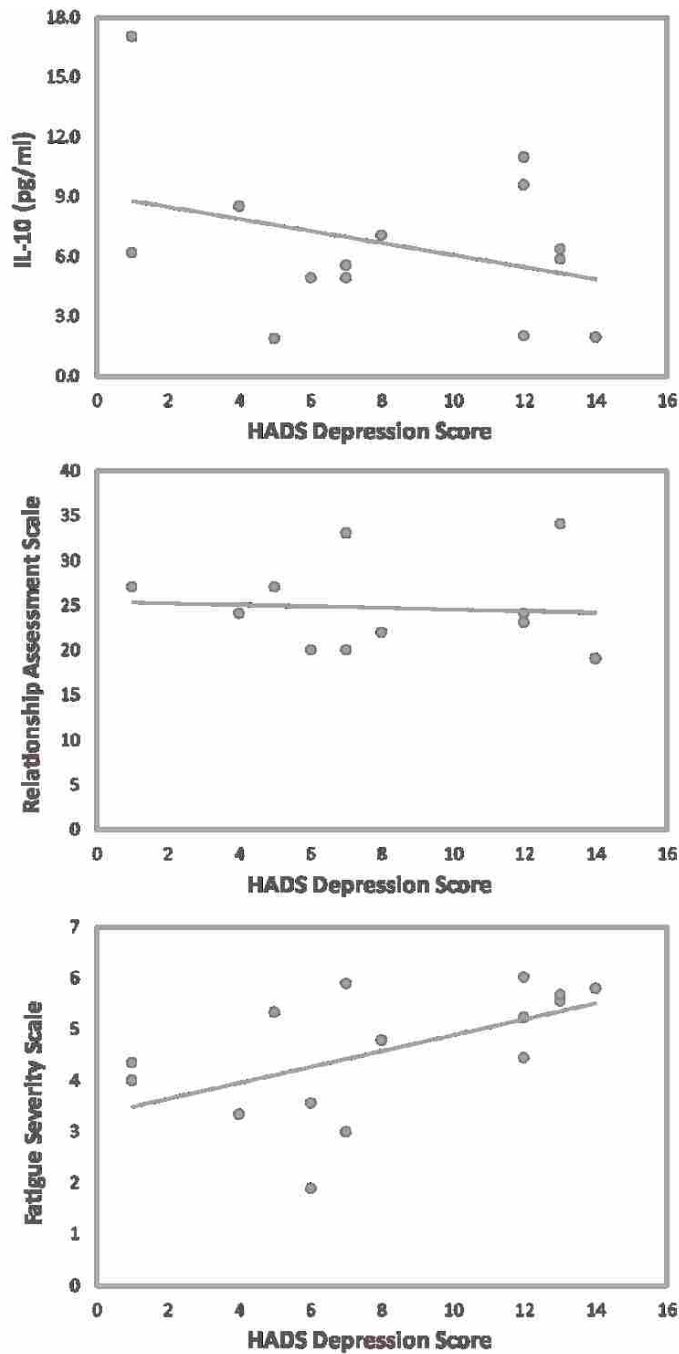
Figure 17. Linear regression identifies clinical and psychological assessments that are correlated with depression in SLE patients.

Univariate analysis found IL-10, relationship assessment scale, and fatigue severity scale values to be correlated with depression in our SLE cohort. IL-10 and relationship satisfaction are negatively correlated with depression while fatigue severity is positively correlated.

0.015, respectively). This was a surprise considering that the RA patients, also suffering from a chronic and debilitating autoimmune disease, had a mean relationship assessment scale score of 30.82 ± 5.69. It was also recognized that the reported scores for SLE patients from the relationship assessment scale showed a distinct negative correlation with HADS depression scores ($p = 0.007$) (Table 15) (Figure 17).

To measure the combination of both physical and mental fatigue that the patients were experiencing, the fatigue severity scales was utilized. The assessment assigns a higher score the higher the fatigue felt by the patient. The SLE patients reported a mean score of 4.6 ± 1.30 which was similar to the arthritis patients who reported a fatigue score of 4.93 ± 1.62 (Table 14). However, it was significantly more than the healthy control subjects ($p = 0.002$) (Figure 11). Of interest, is the fact that the depressed patients did not have significantly lower fatigue than the SLE subjects ($p = 0.234$). The distribution of those in the cohort that experienced heightened fatigue was similar between the SLE and RA subgroups. In contrast, the SLE patients were nearly 4.57 times more likely to have heightened levels of fatigue compared to the healthy control patients. Further, a direct positive correlation was observed between fatigue severity scale scores and depression levels in the SLE patients ($p = 0.001$) (Table 15) (Figure 17).

Multivariate analysis

Of the data collected from the clinical and psychosocial assessment of the SLE patients, we investigated which characteristics correlated with depression could best indicate depression. Due to the relatively small SLE cohort of this study, the model could not compensate for age or socioeconomic status as would be appropriate for a larger cohort. However, it can still indicate which variables among those identified in the study show the closest correlation with depression among the patients and thus might be the best indicators of depression for the patients. Candidate

variables that showed a direct correlation with depression or were significantly different from the healthy control and arthritis patients, but not different from the depressed patients, were considered for the multivariate model. Anxiety was not included as a candidate of for the multivariate model due to the co-relatedness with the HADS depression score and because it was derived from the same assessment as the depression score. Of the variables significantly different from the healthy control subjects and correlated with depression in the univariate models, both the fatigue severity (p = 0.028) and relationship assessment (p = 0.042) scales were indicated as strong correlates and predictors of depression among the SLE cohort (Table 16).

Table 16. Multivariant model for SLE Study Cohort 2

Variables	Odds Ratio (95% CI)	p-value
Fatigue Severity Scale	1.21 (1.03-1.45)	0.028
Relationship Assessment Scale	0.95 (0.91-1.00)	0.042
	pseudo R^2	AICc
Model Fit Summary	0.952	67.400

4.5 Discussion

The study utilized the comparison of multiple control groups as well as linear regression to identify potential indicators of depression in patients with systemic lupus erythematosus. The multiple subgroups of the cohort used as controls allows unique comparisons with the SLE subgroup. By considering what is similar between the arthritis patients and the SLE patients we can understand what might be a result of a chronic inflammatory disease. With the depression group acting as a control, indicating what factors could be the result of or related to psychological illness. Taken together, the control groups offer a new perspective on factors that could be relevant to depression in the SLE patients. As this is the first study of its kind to

approach depression in an SLE population using these groups as comparison controls, it offers credence to results gleaned from other studies.

Our results support other studies in that our SLE cohort had increased pain levels compared to the healthy control cohort, but not to either the depressed or arthritis patients. This offers support for the idea that pain and depression are strongly interwoven. Pain has often been correlated with depression in past studies (Karol et al., 2013; Xie et al., 2016). While we did not observe a direct correlation between pain and depression in the study, it is possible this could be due to the small sample size of the SLE cohort.

We observed elevated levels of IL-6 in our SLE cohort compared to the healthy patients. Increased levels of IL-6 in SLE and RA patients provides supporting evidence for past studies, but it was surprising that they are not significantly different from the depressed cohort (Ishihara & Hirano, 2002; Linker-Israeli et al., 1991). This is likely due to the large standard deviation in the depressed patient cohort. While most depressed patients had levels like those observed in the healthy control group, several had elevated IL-6 levels. We do not believe that IL-6 would be a good indicator of depression as nearly all of the depressed patients display relatively normal levels of IL-6 and there was no direct correlation observed between. Also, we did not observe any correlation with TNF-α and depression in the SLE cohort. TNF-α serum concentrations, while certainly higher on average in SLE and RA patients, was not significantly different than those levels observed in the healthy and depressed subjects. This is different than was observed in a study conducted by Postal and associates who recorded a direct correlation between TNF-α serum concentrations and depression and anxiety as measured by the Beck Depression Inventory (BDI) (Postal et al., 2016). However, their study had a larger SLE study cohort, measured depression using a different depression and anxiety assessment, and utilized spearman

correlation to determine statistically significant correlation relationships. While we did not observe the same trend, this could be due to a variety of differences between the studies.

The study suggests a negative trend associated with IL-10 and depression, where decreasing levels of IL-10 predict a higher risk for depression. Of the clinical data collected, IL-10 results were of great interest. The SLE cohort displayed increased serum concentrations of IL-10 compared to the healthy control subjects. However, the IL-10 levels measured from our SLE patients were actually lower on average than those measured from our depressed subjects, although the difference in IL-10 concentrations was not significant. It is still unclear if IL-10 is related to depression as there are conflicting reports in the literature with regard to human studies (Hiles, Baker, de Malmanche, & Attia, 2012). It has been observed in animal models that IL-10 imbalance does affect depressive behaviors (Mesquita et al., 2008). Also, in studies involving SLE patients it has been observed that heightened levels of IL-10 are likely correlated with depression and neuropsychiatric disorders (Efthimiou & Blanco, 2009; Svenungsson et al., 2001). In a recent animal study using MRL/lpr mice, they found that they developed depression like behavior before developing autoantibody titers. What is more, these mice had differing levels of various cytokines including IL-10, suggesting that these imbalanced levels might be good biomarkers for neuropsychiatric SLE (Y. Li et al., 2015). The SLE study cohort demonstrated a direct correlation between depression and IL-10, adding evidence that levels of IL-10 might be a good candidate as a clinical predictor for depression in SLE patients.

The SLE patients presented elevated anxiety scores, significantly more than the healthy and arthritis subjects. This supports observations of other studies (Mak et al., 2013). Anxiety scores, although slightly higher on average in the lupus patients, were not drastically different from the depressed patients. These results indicate that the increased levels of anxiety are not

likely due to chronic inflammation or disease etiologies that are similar between SLE and RA. More research is certainly necessary to corroborate these findings and help narrow down the potential factors involved in causing the high incidence of anxiety observed in lupus patients.

Unique to this study was the correlation observed between relationship assessment scale (RAS) and HADS depression scores. It is of interest that the SLE patients have the lowest average RAS score between all the subgroups in the study, significantly lower than the healthy and arthritis patients. In addition, the RAS scores reported from the SLE patients were significantly correlated to depression in the univariate and multivariate analysis (Table 15 & 16). This is the first study to the knowledge that demonstrates a strong correlation between the relationship assessment scale score and depression. While a large quantity of studies have observed that quality of life is negatively affected in SLE patients and is correlated with depression, none have utilized the relationship assessment scale to quantify relationship satisfaction. The finding that the RA patients have high relationship satisfaction indicates that it is likely the psychological symptoms of lupus that affect the relationship rather than the physical limitations of chronic disease. The directionality of the correlation is still unknown, is it that depression that is so common among SLE patients results in lower relationship satisfaction? Or is it that due to disease conditions, relationship satisfaction decreases and contributes to factors causing depression? Whatever the case may be, this study provides evidence that depression and relationship satisfaction show a correlation in the SLE patients.

A final relationship that we observed in the study was between the fatigue severity scale and depression in the SLE patients. Markedly higher in the SLE patients than in either the healthy or arthritis subjects, the fatigue severity scale shows correlation with depression in both the univariate and multivariate analysis (Table 15 & 16). Studies have shown that fatigue is

typically higher in SLE patients that in the general population(Wiseman et al., 2017; Yilmaz-Oner et al., 2016). Multiple studies have also observed that there is a correlation between fatigue and depression among lupus patients. In a recent cross-sectional study of Chinese SLE patients, it was observed that higher levels of fatigue predicted higher risk of depression(Xie et al., 2016). Our results support the findings from other human studies, that fatigue is highly correlated to depression in SLE populations (van Exel et al., 2013). With such a high prevalence of fatigue in SLE patients, up to 76 % reported by some studies, the necessity for improved management of fatigue in SLE patients seems paramount to helping prevent and/or improve patient care and quality of life(Ahn & Ramsey-Goldman, 2012; Morris et al., 2016). Unfortunately, most of the factors that contribute to fatigue in SLE patients have yet to be identified, making treatment of fatigue along with other symptoms quite challenging. There is evidence implicating increased levels of proinflammatory cytokines, oxidative and nitrosative stresses, mitochondrial dysfunction, and increased activation of toll-like receptors via pathogen and damage associated molecular patterns as causes for increased stress in patients with chronic fatigue (Maes, Kubera, Leunis, & Berk, 2012; Morris & Maes, 2013, 2014; Perl, 2013). Poor sleep quality can contribute to fatigue as well (Inoue et al., 2017). In this study we found the SLE cohort to have higher PSQI values, indicating poor sleep compared to the healthy control group, but these values were not significantly different from the arthritic and depressed patients. These results suggest that the sleep quality of SLE patients, while not good, do not likely contribute directly to depression. For the time being, treatment of fatigue remains an obstacle for patients and health care professionals to continue surmounting in tandem.

In summary, the study has proffered further evidence supporting various other SLE studies indicating correlator relationships between IL-10 serum concentrations, anxiety, fatigue

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severity scale and depression. In addition, this research cohort has identified a negative

correlation between relationship satisfaction, utilizing the relationship assessment scale, and

depression. This research emphasizes the need for more research into predictors of depression

and improved treatment options for fatigue in SLE patients.

Chapter 5: Concluding Remarks and Future Directions

This work highlights several key factors that potentially influence systemic lupus erythematosus disease progression. First, our studies have confirmed recent findings of several indicators of depression and anxiety in SLE patients, as well as providing evidence for several psychosocial tests that might better indicate depressive behavior in patients that could be utilized by care providing personnel. This research provides evidence for several clinical variables that can be measured and potentially used to indicate the presence of the neuropsychiatric disorder of depression. Finally, our studies have identified that EBV does indeed regulate EBI2 in human naïve B lymphocytes and that it does this through the viral gene product Na. Additionally, we postulate how this likely contributes to disease onset and immune system mis-regulation.

5.1 Effects of Epstein-Barr virus on B Lymphocyte Chemotactic Receptor EBI2 Expression via Viral BRRF1

EBI2 is vital in B lymphocytes for the detection of 7α,25-OHC and the subsequent migration to peripheral areas of secondary lymph tissue (Cyster, 2010). In murine models it has been found that when EBI2 is not expressed in B lymphocytes, they remained in follicle centers, failing to migrate to other areas of secondary lymph tissue (Pereira et al., 2009). Further, in that same study they observed that when wild type murine B cell EBI2 expression was antagonized, their participation in germinal centers was impaired. Another murine study found that upregulation of EBI2 in B lymphocytes had the outcome of increasing B cell proliferation (Benned-Jensen et al., 2011). As with the regulation of many genes, EBI2 expression, impaired

or overexpressed, likely results in mis-regulation of B lymphocytes affecting chemotaxis, germinal center formation, and proliferation.

Our study has demonstrated that EBV modulates EBI2 expression during viral infection of naïve B lymphocytes and that EBI2 is only upregulated beyond normal levels of expression upon infection with active EBV. In addition, we have identified that the EBV gene product Na is necessary and sufficient alone to induce expression of EBI2 in human naïve B lymphocytes (Cornaby et al., 2017).

Understanding that by adulthood, nearly 90% of any given human population has been infected with EBV, it is of interest to know how the modulation of EBI2 by EBV affects the immune systems of the majority of the human race. We have proposed that the regulation of EBI2 in EBV infected B cells results in these cells spending extensive amounts of time in the peripheral areas of secondary lymph tissue. While it is unknown exactly how this would benefit a virally infected B cell, we hypothesize that such positioning of infected B lymphocytes might aid in immune evasion. However, the effects of EBI2 upregulation and subsequent down regulation in B lymphocytes days later might have effects that have yet to be recognized as important for the viral life cycle. We suspect that increased time in the peripheral regions of secondary lymph tissue might provide increased risk of activating B cells by self-antigens that typically would not induce B cell activation because of the brevity of B lymphocyte presence in peripheral secondary lymph tissue.

Future research into these hypotheses and assumptions is certainly necessary to provide clarification. The mechanism by which EBV regulates EBI2 would be important to understand. Does the virus directly modulate EBI2 utilizing the BRRF1 gene product Na to induce expression? Or is there a more complex pathway involved? A Chromatin Immunoprecipitation

(ChIP) assay could be used to confirm or exclude the former hypothesis and would likely be the next logical step in whittling down this question. ChIP is a type of protein foot printing assay where you first fix the cell with formaldehyde, lyse the cells, digest the DNA that is not bound by the protein, harvest the protein of interest using a protein specific antibody, separate the remaining DNA from the protein of interest, and sequence it. This could be done for BRRF1 to determine what DNA site(s) the gene product Na recognizes.

Since we already have lentivirus constructs containing the gene BRRF1, it would not be necessary to infect human B lymphocytes with EBV, just transduce them with the lentivirus containing BRRF1, pUltra+BRRF1. In our studies we have been able to produce high titer lentivirus stocks that have been able to transduce 20 to 50 percent of naïve B lymphocytes. Seeding a plate with about $2x10^6$ to $4x10^6$ B lymphocytes and then transducing them with pUltra+BRRF1 should generate cells containing enough Na to isolate using Na specific antibodies, the appropriate bead congregated antibodies, and a magnetic bead isolation column post formaldehyde fixation, cell lysis, and sample treatment with Micrococcal Nuclease.

Another possible research objective would include confirming that increased EBI2 expression results in increased cell migration following the 7α,25-OHC gradient that is present in secondary lymph tissue. Our lab has tried these studies using naïve B cells overexpressing EBI2 in trans-well migration plates, however, naïve B cell migration was very poor, even for the positive control. Thus, if there was an increase in migration, this method was not sensitive enough to detect it due to the lack of naïve B cell migration using these trans-well migration plates. Another method would be to use a different type of B cell overexpressing EBI2, either primary cells or a specific B cell line.

It would be preferable to use primary human B lymphocytes, however, there are various B cell lines that would likely work well including LCL, Raji, and Ramos, as all these cell lines express little EBI2. Since all B lymphocytes are susceptible to infection by EBV, any mature primary B cells should also work well. A good general protocol for mature B cell isolation and measurement of chemotaxis via transwell migration plates (Costar) is outlined by Corcione et al. in their work demonstrating that mesenchymal stem cells modulate B-cell activity (Corcione et al., 2006). In short, isolated B lymphocytes could be transduced with our already functional and tested lentiviruses that contain EBI2 or EBI2 siRNA. This would allow for EBI2 overexpression or silencing in the transduced cells. Upon transduction with the lentivirus about $5x10^5$ isolated B lymphocytes would be seeded to the upper chamber of each well. Chemotaxis would be observed in response to various chemokines including CXCL13, 7α,25-OHC, and CCL21. Cell migration to the lower well would be measured via flow cytometry and transduced cells, with overexpressed or silenced EBI2, could be identified as all the lentivirus vectors contain eGFP reporters. EBI2 overexpression and silencing conditions in mature B lymphocytes could be compared to control conditions to determine if increased EBI2 results in increased chemotaxis as hypothesized. Further, mature B lymphocytes could also be transduced with pUltra+BRRF1 which also increased EBI2 expression due to BRRF1 expression, these treated cells could also be utilized in the transwell migration assay as further verification that BRRF1 induces EBI2 and affects B cell migration.

An additional option would be to use another tool to measure migration, such as visual analysis of cellular migration in a gap closer assay. In this assay cells are placed on one point of a slide and the chemokines at another. Using a microscope to record and software to track cell movement, it can be used to quantify the rate cells move and the number of cells that migrate in

response to chemokines. This assay might even be more sensitive to subtle changes in cell migration as you are able to accurately count individual cells during the assay. To perform this assay transduced cells overexpressing EBI2 or with EBI2 expression silenced would need to be sorted from non-transduced cells using the FACS cell sorter.

5.2 Depression in Patients with Systemic Lupus Erythematosus

Depression in patients with chronic diseases is strongly associated with poor clinical outcomes, as well as higher health costs (Julian et al., 2009; Panopalis et al., 2008). The recognition of neuropsychiatric disorders in systemic lupus erythematosus patients and the appropriate treatment of these disorders has been shown to improve clinical end points, decrease perceived pain, and improve the overall quality of life (Katon et al., 2010; E. H. Lin et al., 2003). These observations emphasize the importance of better understanding the biological mechanisms involved in neuropsychiatric disorders in SLE patients and improve accurate diagnosis of these disorders.

This being the case, it is necessary to identify potential clinical or psychosocial variables that might denote neuropsychiatric disorders in our patient population. Clinical variables in particular would be useful as regular visits to a clinician or rheumatologist along with regular clinical tests is common for SLE patients and necessary to insure proper medical care. Various potential biomarkers for depression have been suggested including: IL-2, IL-6, IL-8, IL-10, TNF-α, and INF among others (Efthimiou & Blanco, 2009). A recent murine study found that NP-SLE mice exhibited increased levels of IL-10, Haptoglobin, CXCL-10, Lymphotactin, CCL-19, CCL-2, CCL-7, CCL-12, VCAM-1, CD-40, IL-1β, and FGF-basic (Y. Li et al., 2015). Several studies, previously published, looking into this question have found that depression in

SLE patients is correlated with some of these biomarkers. A study conducted in Sweden found that TNF-α, INF-γ, and IL-10 were significantly higher in NP-SLE patients (Svenungsson et al., 2001). Additionally, another study conducted by researchers at Duke University Medical Center identified low complement levels and ds-DNA concentrations to be associated with depression in SLE patients (Karol et al., 2013).

Our studies, contained in chapters 2 and 3, conducted with collaborators at the University of Porto, Instituto de Investigação e Inovação em Saúde, and Hospital of São João EPE, found several clinical variables demonstrate a correlation with depression in SLE patients. Univariate analysis revealed during these studies that pain, total complement, BMI, and IL-10 showed correlation with depression in our SLE patients. While other suggested variables that were recorded did not, such as IL-6, TNF-α, SV, CRP, lyeukocyte count, lymphocyte percentages, and anti-dsDNA antibodies among other clinically relevant measurements. These results provide evidence that IL-10 and complement might be ready indicators of depression in SLE patients. Further research is necessary to better understand how well recorded levels of these biomarkers correlate to neuropsychiatric disease progression and severity. It would be particularly good to conduct studies with more longitudinal scope, allowing for the measurement of biomarkers over time to determine their sensitivity and accuracy.

A quandary of interest to those involved in researching depression among SLE patients is if the increased prevalence of depression and anxiety is due to chronic inflammation or unique to lupus pathophysiology. Several studies have investigated the association of SLE disease severity with depression to shed some light on this dilemma, however, there seems to be conflicting evidence as some studies found that disease severity was correlated with depression among SLE patients while others did not (Karol et al., 2013; van Exel et al., 2013). Our cohort study sought

to investigate this question of interest by including a group of RA patients to compare alongside our SLE, healthy, and depressed patients. We found depression scores to be higher in our SLE patients and to have higher prevalence when compared to the RA patients. Further, anxiety scores were also higher and more prevalent among SLE patients compared to RA subjects. However, these scores were not drastically different from our subjects being treated for depression. These results would indicate that it is not just chronic inflammation responsible for depression in SLE patients. Certainly, more research is necessary to clarify this question further and isolate what factors specifically are responsible for the increased prevalence of depression and anxiety in SLE patients.

In the future, it would also be interesting to investigate if biologics influenced depression in patients with lupus, and if certain treatments had stronger associations with depression prevalence. The understanding of which treatment types could lead to neuropsychiatric disorders would allow physicians to monitor their patient's mental health and better anticipate potential problems.

Outlining several factors involved in SLE disease activity, this work provides a foot hold for future research that can continue to elucidate disease pathophysiology. It is predicted that investigations into these topics can help identify key ways to continue improving care for SLE patients and increase our understanding of the biology responsible for the pathophysiology of SLE.

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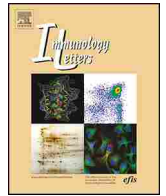
Review

B cell epitope spreading: Mechanisms and contribution to autoimmune diseases

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While a variety of factors act to trigger or initiate autoimmune diseases, the process of epitope spreading is an important contributor in their development. Epitope spreading is a diversification of the epitopes recognized by the immune system. This process happens to both T and B cells, with this review focusing on B cells. Such spreading can progress among multiple epitopes on a single antigen, or from one antigenic molecule to another. Systemic lupus erythematosus, multiple sclerosis, pemphigus, bullous pemphigoid and other autoimmune diseases, are all influenced by intermolecular and intramolecular B cell epitope spreading. Endocytic processing, antigen presentation, and somatic hypermutation act as molecular mechanisms that assist in driving epitope spreading and broadening the immune response in autoimmune diseases. The purpose of this review is to summarize our current understanding of B cell epitope spreading with regard to autoimmunity, how it contributes during the progression of various autoimmune diseases, and treatment options available.

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1. Introduction

In many immune responses, the initial antigens, and epitopes of those antigens (Ag)s, that are recognized by the adaptive immune system are limited. Over time, the T and B cell response can grow to include many other epitopes and even additional antigenic molecules. This process is known as epitope spreading. This process is an important contributor to the efficiency and breadth of the immune system. However, epitope spreading is also implicated in multiple autoimmune diseases [1,2]. Epitope spreading is known to be a major component of systemic lupus erythematosus and bullous pemphigous, and is implicated in multiple other autoimmune diseases ranging from multiple sclerosis to diabetes (Table 1). In B cell epitope spreading, the antibody (Ab) specificity develops from the initial Ag that triggered the response to include other epitopes of these Ags, or other Ags altogether. B cell epitope spreading can potentially occur through many mechanisms, including interactions with T cells, endocytic processing of antigens, and somatic hypermutation in the B cell.

The contribution of epitope spreading in the progression of autoimmune disease was first recognized in print during the study of experimental autoimmune encephalomyelitis (EAE) in Lewis rat and mouse models [3,4]. In these studies it was found that rats and mice immunized with Myelin oligodendrocyte glycoprotein (MOG) induced a T cell encephalitogenic and B cell Ab immune response resulting in demyelination. During this study it was demonstrated that the antigenic targets of the Abs changed and diversified during the course of EAE. It was later shown that epitope spreading was the cause of the Ab diversification. We now understand that B cell epitope spreading assists in the progression of many autoimmune diseases, acting as a two edged sword for many that are affected by autoimmune diseases [1,2]. Epitope spreading is essential for an effective adaptive immune response, at the same time contributing to the progression of self-targeting disorders. It is important to better understand epitope spreading due to its influence in autoimmune progression and the potential to develop more advanced strategies to assist in the treatment and prevention of autoimmune diseases.

While much research has been done to determine the significance of T cell epitope spreading in disease, far less is known about B cell epitope spreading. This review will address B cell epitope spreading and its involvement in the progression of various autoimmune diseases. The current understanding about factors contributing to B cell epitope spreading and intracellular

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Table 1
Compiled evidence implicating B cell epitope spreading in various autoimmune diseases. The autoimmune diseases are listed with the completed cumulative research that provided provocative evidence for B cell epitope spreading. It is specified for each evidenced example of epitope spreading a sentence summary of the example, what type of experiment was performed to obtain the data, the type of epitope spreading involved, and the reference for that example.

Disease	Evidence of epitope spreading	Reference	Type of experiment	Type of B cell epitope spreading
Bullous pemphigoid	Ab diversification to epitopes of BP180 and BP230	[108,130]	Clinical study	Intramolecular and intermolecular
	Ab spread from collagen XVII to collagen VII	[109]	Clinical report	Intermolecular
	Bullous pemphigoid develops after pemphigus foliaceus	[13,14]	Clinical report	Intermolecular
Cardiomyopathy	Immunization with CC2 peptides induces EAC, then DCM	[91]	Lewis rat model	Intermolecular
Diabetes (Type 1)	Diversification of Abs to include ICA, GADA, IA-2, and IAA	[56]	Clincal study	Intermolecular
Graves' disease	Ab diversification from thyroglobulin AA2471-2490 to other epitopes	[6]	NZW rabbit model	Intramolecular
	Intramolecular epitope spreading on TSHR protein molecule	[78,79]	BALB/c mice model	Intramolecular
	Ab diversification on the TSHR protein molecule	[80,81]	Transgenic mice model	Intramolecular
Heymann nephritis	Ab spreading to various epitopes on Megalin protein	[94]	Lewis rat model	Intramolecular
Multiple sclerosis	Ab spreading from MOG peptide 92–106 to intramolecular epitopes	[60]	RR-EAE mice model	Intramolecular
	PLP Abs spread to target other myelin proteins	[52]	EAE mice model	Intermolecular
Pemphigus	Ab diversification to various regions of desmoglein 1 and 3	[100]	Clinical study	Intramolecular and intermolecular
	Development of paraneoplastic pemphigus from Lichenoid Dermatitis	[101,102]	Clinical report	Intermolecular
Rueumtoid arthritis	Abs diversify from ACPA to include anti-CCP	[46]	Serum collection analysis	Intermolecular
Scleroderma	Auto-Abs to differing centrosome domains were present in the same sera	[85]	Serum collection analysis	Intramolecular
Sjogrens syndrome	Ab spread from Ro/SSA to La/SSB and to unimmunized epitopes of Ro/SSA	[71]	BALB/c mice model	Intramolecular and intermolecular
Systemic lupus erythematosis	Ab diversification from 1.5 to 3 out of 7 auto-Ags screened	[65]	Serum collection analysis	Intermolecular
	Ab spread from SmD to SmB and A-RNP molecules	[66]	A/J mice model	Intermolecular
	Intermolecular more common than intramolecular epitope spreading	[7]	A/J mice model	Intramolecular and intermolecular

mechanisms involved in this process will be discussed. Areas for future research and gaps in our understanding of B cell epitope spreading will also be commented upon. The purpose of this review is to provide a summary of our current understanding about B cell epitope spreading mechanisms, involvement in autoimmune disease, and the importance of epitope spreading in treatment options.

2. Potential mechanisms of B lymphocyte epitope spreading

2.1. Types of epitope spreading

There are two different types of epitope spreading, intermolecular and intramolecular [5]. The latter describes a type of spreading where the immune response is directed against different epitopes of the same molecule, while the former is a diversification of the immune response against two or more different molecules [6]. By expanding the antigenic epitopes that the immune system recognizes, responses to these foreign Ags becomes optimized, allowing for neutralization via Abs, recognition by various immune cells, and clearance of the pathogen.

Intermolecular epitope spreading is not dependent on intramolecular epitope spreading [7]; rather these different types of epitope spreading are independent of each other. Diversification takes place only after Ag recognition by cells of the immune system. B cell activation by CD 4 + T cells or by surface clustering immunoglobin bound to the specific Ag epitope initiate this process. Through clonal expansion and affinity maturation, the spreading of epitopes for a given Ag expand and continue to broaden the longer the immune response continues. It has been shown that there are two ways to initiate B cell epitope spreading in autoimmune diseases. McCluskey et al. describe the first to be independent of a physical association with immune cells presenting the Ag, while the second is dependent [1]. In an Ag presenting cell (APC) independent scenario, inflammation and activation cytokines are sufficient enough to allow T cells to recognize cryptic epitopes and activate complementary B cells. This is observed in non-obese diabetic (NOD) and EAE animal models [8]. On the other hand, a dependent response happens typically when there is no tissue destruction or it is delayed, relying on processing and presentation to achieve activated T cells that, through intrastructural T cell help, activate reciprocal B cells. In fine, the independent scenario exists when there are environmental conditions such that T cells can recognize cryptic, or novel, epitopes without the processing and presentation by APCS, such as B cells and dendritic cells. The dependent circumstance relies upon APC processing and presentation to T cells in order for the immune system to recognize cryptic epitopes.

2.2. Intermolecular epitope spreading

Many of the Ags recognized in autoimmune diseases are part of multi-antigenic complexes. For example, the splicesome is a common target for autoAbs in lupus, and consists of multiple proteins and nucleic acids that are associated with each other. T cells specific for one epitope of a multi-antigenic complex can activate B cells that are specific for other Ags of the complex [9]. T cell help allows B cells to target a range of Ags to proliferate, differentiate, and produce Abs, even against Ags that were not originally involved in either the B cell or the T cell response [10,11].

An experimental example of this concept was illustrated by Deshmukh et al. during their study of Systemic Lupus Erythematosus [7]. To induce lupus-like symptoms in a mouse model, female A/J mice were immunized with various SmD peptides. This stimulates an immune response against the small nuclear ribonucleoprotein (snRNP) complex that is characteristic of systemic lupus erythematosus. Immunoprecipitation assays were used to determine Ab reactivity to SmD, SmB, and associated ribonecleoprotein (A-RNP) molecules. It was found that immunization with the SmD_{52-66} lead to the development of Abs against not only regions of the SmD protein, but also to the U1 A-RNP. The Ab diversification from the SmD protein to the A-RNP is a demonstration of intermolecular epitope spreading. During their study of viral triggers leading to SLE, Poole et al. observed a similar example of intermolecular epitope spreading [12]. Immunization of rabbits with the protein peptide PPPGMRPP from Epstein–Barr virus (EBV) protein EBNA-1, lead to the development of antibodies to SmB, SmD, nRNPs and La/SSB.

Clinical studies and reports continue to provide evidence for this process. Maeda et al. described a case where a 64 year old Brazilian male developed bullous pemphigoid twelve years after being diagnosed with pemphigus foliaceus [13]. He was diagnosed with bullous pemphigoid when subepidermal blistering was observed and immunoblotting assays revealed Ab activity against BP180. A congruous event was recorded by Peterson et al. where they observed similar results from a 86 year old African American male [14]. This development of bullous pemphigoid after pemphigus foliaceus is a clinical manifestation of intermolecular spreading. Autoimmune targets resulting in pemphigus foliaceus are typically desmoglein proteins, particularly desmoglein 1. The cause of bullous pemphigoid is an autoimmune reaction against several bullous pemphigoid proteins (BP180 and BP230) of the hemidesmosome. In fine, the incidences reported above indicate an intermolecular epitope spreading event from the desmoglein proteins to the bullous pemphigoid proteins.

It does not take a large variety of T cells to drive a diverse B cell response leading to intermolecular epitope spreading. An example of this was shown by Milich et al. in using mouse T cells primed for the Hepatitis B nucleocapsid to invoke an immune response against the Hepatitis B virus. The result was Ab production against Hepatitis B surface Ags [15]. This demonstrates CD 4 + T cells specific for priming recognition of just one region, leading to an immune response that diversified to include the surface Ag and nucleocapsid Ag of Hepatitis B. Intrastructural T cell help is necessary for B cell epitope spreading to elicit an immune response against pathogens and contributes during development of immune diseases [16,17].

2.3. Intramolecular epitope spreading

After B cells are activated in response to Ag and T cell stimulation, they respond by progressing through clonal expansion and affinity maturation. Upon processing of the Ag, these cells can in turn present novel cryptic epitopes to CD 4+ T cells in the context of MHC class II molecules. Cryptic eptitopes are understood to be epitopes that were previously un-recognized by the immune system. These cryptic epitopes allow for the priming of reciprocal T cells and the activation of complementary B cells. This process allows the immune response to recognize various epitopes from the same molecule, or associated molecules, and thus contributes to epitope spreading. Affinity maturation allows for selection of the clonally expanded B cell population for those cells that have a higher affinity for the Ag. Because of this selection, intramolecular epitope spreading can occur as B cells with higher affinity for a different epitope of the Ag are selected [9].

Endocytic processing and the subsequent MHC class II presentation is a further mechanism that can promote intramolecular epitope spreading. During endocytic antigen processing the antigen is endocytized, selectively cleaved and loaded into a compatible MHC class II complex that is then displayed on the cell surface. This process allows for presentation of previously unrecognized epitopes and the broadening of the immune response to various epitopes on the endocytized molecule (Fig. 1).

A clinical study of pemphigus foliaceus in Brazil provided evidence that intramolecular epitope spreading contributes to autoimmune disease development [18]. Sera from patients were collected pre-disease onset and while the patients were exhibiting clinical symptoms of skin lesions. Using immunoprecipitation assays to analyze the sera from the patients, several interesting observations came to light. Patients still in the pre-clinical phase of the disease developed Abs for the COOH-terminus region of the desmoglein 1 protein. During the study several patients progressed from the pre-clinical phase to the clinical phase of the disease. Testing of the serum during clinical onset of pemphigus foliaceus revealed Abs against the EC1 and EC2 domains of the NH_2 terminal
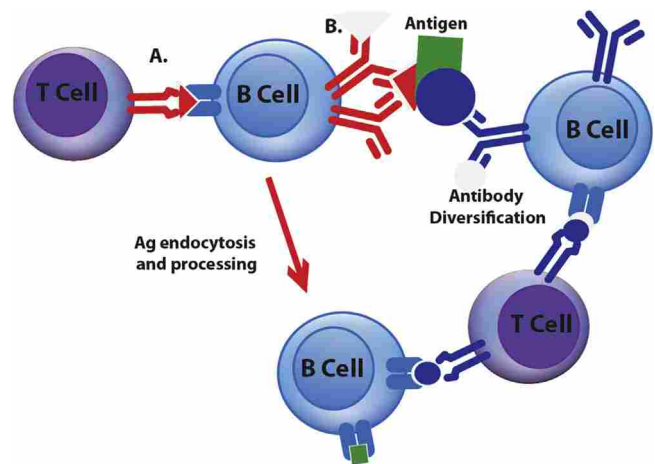
Fig. 1. Intramolecular B cell epitope spreading. (A) Once activated through T cell intrastructural help or (B) clustering of the BCRs attached to a specific epitope on the Ag, B cells endocytise the Ag, selectively cleave it, load selected peptides in MHC II molecules and display it in this context to T cells. Once a T cell is activated by the B cell for a new epitope presented via MHC II, it can then activate a reciprocal B cell which will then express Abs specific for that epitope. This process is known as intramolecular B cell epitope spreading.

region of the desmoglein 1 protein. Another example is presented by Poole et al. In a serum analysis study of SLE patients collected at the Oklahoma Clinical Immunology Serum Repository, it was found that intramolecular epitope spreading occurred during the progression to SLE [19]. Using autoantibody analysis of the extensive serum samples, it was observed that antibody diversification happened over time during disease progression to various epitopes on the nRNP-A and nRNP-C proteins. Anti-nRNP-A antibodies were found to bind to the N-terminus of the protein more frequently in later samples when compared to the initial samples. This diversification of Abs from one region of the protein to the other region demonstrates intramolecular epitope spreading and provides evidence of the role it plays in the progression of autoimmune disease.

2.4. Molecular mimicry and cross-reactivity

B cell epitope spreading after molecular mimicry is a mechanism by which the body likely begins to break self-tolerance, leading to the development of autoimmune disease. Molecular mimicry has been indicated as a contributing factor in autoimmune diseases such as type I diabetes [20], rheumatoid arthritis, multiple sclerosis, glomerulonephritis [21], and systemic lupus erythematosus.

Molecular mimicry exists when an auto-Ag is similar enough to an antigen from a pathogen or other environmental source that Abs generated against the pathogen will also bind to the autoantigen [21]. Cross-reactivity is a phenomenon that occurs in the immune system when a single Ab reacts with multiple Ags varying in structure and composition. The immunochemical evidence in recent literature suggests that conformation as well as structure is essential for an effective Antigenic mimic [22]. This was demonstrated by Mariuzza et al. at the University of Maryland when they showed that the anti-idiotopic Ab E5.2 was a mimic for lysozyme in the way that it bound to D1.3 [23]. Mariuzza's group further showed that it was a binding group mimic and shared no homologous sequence between the Ag and Ab E5.2 [24]. These findings verified that the binding of Abs is not determined by amino acid sequence alone, but by the conformation of the epitope to which it binds. While certain amino acid sequences tend to display select conformations and structures, there is variability due to protein folding, molecular interaction, and binding. These findings indicate that an Ag, or

auto-Ag, does not need to share homologous amino acid sequences in order to be an effective mimic.

The mechanisms of molecular mimicry and cross-reactivity are thought to be key contributors in B cell epitope spreading. One example of molecular mimicry was explored by Poole at al. in experiments tying Epstein–Barr Virus (EBV) infection to the development of the autoimmune disease systemic lupus erythematosus (SLE). The EBV nuclear Ag-1 (EBNA-1) contains a peptide sequence, PPPGRRP, that closely resembles the PPPGMRPP region on the Smith Ag (Sm) targeted by autoAbs in SLE patients. Immunization with the PPPGRRP sequence from EBNA-1 lead to development of cross-reactive antibodies that recognized both epitopes, as well as further epitope spreading leading to autoimmunity against Sm and nRNP complexes. Also, 5 out of the 6 rabbits immunized developed SLE like symptoms over the course of the experiment indicating that the mechanism of molecular mimicry involving EBNA-1 can lead to development of systemic lupus erythematosus-like disease [25]. Clinical evidence shows similar results with respect to the spreading of epitopes in SLE. After an individual loses tolerance for 60 kDa Ro/SSA and beings to produce Abs that specifically target Ro/SSA, later blood samples reveal Abs against Sm B', SmD1, nRNP A and C, and eventually La/SSB [19].

3. Intracellular mechanisms of B lymphocyte epitope spreading

3.1. Endocytic processing and Ag presentation

B cell endocytosis and Ag presentation to T cells contribute to epitope spreading. Endocytosis takes place after Ag binding to B cell receptors [26]. During this process of B cell endocytosis, Ag processing and presentation determines what peptides will be loaded into MHC class II molecules and displayed on the cell surface. This determines what epitopes will be presented to T cells, ultimately identifying what epitopes should be targeted by the immune system. There are various circumstances that affect this processing.

The first part of endocytosis to affect processing is the BCR to which the Ag is bound [27,28]. Binding to the BCR can affect the selection of epitopes presented to T cells [29], however, it is not known exactly how BCR binding impacts processing of Ags and the loading of class II MHC complexes [30,31]. Thanks to McGovern et al. we now understand that the difference in Ag processing and presentation is due to BCR signaling and not due to the signaling-induced changes in the biology of the cell [30]. It has been suggested that stability between the Ag and the surface immunoglobin (sIg) to which it is bound influences processing [32]. Another proposed reason for the difference in the processing of Ags might be related to targeting as a consequence of the sIg cytoplasmic tail, the Igα/Igβ associated chains, or both [33]. These various components of the BCR play a role in internalization and processing, however, it is unknown how they affect this process in detail. It has been demonstrated that Igα and Igβ selectively target Ags to be loaded into MHC II [34].

A second point of processing selection is due to the selective cleavage of proteases contained in the lysosome. This selective processing of Ag into smaller peptide fragments depends on the proteases present in the lysosome, including their relative concentration and activity which is regulated by various endogenous cytokines and competitive inhibitors [35]. These competitive inhibitors vie with protein that is being digested, regulating what proteases are more active in the cleaving of the endocytized Ag. Cytokines can affect endocytic proteases by adjusting their activity, synthesis, or stability. Various cytokines have been shown to affect the endosomal pH, increasing or decreasing the ability of

protease activity [36,37]. In this way, cytokines can stimulate or inhibit enzymes involved in Ag cleavage and degradation, affecting what peptide segments are available to be loaded into MHC II complexes (Fig. 2).

A final mechanism affecting the presentation of epitopes proposed is the loading of MHC II molecules. HLA-DM and the MHC II complex load only peptides that have an appropriate length and affinity. Peptides need to display specific amino acid motifs to be loaded [38]. Two other factors affecting this loading include endosomal environment, including pH, and the HLA-DM [39,40]. Age of the individual is also a factor that affects cellular efficiency. It is known that Ag presentation is better in younger individuals and declines with age [41]. Recycled and newly synthesized MHC II molecules also play a role in selection. They are known to associate with different peptides [42], thus levels of recycled and newly synthesized MHC class II molecules could affect the peptides selectively loaded. Many questions remain unanswered about peptide selection for MHC class II loading and Ag processing. Future investigation include determining regulatory factors responsible for endocytic compartment targeting to lysosomes in the endocytic pathway and regulation of proteases in the cell, endogenous competitive inhibitors, and cytokines are other areas for research into selective processing of Ag.

It has also been demonstrated that through endocytosis, molecules that are associated together, non-covalently, can be presented in the context of MHC class II molecules as well [1]. The event where an Ag and the associated molecule are processed and part of the Ag associated molecule is presented via MHC II to T cells has been termed intermolecular/intrastructural help [43]. Such processing leads to intermolecular epitope spreading. Understanding of these events in concert with B cell processing and selection is essential for adequate comprehension of how and to what extent epitope spreading contributes to the human immune response, disorders, and diseases.

3.2. Somatic hypermutation

During affinity maturation, B lymphocytes undergo somatic hypermutation (SHM) which can contribute to the spreading of epitopes [9]. This process takes place in germinal centers to improve B cell Ab affinity for an Ag (Fig. 3). At this period of time SHM of the IgV gene region experiences single nucleotide substitutions. These happen at a frequency of about 10^{-3} per base pair in each B lymphocyte generation [44]. The consequence of SHM results in Abs with a higher affinity for the Ag presented by dendritic cells in secondary lymphoid tissue during affinity maturation. It has been suggested that SHM also leading to B cell epitope spreading, contributes in the progression of autoimmune diseases. Autoreactive Abs can be generated against dsDNA through SHM in the development of systemic lupus erythematosus [45]. It is uncertain to what extent SHM contributes to B cell epitope spreading. Investigating this question and determining if SHM contributes to other autoimmune diseases remains an area for future research that is lacking at this time.

4. B cell epitope spreading in autoimmune disease

4.1. Rheumatoid arthritis

The most common inflammatory arthritis worldwide, rheumatoid arthritis (RA) affects nearly 1% of the global population, and can cause severe debilitation and joint deformity within the first few years of clinical symptoms due to joint inflammation and tissue degradation [46–48]. While joint damage is the most characteristic and common symptom, the inflammation produced can also affect organs such as the cardiovascular and pulmonary systems

Fig. 2. The endocytic pathway is a primary mechanism in B cell epitope spreading. (A) The binding of the sIg to the Ag can affect the epitope that is selected for and loaded into MHC II. (B) The endosome fuses with the lysome, containing GILT, cathepsins, and other proteases. These enzymes selectively digest the Ag into peptide segments. This provides another point for selection of epitopes and the exclusion of others based on the cleavage sites. (C) With the disassociation of CLIP, peptides are selectively loaded into the MHC II molecule based on amino acid motifs, pH, HLA-DM association, among other enodsomal environmental factors (D) Once these various selective processes are accomplished the resulting epitope chosen is displayed via the MHC II on the surface of the cell. Because of the many factors involved, various peptides could be displayed.

[48]. Although the specific causes that lead to RA are unclear, it is thought to be the result of interplaying genetic factors, environmental agents, and chance, similarly observed among other autoimmune diseases. Certain Abs, including rheumatoid factor and Anti-Citrullinated Protein Abs (ACPA) are important contributors to RA [47]. Rheumatoid factor is a high-affinity autoAb against the FC region of immunoglobulins [48]. These Abs often develop years before clinical disease, preceding the development of rheumatoid arthritis, with the number of targeted proteins gradually increasing until the time of diagnosis [46,49,50]. Interestingly, increasing Ab targets correlate with increased inflammatory cytokine levels [46] and the disease course of RA. ACPAs can be directed against a multitude of targets [51]. This wide range of targets arises because citrullinated proteins result when arginine residues in proteins are converted to citrulline residues due to a post-translational modification by peptidylargenine deiminase, a process that is not specific to a single protein [52].

Epitope spreading is seen in the development of the ACPA repertoire. It is hypothesized that the neoAgs that result from citrullination could evoke an initial immune response, which then develops into a polyclonal Ab response against the entire protein or protein-complex due to epitope spreading [47]. This process
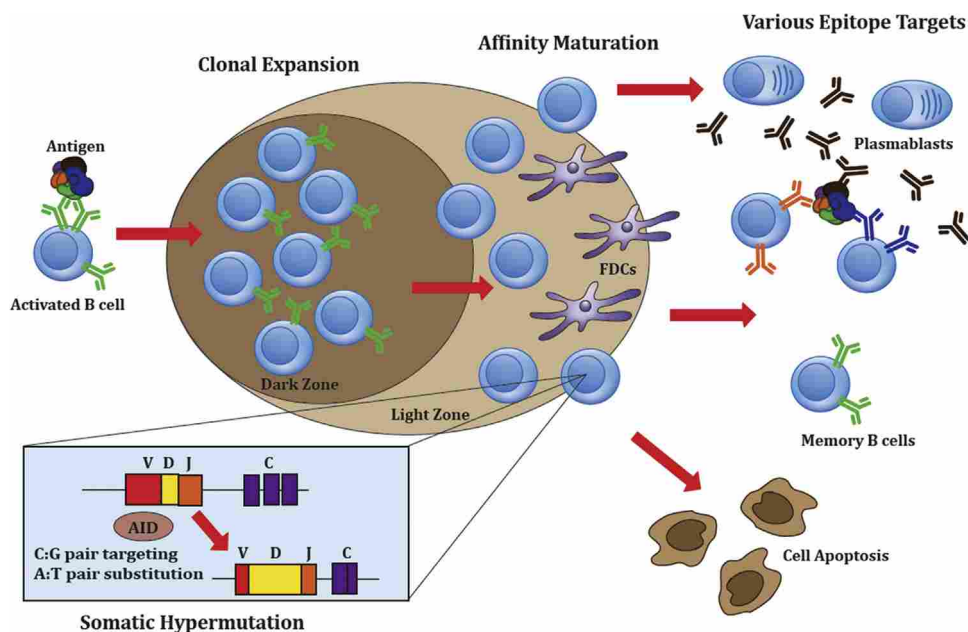
Fig. 3. Somatic Hypermutation (SHM) as a mechanism of B cell epitope spreading. After B cell activation in response to Antigenic detection, in secondary lymph tissue, B cells produces large quantities of daughter cells through the process of clonal expansion. These daughter cells independently induce changes in the variable regions of the immunoglobulin gene. These changes allow for a higher or lower affinity binding to the Ag, higher affinity binding B cells are selected for and proceed to produce various classes of Abs while low affinity binding cells are signaled to apoptose. Because of the induced mutation of the Ab variable region, it is possible to develop Abs that bind to various domains on an Ag after the process of affinity maturation and SHM.

is restricted mainly to genetically susceptible individuals, and the mechanism that triggers the initial citrullination is still unclear [47]. Additionally, the reason why a systemic loss of self-tolerance leads to localized joint inflammation is also unclear [48].

An investigation into United States Armed Services personnel clinically diagnosed with Rheumatoid Arthritis revealed unique information about Ab development and epitope spreading during the development of RA [46]. Using clinical Ab assays and a novel multiplex autoAb assay, it was possible to process serum samples obtained from the Department of Defense Serum Repository and screen for various Abs unique to RA patients. The samples used predated and preceded clinical symptoms and diagnosis of RA. Upon analysis, it was noted that the Ab diversification increases with the progression of RA and occurs before the onset of clinical symptoms. ACPAs were observed prior to the development of anti-cyclic citrullinated peptides (anti-CCP). These findings indicate both intramolecular and intermolecular B cell epitope spreading during the progression of rheumatoid arthritis. It is hypothesized that this epitope spreading was initiated by B cells stimulated by citrullinated fibrinogen complexes [46]. These examples indicate that B cell epitope spreading takes place during disease progression in rheumatoid arthritis and that citrullination of peptides contributes to these epitope spreading events.

4.2. Type 1 diabetes

Type I diabetes (T1D) results when an autoimmune reaction attacks and destroys the insulin producing beta cells of the pancreas. T cells specific for a number of diabetogenic auto-Ags are responsible for the destruction of the pancreatic islet cells. T1D is the most prevalent chronic disorder among children, and the loss of glucose level homeostasis that results can cause polydipsia, polyuria, weight loss, and potential complications of hypoglycemia such as hypoglycemic shock [53].

Autoimmunity may first be triggered against the B9-23 region of insulin [53] and progression to overt disease is mediated by epitope spreading to an array of beta cell Ags. It was shown that non-obese diabetic (NOD) mice that lacked mature B cells could not develop this autoimmunity, suggesting a crucial role of B cells in the mechanism of epitope spreading. In this experiment, two to three week old NOD mice were transfused with naïve T and B cells, or only naïve T cells, and the T cell response to various beta cell Ags was measured at four and ten weeks after transfusion. The TB NOD mice demonstrated progressive autoimmunity development to glutamic acid decarboxylase (GAD), heat shock protein 277 (HSP277) and insulin B-chain, while the T NOD mice showed no T cell response to any of the beta cell Ags tested. Researchers showed that NOD mice B cells have deficient tolerance to auto-Ags, and are relatively resistant to activation-induced cell death, which could explain why autoimmunity was able to develop [54]. In C57BL/KsJ db/db mice, it was found that the retroviral p73 protein, a group-specific Ag, acts as a molecular mimic of insulin [55]. It is speculated that presentation of this retroviral Ag to T-helper cells may stimulate B-lymphocytes producing the cross-reactive anti-p73 Ab, which also bind insulin [55]. Once the initial self-tolerance to insulin is broken, epitope spreading can contribute to the development of autoimmunity. The currently proposed model is that an initial wave of activated T cells causes B cell activation through T–B cell interaction and cytokine release. This enhances the Ag presenting functions of the B cells, causing them to capture and present beta cell Ags to T cells, causing the expansion of T cell autoimmunity [54].

A clinical study further demonstrated epitope spreading as a mechanism leading to disease progression and clinical diagnosis [56]. During an average of 30 months, 25 volunteers at risk for type 1 diabetes donated blood for analysis. These individuals were classified as 'at risk' due to the detection of islet cell Abs (ICA),

Glutamic acid decarboxylase Abs (GADA), islet cell Ab-2 (IA-2), or insulin autoAbs (IAA). Serum samples obtained were tested for the four Abs using specific Ab assays for ICA, GADA, IA-2, and IAA. Out of the 25 at risk volunteers under observation, seven developed clinical type 1 diabetes. All seven of these volunteers displayed serological evidence of intermolecular B cell epitope spreading. One patient, during pre-clinical T1D only displayed ICA in the serum. By the end of the study the B cell repertoire had diversified to include all four Abs. Other at risk volunteers not diagnosed with clinical type 1 diabetes also displayed evidence of intermolecular B cell epitope spreading [56].

4.3. Multiple sclerosis

An autoimmune disease of the central nervous system, multiple sclerosis results from the degradation of myelin proteins that sheath the neurons. The demyelization is caused by the attacking immune cells, particularly T and B cells, causing inflammation and a subsequent autoimmune response. With the destruction of the myelin sheath, biochemical electrical impulses from the brain are not transmitted efficiently. A variety of symptoms result including: fatigue, pain, movement, coordination, cognitive, and visual problems [57]. Citrullination, via peptidylargenine deiminase, is thought to be caused by inflammation of target tissues and occurs in many circumstances normally. Myelin basic protein (MBP) is partially citrullinated in healthy brain tissue, but in those with MS there is an increase of citrullination. It is hypothesized that citrullination contributes to the progression of multiple sclerosis [58]. AutoAbs derived during mouse model studies were between two and 20-fold more reactive against citrullinated MBP compared to non-citrullinated MBP [52]. In addition, these autoAbs were found to be reactive against epitopes derived from αB-crystalline, the most abundant early gene transcript. Recent studies have shown that development of B cells into tertiary lymphoid tissue near CNS regions further contribute to the progression of MS [59].

The essential role of B cell epitope spreading in MS was demonstrated clearly in SJL/J mice with remitting-relapsing experimental autoimmune enchephalomylitis (RR-EAE) [60]. These transgenic mice spontaneously develop RR-EAE affecting various central nervous system tissues and have a TCR specific for mouse myelin oligodendrocyte glycoprotein (MOG) peptide 92–106. The study showed that with the SJL/J mice, the endogenous B cells secreted Abs for new epitopes besides the peptide sequence the T cells targeted. Their findings also showed that there was no spontaneous EAE development in B cell deficient mice and that B cells enhanced the pathogenesis of EAE in the RR-EAE mouse model. The spreading of the B cell repertoire to other MOG epitopes besides the targeted amino acid sequence suggests that in EAE, the mouse model equivalent of multiple sclerosis, B cells are essential for the development of the disease, with a likely role for epitope spreading [60].

A different study conducted using EAE mice models contributed further to our understanding by showing that epitope spreading was found to directly correlate with disease progression [52]. In a mouse model, proteiolipid proteins (PLP) where used to induce EAE. Various regions of these PLPs became targets for Abs, along with the targeting of additional epitopes on other myelin proteins, both citrullinated and non-citrullinated. This is indicative of both intramolecular and intermolecular B cell epitope spreading. These results add credence to previous research demonstrating the development of cryptic epitopes during disease development and provides an explanation for why this occurs [61]. Epitope spreading has been shown to lead to the development of multiple sclerosis in mice during infection by Theiler's virus [62], indicating viruses as a potential trigger for the development of CNS autoimmune diseases.

4.4. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease caused by immune reacting with targets at diverse locations in the human body leading to a variety of clinical manifestations. SLE affects approximately 1 in every 2000 individuals with a 9:1 ratio for female vs. male diagnosis respectively [63]. Auto-Abs in SLE are often raised against double-stranded DNA as well as ribonuclear-proteins (RNPs) of the Sm complex found in splicesomes [5,25,63]. This autoimmune response causes an accumulation of immune complexes and immune-mediated attack of various organs. Symptoms of SLE include: butterfly rash, chest pain, sensitivity to light, joint pain, and kidney damage [9,64].

Arbuckle et al. explored the clinical evidences of B cell epitope spreading by comparing the presence of autoAbs from the serum of pre-diagnosed and post diagnosed SLE patients [65]. This was made possible through The Department of Defense Serum Repository, containing over 30 million serum samples from members of the U.S. Armed Forces. Serum is collected at the time of enlistment and on average every year thereafter. This allowed Arbuckle et al. to identify SLE patients and then go back and observe the development of autoAbs prior to SLE diagnosis. 130 SLE patients were selected and then tested for the presence of the major SLE autoantibodies including: anti-double-stranded DNA, anti-Ro, anti-La, anti-Sm, anti-nuclear ribonucleoprotein, and antiphospholipid. The presence of auto-Abs was confirmed via indirect immunofluorescence with HEp-2000 cells and ELISA assays. Arbuckle at el. observed that pre-diagnosis of SLE patients autoAb detection recognized on average 1.5 of 7 auto-Ags associated with SLE. At the time of clinical SLE diagnosis typically 3 of 7 auto-Ags were recognized, on average, by the Abs showing a spread of autoAb specificity over time [65]. These results indicate the occurrence of intermolecular B cell epitope spreading during the progression of preclinical to clinical autoimmune manifestation as well as during the progression of the pathogenesis of SLE.

Another investigation demonstrated the role of epitope spreading in SLE by studying autoAb diversification in response to small nuclear ribonuclearproteins (snRNP's) Ags [66]. SLE patients often show diverse autoAb reactivity to snRNP's. To demonstrate this phenomenon, A/J mice were vaccinated with recombinant Smith D (SmD), Smith B (SmB), and A ribonuclearprotein (A-RNP) and the specificity of the Abs that developed were observed. Using western blots and immunoprecipitation assays to identify Ab specificity, it was observed that mice immunized with SmD also developed Abs specific against SmB and A-RNP. Likewise, mice immunized with SmB showed a development of Ab specificity against A-RNP and A-RNP immunized mice developed Abs against SmB as well as the 70-kDa protein A of the U1-snRNP. These results demonstrated that autoAb reactivity can spread from a single Antigenic site in a multi protein complex to associated complex proteins [66].

Studies by van Bavel et al. have investigated new antigenic epitopes presented after cell apoptosis that have been linked with SLE [67]. They found that acetylated epitopes of chromatin could allow for increased pathogenesis and epitope spreading. Scofield and associates studied a related line of research that indicate modification of Ro/SSA with lipid oxidation products, 4-hydroxy-2-alkenals in particular, increases the antigenicity and opportunity for B cell epitope spreading [68]. Research using A/J mice has also lead to the understanding that in some cases it is possible that intermolecular epitope spreading is more likely to occur than intramolecular epitope spreading [7]. As has been mentioned previously, environmental factors have been implicated leading to the breaking of tolerance and subsequent initiation of epitope spreading via molecular mimicry [25]. Thanks to recent and past research, it is apparent that B cell epitope spreading plays a crucial role in the pathogenesis of SLE once self-tolerance is broken.

4.5. Sjogrens syndrome

Sjogren's syndrome (SS) is an autoimmune disease that is associated with lymphocytic infiltration of the salivary and lacrimal glands resulting in dry mouth and eyes [69]. The immune response can result in the damage and destruction of the glands. When only exocrine glands are involved in the autoimmune response it is considered primary Sjogren's syndrome, after other connective tissue becomes involved it is considered secondary Sjogren's syndrome. In SS, autoAbs commonly develop against the Ro/SSA and La/SSB ribonucleoprotein particle linking SS to SLE through a common autoantigenic site. SS is the second most common rheumatic disease but frequently goes without diagnosis due to its common symptoms. While a portion of the disease can be contributed to genetic risk factors, other stimuli are hypothesized to play a role in the development of SS, such as hormone imbalance and stress. Several environmental factors are thought to be triggers for SS including Epstein–Barr virus, Hepatitis C, tuberculosis, and malaria among others [70].

Scofield et al. explored the role of epitope spreading in SS by vaccinating BALB/c mice with different amino acid sequences from 60-kDa Ro/SSA and observed whether autoAbs against the entire 60-kDa Ro/SSA protein would develop [71]. In the experiment 10 mice were vaccinated with Ro/SSA 480 and 10 separate mice were vaccinated with Ro/SSA 274. The results showed that mice developed Abs against their respective peptide sequence in 2–3 weeks and then over the course of several months developed autoAbs that were reactive against the entire Ro/SSA and La/SSB complex. They further observed that mice vaccinated with 60-kDa Ro peptides not only developed SS associated autoAbs but that the vaccinated mice developed salivary gland lymphocyte infiltrates and salivary gland dysfunction that resembled symptoms seen in human SS [71].

It is important to continue research investigating risk factors related to the development of SS. Recent studies have reported HLA class II specific genetic markers as risk factor for SS and leading to B cell epitope spreading [69,72,73]. Routsias et al. recently suggested that intermolecular B cell epitope spreading in SS may begin via molecular mimicry in the RRM region of La/SSB [74]. Further studies on possible risk factors and mechanisms of epitope spreading are needed to better understand the initiation and advancement of SS. Increased understanding in these areas would allow for the potential development of therapeutic treatments.

4.6. Graves disease

Also known as Basedow disease, Graves' disease is a condition where Abs develop that bind thyrotrophin receptors of the thyroid causing their activation and a subsequent up regulation of cyclic adenosine monophosphate and thyroid hormone synthesis [75,76]. This stimulation leads to hyperthyroidism and hyperplasia of the thyroid. Pathological symptoms include goiter development, insomnia, pretibial myxedema, muscle weakness, itching and Graves' ophthalmopathy [76,77].

B cell epitope spreading has been related to thyroid diseases in clinical settings. Kim et al. found that in the serum analyzed during their study, Ab targets changed during disease progression. Since that time various experimental animal models have provided evidence of B cell epitope spreading in Graves' disease. Using New Zealand White rabbits in a study to determine if B cell epitope spreading plays a role in the pathogenesis of Graves' disease, Thrasvoulides et al. immunized their rabbits with three different peptides from thyroglobulin [6]. Using affinity chromatography and ELISA assays, they confirmed that when they immunized the rabbits with peptide AA2471-2490, after a period of several weeks there

was intramolecular epitope spreading resulting in Abs binding to other epitopes on the thyroglobulin protein other than the initial peptide used during immunization.

Using Balb/c mice, evidence was found indicating intramolecular B cell epitope spreading [78]. In later mouse model studies, similar results were noted. When Balb/c and C57BL/6 mice where immunized with TSH receptor protein (TSHR), the Ab diversified from recognizing only the N-terminus domain to additional epitopes including the TSHR ectodomain [79]. Using more specific methods, Inaba et al. immunized breeding stock mice transgenic for HLA-DR3 and HLADR2 with 41 different TSHR peptides [80]. When mice where immunized with peptides AA70-88, AA83-102, or AA105-118 there was an Ab diversification outside of the original peptide epitope presented. More recently, it has been demonstrated that B cell epitope spreading occurs more rapidly than T cell epitope spreading [81]. The variety of research using animal and clinical means establishes B cell epitope spreading as a participant process in the development of Graves' disease.

4.7. Scleroderma

Scleroderma, or systemic sclerosis, is an autoimmune disease that involves connective tissue damage, most notably endothelial cells and fibroblasts of the extracellular matrix [82]. Scleroderma is characterized by fibrosis of various organs throughout the body. The hardening of the organs is thought to be caused by an overproduction of collagen. An early symptom indicative of scleroderma, termed Raynaud's phenomenon, is when extremities such as fingers or toes, experience an exaggerated response to temperatures and distress. These areas will often feel numb or pained and may change color. B cells have often been shown to over express CD19, an activating receptor, during autoimmune progression. AutoAbs for various targets are seen in patients with scleroderma including anti-endothelial cell, anti-fibroblast, anti-MMP, and anti-fibrillin-1 among others, which involve targeting many DNA binding proteins including DNA topoisomerase [82,83]. These autoimmune responses results in inflammation and tissue damage in the affected regions [84].

In scleroderma, B cell epitope spreading is initiated for a variety of reasons such as molecular mimicry or displays of previously cryptic domains which are displayed by enzymatic degradation, in which portions of the protein are degraded and novel domains are available for recognition. Furthermore, modification of target Ags may occur, such as the modification of U1-70 kd Ag modified by reactive oxygen species [8,82]. Cryptic domains may also be exposed after vascular spasms, and the release of reactive oxygen [85]. Few studies in recent years have investigated into epitope spreading and the development of scleroderma. In two studies, autoAbs to differing centrosome domains were present in the same sera, suggesting intermolecular spreading [85].

B cell epitope spreading has been observed as novel Ags are identified in patients with scleroderma. Of particular note is anti-PM/Scl Abs, antinucleolar Abs. Though further studies are necessary, it is thought that novel auto-Ags are targeted during disease progression. In a study conducted by Henry et al., autoAbs were shown to have higher incidences to certain auto-Ags than to other target auto-Ags [83]. For example, reactivity toward Scl-70 was much higher than topoisomerase I epitopes. These differences were concluded to occur because of the greater number of conformational determinants, which are larger than linear domains. This could explain the development of auto-Ags later in disease development resulting from the protein preference and subsequent epitope spreading events. The continued identification of novel auto-Ags and how B cell epitope spreading contributes to the pathogenesis of scleroderma remain areas for substantial future research.

4.8. Cardiomyopathy

A disease resulting in the weakening and enlarging of the myocardium, dilated cardiomyopathy (DCM) is a fatal disorder causing various cardiovascular problems and a frequent culprit of heart failure. Dilated cardiomyopathy generated as an autoimmune response is a consequence of Abs reactive to heart associated protein [86]. Anti-myosin IgG Abs are commonly observed in patients with autoimmune cardiomyopathy along with other auto Abs including anti-C protein Abs [87–89]. A clinical familial study conducted by Catorio et al. found that the production of cardiac specific Abs usually precludes disease development [90]. The occurrence of dilated cardiomyopathy is due to several factors both genetic and environmental. Potential triggers for dilated cardiomyopathy include enteroviruses and other myopathic diseases [62,88].

One of the few examples showing the involvement of B cell epitope spreading in cardiomyopathy, Matsumoto et al., observed this event in Lewis rats immunized with C protein fragment 2 (CC2) [91]. During their study they immunized Lewis rats with various peptides derived from CC2, peptide segments P1 through P12. All CC2 peptide mixes induced experimental autoimmune carditis (EAC) and lead to DCM, with the exception of P12, which only lead to the development of EAC. Mixes of P1 to P3, P4 to P6, P7 to P9, P10 to P12, and several individual peptide segments were used to inoculate the Lewis rats. After immunization, it was observed that the Ab response diversified to include not only the Abs against the mix of segments used to immunize the rats, but also against various other peptide segments on CC2. All of the Lewis rats immunized P1 through P11 demonstrated this diversification and eventually progressed from EAC to DCM. While those Lewis rats immunized with CC2P12 only developed Abs against that region and did not develop DCM, but remained expressing EAC symptoms [91]. The study evidences the influence that B cell epitope spreading plays in the progression of dilated cardiomyopathy.

4.9. Heymann nephritis

Heymann nephritis (HN) disease models using Lewis rats are one of the most valuable tools for researchers to use in understanding the immunopathology of glomerular subepithelial immune deposit formation and mechanisms by which such deposits injure glomeruli. The Lewis rat model of induced active Heymann nephritis first described in 1959 closely resembles that of glomerular injury and nephropathy in humans. Mechanisms of renal sodium retention, edema formation, and metabolic abnormalities have been observed as a guide to treating primary and secondary human glomerulonephritis diseases [92,93]. Inducing active HN in rats is conducted by immunizing susceptible strains of rats with certain fractions of homologous or heterologous proximal tubular brush border. Granular glomerular capillary wall deposits of rat IgG and subepithelial electron-dense deposits characterize the disease after three to four weeks. Primary manifestations of HN include proteinuria (developing in 30–80% of induced rats), changes in glomerular hemodynamics, tubular function, and alterations in renal hormone production. These disease manifestations are closely related to human nephropathy disease manifestations, and demonstrate that active HN is indeed an autoimmune-induced disease [92–94].

Megalin, or more specifically gp330, was found in 1982 and determined to be the glycoprotein and target Ag within the pits of glomerular and proximal tubular epithelia. The pathogenesis of HN includes the binding of circulating Abs to glomerular components. Megalin and RAP (receptor associated protein – 44kd) are the target Ags for nephritic activity and it was determined that RAP binds to Megalin [94,95].

Megalin is a complex Ag with four discrete ligand-binding domains (LBDs) that may contain epitopes to which pathogenic

autoAbs are directed. In a recent study a 236-residue N-terminal fragment (termed "L6") that spans the first LBD was shown to induce autoAbs and severe disease in Megalin-immunized HN rats [94]. Sera obtained from test results of rats that had received an L6 fragment showed reactivity only with the first LBD after a 4-week period. However, after 8 weeks the same L6 fragment showed reactivity with all four recombinant LBDs. The study demonstrated that the L6 immunogen did not contain the epitopes responsible for reactivity with the LBD fragments and therefore suggest intramolecular epitope spreading as the key variant to the increased severity and reactivity of HN rats. Further correlation of epitope spreading to the pathogenesis of HN disease indicated by the study was the onset of proteinuria. Proteinuria in rats immunized with L6 was seen at 6–8 weeks. ELISA of antisera indicated peak titers against L6 at weeks 4–6, however reactivity of LBDs arose after week 6 and had maximal values at week 9. Thus induction of proteinuria with serum reactivity was indicative of epitope spreading [94,95]. Treatment in HN Lewis rats with DNA vaccinations resulted in lowered proteinuria and promise for future treatment [96,97].

4.10. Pemphigus

Pemphigus is classified as a rare form of skin blistering caused by an autoimmune response against desmosomal adhesion proteins. These proteins are responsible for the homeostasis and attachment of epidermal cell layers [98]. There are four distinct forms and each is classified according to clinical signs and symptoms. Pemphigus vulgarius is the most common and is diagnosed by the presence of anti-Desmoglein 3 protein IgG Abs, with the initiation of sores in the oral mucosa and spreading to blisters on the face, limbs, and trunk of the body [99]. Pemphigus foliaceus is characterized by Desmoglein 1 autoAbs and superficial blister formation on the extremities. P. foliaceus tends to be one of the least severe forms of pemphigus. Immunoglobulin A pemphigus is identified by the characteristic IgA Abs directed against desmoglein, and in some cases, desmocollin, proteins. The final and most severe form, Paraneoplastic pemphigus, is often associated with a secondary malignancy. The product is a wide spread autoimmune response causing severe cutaneous blistering in potentially any epidermal or mucosal tissue of the body. The cause of the autoimmune response in this case is unknown, although it is suspect that disease initiation may be related to insect bites [99].

To demonstrate that intermolecular and intramolecular epitope spreading occurs in Pemphigus, Valerie K. Salato, Mong-Shang Lin, and their associates at the Medical College of Wisconsin collected and analyzed sera from pemphigus vulgaris (PV) positive volunteers [100]. These samples were taken at various times during the course of disease progression. In their study 14% of their volunteers transitioned from mucosal PV to mucocutaneous PV. They evaluated their Ab profiles during the progression of PV by immunoprecipitation and competition indirect immunoflorescence assays. Giving an example of one of these patients, early assays and detailed epitope mapping showed that they had Abs autoreactive for region AA405-566 of Desmoglein 3. Several years later that same patient transitioned to produce auto-Abs that reacted with regions AA1-88 and AA87-566 of Desmoglein 3, demonstrating the spread of the B cell repertoire and offering evidence of intramolecular spreading. This same patient also showed evidence of intermolecular epitope spreading when they developed Abs that were auto-reactive with human skin cells. Samples collected earlier in the study showed no human skin auto-Abs while six years later, after the patient had transitioned to mucocutaneous PV, samples taken exhibited these auto-Abs. This hypothesis of intermolecular spreading was further supported when they confirmed the Abs auto-reactive for desmoglein 1 in later samples [100]. Their study demonstrates how B cell epitope spreading contributes to the progression of pemphigus vulgarius from displaying strictly mucosal legions to cutaneous legions.

Further observations give credence to the phenomenon of epitope spreading and its role in the development of pemphigus when Bowen et al. described the development of paraneoplastic pemphigus from patients displaying Lichenoid Dermatitis [101]. They speculate that the intramolecular spreading could be due to lichenoid sores and the subsequential inflammation predisposing patients via a heightened immune response. This theory was further compared to other leading ideas as to the cause for disease progression in paraneoplastic pemphigus and was suggested as a possible area for future research [102]. In a study done in a Brazilian community it was proposed that epitope spreading was the cause for the development of pemphigus foliaceus in patients diagnosed with Fogo Selvagem, loosely translated as "wild fire" disease [18]. In their study, Li et al. proposed that a genetic predisposition may contribute along with epitope spreading leading to pemphigus foliaceus. In a unique case, clinical evidence suggests that B cell epitope spreading can contribute to the progression of pemphigus to bullous pemphigoid [14]. B cell epitope spreading has been observed to play a role in the progression of pemphigus and contribute in the development to more severe symptoms.

4.11. Bullous pemphigoid

Similar to pemphigus, bullous pemphigoid is a cutaneous blistering autoimmune disease. Patients suffering from bullous pemphigoid do not typically experience mucosal lesions and the disease is often marked by pruritic blisters on the trunk of the body, epithelial areas of the joints, and the extremities. The autoimmune response in this case is characterized by the development of Abs against bullous pemphigoid Ag 1 and 2 (BP230 and BP180 respectively), also known as BPAG 1 and BPAG2, which compose part of the hemidesmosome and assist with cell to matrix adhesion in the epithelium [103,104]. As a result, autoimmune response to differing collegen types is characteristically seen in bullous pemphigoid. It is documented to have a higher incidence among the elderly and is estimated to have an incidence in the general population of 6–7 cases per million. This estimate increases to 150–330 cases per million in the population older than 80 years of age [105,106]. With respect to the environmental or genetic factors that could initiate an autoimmune response, there remains little information on triggers. Our current understanding indicates that triggers for bullous pemphigoid could include vaccines, medications, and possibly a variety of infections [107].

A clinical study was conducted by Giovanni Di Zenzo and his international associates with the specific aim to appraise the development of IgG Abs during the course of bullous pemphigoid development [108]. Thirty-five patients volunteers diagnosed with bullous pemphigoid were selected for this multicenter study along with a control group of 50 volunteers including both bullous pemphigoid patients and healthy individuals. All volunteers were assessed at the initiation of the study as well as one, three, six, and twelve months during the study while undergoing treatment. ELISA and immunoblotting assays specific for various domains on BP180 and BP230 were utilized to assess the Abs present in patient sera. Their study found that in three of the patients, BP180 Abs recognized the ectodomains of the protein before developing Abs specific for the intracellular domains. Intermolecular epitope spreading was also observed to occur during the course of the study. 17.6 percent of the volunteers developed an IgG response that initially recognized either BP180 or BP230, to recognizing both proteins. Analyzing the data collected during the study, it was observed that epitope spreading incidence was higher during the first three months after diagnosis compared to any other time during the study. It was noted that the spreading from ectodomains to

intracellular domains and the incidence rates are correlated with the severity of bullous pemphigoid [108].

During a previous clinical study, Epitope spreading was observed in the Ab response progressing from collagen XVII to collagen VII [109]. As has been previously mentioned, bullous pemphigoid has been observed to develop after diagnosis of pemphigus foliaceus [13,14]. Another clinical report was submitted several years later with similar observations of intermolecular epitope spreading resulting in the development of bullous pemphigoid from pemphigus foliaceus, but with the additional information that the patient exhibited only IgG3 autoAbs, not IgG1 [110]. Antibody isotype switching was not noted during the 13 year course of clinical observation as normally would be the case. This remains an area for further research with regard to the role isotype switching, or the lack there of, has in epitope spreading.

5. Treatment strategies for B cell epitope spreading: current concepts and perspectives

Currently there exist few options for treatment of autoimmune diseases. Clinicians and researchers rely on generalized immunosuppressive treatments that are designed to block a broad-spectrum of potential risk factors associated with such diseases. Consideration of both efficacy and side effects of such treatments is vital. In a review published by Vanderlugt and Miller discuss the role of tactics that can be designed to slow and/or neutralize the effects of epitope spreading in autoimmune function [2]. Due to the unknown biologic pathways and change in antigenic specificity, it is difficult to design drugs that can treat epitope spreading. Very little, if any, research has been done investigating the effects of these therapeutics on epitope spreading. Because these treatments each have specific effects on B cells, from killing them to inhibiting specific signaling pathways, investigations into their effects on epitope spreading could reveal insights into the mechanisms and function of this important immune process.

The use of various drugs to control and inhibit both B and T cell activity is currently the most commonly used approach to autoimmune diseases, however these drugs typically suppress the entire immune system and are non-specific in their approach [2,111]. T-cells play a major role in recognizing foreign and self-Ags and thus promote a cytokine response due to changes in epitope specificity leading to autoimmune dysfunction. Vanderlugt et al., utilizing EAE (experimental autoimmune encephalomyelitis) induced SJL mice and focusing on co-stimulatory signaling centers of CD28-CD80/86 and CD154-CD40, established anti-CD80 Ab fragments and monoclonal anti-CD154 Abs that were administered during peak phase or during relapse of EAE. Both anti-CD80 and anti-CD154 Abs showed significant inhibition of disease relapse. Epitope spreading has been thoroughly studied in EAE mice, which serve as a model for multiple sclerosis. Other drugs such as interferon pathway regulatory agents could provide a further targeted approach to management of autoimmune activity [112,113]. Most recently researchers are working to develop Th2 bystander suppression drugs to combat epitope spreading, disease-specific immunologic drugs, and targeted cytokine inhibitors [2]. These treatments are not in common use at this point, and there is very little available data regarding their effects on epitope spreading.

5.1. Rituximab and B cell depletion therapy

Rituximab is becoming a commonly used immunosuppressive. It utilizes a CD20 binding protein designed to deplete mature B cells. In one report discussing the multi-type efficacy of Rituximab, patients with pemphigus, a severe autoimmune disease resulting in blisters of the skin and skin and mucosal erosion, were treated with rheumatoid arthritis (RA) dosing schedules of Rituximab. At 6 months of dosage treatment 90% of patients achieved remission. At 22 months there was a 67% relapse rate with patients, however Global CD 4 + T cell numbers were preserved up to 3 months after treatment [114]. In conjunction with corticosteroids and other cytotoxic agents, Rituximab has also been used to treat SLE and other Ab mediated autoimmune diseases [115,116].

Other B cell depleting therapies include ocrelizumab and epratuzumab, which bind CD20 and CD22 respectively [117]. Use of B-cell depletion therapy may be helpful for patients that don't respond to other immunosuppression therapies [2,115,118]. Use of rituximab could potentially inhibit or impede B cell epitope spreading due to the depletion of B cells during the course of therapeutic treatment. However, there is no current evidence to support that B cell depletion significantly disrupts B cell epitope spreading.

5.2. Belimumab and cytokine inhibition therapy

A promising treatment for SLE, Belimumab is a monoclonal Ab that binds B lymphocyte stimulators (BLyS). This type of therapy prevents the differentiation and survival of B cells. Used in various clinical studies, Belimumab has become recognized as a treatment with high efficacy and tolerability [117,119,120]. A large multi-center phase III study performed on Belimumab by Furie et al. demonstrated the efficacy of the therapy. 819 anti-nuclear Ab or anti-double stranded DNA Ab positive patients participated in the randomized placebo controlled study over a period of 52 weeks. Those treated with non-placebo participants were randomly given a high or low dose of Belimumab, 10 mg/kg and 1 mg/kg respectively. At one year post treatment start of the study it was found that the risk of severe flares was reduced by 34% ($p = 0.023$) for the low dose group and 23% ($p = 0.13$) for the high dose group. It was also calculated that the SRI rates were 44%, 51% ($p = 0.0129$), and 58% ($p = 0.0006$) for the placebo, low, and high dose groups respectively [120]. Similar results have been observed in other studies and suggest that Belimumab could be a good alternate treatment option for SLE patients.

Blisibimod and Tabalumab are two other BLyS targeting Abs that are currently in clinical trials and show promise for the treatment of SLE. In a recent randomized, placebo controlled clinical phase II study, 547 SLE patients were treated with Blisibimod at one of three dose levels and monitored over a period of 24 weeks. It was reported that the most effective dose was the highest at 200 mg/kg and that it significantly reduced levels of anti-double stranded DNA Abs ($p < 0.001$) after 24 weeks. This dosage also improved levels of C3 and C4 ($p < 0.01$ and $p < 0.001$, respectively). It was also observed that the treatment significantly lowered levels of B cells ($p < 0.001$). The results of the study suggests that at 200 mg/kg, Blisibimod is most efficacious for patients with severe SLE [121].

Atacicept is a biologic treatment option that is in clinical trials and has been studied for the treatment of SLE, RA, and MS [122–124]. Due to its fusion protein construction it inhibits both BLyS and A proliferation inducing ligand (APRIL), which results in the prevention of symptoms similar to other BLyS targeting therapies, inhibiting B cell differentiation. A phase II clinical study published by Genovese et al. reported that Atacicept showed no significant efficacy compared to the placebo group used in the study [123]. But, they did demonstrate that Antacicept lowered rheumatoid factor and immunoglobulin levels even though it did not decrease the levels of anti-citrullinated protein Abs. It was observed in a recently published double blind, randomized, placebo controlled clinical phase II study of Atacicept for the treatment of MS, that the treatment adversely affected the patients treated compared to the control group [124]. The results of a randomized, double blind, placebo controlled clinical study to determine the efficacy in treating SLE patients were recently published by

Isenberg et al. In this 52 week study 461 SLE patients were treated with 75 mg/kg, 150 mg/kg, or the placebo. They reported that the high dose group, 150 mg/kg, demonstrated lower flare rates verses the placebo group ($p = 0.002$). Both the low and high dose treated groups showed reduced levels of total Ig and anti-double stranded DNA Abs [122]. The results of these recent studies suggest that Atacicept could be a possible biologic treatment option for SLE. There is no published evidence to demonstrate that BLyS and APRIL therapeutic strategies inhibit or impede B cell epitope spreading, aside from the lack of clinical signs and symptoms demonstrated by patients during the clinical trials. It does stand to reason that such treatment options would prevent or disrupt epitope spreading, inhibiting disease progression.

5.3. Abatacept and co-receptor inhibition

A further strategy of treatment is utilized by Abatacept, which inhibits T cell activation by binding the CD28 receptor on B cells. Approved by the FDA in 2005, Abatacept is currently used in the treatment of RA and to slow the progression of joint damage. In a recent phase III clinical study comparing Abatacept to Adalimumab, a tumor necrosis factor α (TNF-α) inhibitor approved for the treatment of RA in 2002, demonstrated that subcutaneous Abatacept produced similar results in the treatment of RA [125]. In a multicenter, phase IIb, randomized, double-blind, placebo controlled study of Abatacept in the treatment of SLE it was observed that patients treated experienced a similar frequency of adverse events, and that these adverse events were typically worse in the treatment group compared to the placebo group [126]. While there is no evidence demonstrating this treatment inhibits epitope spreading, such a strategy shows promise as an effective way to inhibit disease progression and possibly impede B cell epitope spreading in patients with RA [127].

6. Concluding remarks

Over the past two decades our understanding of B cell epitope spreading has broadened substantially. It is well understood how the process of epitope spreading contributes to pathogen clearance and several factors that contribute to its effectiveness. Molecular mimicry and Intrastructural T cell help allow B cells to expand their Ab repertoire, which diversity results in higher affinity Abs and a more effective immune response. Through various intracellular processes, stimulated B cells can generate novel target epitopes and present them in the context of MHC class II molecules to T cells. During B cell endocytosis, an Ag is endocytosed after stimulation of sIg molecules on the cell surface. GILT, endocytic proteases, and lysosomal cathepsins selectively process the Ag. After which a digested peptide is selectively loaded into the MHC II molecule post LI degradation and CLIP dissociation, allowing for various epitopes to be displayed by MHC class II molecules. Through somatic hypermutation a variety of higher affinity Abs are produced granting opportunity for further diversification of epitope targets.

While this process contributes positively to our systemic defense against pathogenic organisms and macromolecules, it is also responsible for the progression of autoimmune diseases after self-tolerance is broken. Evidence from clinical studies and reports, a variety of animal models, and serological collection analysis has yielded substantial evidence indicating such. Both intermolecular and intramolecular epitope spreading are observed to happen congruously with the progression of autoimmune disease. Potential auto-Ags are more likely to become Antigenic targets to initiate epitope spreading after modification by reactive oxygen species [8,82] and lipid oxidation products [68] or abnormal apoptosis [67] and citrullination [52]. Various other environmental triggers for initiating epitope spreading in autoimmune afflictions have been identified including assorted viruses, bacterial infections, and stress [25,70]. Currently we lack treatments that can effectively stop detrimental B cell epitope spreading. It can be considered that various treatments, B cell depletion and immune repression included, partial impede epitope spreading [128].

There are many aspects that still remain to be explored and would contribute to future understanding of epitope spreading, while the role of T cell interaction in epitope spreading is fairly well understood, less work has been done investigating the role of B cells in epitope spreading. Future research opportunities regarding B cell endocytosis include investigating into the currently unknown regulatory factors that are involved in the loading of MHC class II molecules and competitive inhibitory processes that regulate the selective processing of Antigenic peptides by cathepsins. It is also unclear how various compartments in the endocytic pathway target each other. The extent to which B cell epitope spreading contributes in the progression of many autoimmune diseases is still not well understood and could be better researched. Comprehension of patterns involved in epitope spreading to allow the prediction and potential therapy of epitope spreading in autoimmune diseases is also lacking. Treatments to inhibit epitope spreading or eliminate the detrimental aspects of epitope spreading are areas where future research could be conducted.

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Review

Piracy on the molecular level: human herpesviruses manipulate cellular chemotaxis

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Cellular chemotaxis is important to tissue homeostasis and proper development. Human herpesvirus species influence cellular chemotaxis by regulating cellular chemokines and chemokine receptors. Herpesviruses also express various viral chemokines and chemokine receptors during infection. These changes to chemokine concentrations and receptor availability assist in the pathogenesis of herpesviruses and contribute to a variety of diseases and malignancies. By interfering with the positioning of host cells during herpesvirus infection, viral spread is assisted, latency can be established and the immune system is prevented from eradicating viral infection.

INTRODUCTION

Cells respond to a variety of cytokines and chemokines that allow them to migrate in different areas in the body depending on where they are needed. This process is essential for appropriate tissue maintenance, homeostasis, formation, repair and pathogen clearance (Turner *et al.*, 2014; Zhou *et al.*, 2014). Dysregulation of the delicate balance of cellular signals and/or improper positioning could impede these processes. Aside from being related to a range of diseases, viral-induced chemotaxis contributes to the epidemiology and persistence of human herpesviruses. These viruses regulate a multitude of cellular genes that direct cellular chemotaxis, thereby manipulating these genes for the benefit of the invading virus. Herpesviruses also produce various chemokines and chemokine receptors from genes in the viral genome, further affecting cellular chemotaxis. In essence, viral infection results in the piracy of cellular function as it directs cell movement in both infected and uninfected cell types.

The family *Herpesviridae* is divided into various subfamilies including *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* (Flint & American Society for Microbiology, 2009; Yoshida & Yamada, 2006). The nine human herpesviruses (HHVs) include herpes simplex virus type 1 (HHV-1 or HSV-1) and 2 (HHV-2 or HSV-2), varicella–zoster virus (HHV-3 or VZV), Epstein–Barr virus (HHV-4 or EBV), human cytomegalovirus (HHV-5 or hCMV), human herpesvirus 6A (HHV-6A) and 6B (HHV-6B or roseola virus), human herpesvirus 7 (HHV-7) and Kaposi's sarcoma-associated herpesvirus (HHV-8 or KSHV) (Siakallis *et al.*, 2009). All of these viral species share similar structural characteristics with a genome composed of double-stranded DNA, an icosahedral capsid, an envelope studded with a variety of viral and host proteins, and viral tegument proteins in an amorphous layer between the capsid and envelope (Flint & American Society for Microbiology, 2009). Herpesviruses are able to remain latent in host cells for the life of the individual, during which time viral particles are undetectable but viral nucleic acids can be found, and viral gene expression is very limited. Various stimuli can cause viral reactivation, wherein viral gene expression recommences and infectious particles can be detected and shed to new hosts. Herpesviruses encode a complex assortment of proteins that manipulate cellular functions during infection in order to promote viral persistence. Human herpesviruses are an integral part of human existence, with over 90% of adults being persistently infected with one or more of these nine herpesviruses in their lifetimes. Although the incidence of serious herpesvirus-induced diseases is rare in most cases, the prevalence of infection is so high that the overall disease burden takes a toll on society.

It has been hypothesized that several herpesvirus species affect development or progression of diseases, including lymphomas, atherosclerosis, autoimmune disorders, and disruption of angiogenesis, through interference with cellular chemotaxis (Ehlin-Henriksson *et al.*, 2009; Franciotta *et al.*, 2008; Rosenkilde & Schwartz, 2004; Stern & Slobedman, 2008; Streblow *et al.*, 2001). In this review we will elaborate on the known human HHV mechanisms and pathways that influence cellular chemotaxis during viral infection. Potential benefits to herpesviruses in evolving these mechanisms will be presented as well as the resulting potential for their roles in disease development.

ALPHAHERPESVIRINAE

HSV-1, HSV-2 and VZV encompass the human pathogens of the subfamily *Alphaherpesvirinae*, typically showing lytic replication in epithelial cells and harboured as a latent infection in neuronal cells. HSV-1 is quite common in industrialized countries, with a seroprevalence of around 90% (Viejo-Borbolla *et al.*, 2012) in the adult population. Symptoms of viral infection include cold sores and redness of the skin; however, many infections are asymptomatic. HSV-1 transmittance only occurs when viral replication takes place, either during primary infection or in a reactivation event. The most common methods of transferring HSV-1 include direct skin contact and via saliva. Similar to HSV-1, HSV-2 can mask its presence from the host's immune system, demonstrating a preference to lie dormant in the sacral ganglia (HSV-1 in trigeminal ganglia) and manifest occasional lytic outbreaks, typically in the genital area. HSV-2 is one of the most common sexually transmitted diseases, with a seroprevalence of 12–20% in the USA. HSV-2 infection is of greater concern in developing countries, where seroprevalence is much higher (Weiss, 2004; Xu *et al.*, 2006). VZV primary infection results in the common childhood disease varicella (chickenpox) after which the virus establishes latency in the ganglia of a variety of neurons (Gilden *et al.*, 2014). Reactivation of the virus results in zoster (shingles) and other chronic pain diseases, which can be manifest in various places on the epithelium (Gilden *et al.*, 2014).

Until recently, not much was known about HSV and how it affects chemotaxis; however, current work has demonstrated that HSV infection has a strong influence on chemotaxis (see Table 1). Viejo-Borbolla *et al.* (2012) showed that a secreted form of viral glycoprotein G (SgG) from both HSV-1 and HSV-2 binds chemokines with high affinity. Membrane-bound glycoprotein G (gG) was shown to be necessary for chemokine-binding activity. They found that HSV SgG in both HSV-1 and HSV-2 increased chemotaxis of monocytes in infected individuals towards CXCL12 and that gG attaches to glycosaminoglycans (GAGs) at the surface of cells without negative effects on G-protein-coupled receptors (GPCRs). Another, more recent, study further investigated the mechanism by which viral SgG enhances chemotaxis. It was found that gG binds to GAGs, which induces lipid raft clustering, leading to increased CXCR4 incorporation. The conformational change causes an increase in

functional chemokine–receptor complexes at the cell surface (Martinez-Martin *et al.*, 2015). CXCL12 is the natural ligand for CXCR4 and is secreted constitutively in a variety of tissues, including the lymph nodes, bone marrow, lungs and adrenal glands (Alkhatib, 2009; Luker & Luker, 2006). It is also known that CXCR4 signalling is important in modulating the survival of neuronal cells and modulating synaptic function (Nash & Meucci, 2014). The increased functionality of CXCR4 could potentially allow infected cells to migrate to these areas *in vivo*. By migrating to areas secreting CXCL12, infected cells could come into contact with more target cells. Similar results had been observed by Bellner *et al.* (2005) when they tested the chemotactic ability of HSV-2 gG (gG-2p20). These authors found that isolated human neutrophils and monocytes followed a gradient of gG-2p20 via binding of the formyl peptide receptor (FPR) on the surface of these cells. While the chemoattractant properties have never been displayed using the full-length gG2 protein, several speculations can be made based on the findings that suggest that neutrophils and monocytes could be attracted to areas with infected cells expressing gG-2p20. This could possibly be beneficial for HSV-2 infection. Attracting a large number of phagocytic cells would increase tissue damage and activated cells, potentially enabling viral spread and propagation (Bellner *et al.*, 2005). It was shown that gG-2p20 is an FPR-activating agonist. Activation of FPR *in vivo* led to the downregulation of other chemotactic receptors. These observations suggest the possibility that the change in expression could lead to impaired clearance of HSV-2 during infection (Bellner *et al.*, 2005). In summary, a variety of studies have demonstrated the effectiveness of HSV-1 and HSV-2 in manipulating CXCR4 in infected cells.

HSV-2 has demonstrated the ability to manipulate chemotaxis via a host chemokine as well. A study performed by Huang *et al.* (2012) demonstrated an elevated expression of CXCL9 in the cervical mucosa of HSV-2-positive women. Further research confirmed that HSV-2 regulated the expression of CXCL9 in human cervical epithelial cells by inducing the phosphorylation and translocation of C/EBP-β to the nucleus, where it transactivates CXCL9. The known receptor for CXCL9 is CXCR3, which is expressed predominantly in non-resting T cells (Van Raemdonck *et al.*, 2015). Expression has also been observed in epithelial, endothelial, fibroblast and smooth muscle cells (SMCs) (Billottet *et al.*, 2013; Van Raemdonck

Table 1. Alphaherpesviruses change cellular receptors/chemokines

Cell type	Receptor or chemokine	Virus	Amount or functionality	References
Monocyte	CXCR4	HSV-1, HSV-2	Increase	Bellner *et al.* (2005); Viejo-Borbolla *et al.* (2012)
Neutrophil	CXCR4	HSV-1, HSV-2	Increase	Bellner *et al.* (2005)
Epithelial	CXCL9	HSV-2	Increase	Huang *et al.* (2012)

et al., 2015). This upregulation of CXCL9 was shown to result in increased migration of activated peripheral blood leukocytes (PBLs) and CD4+T lymphocytes (Huang et al., 2012). Huang and associates postulate that HSV-2 is responsible for upregulating CXCL9; however, it was not shown what viral protein induced the expression or if the increase in CXCL9 expression was a cellular response to viral infection. The viral benefits for inducing migration of CD4+T cells and PBLs to sites of infection are unclear. The ability of HSV-2 to regulate CXCL9 could be investigated more in depth as this is the only study demonstrating this type of subversion in epithelial cells.

Past research has also suggested that VZV could utilize glycoproteins as chemoattractants, inducing migration of polymorphonuclear leukocytes (Ihara et al., 1991). No other recent research has been conducted to determine if VZV affects chemotaxis of other infected cell types, although several studies do provide evidence for how VZV might influence cellular chemotaxis (Desloges et al., 2008; Shavit et al., 1999; Steain et al., 2011). We now understand that HSV-1 and HSV-2 can manipulate monocytes through increasing the functionality of CXCR4 by making lipid rafts with the viral SgG protein. HSV-2 can further change the migration of cells by increasing the expression of CXCL9 in infected epithelial cells, potentially attracting CD4+T cells and PBLs to sites of infection.

BETAHERPESVIRINAE

Human cytomegalovirus (hCMV)

Also known as human herpesvirus 5, hCMV is a prominent member of the Betaherpesvirinae subfamily. With a sero-prevalence worldwide ranging from 45 to 100%, hCMV is a common human pathogen that is often asymptomatic in infected adults and children (Cannon et al., 2010; Chen et al., 1999; McGavran & Smith, 1965). hCMV has gained public scrutiny and awareness owing to further understanding of its prevalence in causing congenital infections leading to birth defects (Bialas et al., 2015). In the USA it is a more common cause of birth defects than many other causes, including fetal alcohol syndrome, Down syndrome, spina bifida, HIV/AIDS, Haemophilus influenzae type B and congenital rubella syndrome (Cannon & Davis, 2005). Like other herpesviruses, hCMV is associated with various post-transplant complications and is a main viral cause of solid organ transplant and haematopoietic stem cell transplant morbidity and mortality (Ariza-Heredia et al., 2014; Gandhi & Khanna, 2004). It is also known to cause severe disease in other immunocompromised individuals, such as AIDS patients. Viral shedding can occur via saliva, urine, breast milk, semen and tears. hCMV is known to infect various cell types, including epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, dendritic cells and lymphocytes, the latter cell type typically remaining latently infected for the life of the host.

Using a variety of viral proteins to manipulate migration of host cells and potential target cells, hCMV uses both surface receptors and secreted chemokines (see Tables 2 and 3). Among the viral chemokines secreted by hCMV-infected cells are the products of the UL128 and UL146 genes. Numerous studies have been performed demonstrating how these viral gene products affect the migration of hCMV-infected cells. It has been noted that hCMV-infected monocytes demonstrate a reduced chemotactic ability owing to a downregulation of CCR1, CCR2 and CCR5 (Frascaroli et al., 2006). A similar downregulation of various chemokines was observed along with an increase in migratory inhibitory factor in hCMV-infected macrophages, resulting in a lack of motility (Frascaroli et al., 2009).

Table 2. Human herpesvirus-encoded chemokine receptors

Virus	Viral receptor	Result	References
hCMV	US27	Potentiates CXCR4, increases migration to various tissues	Arnolds et al. (2013)
	US28	Migration of infected cells to areas of inflammation	Streblow et al. (1999); Vomaske et al. (2009)
	UL33 and UL78	Prevents migration to sites of inflammation and certain tissues	Tadagaki et al. (2012); Tschische et al. (2011)
HHV-6A	U51	Prevents NK cell interaction and prevents apoptotic signals	Catusse et al. (2008); Fitzsimons et al. (2006)
HHV-6B	U12	Migrates to inflammatory and T cell-rich zones	Isegawa et al. (1998)
HHV-7	U51 U12	Migration of infected cells to T cell-rich and inflammatory areas	Nicholas (1996); Tadagaki et al. (2005)
KSHV	KSHV-GPCR	Increases cell survival	Couty et al. (2009); Pati et al. (2001); Shepard et al. (2001)

Table 3. Human herpesvirus-encoded chemokines

Virus	Viral chemokine	Result	References
VZV	GP I and GP II	Chemoattractant for polymorphonuclear leukocytes	Ihara *et al.* (1991)
HSV	gG2	Chemoattractant for monocytes	Bellner *et al.* (2005)
hCMV	UL146	Chemoattractant for neutrophils	Lüttichau, (2010); Penfold *et al.* (1999)
hCMV	UL128 and UL146	Attracts PBMCs and prevents monocyte migration	Frascaroli *et al.* (2006); Gao *et al.* (2013); Straschewski *et al.* (2011)
HHV-6A	U83A	Chemoattractant for T cells, monocytes and immature dendritic cells	Catusse *et al.* (2007); Dewin *et al.* (2006)
HHV-6B	U83B	Chemoattractant for monocytes	Clark *et al.* (2013); Lüttichau *et al.* (2003)
KSHV	vCCL1	Chemoattractant for monocytes	Nakano *et al.* (2003); Weber *et al.* (2001)
KSHV	vCCL2	Prevents Th1 T cell chemotaxis and attracts monocytes	Nakano *et al.* (2003); Weber *et al.* (2001)
KSHV	vCCL3	Inhibits chemotaxis of Th1 T cells and NK cells	Lüttichau *et al.* (2007)
KSHV	vIL-6	Attracts endothelial cells	Wu *et al.* (2014)

Later it was demonstrated by Frascaroli and associates that in the presence of UL128 there was a resulting downregulation of CCR1, CCR2 and CCR5 in monocytes (Straschewski *et al.*, 2011). Because of this impairment, monocytes could no longer migrate following the chemokines CCL5 and CCL2, which are ligands of the aforementioned receptors. CCL2 and CCL5 are known to be involved in the recruitment of monocytes and T cells and are secreted as pro-inflammatory cytokines in response to tissue damage or viral detection (Ansari *et al.*, 2013; Soria & Ben-Baruch, 2008). Recently, using a *UL128*-transfected cell line (CHO-UL128) to produce UL128, Gao *et al.* (2013) studied the effects of this β chemokine on cell migration. They found that UL128 acted as a chemoattractant for peripheral blood mononuclear cells (PBMCs) *in vitro* and functioned similarly to CCL3 as a chemoattractant. These results suggest that UL128 could act to prevent chemotaxis of monocytes following other gradients, such as CCL5 and CCL2, and could use a separate receptor to attract the monocytes to areas of infected cells (Gao *et al.*, 2013). This increases the cells available to be infected by hCMV, potentially furthering viral spread. The other known chemokine produced, UL146, codes for an α chemokine and viral homologue to CXCL1 (vCXCL1) (Penfold *et al.*, 1999). Two studies demonstrated that vCXCL1 could induce the chemotaxis of neutrophils *in vitro* (Lüttichau, 2010; Penfold *et al.*, 1999). Using calcium mobilization, chemotaxis and phosphatidylinositol turnover assays, it was found that vCXCL1 was a ligand for CXCR1 and CXCR2. CXCR1 and CXCR2 are both expressed on neutrophils, and it is expected that hCMV-infected endothelial cells express vCXCL1 as a chemoattractant to increase the numbers of neutrophils and assist in viral spread to other endothelial cells (Lüttichau, 2010). In a study conducted by Smith *et al.* (2004), it was observed that hCMV-infected monocytes induced

transendothelial migration *in vitro*, although the viral mechanism is unknown (Smith *et al.*, 2004).

Regulating host cell chemokines can also result in chemotactic changes (see Table 4). hCMV UL144 is a viral protein that activates NF-κB (Poole *et al.*, 2006). This leads to a cascade of multiple pathways, including induced expression of host CCL22, which acts as a chemoattractant for Th2 and regulatory T cells (Tregs). By recruiting these cells to sites of viral infection it is possible to suppress T helper and CD8 + T cells, tapering the immune response (Fielding, 2015). It has also been found that granulocyte macrophage progenitors (GMPs) latently infected with hCMV demonstrate increased expression of CCL2 (Stern & Slobedman, 2008). CCL2 is a pro-inflammatory cytokine that acts as a chemoattractant to monocytes, macrophages, dendritic cells and T cells expressing CCR2. This increase in CCL2 acts to attract CD14 + monocytes to latently infected GMPs (Stern & Slobedman, 2008). This behaviour of latently infected GMPs is likely a viral strategy employed to recruit new leukocytes to be infected; however, too little is known about *in vivo* hCMV reactivation to know if this spread and reactivation occurs before or after GMPs develop into macrophages. Further research could be done into the manipulation of hCMV-infected GMPs as there is currently just one study demonstrating this change in chemotaxis.

There are a variety of hCMV chemokine receptors shown to affect cell migration, including US27, US28, UL33 and UL78 (Fielding, 2015), all of which are homologous to human GPCRs. US27 is expressed late during lytic infection and has no known ligand (Fraile-Ramos *et al.*, 2002; Stapleton *et al.*, 2012). However, it was found to potentiate CXCR4-mediated chemotaxis, increasing the expression and amount of surface CXCR4 (Arnolds *et al.*, 2013). As previously explained, CXCR4 is a seven-membrane-

Table 4. Betaherpesviruses change cellular receptors/chemokines

Cell type	Receptor or chemokine	Virus	Amount or functionality	References
Dendritic cell	CCR7	hCMV	Decrease	Moutaftsi *et al.* (2004)
	CCR1	hCMV	Decrease	Varani *et al.* (2005)
	CCR5	hCMV	Decrease	Varani *et al.* (2005)
GMP	CCL2	hCMV	Increase	Stern & Slobedman (2008)
Monocyte	CCR1	hCMV	Decrease	Frascaroli *et al.* (2006, 2009);
	CCR2	hCMV	Decrease	Straschewski *et al.* (2011)
	CCR5	hCMV	Decrease	
	CCL22	hCMV	Increase	Poole *et al.* (2006)
T cell	CCL5	HHV-6A	Decrease/increase	Cerdan *et al.* (2001)
	CCR7	HHV-6	Increase	Hasegawa *et al.* (1994)
	CXCR4	HHV-6	Decrease	Yasukawa *et al.* (1999)
	CXCR4	HHV-7	Decrease	Yasukawa *et al.* (1999)
	CCR7	HHV-7	Increase	Hasegawa *et al.* (1994)

spanning GPCR that allows the cell to follow the chemokine gradient of its natural ligand, CXCL12, which is secreted constitutively in a variety of tissues, including the lymph nodes, thymus, bone marrow, lungs and adrenal glands (Alkhatib, 2009; Luker & Luker, 2006). The potentiation of CXCR4 resulted in increased migration to CXCL12 during *in vitro* migration assays (Arnolds *et al.*, 2013). It has been speculated that increased CXCR4 levels at appropriate times could allow hCMV-infected cells to migrate to bone marrow or lymph nodes, where there would be an increased opportunity to spread to susceptible cells (Arnolds *et al.*, 2013).

US28 was first shown to affect migration in vascular SMCs (Streblow *et al.*, 1999). It was found that US28 directed cell migration following the chemokines CCL2 and possibly CCL5. In the absence of CCL2, there was no migration of hCMV-infected SMCs (Streblow *et al.*, 1999). This would allow infected SMCs to migrate to areas of inflammation, potentially providing opportunity for viral spread to leukocytes. Later it was demonstrated that US28 acted to control migration of both infected SMCs and infected macrophages. Kledal *et al.* (1998) found that US28 also bound CX3CL1, which is a chemokine that is found on the cell surface and extracellularly in a secreted form; this work was later followed up by others (Murphy *et al.*, 2008; Vomaske *et al.*, 2009). CX3CL1 is only known to be produced by endothelial cells and results in the recruitment of inflammatory cells (Bazan *et al.*, 1997; Vomaske *et al.*, 2009). These authors found that the presence of CX3CL1 inhibited the migration of hCMV-infected SMCs, but induced the migration of hCMV-infected macrophages. It was also demonstrated that the inverse was true, in the presence of CCL5, hCMV-infected macrophages US28-mediated migration was inhibited, but hCMV-infected SMCs demonstrated normal chemotaxis, as expected (Vomaske *et al.*, 2009). This makes the viral GPCR US28 unique in that it is chemokine- and cell-type-specific.

It seems important for the virus to control cellular migration during hCMV infection. Evidence for how US28 functions was provided by Tschische *et al.* (2011), when they found that hCMV chemokine receptors heteromerize with each other. It was observed that UL33 and UL78 heteromerization resulted in silencing of US28-mediated activation of the NF-κB pathway. Tadagaki *et al.* (2012) investigated UL33 and UL78, and found that these two GPCR homologues formed heteromers with CCR5 and CXCR4 on the surface of infected THP-1 cells. This was found to prevent cell chemotaxis facilitated by CCR5 and CXCR4 *in vitro*. CCR5 allows the cell to follow a variety of chemokines, including CCL3, CCL4 and CCL5, these being the best agonists, while CXCR4 is known to be chemoattracted to CXCL12 (Alkhatib, 2009). The majority of chemokines that act as CCR5 ligands are pro-inflammatory.

During hCMV infection the virus is able to regulate host receptors in various ways to prevent chemotaxis. It has been demonstrated that hCMV prevents CCR7 expression in monocyte-derived dendritic cells, preventing chemotaxis following CCL19 and CCL21 chemokine gradients *in vitro* (Moutaftsi *et al.*, 2004). hCMV-infected Langerhans cells also demonstrate reduced chemotaxis in response to lymphoid chemokines (Lee *et al.*, 2006). After these observations it was found by Wagner *et al.* (2008) that hCMV UL18 inhibited chemotaxis of dendritic cells *in vitro*. The extracellular UL18 is expressed late in hCMV infection and binds the leukocyte immunoglobulin-like receptor 1 molecule on the surface of dendritic cells. This results in various changes, including reduced chemotaxis, increased pro-inflammatory cytokine production, upregulation of CD83 and inhibition of CD40 (Park *et al.*, 2002; Wagner *et al.*, 2008). It was also observed that hCMV-infected dendritic cells showed downregulated chemokine expression and inhibited maturation due to vIL-10, a product of the hCMV *UL111A* gene. Dendritic cells that were able to

mature during hCMV infection showed an increase in chemotactic ability to follow the lymph node homing chemokine (Chang *et al.*, 2004). It has further been observed that chemotaxis is disrupted in infected endothelial cells. Reinhardt *et al.* (2014) demonstrated how hCMV-infected human coronary artery endothelial cell (HCAEC) chemotaxis to vascular endothelial growth factor is inhibited. HCAEC migration is important for repair post-vascular injury (Deanfield *et al.*, 2007; Waltenberger, 2007). While the observation explains how hCMV can play a role in contributing to pro-atherosclerotic phenotypes, the viral strategy for inhibiting HCAEC migration remains unknown. A further way to inhibit chemotaxis of cells is by secreting chemokine-binding proteins. hCMV-produced UL21.5 acts in this capacity by binding CCL5, acting as a chemokine sink or decoy receptor (Wang *et al.*, 2004a). This would prevent the cellular receptor from being able to bind CCL5 and follow the chemoattractant. hCMV also utilizes miR-UL148D to silence CCL5 protein synthesis in infected cells (Kim *et al.*, 2012). These studies emphasize the importance of CCL5 regulation during hCMV infection. Preventing immune cell production and detection of

CCL5 would assist in preventing the attraction of monocytes and T cells to areas of hCMV infection. While a certain number of monocytes would be beneficial for viral spread, an overabundance of monocytes and the presence of T cells could result in the impairment of viral spread.

To better enhance viral spread, hCMV uses virally encoded chemokines UL128, UL146 and vCXCL1 to attract target immune cells. The piracy of host chemokine CCL22 further assists in this process. By upregulation of CCL22 in infected monocytes, Tregs are attracted, and could assist in downregulation of an immune response to viral infection. By increasing the functionality of CXCR4, chemotaxis of virally infected cells to other tissues could be encouraged. By manipulating host chemokine receptor CCR7, hCMV can avoid migration to primary and secondary lymph tissue, evading possible detection. The dysregulation of virus-infected cell movement appears to allow hCMV the edge in evading immune detection and increase the opportunity for viral spread (see Fig. 1). Future studies investigating the function of viral chemokines and chemokine receptors could examine their effects *in vivo* utilizing
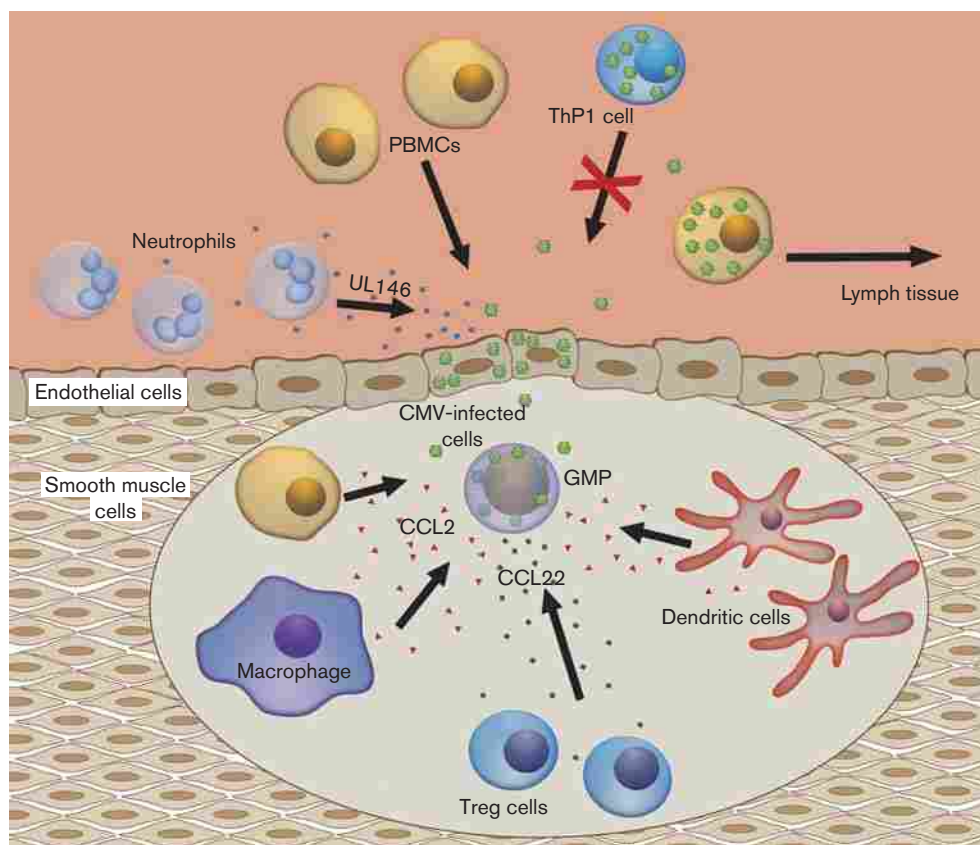
Fig. 1. Changes in cellular chemotaxis resulting from hCMV infection. hCMV uses virally encoded chemokines UL128, UL146 and vCXCL1 to attract target immune cells such as neutrophils and PBMCs. Increases in CCL2 expression further assist in attracting monocytes, macrophages and dendritic cells. Upregulation of CCL22 in infected monocytes attracts Treg cells, which can assist in downregulation of an effective immune response to viral infection. Increasing the functionality of CXCR4 allows virally infected cells to migrate to other tissues, including secondary lymphoid tissues.

animal models, as has been done with hCMV US28 (Bongers *et al.*, 2010).

Human herpesvirus 6 (HHV-6)

HHV-6, initially named human B lymphotropic virus, was first discovered in 1986 in patients with lymphoproliferative disorders (Salahuddin *et al.*, 1986). HHV-6A and HHV-6B were recognized as different variants of the same species in 1992, and in 2012 the International Committee on Taxonomy of Viruses classified them as two distinct viruses (Ablashi *et al.*, 1993; Adams & Carstens, 2012). Because classification as two distinct viruses has come relatively recently, it makes it difficult to distinguish between HHV-6A and HHV-6B in some of the early literature. The seroprevalence of HHV-6 in adults worldwide is approximately 83–100% (Hall *et al.*, 2006).

HHV-6A

HHV-6A is a betaherpesvirus that has primary tropism for CD4 + T cells and can also infect CD8 + T cells, natural killer (NK) cells, gamma/delta T cells, human neural stem cells, human progenitor-derived astrocytes, and oligodendrocyte progenitor cells (Ablashi *et al.*, 2014; Lusso *et al.*, 1991, 1993, 1995). It has also been shown to lytically infect B cells that have been immortalized with EBV (Ablashi *et al.*, 1989). HHV-6A can alter the expression of different cellular markers involved in cellular homing and trafficking, which causes significant disruption to immune cell function and viability. The virus has been implicated in a number of diseases including multiple sclerosis, Hashimoto's thyroiditis, and AIDS. *In vitro* studies show that HHV-6A causes upregulation of CD4 on cells that do not typically express this marker, making these cells susceptible to HIV infection and possibly contributing in the progression to AIDS (Lusso *et al.*, 1991, 2007).

HHV-6 has one functional chemokine-like protein, U83 (see Table 3). The viral chemokine U83A from HHV-6A is involved in chemoattraction and has selective specificity for receptors CCR1, CCR4, CCR5, CCR6 and CCR8. These are found on T cells, monocytes/macrophages and activated T lymphocytes (CCR1, CCR5, CCR8), skin-homing T lymphocytes (CCR4, CCR8), immature dendritic cells (CCR1, CCR6) and NK cells (CCR8) (Ablashi *et al.*, 2014; Catusse *et al.*, 2008; Dewin *et al.*, 2006). The difference in specificity of U83A (from HHV-6A) and U83B (from HHV-6B) to attract diverse cell types (see Table 3) could account for the variable tropism of the two viruses (Clark *et al.*, 2013). U83A is found in a full-length form as well as a truncated splice variant (French *et al.*, 1999). It is thought that, because of the different forms of the peptide, U83A could block both innate and adaptive immune responses, as well as attract the cells involved in these responses for further infection (Dewin *et al.*, 2006). U83A induces chemotaxis and morphological changes in cells

expressing CCR5 in a manner similar to CCL4, but with a significantly delayed internalization of CCR5 compared with CCL4. Interestingly, binding of U83A to CCR5 has been shown to inhibit CCR5 tropic HIV-1 infection (Catusse *et al.*, 2007).

HHV-6 has two GPCRs, U12 and U51, which encode chemokine receptors (see Table 2). U51, known to affect migration in HHV-6A infected cells, is expressed at early time points post-infection, whereas U12 is expressed late and influences chemotaxis of HHV-6B-infected cells. HHV-6A U51A has novel specificity for CCL5 and can also bind CCL2, CCL11, CCL7 and CCL13. This makes U51A unique among viral and cellular receptors in that it overlaps activity with CCR1, CCR2, CCR3 and CCR5 in the binding of CCL5 (Catusse *et al.*, 2008). There is also overlap with CCR2, CCR4, US28, UL12, D6 and Duffy in the binding of CCL2; CCR3 and E1 in the binding of CCL11; CCR1, CCR3, US28 and D6 in the binding of CCL7; and CCR2 and CCR3 in the binding of CCL13. Unlike many viral GPCRs that have constitutive signalling, U51A has been shown to perform both inducible and constitutive signalling (Catusse *et al.*, 2008; Fitzsimons *et al.*, 2006).

U51A expression has been shown to cause a reduction of CCL5 expression using the Hut78 human CD4 + T lymphocyte cell line. U51A has high relative affinity for XCL1, which normally binds human receptor XCR1 found on NK cells and T lymphocytes. This binding could have a number of effects, including: preventing infected cells from interacting with NK cells; inducing chemotaxis to T lymphocytes, which could spread infection; and preventing apoptotic signals within infected cells (Cerdan *et al.*, 2001). CCL19, normally bound by human receptor CCR7, can also be bound by U51A. This could cause infected cells to migrate to the T cell-rich lymph node, promoting viral spread. HHV-6A U83A chemokine does not bind U51A. Expression of U51A ligands in the brain could also allow migration of infected cells into the central nervous system. Damaged epithelial lung cells and airway parasympathetic nerves express CCL2 and CCL11, which both bind U51A, and could promote migration of the infected cells to these areas to be transmitted to new hosts.

CCR7, which is expressed in various lymphoid tissues, is another receptor that is modulated by herpesviruses (see Table 4). HHV-6A and HHV-6B upregulate CCR7 expression in CD4 + T cells (Hasegawa *et al.*, 1994). CCR7 is specific for CCL19 and CCL21 and plays roles in cell migration and proliferation (Tadagaki *et al.*, 2005). This upregulation of CCR7 could be an important aspect of HHV-6 pathogenesis as upregulation of CCR7 promotes migration of T cells and dendritic cells to the paracortex in lymph nodes (where T cell priming occurs) and the periarteriolar lymphoid sheath in the spleen, both of which are T cell-rich (Comerford *et al.*, 2013).

As mentioned previously, HHV-6A can also downregulate cellular receptors. Along with downregulation of CD46 (its entry receptor) and CD3 (Grivel et al., 2003), CXCR4 is downregulated by HHV-6A in primary CD4 + T lymphocytes and the Jjhan T cell line, which affect the chemotactic response of the cells to CXCL12, the natural ligand of CXCR4 (Yasukawa et al., 1999). The disruption of CXCR4/CXCL12 signalling by downregulation of CXCR4 by HHV-6A could prevent the retention of haematopoietic stem/progenitor cells (HSPCs) and more mature leukocytes in the bone marrow, allowing these cells to be mobilized and enter into circulation (Karpova & Bonig, 2015). The migration of CXCR4-expressing thymocytes out of the thymus was shown to occur in a CXCL12-dependent manner (Poznansky et al., 2002; Weinreich & Hogquist, 2008); so the downregulation of CXCR4 by HHV-6A could be another way the virus prevents migration away from areas where target cells are present. Additionally, the downregulation of CXCR4 by HHV-6A could prevent homing of bone marrow-derived precursor cells to the thymus (Calderón & Boehm, 2011), possibly preventing positive and negative selection from occurring in these cells.

HHV-6 has been shown to cause modulations to CCL5 expression. This chemokine has selective chemoattractive activity on resting CD4 + memory T cells (Hasegawa et al., 1994) and has been shown to be upregulated by HHV-6 in an ex vivo study where human tonsil blocks were infected with both HHV-6 and HIV-1. This upregulation of CCL5 was shown to suppress HIV-1 CCR5-tropic variants and possibly to stimulate replication of CXCR4-utilizing variants, which gives evidence that HHV-6 may play a role in HIV pathogenesis by promoting the switch between CCR5-tropic to CXCR4-tropic HIV-1 (Grivel et al., 2001). In contrast, CCL5 expression in epithelial cells is downregulated by U51A from HHV-6A (Milne et al., 2000). Epithelial cells expressing U51A also had morphological changes and exhibited increased spreading and flattening, which could increase the ability of HHV-6 to spread to uninfected cells as it is primarily spread by cell-to-cell contact (Milne et al., 2000). As has been observed with other viral chemokines and chemokine receptors, their functions could be multipurpose in attracting cells to the area of infection, and also in evading the immune cells of the host so replication and latency can take place.

As described above, HHV-6A alters the expression of different cellular markers. Many of these markers are involved in cellular homing and tracking to specific areas of the body, and when altered can cause significant disruption to immune cell function and viability. Further research into HHV-6A effects on cellular trafficking could serve as a critical guide for developing new treatments to prevent these disease-causing disruptions.

HHV-6B

HHV-6B causes exanthem subitum (roseola) (Yamanishi et al., 1988) and is found in approximately 95–100% of adults worldwide. Unlike HHV-6A, HHV-6B has very little to no ability to infect CD8 + T cells, NK cells and gamma/delta T cells (Grivel et al., 2003; Martin et al., 2012). The cellular receptor for HHV-6B is CD134 which, like the cellular receptor for HHV-6A, CD46, is expressed on almost all human cells (Tang et al., 2013), indicating that other factors are required for effective viral replication.

The HHV-6B viral chemokine U83B is specific for CCR2 and can cause chemoattraction of CCR2-expressing cells (classical and intermediate monocytes) for infection (Ablashi et al., 2014; Clark et al., 2013; Lüttichau et al., 2003) (see Table 3). U83 from HHV-6B induced transient calcium mobilization and efficient migration in THP-1 cells (a monocyte cell line derived from monocytic leukaemia) (Zou et al., 1999). U83B has been shown to have a different specificity from U83A as U83B chemoattracts CCR2-expressing monocytes, whereas U83A has a broader but still selective specificity as mentioned previously (Catusse et al., 2008; Dewin et al., 2006). The specificity of U83B for CCR2 appears to be due to its N-terminal region. Human chemokines can induce rapid internalization of CCR2 upon binding, whereas in vitro experiments show U83B does not cause CCR2 internalization. This finding is similar to the delayed internalization of CCR5 observed with U83A. CCR2 expression is induced in pro-inflammatory conditions and, interestingly, HHV-6B is associated with inflammatory diseases such as encephalitis and myocarditis (Clark et al., 2013).

The HHV-6B GPCR U12 efficiently binds CCL2, CCL5 and CCL4, so it has overlapping activity with the receptors for CCL2 and CCL5 as in HHV-6A, but also has overlapping activity with the receptors for CCL4 (Balkwill, 2004; Isegawa et al., 1998) (see Table 2). The exact role of chemokine receptors with these viruses is still unknown, but they could be multipurpose, in that they could have been developed for immune evasion to intercept chemokines that would otherwise be attracting immune cells to the area of infection, to attract uninfected cells that could then be infected, to induce latency, or to transition from latency to active replication.

Similar to HHV-6A, HHV-6B was shown to downregulate CXCR4 in CD4 + T lymphocytes as well as MT-4 cells. This downregulation impaired the chemotactic response of the cells to the natural ligand, CXCL12 (Yasukawa et al., 1999). Similar to HHV-6A, this could induce mobilization of HSPCs into the circulation as well as prevent migration of cells out of the thymus, both of which aid in the propagation and survival of the virus.

Human herpesvirus 7 (HHV-7)

As part of the same subfamily as HHV-6A and -6B, HHV-7 shares similar characteristics, including also being a T-lymphotropic virus, although it can infect other cell types (Ablashi et al., 1995; Ward, 2005). Like other human

herpesviruses, once HHV-7 is acquired, the host is infected for life. The virus is shed in saliva and spread through this route of transmission. Compared with the other human herpesviruses, much less research has been conducted on HHV-7 infection and pathogenesis. Clinically it has been associated with the development of pityriasis rosea, post-infectious myeloradiculoneuropathy, encephalopathy and other syndromes. There is some speculation on the involvement of HHV-7 in the development and progression of these diseases (Chuh et al., 2004; Mihara et al., 2005; van den Berg et al., 1999). HHV-7 infections can have a variety of symptoms, including fever, rash, febrile respiratory problems, vomiting and diarrhoea (Clark et al., 1997; van den Berg et al., 1999). Infections typically occur in children and are most often asymptomatic (Ward, 2005).

HHV-7 has been shown to influence migration in human cells in a variety of ways (see Tables 1 and 4). Yasukawa et al. (1999) showed that it downregulated transcription and surface expression of CXCR4 in CD4+T cells. As described before, CXCR4 is the receptor for CXCL12, which is secreted by various cells in the lymph nodes, bone marrow, etc. With CXCR4 assistance, T cells can follow a CXCL12 gradient to sites of inflammation (Domanska et al., 2013). After infection with HHV-7, Yasukawa et al. (1999) tested the migration and intracellular levels of Ca^{2+} of CD4+T cells. It was found that infected cells demonstrated less migration following the CXCL12 gradient and decreased levels of intracellular Ca^{2+} compared with the mock-infected cells used as controls. It is currently unknown what viral factor(s) contribute to the downregulation of CXCR4. It has been demonstrated that lower levels of CXCR4 in HHV-7-positive T lymphocytes prevent infection by CXCR5-tropic HIV-1 (Yasukawa et al., 1999). Future research could explore how HHV-7 manipulates CXCR4 in infected cells and further confirm the findings of Yasukawa et al. (1999), as theirs is the only study investigating this change in chemotaxis.

While CXCR4 is a cellular GPCR that is influenced post-viral infection, HHV-7 has two known viral chemokine receptors, products of the U12 and U51 genes. These genes were identified as GPCR homologues and later Tadagaki et al. investigated the functionality of the protein products of these genes (Nicholas, 1996; Tadagaki et al., 2005). They verified that these proteins do accumulate on the surface of the cell. Further, they verified that they could act as functional chemokine signal receptors. Cells expressing U12 and U51 expressed heightened levels of intracellular Ca^{2+} after appropriate signalling through the U12 and U51 GPCRs. Testing the chemotactic effect of the expression of these proteins in the Jurkat T cell line using microchannel migration techniques, it was found that cells expressing U12 migrated effectively following a gradient of CCL19 and CCL21. This would make U12 a viral homologue of the cellular GPCR CCR7, as it also responds to both CCL19 and CCL21. Both of these chemokines are strongly expressed in the T cell zone of secondary lymphoid

tissues and are important in lymphocyte homing and migration (Nomura et al., 2001). It has also been observed that CCR7 expression is upregulated during HHV-7 infection (Hasegawa et al., 1994). While the strategy behind the manipulation of cellular chemotaxis following these ligand chemokines is still unclear, it could be speculated that migration to such areas could be beneficial for HHV-7 transmission as T cells are preferential targets of infection. Tadagaki et al. (2005) also speculated that expression of these viral proteins could aid in immune evasion and viral replication. Further research in murine L1.2 cells showed that U12 and U51 products could respond to CCL22 and CCL19, respectively (Tadagaki et al., 2007). Gene products U12 and U51 could act with CCR4 and CCR7, respectively, to direct migration in this cell line in response to CCL22 and CCL19 (Luther et al., 2002). If this were to hold true in human cells infected with HHV-7, then infected cells would be expected to migrate more to areas of inflammation, as CCL22 is a pro-inflammatory chemokine secreted by a wide variety of cells, and areas of high T cell density as CCL19 is constitutively expressed by stromal cells in the T cell zone (Luther et al., 2002). These areas would be attractive locations for the viral spread of HHV-7.

GAMMAHERPESVIRINAE

Epstein–Barr virus (EBV)

The main cause of viral mononucleosis, EBV infects nasopharyngeal epithelial cells and B lymphocytes (Balfour et al., 2005; Cohen, 2000). Viral spread is mainly accomplished through shedding in saliva (Balfour et al., 2005). EBV gains access to appropriate host cells by using viral gp350 to bind CD21 (a type 2 complement receptor) on the cell surface; the viral envelope then fuses with the cell membrane, releasing the viral capsid and associated tegument proteins into the cytoplasm (Toussirot & Roudier, 2008). The virus uses major histocompatibility complex class II molecules as cofactors when infecting B lymphocytes (Li et al., 1997). During its latent infection of host B cells, EBV expresses one of four possible latency programmes, depending on cellular development and conditions (Young & Rickinson, 2004). It is likely that reactivation in vivo of latent virus is due to the differentiation of infected memory B lymphocytes (Amon & Farrell, 2005; Hochberg et al., 2004b). EBV is associated with a variety of malignancies owing to its ability to regulate cell proliferation, including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, gastric carcinoma, and post-transplant lymphoproliferative disorder (Hochberg et al., 2004a; Kutok & Wang, 2006; Shibata & Weiss, 1992; Young & Rickinson, 2004).

During infection of B cells, EBV controls the expression of various endogenous chemokines and chemokine receptors (see Table 5). One such manipulated receptor that is shown to affect migration is CXCR4. As previously described it is

Table 5. Gammaherpesvirus changes cellular receptors/chemokines

Cell type	Receptor or chemokine	Virus	Amount or functionality	References
B cell	CXCR4	EBV	Decrease	Ehlin-Henriksson *et al.* (2006, 2009);
	CCR7	EBV	Decrease/increase	Nakayama *et al.* (2002)
	CCR6	EBV	Increase	Nakayama *et al.* (2002)
	CCR10	EBV	Increase	
	CXCR5	EBV	Decrease	Ehlin-Henriksson *et al.* (2009); Nakayama *et al.* (2002)
	EBI2	EBV	Increase	Birkenbach *et al.* (1993); Kelly *et al.* (2011)
Endothelial	CXCL8	KSHV	Increase	Wang *et al.* (2004b)
	CCL2	KSHV	Increase	Pati *et al.* (2001); Xu & Ganem (2007)
	CCL5	KSHV	Increase	
	CXCL7	KSHV	Increase	
	CXCL16	KSHV	Increase	

the receptor for CXCL12, which is secreted by various cells in a number of organs, including the lymph nodes, lungs, liver, kidneys, heart and bone marrow (Teicher & Fricker, 2010). Ehlin-Henriksson *et al.* (2006) demonstrated that tonsillar B cells infected with EBV showed reduced expression of CXCR4 (Ehlin-Henriksson *et al.*, 2006). Assays of chemotactic migration further showed that infected tonsillar B cells had decreased ability to migrate towards CXCL12. This decreased expression and the subsequent lack of chemotaxis was demonstrated in EBV-immortalized B cells as well (Nakayama *et al.*, 2002). The inability to follow the CXCL12 gradient would prevent infected B cells from migrating to tissues expressing only this chemokine. CCR7 is another host receptor that is virally regulated during EBV infection. In a later study by Ehlin-Henriksson *et al.* (2009), it was found that CCR7 is downregulated in tonsillar B cells post-infection. This change in expression led to decreased migration following the natural chemokine ligand CCL21. CCL21 is produced by stromal cells in primary and secondary lymphoid tissues and lymphatic endothelial cells in the peripheral tissue (Comerford *et al.*, 2013). It is critical for directing the formation of secondary lymphoid tissues such as spleen, Peyer's patches, and lymph nodes (Ohl *et al.*, 2003). It has also been surmised that CCR7 ligands are influential in tertiary lymphoid organs (Comerford *et al.*, 2013). Immortalized B lymphoblast cell lines (LCLs) have been shown to have an increased expression of CCR7 compared with uninfected cells. This upregulation resulted in increased migration following a CCL21 gradient in assays of chemotaxis (Nakayama *et al.*, 2002). While these results may seem to be paradoxical, it is possible that the difference in expression could be a result of a different latency programme or stage of viral infection. Ehlin-Henriksson *et al.* (2009) used harvested tonsillar B cells and measured CCR7 expression and cellular migration 7 days post-infection. In contrast, LCLs are a result of EBV immortalization of B lymphocytes, the process taking several weeks to establish the cell line and expressing a type III latency programme (Young & Rickinson, 2004). The difference in expression could be a result of either the

length of infection or the latency programme employed by the virus post-infection.

In that same study of LCLs, it was found that they expressed increased amounts of CCR6 and CCR10, the natural ligands of which are CCL20 and CCL28, respectively. Migration assays confirmed that this change resulted in increased chemotaxis towards CCL20 and CCL28 chemokine gradients (Nakayama *et al.*, 2002). CCL20 is an inflammatory chemokine involved in the recruitment of dendritic cells, CD4 + T lymphocytes and B lymphocytes (Zhao *et al.*, 2014). CCL28 is secreted by epithelial cells that line the mucosa and is used to recruit IgA$^+$ plasma cells (Vazquez *et al.*, 2015; Wilson & Butcher, 2004). CCL28 expression is highest in the salivary glands (Liu *et al.*, 2012). It would be in the best interest of EBV to regulate these receptors, allowing the virus to migrate to mucosal tissues, such as the salivary gland, for effective viral spread. Chemotaxis to sites of inflammation could result in viral reactivation and increased targets for further infection. A final cellular receptor that is downregulated during infection, effecting a change in chemotaxis, is CXCR5. The inability to migrate owing to lowered levels of CXCR5 was observed in LCLs and infected tonsillar B cells (Ehlin-Henriksson *et al.*, 2009; Nakayama *et al.*, 2002). CXCR5 allows B cells to migrate in response to CXCL13 (Carlsen *et al.*, 2004). CXCL13 is an important chemokine for secondary lymphoid tissue development, and the main cells responsible for secretion of CXCL13 are follicular dendritic cells (Cyster *et al.*, 2000; Legler *et al.*, 1998). It is expressed in vascular tissue, Peyer's patches, and inflamed lymphoid tissue (Ebisuno *et al.*, 2003; Mazzucchelli *et al.*, 1999; Okada *et al.*, 2002; Shi *et al.*, 2001). A recent study of murine B lymphocyte positioning in CXCR5-negative mice demonstrated that CXCR5 is important for the retention of B cells in Peyer's patches (Schmidt & Zillikens, 2013). While avoiding tissue types expressing CXCL13 could be beneficial for the virus, possibly assisting in immune avoidance, the exact reason for regulating CXCR5 is still unclear. Another receptor thought to be

influenced by EBV is EBV-induced gene 2 (*EBI2*). Infected B cells display a heightened expression of EBI2 (Birkenbach *et al.*, 1993; Kelly *et al.*, 2011). While it remains unknown how EBV manipulates EBI2 expression in B lymphocytes, it has been observed that EBI2^{+} cells migrate following a 7α-OHC gradient (Liu *et al.*, 2011). 7α-OHC is the natural ligand of EBI2 and is expressed by stromal cells of secondary lymph tissue, assisting in directed migration during cell chemotaxis in these areas (Gatto & Brink, 2013; Hannedouche *et al.*, 2011). Exaggerated expression of EBI2 by EBV could result in migration to the outer follicular zone in secondary lymph tissue, preventing migration toward T cell zones and germinal centres (Cyster, 2010).

The viral regulation of host lymphocytes extends to controlling various chemokines produced during infection, resulting in a change in the chemotaxis of uninfected cells. EBNA-3C, a viral product essential in establishing latency and immortalization of B cells, acts to regulate two host-produced chemokines, CXCL10 and CXCL11 (McClellan *et al.*, 2012). EBNA-3C has been found to interact with both transcriptional co-repressors and co-activators (Cotter & Robertson, 2000; Radkov *et al.*, 1999; Touitou *et al.*, 2001). Using the EBV-negative BJAB cell line, McClellan *et al.* (2012) showed that expression of EBNA-3C reduces expression of these two chemokines. The result is decreased migration of CXCR3^{+} cells (McClellan *et al.*, 2012). Cells that express and migrate in response to CXCL10 and CXCL11 via CXCR3 include various T lymphocytes, including CD8+T cells. CXCL10 and CXCL11 are typically expressed to attract Th1 cells in response to infection. EBV has also demonstrated the ability to influence the expression of chemokines via microRNAs (miRNAs). miR-BHRF1-3, an EBV-produced miRNA, has the ability to silence CXCL11 protein synthesis (Xia *et al.*, 2008). Downregulation of these chemokines suggests that immune avoidance could be a reason behind viral manipulation. Repression of CXCL11 would prevent attraction of cytotoxic T cells that might recognize virally infected B lymphocytes.

The chemokine receptor CXCR4 is a popular target for manipulation, and EBV, like other herpesviruses, uses it to prevent cell migration to certain tissue areas, probably to avoid immune detection. To achieve this same purpose, EBV also downregulates CCR7. During infection, the virus increases the host chemokine CCR6, allowing infected cells to more readily migrate to areas of inflammation. CCR10 function is also pirated, allowing infected cells to migrate toward epithelial cells, such as mucosal epithelial cells. This is likely vital for the spread of EBV. Reduction in expression of CXCL10 and CXCL11 could help in immune avoidance by suppressing the ability to attract T lymphocytes via these chemokines (see Fig. 2).

## Kaposi's sarcoma herpesvirus (KSHV)

KSHV, also known as human herpesvirus 8 (HHV-8), is named after Moritz Kaposi, who originally described a unique skin lesion in the 1870s. The discovery of the association of herpesviral DNA sequences in Kaposi's sarcoma (KS) did not occur until 1994 (Chang *et al.*, 1994; Ganem, 2010). KS presents as tumours most often found in the dermis but can also be found in lungs, liver and intestines (Moore & Chang, 2003). KSHV is also linked to primary effusion lymphoma and multicentric Castleman's disease (Avey *et al.*, 2015; Cesarman *et al.*, 1995; Soulier *et al.*, 1995).

KSHV encodes three secreted chemokines; vCCL1 (ORF K6 or vMIP-I/MIP-1a), vCCL2 (ORF K4 or vMIP-II/MIP-1b) and vCCL3 (ORF K4.1 or vMIP-III/BCK), which activate CCR8, CCR3 and CCR4, respectively (see Table 3). This set of chemokines antagonizes the recruitment of Th1 and NK cells. This redirects the immune response from a Th1-like response towards a Th2 profile. vCCL2 has also been shown to prevent CCL5-mediated chemotaxis of Th1-like lymphocytes (Moore & Chang, 2003; Stebbing *et al.*, 2003; Weber *et al.*, 2001). The receptor XCR1, which normally binds the ligand XCL1 and is involved in T-cell recruitment, is selectively activated by vCCL3 but is also blocked by vCCL2. The opposing function and differing time of expression of the two viral chemokines could indicate the importance of the regulation of the XCR1 receptor in KSHV infection and pathogenesis. Neutrophils have high levels of XCR1, and vCCL3 chemoattracts these cells, which may indicate that neutrophils play a role in viral spread (Lüttichau *et al.*, 2007). vCCL1 and vCCL2 expression were also shown to induce migration of monocytes. This could play a role in the process of tumour development in KS as circulating monocytes could be recruited to KSHV-infected cells, thus propagating the infection (Nakano *et al.*, 2003).

KSHV encodes a GPCR (vGPCR or ORF74) that is homologous to CXCR2 and has a high level of constitutive activity (Arvanitakis *et al.*, 1997; Cesarman *et al.*, 1996; Hensbergen *et al.*, 2004; Pati *et al.*, 2001) (see Table 2). Constitutive expression of ORF74 in microvascular lung endothelial cells inhibits migration and increases cell survival. This inhibitory effect on migration can be reversed by endogenous chemokines CXCL10 and CXCL12. These act as inverse agonists of ORF74, as seen in an *in vitro* wound closure assay, where CXCL10 increased migration of ORF74-expressing cells. Limiting migration of infected cells may aid in immune evasion and KSHV survival. Constitutive expression of ORF74 has also been shown to attract uninfected endothelial cells, which could then be infected and propagate the infection (Couty *et al.*, 2009).

ORF74 has been shown to activate the transcriptional activators NF-κB and activator protein 1 (AP-1), leading to the downstream production of signals including IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and CCL5 (Pati *et al.*, 2001; Schwarz & Murphy, 2001; Shepard *et al.*, 2001). Elevated levels of CXCL8 are observed in KS patients and can activate KSHV-infected cell growth and induce chemotaxis (Wang
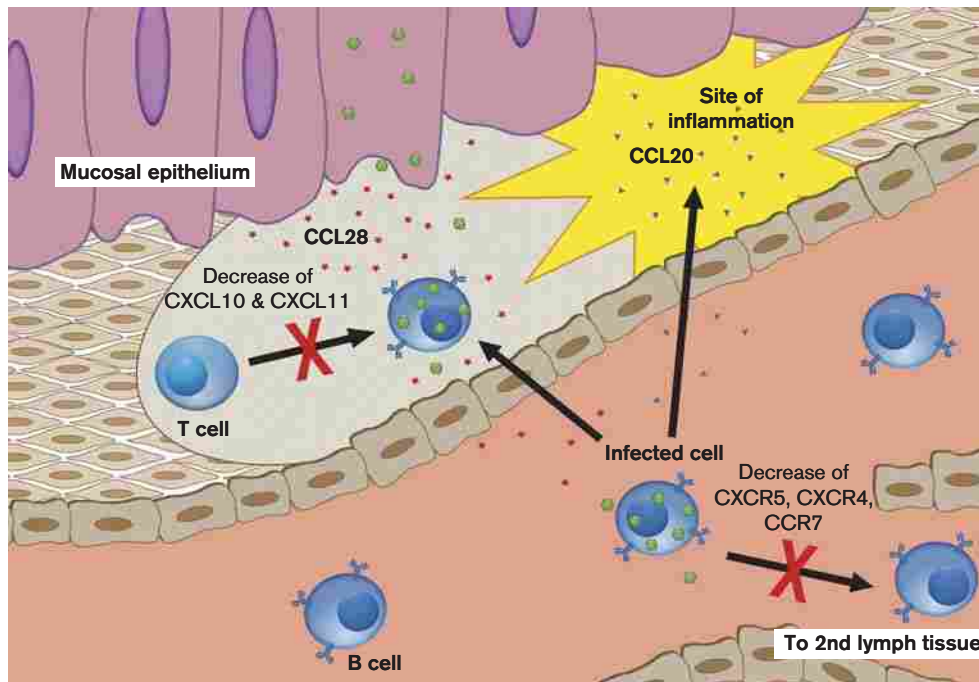
Fig. 2. Changes in cellular chemotaxis resulting from EBV infection. EBV decreases expression of cellular CXCR4 and CCR7 to prevent migration to certain tissue areas, probably to avoid immune detection. Increases in host chemokine CCR6 allow infected cells to migrate to areas of inflammation. CCR10 function is also pirated, allowing infected cells to migrate toward epithelial cells, such as mucosal epithelial cells. Downregulation of CXCL10 and CXCL11 could help in immune avoidance by suppressing the ability of infected cells to attract T lymphocytes.

et al., 2004b). CXCR1 and CXCR2, receptors that bind CXCL8, have been found to be expressed in KS lesions. CXCR4 has also been found to be expressed on cells in these lesions, which is important as this receptor acts as a co-receptor for CXCR4-tropic strains of HIV (Masood *et al.*, 2001; Pati *et al.*, 2001; Wang *et al.*, 2004b). These combined effects of ORF74 could stimulate the proliferation, migration and chemotaxis of endothelial cells in KS.

KSHV encodes a homologue of IL-6, vIL-6, that has been shown to promote migration of endothelial cells in both autocrine and paracrine fashions. Inhibition of this migration can be specifically inhibited by a DNA methyltransferase 1 (DNMT1) inhibitor, suggesting that the mechanism of vIL-6 is dependent on enhancing expression of DNMT1. As the control of DNA methylation is crucial for gene expression and other cellular processes, disruption of methylation could be a mechanism for KS tumorigenesis (Wu *et al.*, 2014).

There are a number of different cellular chemotactic proteins shown to be upregulated by KSHV, including: CCL2, CXCL7, CCL5, GM-CSF, CXCL16 and angiogenin (Xu & Ganem, 2007) (see Table 5). Some of these, such as CCL5 and GM-CSF, likely increase migration of endothelial cells toward KSHV-GPCR-expressing KS cells

(Bussolino *et al.*, 1989; Pati *et al.*, 2001). In contrast, CXCL16 appears to play an indirect role in tumour growth and expansion through migration of activated T cells (Xu & Ganem, 2007). KSHV also causes downregulation of certain genes. The KSHV miRNA miR-K12-10a downregulates the cytokine receptor TNF-like weak inducer of apoptosis (TWEAK) receptor. This inhibits the pro-inflammatory response and also provides protection from TWEAK-induced apoptosis (Abend *et al.*, 2010).

Latency-associated nuclear antigen 1 (LANA-1; encoded by ORF73), a latently expressed gene, has been shown to hinder neutrophil chemotaxis, which interferes with recruitment of neutrophils to infected areas and could be a way in which latent KSHV survives host-induced acute inflammation. Neutrophil recruitment is restored in LANA-1 knockdowns, although not to the level of uninfected cells, indicating that KSHV has other mechanisms to repress neutrophil recruitment (Li *et al.*, 2011).

As seen with other herpesviruses, KSHV can induce or inhibit cellular migration, according to what is most beneficial for viral infection at the given stage. It not only interferes with cellular marker expression, it also induces increased production of specific chemokines and cytokines, which leads to other issues in cellular function and trafficking.

CONCLUDING REMARKS

With exposure to human herpesviruses being very common, it is important to understand how these infectious human pathogens influence infected and uninfected cell types. The viruses of the subfamily *Alphaherpesvirinae* use gG to increase the functionality of CXCR4, leading to increased chemotaxis to a variety of tissues while being able to manipulate cellular chemokines, such as CXCL9, to attract PBLs. Viruses of the subfamily *Betaherpesvirinae*, also capable of producing and manipulating chemokine and chemokine receptors, influence a variety of cells during infection. hCMV inhibits migration in infected monocytes and potentially attracts monocytes, PBMCs, macrophages, dendritic cells and regulatory T cells to sites of infection. Similarly, through its ability to attract target cells, HHV-6 is able to induce chemotaxis of T lymphocytes, monocytes, immature dendritic cells, and NK cells to areas of infected cells using viral U83. T cells infected with HHV-6 are further manipulated, as viral and cellular GPCRs allow cells to migrate to sites of inflammation and areas rich in T cells. Also manipulating T cells, HHV-7 prevents infected T lymphocytes from migrating to various organs and tissues by downregulating CXCR4. However, it too potentially encourages migration to inflammatory sites and locations high in T cells by inducing cells to follow chemokine gradients of CCR7, CCL21, CCL22 and CCL19.

Further masters of cellular piracy, viruses of the subfamily *Gammaherpesvirinae* also influence cellular chemotaxis to avoid immune detection and spread viral infection throughout the host until latency can be established. To prevent newly infected cells from potentially migrating to lymph tissue and other organs, EBV reduces expression of CXCR4, CCR7 and CXCR5. By downregulating the chemokines CXCL10 and CXCL11, EBV could prevent infected cells from attracting cytotoxic T cells. To regulate chemotaxis of cells during infection, KSHV regulates the attraction or avoidance of neutrophils and monocytes by viral chemokines vCCL1, 2 and 3. KSHV would be able to induce the chemotaxis of uninfected endothelial cells by upregulating various cellular chemokines and activating the NF-κB pathway, enabling viral spread.

Though our current understanding of how human herpesviruses affect host cell migration during infection is rather expansive, there still remain various areas for future research opportunities. In this review we have elaborated on the cells potentially affected by virally encoded and virally induced chemokines and chemokine receptors. However, the full range of cells affected by these chemokines remains to be tested and investigated further. Several virally regulated cell chemokine receptors suspected of influencing viral spread and immune avoidance are in need of confirmatory scientific inquiry. Several human herpesviruses have not been studied extensively for effects on cellular chemotaxis, such as the viruses of the subfamily *Alphaherpesvirinae* and HHV-7. These areas leave a variety of opportunities for future research that could contribute to our understanding of how these viruses lead to disease pathogenesis and progression.

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EBI2 expression in B lymphocytes is controlled by the Epstein–Barr virus transcription factor, BRRF1 (Na), during viral infection

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Abstract

Epstein–Barr virus-induced gene 2 (EBI2) is an important chemotactic receptor that is involved in proper B-cell T-cell interactions. Epstein–Barr virus (EBV) has been shown to upregulate this gene upon infection of cell lines, but the timing and mechanism of this upregulation, as well as its importance to EBV infection, remain unknown. This work investigated EBV's manipulation of EBI2 expression of primary naive B cells. EBV infection induces EBI2 expression resulting in elevated levels of EBI2 after 24 h until 7 days post-infection, followed by a dramatic decline (P=0.027). Increased EBI2 expression was not found in non-specifically stimulated B cells or when irradiated virus was used. The EBV lytic gene BRRF1 exhibited a similar expression pattern to EBI2 (R^2=0.4622). BRRF1-deficient EBV could not induce EBI2. However, B cells transduced with BRRF1 showed elevated expression of EBI2 (P=0.042), a result that was not seen with transduction of a different EBV lytic transfection factor, BRLF1. Based on these results, we conclude that EBI2 expression is directly influenced by EBV infection and that BRRF1 is necessary and sufficient for EBI2 upregulation during infection.

INTRODUCTION

Epstein–Barr virus (EBV), a human B-lymphotropic herpes virus, infects over 90 % of adults [1–3]. Primary EBV infection usually occurs in childhood [4]. Although most infections are asymptomatic, delayed infection into adolescence commonly causes infectious mononucleosis [5, 6]. In this case, the virus is spread via saliva and infects permissive epithelial cells of the oropharynx [7]. During this time, the virus also infects mucosal B cells. Once B cells are infected, the virus enters a stage of latency. This results in the expansion of an lymphoblastoid cell lines (LCL)-like population of B lymphocytes in the tonsils [8]. This also allows for the virus to avoid immune detection and remain with the host for the rest of their life, persisting in the B-lymphocyte population. Infection with EBV is also associated with and suspected of being responsible for several types of cancers [9]. It is a strongly associated agent of Burkitt's lymphoma, as well as various types of non-Hodgkin's lymphomas, nasopharyngeal carcinoma and immunoproliferative disorders, and is associated as an environmental trigger in autoimmune diseases such as systemic lupus erythematosus [10–14]. Post-infection (p.i.), EBV is adept at controlling lymphocyte gene expression, likely manipulating EBV-induced

gene 2 (EBI2) expression, among others, as a means of controlling B-lymphocyte migration. However, after a time, the virus lowers the expression of most viral genes and enters a latent state.

Following the establishment of latency, it is possible for EBV to reactivate due to environmentally induced stimulation. This is a problem of particular concern during tissue and organ transplants and can result in post-transplant lymphoproliferative disease [15]. Key to this process of reactivation are the various EBV lytic transcription factors, mainly BZLF1, BRLF1 and BRRF1, that assist in initiating the cascade of transcription needed to achieve viral lytic replication [16, 17]. These and various other EBV genes can also be transiently expressed early after infection. This transient expression of EBV genes before latency is often called pre-latency [18]. Pre-latency is thought to be important for viral immune evasion as various pre-latently expressed proteins assist in immune avoidance by suppressing intracellular and extracellular detection.

EBI2 is a member of the rhodopsin-like subfamily of seven-transmembrane G protein-coupled receptors. Sequence alignments have grouped EBI2 with lipid receptors, and it shows

highest similarity with GPR18 [19, 20]. EBI2 expression is found to be naturally highest in secondary lymphoid tissues [20, 21]. The natural ligand of EBI2 is an oxysterol, 7α,25-dihydroxycholesterol (7α,25-OHC), which is expressed primarily in secondary lymph tissue by stromal cells. Chemotaxis of naive B cells within the follicle is mediated through the expression of EBI2, CCR7 and CXCR5. Varying expression levels of EBI2 affect the positioning and migration of the B cells throughout the follicle [22–24]. When EBI2 expression is highest, the B cell migrates to the outer areas of the B-cell follicles [22]. Likewise, low levels of EBI2 expression lead B cells to accumulate in the Follicular dendritic cell (FDC)-dense region in the centre of the follicle or the interface of the T-cell zone [23]. These expression patterns are kept in a fine balance as positioning within the follicle, which directly affects both B-cell proliferation and differentiation [25].

EBV controls expression of EBI2 upon infection. This control could affect EBV-infected cells in several ways. These include B-cell chemotaxis, improper positioning of B cells within the follicle and immune deficiency or other disruptions to cell haematopoiesis and function [26]. Altered positioning and chemotaxis of B cells is also thought to be necessary for the development and progression of lymphomas [27]. Understanding how EBV controls lymphocyte positioning could allow for improved control of infection and response to EBV-induced malignancies. To enhance our understanding of EBV infection, we investigated the expression profile of EBI2 during EBV infection and potential viral mechanisms responsible for tampering with EBI2 expression.

RESULTS

Time course of EBI2 expression after EBV infection

EBI2 is vital in directing migration of primary B cells to areas in secondary lymph tissue where they can potentially detect antigenic proteins [28]. To investigate the way EBI2 expression was influenced by viral infection, naive human B cells were infected with EBV. Levels of EBI2 expression were measured and compared to uninfected control naive B cells (Fig. 1). EBV-infected cells showed, on average, a threefold higher relative mRNA expression of EBI2 than uninfected cells. When the EBI2 expression found in infected B cells is compared to the expression in cell lines that contain EBV, there is a significant difference of gene expression. Freshly isolated naive B cells demonstrated a much higher expression of EBI2 when compared to the expression observed in the EBV-positive LCL (P=0.012) and Raji cell lines (P=0.027), which are primarily in a latent state.

To determine the pattern of EBI2 expression during the course of EBV infection, isolated naive B cells were infected with EBV and EBI2 expression was measured at intervals over a 21-day period of time. An expression time line was constructed starting with pre-infection and ending 21 days p.i. Between 3 and 6 h p.i., there was a significant decrease in EBI2 expression (P=0.002). There is also a significant increase in expression at 24 h p.i. (P=0.027) (Fig. 1). This

heightened level of EBI2 expression persisted for as long as 7 days p.i. At this point, there was a large variance in the samples. This could be indicative of EBV induction of EBI2 ending prior to this time in some samples received from different donors, while others are still maintained. However, by days 8 and 9 p.i., the levels of EBI2 expression have decreased to levels equal to or lower than those observed in uninfected control naive B cells (Fig. 1).

Non-specific gene regulation in infected cells was controlled for by measuring the expression of the REEP5 gene at every time point. This gene expression was stable throughout the time course of infection, except that it decreased between 3 and 6 h p.i., as did EBI2. This suggests that some genes are downregulated immediately upon EBV infection, and the initial dip in EBI2 may be part of an overall pattern of gene expression that is separate from the increase seen by 24 h.

To determine if increased RNA expression corresponded to increased protein levels, naive B cells were infected with EBV and proteins were harvested at 24 h p.i. Western blots were performed to determine the ratio of EBI2 protein between the control and infected cells (Fig. 2). At 24 h p.i., the EBV-infected cells displayed more than twice the amount of protein compared to uninfected cells (P=0.015).
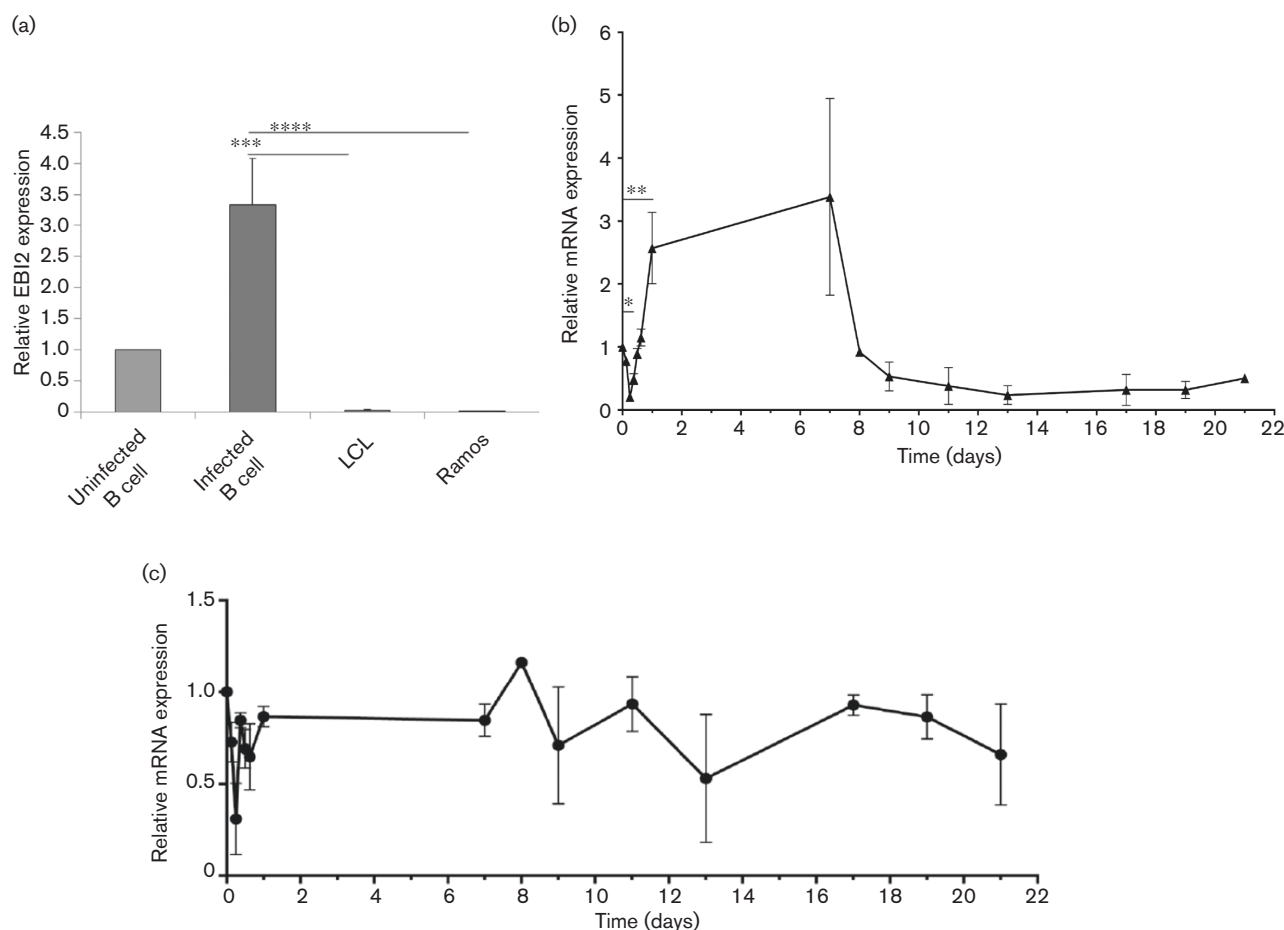
Cell activation alone is not sufficient to increase EBI2 expression

It is important to establish if EBI2 expression could possibly be induced by a mechanism such as B-cell activation due to viral infection. For this purpose, isolated naive B cells were treated with imiquimod. Imiquimod binds to Toll-like receptor 7 (TLR7), which normally recognizes ssRNA, and leads to B-cell activation [29]. We used imiquimod to stimulate naive B cells instead of using other methods, such as CD40 and IL-4, because we wanted to investigate whether the TLR pathway could be responsible for the induction of EBI2 [30, 31]. The imiquimod treatment activated the B cells, as shown by increased IL-1 and IL-6 transcription after treatment, but no increase in EBI2 expression was noted (Fig. 2).

To further establish that upregulation of EBI2 expression was due to EBV genes expressed during infection, naive B cells were treated with UV-irradiated EBV. Media containing EBV was UV irradiated as described and then used to treat naive B cells. The irradiation inactivates viral particles, preventing replication after entering the cell due to DNA damage. After incubation with the UV-irradiated EBV, EBI2 expression was measured. The relative mRNA expression in the B cells treated with the UV-irradiated EBV did not differ from that found in uninfected naive B cells (Fig. 2).

BRRF1 demonstrates a similar pattern of expression as EBI2 during EBV infection of B cells

EBV uses various viral proteins to regulate cellular gene expression [32, 33]. Significant regulation of EBI2 was observed beginning between 3 and 6 h p.i., indicating that
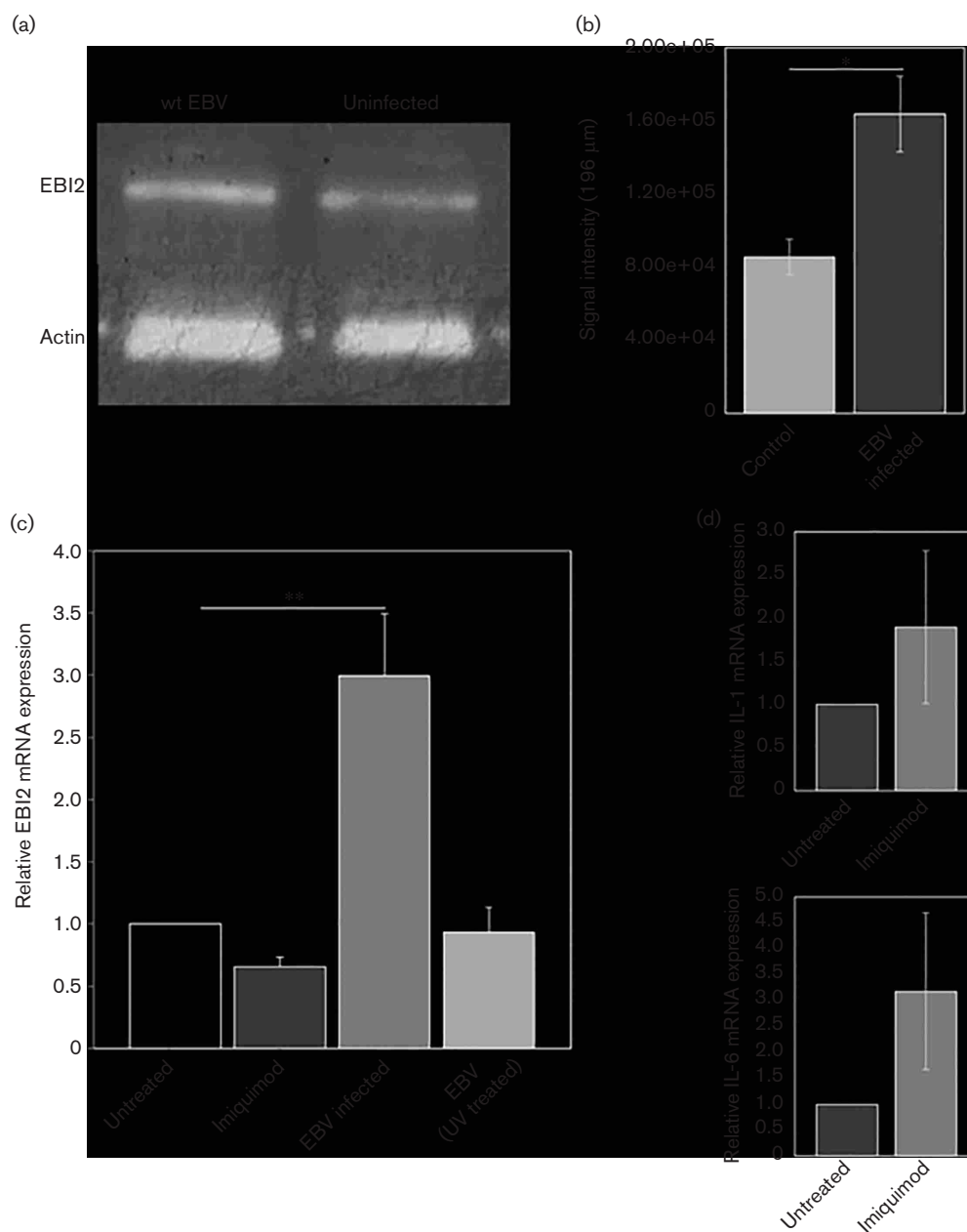
Fig. 1. EBI2 expression is modulated during the course of EBV infection. (a) Twenty-four hours after infection, EBI2 expression in infected isolated naive B cells increased by a mean of threefold over that of uninfected B cells. Expression of EBI2 in EBV-infected B cells was significantly higher than in the EBV-containing LCL B-cell-derived cell lines (***P=0.012) and the EBV-containing Ramos cell line (****P=0.027), *n*=4. (b) Isolated naive B cells demonstrated significantly decreased expression of EBI2 by 6 h p.i. with EBV. After this initial downregulation, EBI2 expression increased to remain at a heightened level of expression for several days (*P<0.007, **P≤0.027). Seven days p.i., EBI2 expression decreased to levels equal to or lower than those observed prior to EBV infection. (c) Non-specific gene regulation was controlled by measuring the expression of the REEP5 gene at all time points, *n*=5. Error bars indicate standard error.

an immediate early or early gene product would most likely be responsible for regulating EBI2 expression. Various EBV genes that are expressed during this period were selected as candidates for investigation. These included BARF1, BHRF1, BRRF1, BMLF1, LMP1, LMP2, BRLF1 and BZLF1. Analysis was first begun on these genes, with the intention of examining other viral genes if strong correlation values could not be found upon comparing the expression pattern of these EBV genes to EBI2.

Expression of these genes at three different time points during naive B-cell infection by EBV was measured using reverse-transcriptase quantitative PCR (RT-qPCR) and a pattern of expression was established for each one (Fig. 3). These time points were chosen due to the distinct difference of EBI2 expression observed during EBV infection. Epstein–Barr virus nuclear antigen 1 (EBNA1) was used to control

for viral gene expression as it is expressed throughout EBV infection. It was also chosen as the control to standardize for viral infection, and even though the m.o.i. used was the same for each experiment, there is still variation observed in the samples treated with EBV. Therefore, all viral gene levels are relative to EBNA1 in this experiment. Upon comparing the expression patterns of the viral genes to the expression pattern of EBI2, BRRF1 demonstrated a similar expression pattern with the highest R^2 value (Fig. 3). Linear regression analysis comparing EBI2 gene expression to the mRNA expression of the various viral genes revealed that BRRF1 shared more similarity than any other gene screened (R^2 =0.866). Between 3 and 6 h p.i., there is a significant decrease in expression (*P*=0.033) and by 12 h p.i., expression had increased to a level similar to that observed at 3 h p.i. These results suggest a possible connection between BRRF1 and EBI2 expression.

Fig. 2. EBI2 protein expression is highly upregulated only by infection with infectious EBV. (a) EBI2 protein levels correlate with RNA levels. Twenty-four hours p.i. of naive B cells infected with EBV demonstrate heightened protein levels compared to uninfected naive B cells. (b) EBI2 protein signal measured was twofold higher than the uninfected cells as observed by Western blot (*P=0.015), n=4. EBI2 was normalized to β-actin, which was used as a loading control. (c) Both Toll-like receptor 7 stimulation with imiquimod and incubation with UV-irradiated EBV showed expression levels of EBI2 similar to unstimulated naive B cells, while EBV-infected naive B lymphocytes demonstrated significantly higher levels of EBI2 mRNA expression (**P<0.028), n=3. (d) IL-1 and IL-6 mRNA expression was measured in all imiquimod-treated samples. Heightened mRNA levels indicate successful imiquimod stimulation compared to unstimulated cells. Error bars indicate standard error, n=3.

BRRF1 induces a heightened expression of EBI2 in B lymphocytes during EBV infection

BRRF1 is an early lytic gene product [17, 34] encoding a transcription factor (Na), which plays an important part in regulating between latent and lytic EBV infection [35]. In high enough conce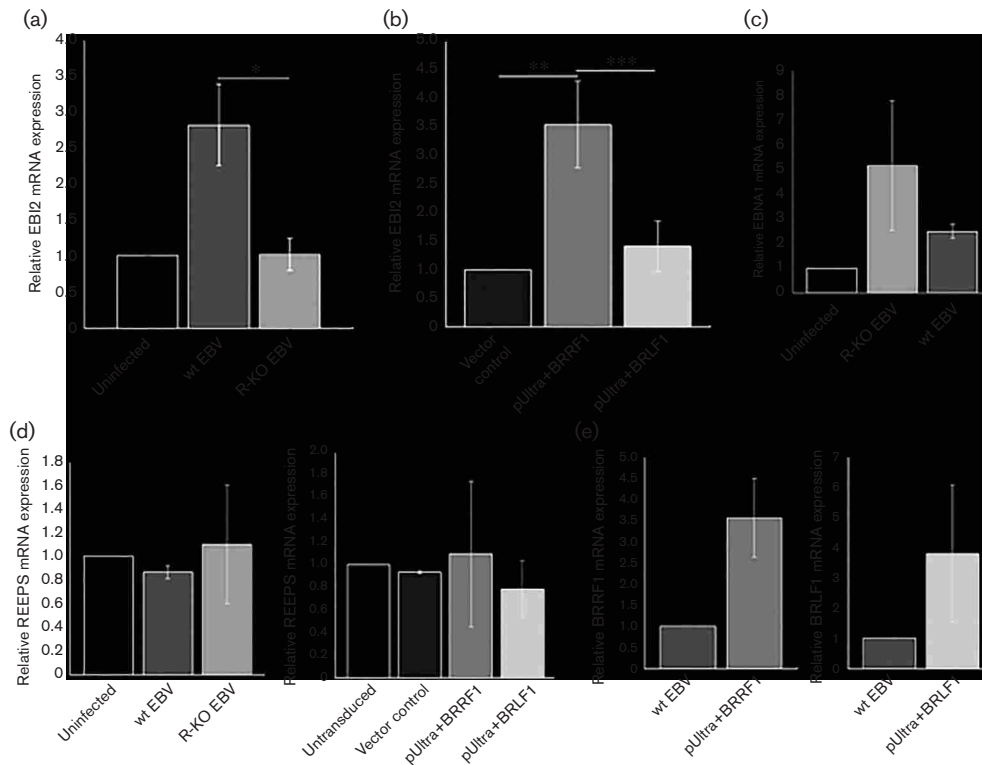ntrations, BRRF1 presence alone has been shown to induce EBV lytic gene expression [35]. To determine if upregulation of EBI2 was caused by BRRF1, naive B lymphocytes were infected with BRRF1-deficient (BRLF1/BRRF1 knockout, R-KO) EBV. The R-KO EBV was used and described in previous studies [17, 35]. To summarize, the R-KO EBV strain is a bac engineered B95.8
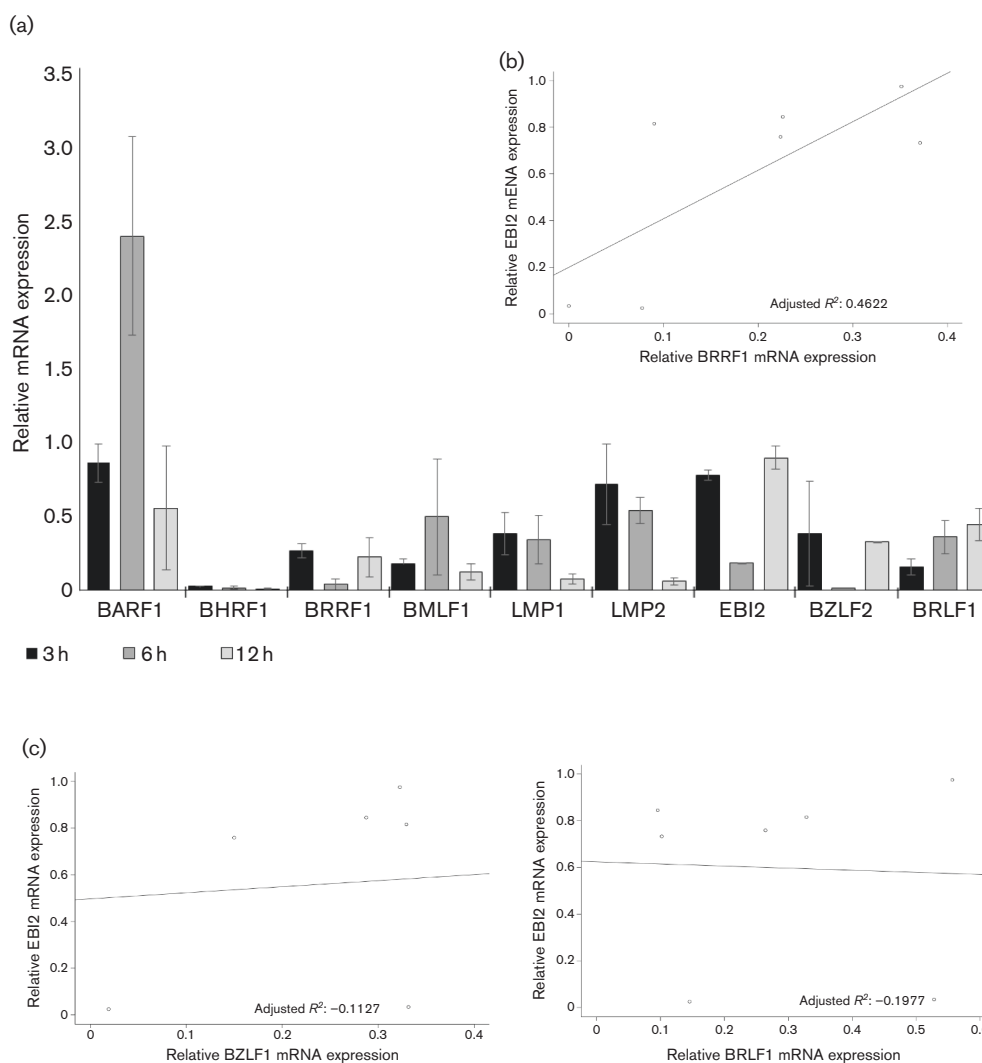
Fig. 3. BRRF1 demonstrates a similar pattern of expression to EBI2 in EBV infected B cells. (a) Several EBV early genes were screened using qPCR to measure relative mRNA expression, *n*=5. (b) BRRF1 demonstrated the most similar pattern of expression to that of EBI2. This correlation (R^2=0.4622) suggests that there might be a connection between the expression of BRRF1 and EBI2. (c) BZLF1 and BRLF1 expression patterns were also compared to EBI2 expression. The resulting correlation plot demonstrates that these other EBV lytic genes demonstrate lower correlation values with EBI2 mRNA expression.

Epstein–Barr virus (V01555). It lacks the ability to express both the BRLF1 and BRRF1 lytic genes resulting in the absence of the subsequent gene products, Rta and Na, respectively. Without the BRLF1 gene, it would be predicted that the virus would not be able to induce expression of various genes that depend on Rta response elements in their promoters including BMLF1, BMRF1, BALF2, BaRF1 and BLRF2 [36]. Rta is one of the main proteins involved in EBV reactivation and is thought to be essential for viral reactivation and lytic cycle induction, along with Zta. Na is known to act with Rta as a co-activator and assists in inducing transcription of BZLF1 and the subsequent protein synthesis of Zta [17, 35]. It has been hypothesized that it helps regulate Rta transcriptional effects [37].

The EBI2 mRNA expression was measured at 0 and 24 h p.i. in naive B lymphocytes with R-KO EBV (Fig. 4). The mRNA was extracted from cells and quantified by qPCR. Following infection of naive B lymphocytes with Na- and Rta-deficient R-KO EBV, no significant change in expression of EBI2 was detected at 24 h p.i. (Fig. 4). To verify that our R-KO infection assays were not yielding low EBI2 levels of expression due to low R-KO EBV infection rate, we measured the mRNA expression of EBNA1 in samples that were EBV or R-KO EBV infected at 24 h p.i. (Fig. 4). If our R-KO EBI2 expression was a result of low viral infection, we would expect the measured EBNA1 expression to be less than that measured in samples from EBV-infected B cells. However, our results show that EBNA1 expression is not lower in

Fig. 4. BRRF1 is necessary and sufficient to induce expression of EBI2 in B lymphocytes. (a) EBI2 expression was measured in naive B lymphocytes infected with the BRRF1-deficient R-KO EBV. There was no significant change in expression at 24 h p.i. with the BRRF1-deficient EBV, *n*=6. (b) Naive B cells were transduced with BRRF1 using a lentiviral vector (pUltra+BRRF1) and EBI2 expression was measured. There was a significant increase in EBI2 expression at 24 h (**P=0.042) post-lentiviral treatment with BRRF1. Naive B cells were transduced with BRLF1 using a lentiviral vector (pUltra+BRLF1) and expression was measured. There was no significant difference in EBI2 expression measured at 24 h post-transfection when BRLF1 was expressed. Error bars indicate standard error (**P=0.049; ***P=0.05), *n*=6. (c) Relative EBNA1 expression measured in EBV and R-KO EBV-infected B cells 24 h p.i. This demonstrates that the R-KO EBV was able to establish an infection in the naive B cells, evidenced by the expression of viral genes, *n*=6. (d) As a negative control, REEP5 mRNA expression was measured and found to be similar in all experiments, demonstrating no significant difference, *n*=6. (e) BRRF1 and BRLF1 mRNA expression was measured in lentiviral-transduced naive B-cell samples and wild-type EBV-infected naive B cells and compared. The levels of BRRF1 and BRLF1 mRNA expression in pUltra+BRRF1 and pUltra+BRLF1-transduced samples, respectively, are similar. Error bars indicate standard error, *n*=6.

samples from R-KO-infected B cells. This suggests that lower levels of EBI2 expression are not a result of a poor R-KO infection.

To confirm our findings from the R-KO EBV assay and verify that low EBI2 levels were a result of the lack of BRRF1 and not due to the lack of expression of other viral genes, such as BRLF1, we first transduced naive B lymphocytes with a lentivirus containing the BRRF1 gene (pUltra+BRRF1). The relative EBI2 expression was measured at 0 and 24 h (see Fig. 4a). Heightened expression of EBI2 was observed at 24 h post-transduction (*P*=0.042) when naive B lymphocytes were transduced with pUltra+BRRF1. Second, to test whether BRRF1 was specifically causing EBI2 upregulation and confirm that the increased levels of EBI2 expression were not the product of transactivation by a viral DNA-binding gene, the viral gene BRLF1 was transduced into naive B cells (pUltra+BRLF1). The relative EBI2

mRNA expression was measured at 0 and 24 h post-transduction. In contrast with BRRF1, there was not a significant increase in EBI2 expression after treatment with BRLF1 (Fig. 4).

It was probable that most of our blood donor volunteers had previously been infected with EBV. It was not expected that EBV reactivation would contribute to the heightened EBI2 expression levels observed since EBV genomes are only present in approximately 1 in 10^6 circulating B cells. However, to verify that latent EBV from prior infection was not interfering with the results from our pUltra+BRRF1- and pUltra+BRLF1-transduced samples, BZLF1 was measured in the EBV-infected naive B cells and the lentivirus-transduced naive B cells. BZLF1 expression was greater, estimated at 2×10^4 fold higher, in EBV-infected B-cell samples compared to the pUltra+BRRF1-transduced B-cell samples. In most cases, BZLF1 expression was not

detectable in B cells transduced with the BRRF1-expressing lentivirus. This would suggest that potential reactivation of EBV by the pUltra+BRRF1 in EBV-positive primary B cells is not responsible for the resulting increase in EBI2 expression observed.

It could also be suggested that perhaps high expression of an EBV lytic gene could non-specifically induce EBI2 expression. To investigate this possibility, we measured BRRF1 transcripts in pU+BRRF1-transduced, EBV-infected and control samples. We found that there was about three-fold more BRRF1 expression in pU+BRRF1-transduced samples compared to EBV-infected samples. This difference can be attributed to the effectiveness of the human ubiquitin promoter used to drive expression of BRRF1 post-transduction compared to the BRLF1 and BRRF1 promoter used by wild-type EBV. These results combined with the lack of upregulation in cells transfected with BRLF1 using the same promoter led us to conclude that BRRF1 expression alone can induce EBI2 and that EBI2 expression is not a result of non-specific binding or latent EBV reactivation.

DISCUSSION

Regulation of EBI2 is crucial to B-cell chemotaxis in secondary lymph tissue [28, 38]. Upregulation of EBI2 generally occurs during cellular migration in secondary lymph tissue [28]. When expressed, EBI2 allows the cell to follow a $7\alpha,25$-OHC gradient [21, 38, 39]. $7\alpha,25$-OHC is produced by stromal cells of secondary lymph tissue and is the only identified natural ligand for the EBI2 chemokine receptor. B cells upregulate EBI2 at key times to manoeuvre the cell away from the follicular region to the outer and inter-follicular regions [24]. It has been suggested that these movements allow the naive B cells to potentially be exposed to any antigens that are present in that region (Fig. 5) [28]. Downregulation of EBI2 allows for the naive B cells to return to the follicular area and migrate to the T-cell zone for a time before exiting the secondary lymph tissue. The migration of B cells through this pattern allows for exposure to areas with different antigens as well as the possibility to be primed by T cells.
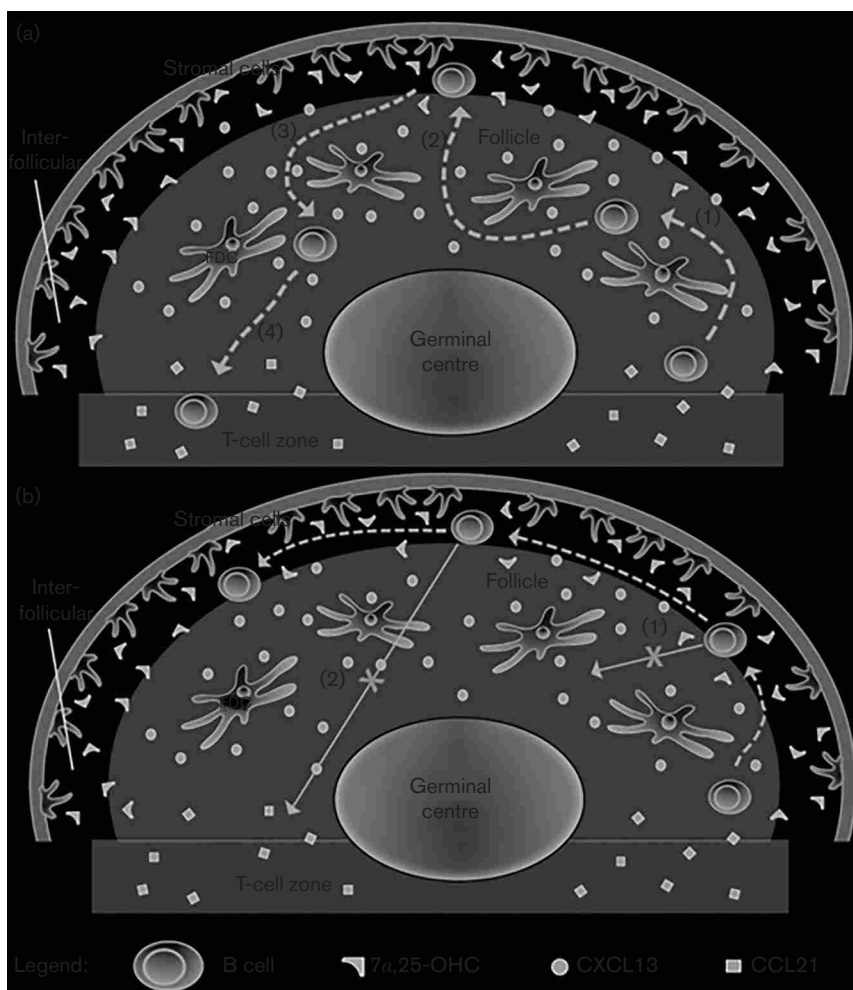
EBV is known to manipulate cellular genes to avoid the immune system, control viral replication and prevent apoptosis [40–42]. EBV had been previously shown to influence EBI2 [43–45]. Various studies have researched EBI2 expression during EBV infection; however, it has yet to be determined if the virus induces EBI2 or if heightened EBI2 expression is a result of the cellular response to viral infection [20, 46–48]. Previously, it has been hypothesized that EBI2 expression is a result of the immune response to viral infection since EBI2 has been shown to be induced during EBV latency program I. Normally, only EBNA1 is highly expressed [20, 43]. It has also been hypothesized that the increase of EBI2 expression observed during EBV infection is a result of viral manipulation to promote a successful persisting EBV infection, allowing for the virus to direct infected cells to areas that would provide a better survival niche for viral persistence [22, 49]. The results of this study provide evidence for the later explanation of why heightened EBI2 expression is observed during EBV infection.

It is possible that the EBV-induced upregulation of EBI2 could have been the result of non-specific activation, as a cellular response to EBV infection. However, the B-cell-stimulating agent imiquimod did not cause an upregulation in EBI2 expression, nor did incubation with inactivated EBV. These findings indicate that EBI2 upregulation is not likely due to cellular activation by TLR7, and that viral tegument or surface proteins are not responsible for the upregulation of EBI2.

Screening for viral gene candidates that could be responsible for the regulation of EBI2 post-EBV infection found that BRRF1 shares a similar mRNA expression pattern as EBI2. BRRF1 encodes a viral transcription factor, Na. Na is responsible for assisting in the activation of viral lytic genes in various latently infected epithelial cells [35] and has been found to associate with human TNF receptor-associated factor 2 in a yeast two-hybrid assay [50]. Due to its function as a transcription factor and its expression pattern being similar to that of EBI2, BRRF1 seemed the best candidate of those screened. To ascertain if BRRF1 expression could directly influence EBI2 expression, the BRRF1-deficient R-KO EBV was used to infect isolated naive B cells. R-KO EBV has been used in several studies and has the R gene, BRF1, knocked out as well as the promoter of BRRF1 resulting in EBV that cannot express BRRF1 [35]. R acts with BZLF1 to activate latent EBV and can bind to various EBV promoters [16, 51, 52]. The results show no upregulation of EBI2 expression upon infection with the BRRF1-negative EBV. This would suggest that BRLF1 or BRRF1 was inducing EBI2 expression. To further verify that BRRF1 could induce EBI2 expression, the pUltra+BRRF1 lentivirus was used to treat isolated naive B cells. This resulted in a significant increase in EBI2 expression. These results demonstrate that the expression of BRRF1 was necessary and sufficient to induce EBI2 expression. While the mechanism that BRRF1 uses to induce this expression in B cells is unknown, it is possible that Na acts as a transcription factor at the EBI2 promoter site. Further research needs to be performed in order to ascertain if this is the case.

It is probable that cell migration would be influenced by the EBV-induced regulation of EBI2. With a heightened concentration of EBI2 on the surface of the infected B cells, it is no stretch to infer that EBI2 might override other migratory signalling pathways and direct the cell to follow a $7\alpha,25$-OHC gradient, which is the natural ligand of chemoattractant receptor EBI2 (see Fig. 5b) [21, 39]. $7\alpha,25$-OHC is only produced by stromal cells of secondary lymph tissue [53]. Following this gradient would cause infected B cells to migrate and remain in the outer follicular and inter-follicular regions of secondary lymph tissue. This type of control over cell migration could allow the virus to avoid immune detection until latency has been established, assisting in immune evasion.

Fig. 5. Proposed effects on B-cell migration during EBV infection due to modulation of EBI2 expression. (a) This illustration demonstrates the regular pattern of naive B-cell migration in the lymph node. (1) CXCR5 is expressed, allowing cells to follow a CXCL13 gradient and enter the follicular region. CXCR5 is constitutively expressed to assist in cell migration. (2) EBI2 is upregulated and the cells follow a 7α,25-OHC gradient to the outer or inter-follicular region. (3) Downregulation of EBI2 allows the naive B cells to return to the follicular area and (4) upregulation of CCR7 permits the cell to follow the CCL21 chemokine gradient to the T-cell zone. If B cells remain inactivated, they will leave via the cortical sinus of the lymph node. If they become activated, they will be directed back to the follicular and germinal centre area. (b) This figure depicts the predicted pattern of migration during EBV infection of naive B cells. (1) Upon entry into the lymph node, the B cell will follow the 7α,25-OHC gradient to the outer and inter-follicular region of the lymph node. (2) The cell will be unable to migrate to the follicular region.

It is also possible that increased levels of EBI2 might influence B-cell proliferation. Benned-Jensen et al. [54] found that overexpression of EBI2 in antibody stimulated murine B cells resulting in increased proliferation. By increasing the proliferation of infected B cells during a specific time, it would increase the chances of viral persistence in the infected host. The results of this study emphasizes the importance of EBI2 regulation during viral infection. It is possible that the use of EBI2 antagonists, or inverse agonists, could be used as EBV antiviral treatment options or to disrupt the viral life cycle in an animal model [54–56].

During our study, we have established that EBI2 is controlled during EBV infection of B cells. Different EBV expression profiles result in different levels of EBI2 expression. Higher levels of EBI2 mRNA expression result in higher levels of EBI2 protein. We have further established that EBI2 is induced by an EBV gene, BRRF1. Potential research for the future can address to what degree heightened expression of EBI2 possibly changes migration of EBV-infected B cells. It would also be beneficial to verify the mechanism used by BRRF1 to induce EBI2 expression.

METHODS

Generation and harvesting of viral stock

Viral stock was generated using the Bac B95-8 EBV producing HEK 293 cell line as previously constructed and described by Delecluse *et al.* [57]. To summarize, the strain has been engineered with an F factor origin of replication, partitioning proteins A and B, chloramphenicol-resistance gene, hygromycin-resistance gene and an EGFP reporter. Cells were seeded in T-75 flasks (Corning) to attain a confluence of 50 to 60 %. The cells were then transfected with pUltra+BZLF1, pUltra+BRRF1 and pUltra+BRLF1 plasmids using calcium-phosphate transfection. Media was changed 16 h post-transfection to RPMI and cells were allowed to incubate for 7–10 days. Viruses were harvested in the RPMI media, filtered using a 0.45 µm filter, collected in 15 ml conical vials and stored at $-80\,°C$. The titre of Epstein–Barr viral infectious units was determined using green Raji cell assays following a previously described protocol [17]. To summarize, 2×10^5 Raji cells were suspended in 0.5 ml of viral supernatant. The cells were allowed to incubate for 3 h and then 1.5 ml of RPMI media was added to the wells. At day 2 p.i., sodium butyrate and phorbol-12-myristate-3-acetate (PMA; ACROS) were added to a final concentration of 3 mM sodium butyrate and 50 ng of PMA ml^{-1}. The cells were allowed to incubate for another 16–24 h and then the GFP-positive cells were quantified by fluorescence microscopy.

The BRLF1- and BRRF1-deficient Epstein–Barr virus (R-KO EBV) was kindly given to us by Dr Henri-Jacques Delecluse from the German Cancer Research Center, having been described and used in previous studies [17, 35, 58]. For specific information on the construction and testing of this virus, the reader is referred to previous publications by Hong *et al.* [17], Hagemeier *et al.* [35] and Feederle *et al.* [59]. R-KO EBV viral stocks were generated by the same method previously described for B95-8 EBV production. The titre of viral infectious units was determined using green Raji cell assays following a previously described protocol [17] and as described.

The lentiviruses pUltra+BZLF1, pUltra+BRRF1 and pUltra+BRLF1 DNA were grown in *Escherichia coli* DH5α cells and extracted using plasmid extraction kits (Qiagen). The plasmid DNA was then transfected into the PHX cell line using calcium-phosphate transfection. The supernatant was harvested and filter sterilized using a 0.45 µm filter. Lentiviral vector concentrations were quantified by placing 100 µl of lentiviral media with 1 ml of Raji cells at a concentration of 1×10^5 cells ml^{-1}. Cells were allowed to incubate for 48 h and then counted by fluorescence microscopy to determine the lentivirus titre.

UV irradiation of EBV

EBV supernatants in 15 ml centrifuge conical tubes were exposed to ultraviolet light (200–280 nm) in a biosafety cabinet hood for a period of 4 h. The UV-irradiated virus was then used to infect isolated B cells.

Human B-cell isolation

Human naive B cells were isolated using lymphocyte separation media (Cellgro) and EASYSEP negative selection magnetic separation (STEMCELL Technology). PBMCs were isolated from 30 ml of peripheral blood collected from healthy volunteers after informed consent using lymphocyte separation medium (Cellgro). PBMCs were then resuspended in PBS +2 % FBS with 1 mM EDTA added. Naive B cells were separated from other lymphocytes by magnetic cell separation using the Human B cell enrichment kit (STEMCELL Technology) following the EASYSEP protocol.

Infection assays and RNA extraction

Using standard 12-well plates, 2×10^6 naive B cells were placed in each well in 1 ml of medium. EBV viral stock was added to each well at an m.o.i. of 15. An equal volume of media without EBV was added to the negative controls. Cells were collected by centrifugation. Using the RNAqueous-Micro RNA extraction kit (Ambion) and procedure, RNA was extracted and suspended in elution solution. Since in naive B cells high rates of infection with EBV are difficult to obtain, we used the highest m.o.i. that was practical. Using an m.o.i. of 15, we typically obtained infection of about 15–25 % of naive B cells. All viral infection experiments were repeated at least three or more times in duplicate.

For infection with R-KO EBV, isolated naive B cells at a concentration of 2×10^6 cells ml^{-1} were incubated with R-KO EBV supernatant at an m.o.i. of 15. At each time point, samples were pelleted and treated as previously described for RNA extraction.
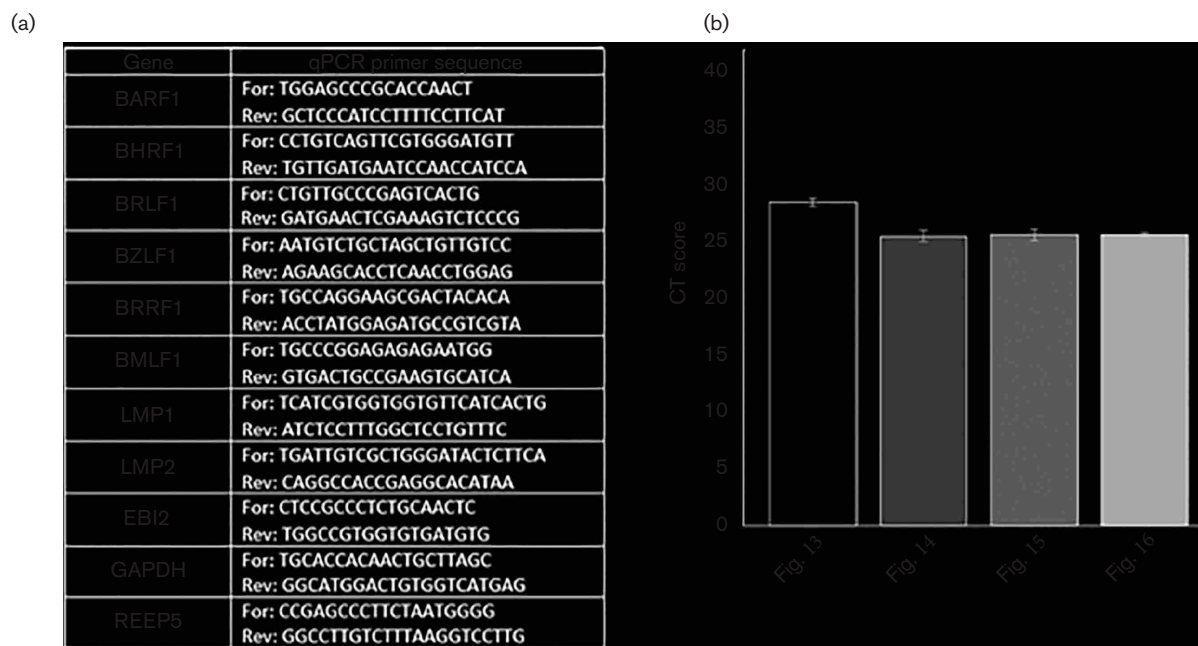
For lentiviral transduction, either pUltra+BRRF1 or pUltra+BRLF1, at an m.o.i. of 15, was added to isolated naive B cells at a concentration of 2×10^6 cells ml^{-1}. At 24 h p.i., the cells were pelleted and treated as previously described for RNA extraction.

Quantification of gene expression by qPCR

Reverse-transcriptase quantitative PCR (RT-qPCR) was performed using StepOne Plus software and equipment with Power SYBR Green PCR master mix (Applied Biosystems). Samples were analysed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. As a control for non-specific gene regulation, levels of the REEP5 gene were also measured using RT-qPCR. qPCR primers for all target genes were designed using Primer Express 3.0 (Applied Biosystems). Primer sequences as well as the consistency between experiments can be found in Fig. 6.

Statistical analysis

In most cases, two-tailed paired *t*-tests and an alpha value of 0.05 were used to determine significant differences in relative mRNA expression levels. Since our experiments were run and analysed in tandem, it allows us to use the paired tests when doing our statistical analysis when comparing variable conditions in the same experiment. There was one exception when determining the significance of the EBI2

(a)

Gene	qPCR primer sequence
BARF1	For: TGGAGCCCGCACCAACT
	Rev: GCTCCCATCCTTTTCCTTCAT
BHRF1	For: CCTGTCAGTTCGTGGGATGTT
	Rev: TGTTGATGAATCCAACCATCCA
BRLF1	For: CTGTTGCCCGAGTCACTG
	Rev: GATGAACTCGAAAGTCTCCCG
BZLF1	For: AATGTCTGCTAGCTGTTGTCC
	Rev: AGAAGCACCTCAACCTGGAG
BRRF1	For: TGCCAGGAAGCGACTACACA
	Rev: ACCTATGGAGATGCCGTCGTA
BMLF1	For: TGCCCGGAGAGAGAATGG
	Rev: GTGACTGCCGAAGTGCATCA
LMP1	For: TCATCGTGGTGGTGTTCATCACTG
	Rev: ATCTCCTTTGGCTCCTGTTTC
LMP2	For: TGATTGTCGCTGGGATACTCTTCA
	Rev: CAGGCCACCGAGGCACATAA
EBI2	For: CTCCGCCCTCTGCAACTC
	Rev: TGGCCGTGGTGTGATGTG
GAPDH	For: TGCACCACAACTGCTTAGC
	Rev: GGCATGGACTGTGGTCATGAG
REEP5	For: CCGAGCCCTTCTAATGGGG
	Rev: GGCCTTGTCTTTAAGGTCCTTG

(b)

Fig. 6. Primers and consistency of RT-qPCR analysis. (a) This table contains the qPCR forward and reverse primer sequences used in the experiments described. (b) GAPDH cycle threshold (CT) scores for the various figures have been compiled to verify that they are similar. There is no significant difference when comparing the GAPDH CT scores from the different experiments.

expression between the pUltra+BRRF1-transduced samples and the pUltra+BRLF1-transduced samples 24 h post-transduction: some of the samples were lost during harvesting and new samples were transduced and harvested. Due to this event, the experiment was analysed using Welch's unpaired *t*-test, and an alpha value of 0.05 was still considered significant. To analyse correlations between the expression of EBV genes and EBI2, linear regression analysis was performed using R statistical analysis software. In this analysis, EBI2 expression was compared to the expression of each gene in turn and R^2 values calculated [EBI2 ~ (EBV gene of interest)].

LCL cell line generation

PBMCs were re-suspended at 2×10^6 cells ml^{-1} in complete RPMI. Then 5 ml of cells suspended in media and 5 ml of B95-8 EBV cell culture supernatant were placed together in a T-25 flask with cyclosporin A or actinomycin D. Cells were incubated for 3 weeks, pipetted weekly to break up cell clumps. LCL cell lines are maintained in complete RPMI and passaged frequently.

Lentivirus generation

The lentiviral vector pUltra+BRRF1 was constructed using pUltra, a third-generation lentivirus obtained from Addgene. The BRRF1 gene was PCR amplified from wild-type B95-8 EBV using forward (TCTAGAATGGCTAGTAGTAACAGAGGAAATG) and reverse (TGATCATTATTTGTATTGCATGGCAGAACAGT) primers with XbaI and BclI restriction site extensions added, respectively. BRRF1

was then cloned into pUltra cut with XbaI and BclI restriction enzymes. The ligated pUltra+BRRF1 was then transfected into *E. coli* DH5α cells. Using a BRRF1-specific forward primer and a pUltra-specific reverse primer, the colony containing the complete pUltra+BRRF1 plasmid was verified by sequencing. The plasmid used for transfecting EBV producing HEK cells pUltra+BZLF1 was produced using pUltra with the BZLF1 gene cloned into the construct. Forward (GTCGACTCAAAGAGAGCCAACAGGAAG) and reverse (GAATTCAAAGGGGAGATGTTAGACAGGT) primers with SalI and EcoRI restriction site extensions were added, respectively. The PCR-amplified BZLF1 gene was cloned into pUltra cut with SalI and EcoRI. The ligated pUltra+BZLF1 was transfected into *E. coli* DH5α cells and verified as described previously using a BZLF1-specific forward primer and a pUltra-specific reverse primer. The lentiviral vector pUltra+BRLF1 was constructed using the same pUltra lentivirus as aforementioned. The BRLF1 gene was PCR amplified from wild-type B95-8 EBV using forward (TCTAGAATGGTCGGGCATTTCCTCTG) and reverse (TGATCACCAAAAGAGGAGGAGGCAGT) primers with XbaI and BclI restriction site extensions added, respectively. The PCR-amplified BRLF1 gene was cloned in to pUltra cut with XbaI and BclI. The ligated construct of pUltra+BRLF1 was then transfected into *E. coli* DH5α cells and verified as described previously. All primers for cloning the desired the PCR products were designed using Primer 3 software. All lentiviral constructs were verified by sequencing and expression of the cloned genes were verified by RT-qPCR.

Western blot

One millilitre of naive B cells at a concentration of 2×10^6 cells ml^{-1} was infected with EBV at an m.o.i. of 15. At the time of sample collection, the cells were pelleted and resuspended in lysis buffer (Thermo Scientific). Cells were then vortexed and passed through a 25-gauge needle, followed by incubation in Laemmli sample buffer (BIO RAD) and 5 % 2-mercaptoethanol (Sigma) for 5 min at 95 °C. The samples were then subjected to electrophoresis in a 12 % polyacrylamide gel and transferred to a nitrocellulose membrane (Thermo Scientific). Blots were blocked with 2.5 % (w/v) non-fat dry milk. EBI2 polyclonal goat IgG obtained from Santa Cruz Biotechnology at a diluted 1 : 1000 was used as the primary Ab. Rabbit anti-goat IgG-HRP obtained from Santa Cruz Biotechnology was used as the secondary Ab. ECL Plus (GE Healthcare) lumigen reagents and C-DiGit blot scanner (LI-COR) were used to image the Western blot. For measuring protein loading, blots were stripped using stripping buffer (100 mM β-mercaptoethanol, 2 % SDS, 62.5 mM Tris/HCl, pH 6.7) and reprobed using goat anti-actin (Abcam) as the primary antibody followed by secondary antibody and visualization as described for EBI2.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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